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Functions of the centromere and kinetochore in chromosome segregation

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Centromeres play essential roles in equal chromosome segregation by directing the assembly of the microtubule binding kinetochore and serving as the cohesion site between sister chromatids. Here, we review the significant recent progress in our understanding of centromere protein assembly and how centromere proteins form the foundation of the kinetochore.

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Introduction

Eukaryotic organisms package their genomic DNA into a number of physically distinct chromosomes, which are replicated and equally segregated during each cell cycle. Accurate chromosome segregation requires each chromosome's centromere to build a kinetochore, a complex structure containing at least 100 different proteins that serves as the microtubule-binding site for the mitotic spindle. Correct centromere formation and specification is therefore essential to cell survival.

In contrast to kinetochores, which specifically assemble and function in mitosis, centromeric chromatin, and a group of 17 proteins that bind centromeric chromatin termed the constitutive centromere-associated network (CCAN), are present throughout the cell cycle [1]. A hallmark of centromeric chromatin in all eukaryotes is the presence of nucleosomes that contain the essential H3 variant CENP-A (CENtromere Protein-A) (Box 1). In metazoans, the underlying DNA appears to be mostly dispensable for centromere function. Instead, centromeric proteins epigenetically define each centromere. While the mechanisms of CENP-A assembly are yet to be fully defined, CENP-A is currently the most attractive candidate for the epigenetic mark.

Three broad criteria must be satisfied for centromere replication and function. First, as DNA replication dilutes CENP-A at centromeres, new CENP-A assembly during each cell cycle must maintain the correct amount of CENP-A chromatin. Second, CENP-A must facilitate CCAN protein recruitment to form the centromere. Third, CCAN proteins must provide the molecular platform for kinetochore formation to facilitate chromosome segregation during cell division.

The mechanisms of CENP-A assembly and CENP-A distribution during DNA replication have been extensively reviewed [2–9]. Therefore, we focus on progress made in our understanding of the precise molecular links between the underlying DNA of the centromere, CENP-A, and the core CCAN. We then discuss how CCAN proteins promote kinetochore formation. Finally, we consider the implications of the recent advances in the understanding of CCAN dynamics.

The DNA–centromere interface: standing on two legs?

A major research focus in recent years has been to establish how core centromere proteins specifically assemble on centromeric DNA to provide a platform for mitotic kinetochore formation. Two constitutive centromere proteins, CENP-N and CENP-C, have been demonstrated to bind directly to CENP-A nucleosomes. CENP-N binds to reconstituted nucleosomes containing CENP-A/H3 chimeras that possess only the CENP-A Targeting Domain (CATD) [10] while CENP-C binds the unique C-terminal tail of CENP-A (Figure 1a and Box 1) [11•]. The interaction of CENP-C with CENP-A's C-terminus is dependent on a central region of CENP-C that also possesses nonspecific DNA-binding activity [11•,12]. This suggests that CENP-C and CENP-N interact with the CENP-A nucleosome independently of one another by recognizing different domains within CENP-A (Figure 1).

Is CENP-A-mediated recruitment of CENP-N and CENP-C sufficient to build a complete centromere? In *Drosophila* cells, overexpression of CENP-A(Cid) results in misincorporation of CENP-A on chromosome arms and causes the formation of ectopic kinetochores [13,14••]. In addition, artificial tethering of CENP-A to chromatin induces the formation of stable centromeres [14••]. In *Xenopus* egg extracts, arrays of reconstituted CENP-A nucleosomes are sufficient to build kinetochores that can bind microtubules [15••]. In vertebrate cells,

Box 1 Our understanding centromere specification centers on what makes CENP-A distinct from canonical histone H3. Briefly, CENP-A contains the CENP-A targeting domain (CATD), a region within its histone fold domain containing 22 amino acid substitutions when compared to H3 [55]. The CATD allows specific recognition and assembly of CENP-A into chromatin by the loading factor HJURP [51••]. CENP-A also contains a distinct extreme C-terminal tail that has been shown to be important for the interaction of CENP-C and for centromere formation in humans and *Xenopus* [11•,15••].

The composition and structure of CENP-A nucleosomes is also a contentious issue (reviewed in [56]). Briefly, a prevalent model, based upon the crystal structure of reconstituted CENP-A nucleosomes, is that CENP-A nucleosomes are octameric and wrap DNA in a left-handed manner, and therefore mimic H3 nucleosomes. An alternative model is that CENP-A nucleosomes consist of one CENP-A/H4 dimer and one H2A/H2B dimer (termed a 'hemisome'), and potentially wrap DNA in a right-handed manner (reviewed in [57]). It is also possible that CENP-A nucleosomes contain a CENP-A/H3/$H4_2$ heterotetramer, and/or the structure of CENP-A nucleosomes changes during the cell cycle. Complicating this, both the timing of CENP-A assembly and overall centromere size and structure differs widely between organisms (reviewed in [2]).

CENP-A overexpression also causes ectopic CENP-A incorporation into chromosome arms and the recruitment of CENP-C and CENP-N to those sites, but not recruitment of other CCAN components [16••,17]. Although the presence of the endogenous centromere may have prevented CCAN assembly at the ectopic site, it is possible that additional components are required to provide a foundation for the centromere in humans. Indeed, CENP-T has recently emerged as a potential bridge between the underlying DNA, the CCAN, and the outer kinetochore. In human cells, ectopically localizing the N-termini of CENP-T and CENP-C to chromatin, using Lac repressor fusions and chromosomally integrated lac operator sequences, recruits sufficient centromere components to drive formation of pseudokinetochores able to bind microtubules and facilitate chromosome segregation [16••].

Affinity purification studies in chicken DT40 cells identified CENP-W and CENP-X as novel binding partners of CENP-T and CENP-S, respectively [18,19]. CENP-T and W dimerize and CENP-S and X form a tetramer through histone fold domains in each protein [20,21]. Significantly, CENP-T/W/S/X can form a heterotetrameric complex that can supercoil DNA *in vitro*, similar to nucleosomal histones [20]. This raises the possibility that the CENP-T/W/S/X complex acts as an additional structural platform on DNA (Figure 1b). Importantly, the role of CENP-T/W appears to be conserved in budding yeast, as Cnn1 and Wip1 were recently identified as functional homologs of CENP-T and CENP-W, respectively [22•,23•].

Despite this progress, the mechanisms behind CENP-T centromere localization remain unclear. Although CENP-T has been suggested to preferentially associate with H3 nucleosomes, at least in chicken DT40 cells [18,24], CENP-T is present in CENP-A immunoprecipitations [25] and CENP-T relies on CENP-A for its centromere localization through an unknown mechanism [18]. There are conflicting reports as to whether CENP-T is lost from centromeres after CENP-C RNAi in human cells [11•,16••], but CENP-C knockout chicken DT40 cells successfully recruit CENP-T to centromeres [18]. While CENP-T turns over in the course of a cell cycle, CENP-A is thought to remain bound to DNA through multiple cell divisions [26,27•,28,59]. It therefore remains likely that CENP-A defines centromere position and provides the initial foundation of the CCAN.

CENP-T/W is essential for cell viability [18]. However, the phenotype of CENP-S/X deficient DT40 cells is less pronounced [19]. Although CENP-T or CENP-W deficient cells fail to recruit CENP-S/X to centromeres [18], and ectopically localized CENP-S-LacI recruits CENP-T [20], depleting CENP-S or CENP-X does not affect CENP-T centromere localization [19]. Indeed, CENP-S/X requires the presence of CENP-K for centromere localization, but CENP-T/W does not [18,19]. As the CENP-T/W dimer and $(CENP\text{-}S/X)_2$ tetramer can bind DNA independently (CENP-S/X are also the Fanconi anemia proteins MHF1/MHF2 [29]), it is possible that CENP-T/W associates with DNA independently of CENP-S/X, and that subsequent recruitment of CENP-S/X results in CENP-T/W/S/X formation. It is also possible that a population of CENP-T/W functions independently of CENP-S/X and *vice versa*. Establishing how, when, and if the CENP-T/W/S/X complex assembles in cells is therefore an immediate aim for the field.

Kinetochore assembly on core CCAN components

In contrast to the CCAN, kinetochore proteins are recruited to centromeres as cells enter mitosis to build the kinetochore for chromosome segregation. Kinetochores attach to spindle microtubules [1], respond to tension generated by stable attachments [30], and when unattached, generate the spindle checkpoint signal that restrains anaphase onset [31]. Here, we focus on advances in our understanding of how the core CCAN components CENP-C and CENP-T recruit the KMN network, which mediates the attachment of spindle microtubules [32].

The KMN network comprises KNL1 (also known as Spc105), the Mis12 complex (Nsl1, Nnf1, Dsn1, and Mis12) and the Ndc80 complex (Ndc80/Hec1, Nuf2, Spc24, and Spc25). While the Ndc80 complex makes load-bearing attachments to microtubules [33], recent evidence suggests that KNL1 may have a surveillance role in reading the state of kinetochore—microtubule interactions [34]. The Mis12 complex lacks microtubule-binding ability, but has been shown to regulate the recruitment of KNL1 and at least a subpopulation

of Ndc80, and is therefore considered as the 'hub' of the KMN network [35].

In *Xenopus*, *Drosophila* and humans, the N-terminal region of CENP-C is responsible for recruiting the KMN network (Figure 1b) [36••,37,38••]. In *Drosophila*, CENP-C directly interacts with the Mis12 complex component Nnf1 (Figure 1b), and mistargeting CENP-C to the centrosome causes centrosome localization of all KMN network subcomplexes [38••]. In addition, CENP-C knockout cells fail to recruit the Mis12 complex to the kinetochore [39]. In humans, a 21-amino acid N-terminal fragment of CENP-C interacts with the Mis12 complex and a 71-amino acid N-terminal fragment of CENP-C, which cannot localize to kinetochores when expressed in cells, disrupts kinetochore targeting of the KNL1 and Mis12 complexes [36••]. Interestingly, only a partial effect is observed on the Ndc80 complex [36••], highlighting the possibility that additional factors may recruit at least a subpopulation of Ndc80 in human cells.

Consistent with this notion, recent insights have shown that human and budding yeast CENP-T plays a role in recruiting the Ndc80 complex to the kinetochore (Figure 1b). In human cells, this interaction is positively regulated by Cdk1 activity. A non-phosphorylatable N-terminal tail mutant of CENP-T fails to recruit Ndc80 and results in a defective kinetochore [16••]. This provides a direct link between spindle microtubule binding (through Ndc80) and DNA binding (through CENP-T), an explanation for how Ndc80 recruitment is cell cycle stage specific, and potentially describes how forces generated by microtubules translate into movement of sister chromatids (Figure 1b). Indeed, CENP-T has been shown to physically stretch in response to tension at the kinetochore [34]. In *Saccharomyces cerevisiae*, CENP-T competes with the Mis12 complex for Ndc80 binding [23•], and therefore CENP-T may inhibit interactions within the KMN network [22•]. In addition, the CENP-T–Ndc80 interaction may be enriched in anaphase, raising the possibility that the interactions between the CCAN and KMN network change during cell division [23•]. Further defining the role and regulation of CENP-T in kinetochore assembly is likely to provide key insight into kinetochore organization and function.

Recruitment and role of outer CCAN complexes

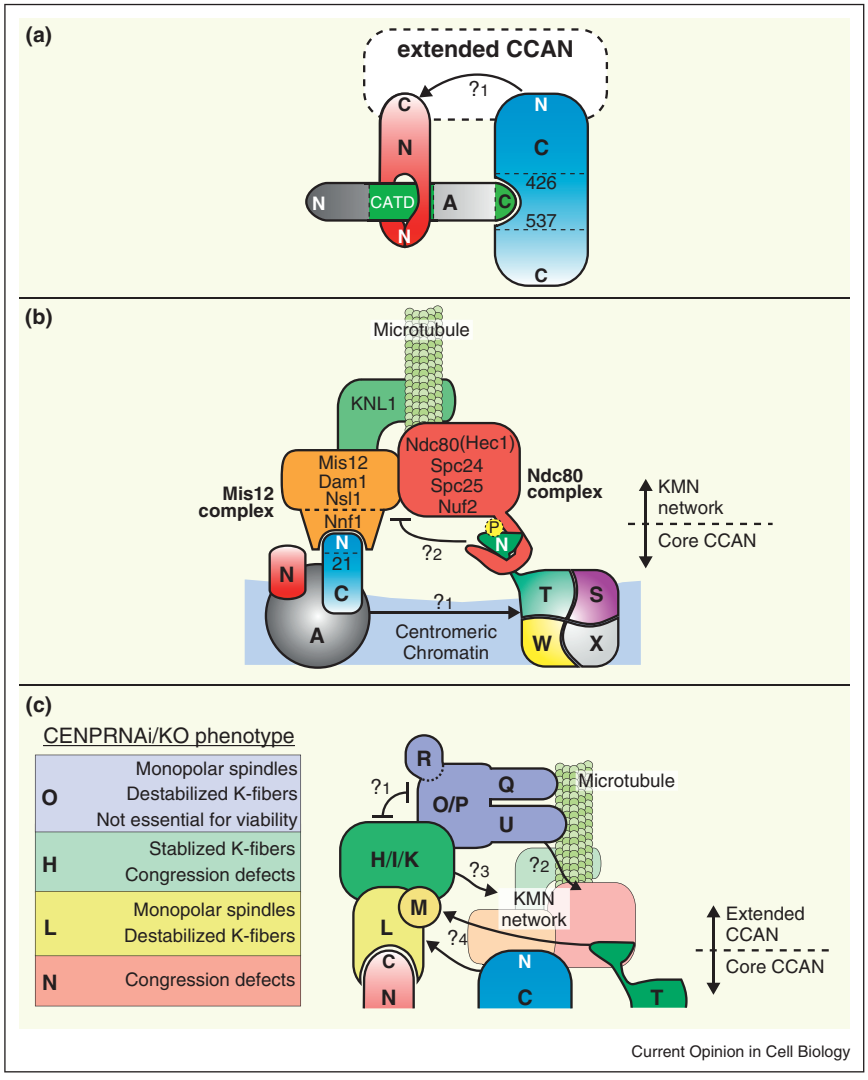
In addition to CENP-A, CENP-C, CENP-N and CENP-T/W/S/X, the other CCAN proteins CENP-H, CENP-I, CENP-K, CENP-L, CENP-M, CENP-O, CENP-P, CENP-Q, CENP-R, and CENP-U, form the extended centromere. These proteins can be divided into the CENP-L/M (and N, see Box 2) complex, CENP-H/I/K (the CENP-H complex) and CENP-O/P/Q/R/U (the CENP-O complex), based upon both biochemical interactions and phenotypic analyses (Figure 1c). CENP-N RNAi causes major defects in centromere function [10,25,40], presumably due to gross losses in CCAN protein centromere recruitment, as CENP-N directly binds CENP-L [10], that is, in turn, required for recruitment of CENP-H/I/K, CENP-M and CENP-O/P/Q/R/U [25,41]. CENP-H depletion prevents recruitment of CENP-O/P/Q/R/U [41], possibly through loss of CENP-K, which has been suggested to directly bind CENP-O [42]. CENP-O depletion selectively affects CENP-P/Q/R and CENP-U recruitment to centromeres, consistent with those proteins participating in a stable protein complex [25,41,43]. Like many large multiprotein complexes, there appear to be multiple reinforcing interactions within the constitutive centromere. For example, loss of the CENP-H/I/K complex (through CENP-H knockout) reduces CENP-L and Ndc80 centromere localization [41], and CENP-U has been suggested to bind Ndc80 [44•]. CENP-T depletion studies suggest that CENP-T may also play a role in bringing CENP-M and CENP-H/I/K to centromeres [18,25]. Finally, although previously discussed here as a core CCAN component, CENP-S was originally identified as part of a CENP-M or CENP-U complex [25]. Thus, while progress has been made in identifying many of the proteins of the centromere, the task of untangling the interdependencies and relationships between CCAN proteins require further systematic and detailed analysis.

Broadly speaking, depletion of any individual extended CCAN protein causes chromosome congression defects, but cells display distinct phenotypes (summarized in Figure 1c). CENP-H depletion increases tubulin turnover at attached kinetochores, stabilizes K-fibers (bundles of kinetochore-attached microtubules), reduces kinetochore oscillations at the metaphase plate, causes congression defects and therefore causes mitotic delay [40,45,46]. In contrast, depletion of CENP-L or CENP-O causes an

Box 2 Is CENP-N a core CCAN or extended CCAN component?

Although discussed above as a 'core' centromere component as it directly interacts with CENP-A, CENP-N could also be considered part of the extended CCAN. CENP-N is found at artificial centromeres specified by CENP-T-LacI and CENP-C-LacI that form independently of CENP-A [16••]. Moreover, although CENP-N binds CENP-A's CATD *in vitro*, in *Xenopus* egg extracts, arrays of nucleosomes containing chimeric histone H3 possessing CENP-A's C-terminal tail (and not the CATD) recruit CENP-N in a CENP-C dependent manner, and CENP-N mutants that do not bind CENP-A *in vitro* can localize to centromeres [10]. In humans, the C-terminus of CENP-N, which is not required for CENP-A association, directly binds CENP-L [10]. The putative fission yeast homolog of CENP-L (Fta1) interacts directly with CENP-C (Cnp3) [58]. If conserved, this would provide a molecular link between CENP-C and CENP-N and provide a potential mechanism for CENP-C-dependent CENP-N centromere recruitment. As the amount of CENP-N at centromeres appears to change during the cell cycle (see main text), CENP-N may be recruited to centromeres *via* at least two temporally distinct mechanisms. Further investigation of CENP-N in cells is required to establish how the CCAN is assembled through the cell cycle.

Figure 1



Schematics of centromere and kinetochore organization. In all figure panels, individual capitalized letters indicate a 'CENP' protein, while smaller letters indicate N-termini and C-termini (N and C, respectively). Arrows with question marks indicate interesting observations that require further investigation. **(a)** Schematic of direct interactions between CENP-A and the CCAN. *In vitro*, CENP-N binds CENP-A's CATD domain and CENP-C binds through CENP-A's distinct C-terminal tail. A fragment of human CENP-C consisting of amino acids 426–537 is sufficient to bind CENP-A. In cells, CENP-N can somehow be recruited to centromeres independently of CENP-A (?1), most likely through interactions within the extended CCAN (see text for details). **(b)** Recruitment of the KMN network by the core CCAN. Recent data suggest that CENP-T/W/S/X and CENP-C provide the foundation for distinct components of the KMN network. The Mis12 component Nnf1 directly interacts with CENP-C in *Drosophila*, and amino acids 1–21 of human CENP-C are sufficient for the binding of CENP-C to the Mis12 complex. In parallel, Cdk1-mediated phosphorylation (yellow 'P') of CENP-T's N-terminal tail facilitates the direct binding of CENP-T to Ndc80. How CENP-T depends on CENP-A for its centromere localization (?1), and how CENP-T may compete with Mis12 for Ndc80 binding (?2), remain unclear. **(c)** Recruitment of the extended CCAN complex by the core CCAN and an overview of CCAN depletion phenotypes. CCAN complexes are recruited to centromeres through their interactions with the chromatin binding proteins CENP-N, CENP-C, and CENP-T as depicted here going from the bottom (CENP-N) to the top (CENP-R). Broadly speaking, removal of any one CCAN component affects all CCAN proteins within its complex and all those above it (but not below). However, CENP-H is required for correct CENP-L recruitment. While considered part of the CENP-O complex, CENP-R depletion does not affect any other known CCAN component. The phenotypes of CCAN depleted cells suggest that the CENP-H and CENP-O complexes antagonize each other (?1). How the extended CCAN influences the microtubule attachments remains unclear, but CENP-Q and CENP-U directly bind microtubules, CENP-U may interact with Ndc80 (Hec1) (?2), and CENP-K has been shown to be partially responsible for Ndc80 recruitment (?3). CENP-C may directly interact with CENP-L, but how CENP-T and CENP-C mediate CCAN assembly remains largely unclear (?4). As summarized in the table depletion (by RNAi or knockout (KO)) of any extended CCAN component results in chromosome congression defects, but distinct phenotypes have been observed. See text for details.

increase in monopolar spindle formation and weakened K-fibers, suggesting that CENP-H/I/K and CENP-O/P/Q/R/U may antagonize each other [40,41,43,47]. The phenotype of CENP-L and CENP-O depletion has been attributed to potential defects in kinetochore-generated pushing forces on centrosomes causes by microtubule polymerization at kinetochores [40,48]. Coupled to recent observations that show a CENP-Q octamer and CENP-U bind microtubules *in vitro* [44•,45], and that CENP-Q/U may be negatively regulated by the mitotic kinase Plk1 [49], it appears that the extended CCAN fine-tunes the kinetochore–microtubule attachment to promote bipolar spindle assembly.

CCAN dynamics

Although we refer to the proteins of the CCAN as constitutive because they are detectable throughout the cell cycle at centromeres, recent data demonstrate that the CCAN is highly dynamic both in individual protein turnover and in overall composition. Both immunofluorescence microscopy and live-cell imaging of EGFP–CENP-N expressing cells suggest CENP-N levels at centromeres increases during S-phase and decreases before mitosis [40,50]. Fluorescence Recovery After Photobleaching (FRAP) studies showed that GFP–CENP-C is more stable during S-phase [26]. While immunofluorescence microscopy studies suggest that centromere levels of CENP-H are constant through the cell cycle [40,46], FRAP of GFP–CENP-H suggests that, like CENP-C, CENP-H is stabilized during S-phase [26]. Interestingly, this may not be a general property of the CENP-H/I/K/M/N proteins as FRAP of GFP–CENP-I, a component of the CENP-H complex, suggested that CENP-I specifically turns over during S-phase. CLIP-tagged CENP-T and CENP-W were recently shown to increase in abundance at centromeres during the later stages of S-phase and G2 [27•]. Finally, recent SNAP–CENP-O experiments suggest that CENP-O is assembled onto centromeres during DNA replication [42], while immunofluorescence studies suggest that CENP-O levels are decreased in mitosis [42,47], perhaps as a result of the aforementioned negative regulation by Plk1 [49]. Like CENP-H and CENP-I, the members of the CENP-O/P/Q/R/U complex also show distinct residence times at centromeres when studied by FRAP [42].

It seems likely that dynamic changes in the CCAN will facilitate cell cycle specific centromere function, namely CENP-A assembly during mitotic exit/G1, CENP-A nucleosome distribution during DNA replication, and kinetochore formation during G2/mitosis. For example, CENP-N binds the same region of CENP-A as the CENP-A assembly factor HJURP [11•,51••], suggesting HJURP association with newly assembled CENP-A may preclude CENP-N centromere recruitment. Consistent with this, the reduction in CENP-N centromere abundance correlates with the timing of CENP-A assembly. In addition, both CENP-H and CENP-K knockout DT40 cells mislocalize CENP-C in interphase, but not in mitosis, suggesting that interactions within the CCAN reorganize concomitant with cell cycle progression [39,46]. Further identifying these changes will undoubtedly shed more light on the mechanisms behind CCAN-mediated kinetochore formation and microtubule regulation.

Concluding remarks

Significant progress has been made in the understanding of how the constitutive centromere assembles and functions. CENP-A directly recruits CENP-C and CENP-N to centromeres, and CENP-C, together with CENP-T, recruits the KMN network. In parallel to this, the extended CCAN protein complexes regulate kinetochore microtubule attachments to promote bipolar spindle formation.

While this provides a simplified framework describing centromere function in humans, it is important to note that significant differences in centromere organization exist between model organisms, both in centromeric chromatin structure and in CCAN composition. Most significantly, homologs of many CCAN proteins have not yet been identified in *Drosophila* and *Caenorhabditis elegans*. In *C. elegans*, depletion of KNL1 abolishes Ndc80 complex recruitment [52], but in human and chicken cells loss of KNL1 does not abolish Ndc80 localization because CENP-K (which appears to be lacking in *C. elegans*) also promotes Ndc80 localization to centromeres [53]. Similarly, in human cells CENP-T also plays a role in recruiting a subpopulation of Ndc80 to kinetochores [16••] while *Drosophila* CENP-C (a *Drosophila* CENP-T homolog has not been found) appears responsible for recruiting the entire KMN network [38••]. It is therefore possible *Drosophila* CENP-C can support many of the functions that other CCAN proteins serve in vertebrates and suggests a simpler CCAN functions in *Drosophila* and *C. elegans*. In contrast, yeast possesses functional homologs of many human CCAN proteins, including CENP-N (Chl4 in *S. cerevisiae* and Mis15 in *Schizosaccharomyces pombe*) and CENP-T (Cnn1 in *S. cerevisiae* and putatively SPBC800 in *S. pombe*) and yet utilizes distinct kinetochore proteins, with the Dam1 microtubule-binding ring complex a prime example [54]. Thus, understanding how the core functions of the centromere and kinetochore changed through evolution is an exciting area of future investigation.

Finally, data suggesting that the number and/or stability of many CCAN proteins depends on the stage of the cell cycle has important implications. To date, many studies have not made this distinction, and therefore paint a picture of a largely stable CCAN. Data mentioned above suggesting CENP-C depends on CENP-H/I/K for centromere recruitment in interphase but not mitosis, and that CENP-N numbers decrease during mitosis and G1,

highlight that this is not the case. Although by definition the CCAN proteins are detectable at centromeres throughout the cell cycle, further investigation of the changes in CCAN relationships will undoubtedly shed considerable light on centromere assembly and function.

Note added in proof

While in press, two independent studies were published describing the interaction between CENP-T and the Spc24/25 portion of the Ndc80 complex in *Saccharomyces cerevisiae* [60•] and in chicken DT40 cells [61•]. These reports confirmed that phosphorylation of CENP-T's N-terminal tail regulates the interaction with Spc24/25, and that CENP-T and the Mis12 complex compete for Spc24/25 binding, potentially revealing phosphorylation dependent changes in Ndc80 complex association with the centromere during mitosis.

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