

Establishment of the vertebrate kinetochores

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Abstract The centromere is essential for accurate chromosome segregation during mitosis and meiosis to achieve transmission of genetic information to daughter cells. To facilitate accurate chromosome segregation, the centromere serves several specific functions, including microtubule binding, spindle-checkpoint control, and sister chromatid cohesion. The kinetochore is formed on the centromere to achieve these functions. To understand kinetochore structure and function, it is critical to identify the protein components of the kinetochore and characterize the functional properties of each component. Here, we review recent progress with regard to the molecular architecture of the kinetochore and discuss the future directions for centromere biology.

Keywords Centromere · Kinetochore · CCAN · KMN

Abbreviations

AFM	Atomic-force microscopy
CCAN	Constitutive centromere-associated network
CENP	Centromere protein
ChIP	Chromatin immunoprecipitation

CPC	Chromosome passenger complex
FACT	Facilitates chromatin transcription
FRAP	Fluorescence recovery after photobleaching
EM	Electron microscope
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HP1	Heterochromatin protein 1
KMN	KNL1 and the Mis12 and Ndc80 complexes
LacO	Lac (lactose operon) operator
LacI	Lac repressor
PP1 γ	Protein phosphatase 1 gamma
RFP	Red fluorescent protein

Introduction

Cell division is a critical biological process for transmitting genetic information to daughter cells in all organisms. As we can observe chromosome behavior during cell division under a classic microscope, studies on chromosome segregation are one of the traditional research areas in biology. For faithful chromosome segregation, each sister chromatid must attach to spindle microtubules. This attachment is mediated by a large proteinaceous structure called the kinetochore that forms on centromeric DNA. Direct observation using an electron microscope (EM) reveals that the kinetochore has a trilaminar structure composed of electron-dense inner and outer layers (Brinkley and Stubblefield 1966; Jokelainen 1967). It is unclear which molecules are involved in the

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formation of this tri-layered structure. Genetic screens and recent biochemical and proteomic analyses have identified more than 100 proteins that localize to the kinetochore, but we are still far from completely understanding how those proteins work coordinately to form a functional kinetochore (Cheeseman and Desai 2008; Perpelescu and Fukagawa 2011; Takeuchi and Fukagawa 2012). To ensure accurate chromosome segregation, the kinetochore has three key activities (Fig. 1): First, the kinetochore must be specified at a single locus on each chromosome in most eukaryotes; if the kinetochore is formed at multiple loci on a chromosome, it is possible for the same chromatid to become simultaneously attached to opposite spindle poles, and this results in chromosome instabilities, which lead to cell death or carcinogenesis. Second, the kinetochore must form an interface to attach to the spindle microtubules; to form a functional kinetochore, various proteins must be assembled and be modified before mitosis. Third, sister kinetochores must be held together by sister-chromatid cohesion. This contributes to proper bipolar attachment of kinetochores to the spindle and silences/satisfies the spindle-checkpoint pathway.

Recent progress has revealed that a group of 16 chromatin-proximal proteins, referred to as the constitutive centromere-associated network (CCAN) (Fig. 1), is largely involved in establishing a foundation for the

kinetochore structure (Perpelescu and Fukagawa 2011; Takeuchi and Fukagawa 2012). External to this, the KMN (KNL1 and the Mis12 and Ndc80 complexes) network recognizes the CCAN to form a functional kinetochore (Fig. 1). In this review, we introduce and discuss the latest insights in kinetochore research, including the CCAN and KMN network and their functional roles in kinetochore formation.

Centromeres are specified by sequence-independent epigenetic mechanisms in vertebrates

For centromere specification, it is easy to imagine that the underlying primary DNA sequence may provide important information. Indeed, the budding yeast *Saccharomyces cerevisiae* has a short, unique 125-bp sequence for specifying the centromere (Clarke and Carbon 1980; Wiens and Sorger 1998). In vertebrates, large arrays of tandem-repeated DNA have been found at specific chromosomal domains, including constitutive heterochromatin and centromeres using classical molecular biological approaches. Although centromeric-satellite DNA is highly divergent in various organisms, unit sequences of repeats are usually similar among the chromosomes of each organism. Human centromeres typically contain mega-base arrays of tandem-repeated 171-bp alpha-

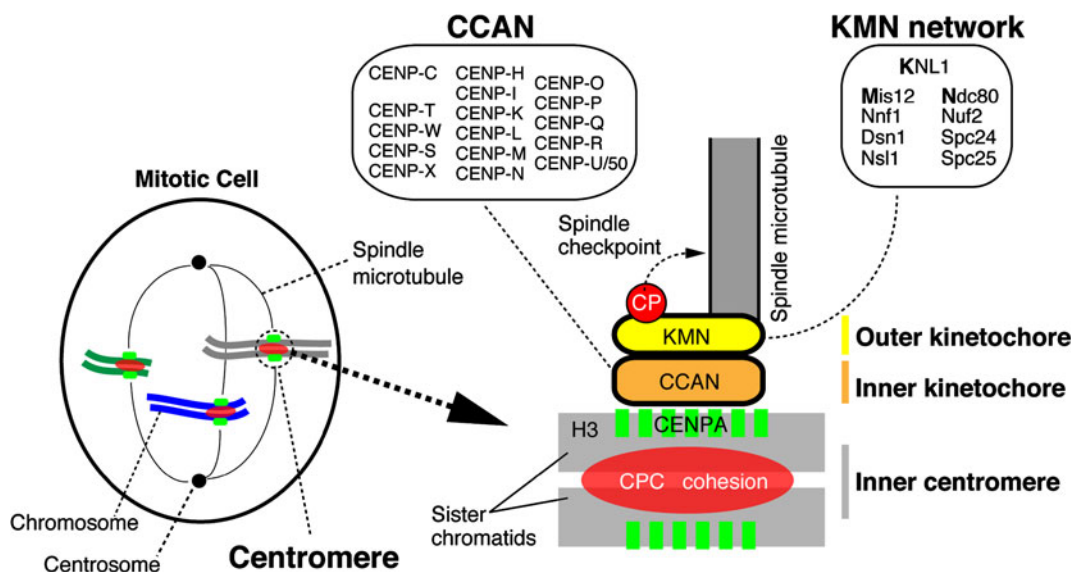


Fig. 1 Architecture of the vertebrate kinetochore in mitotic cells. The kinetochore is assembled on a single locus on each chromosome specified by sequence-independent epigenetic mechanisms. The kinetochore is divided into two major protein

networks: the CCAN and the KMN network. The CCAN localizes to centromeres throughout the cell cycle and provides a structural platform for targeting of the KMN network during mitosis

satellite DNA (Fig. 2) (Willard 1990). Alpha-satellite DNA is recognized by centromere protein B (CENP-B) and contributes to efficient de novo centromere formation in human artificial chromosomes (Harrington et al. 1997; Ikeno et al. 1998; Ohzeki et al. 2002). However, CENP-B is not essential for the maintenance of native centromeres, because CENP-B knockout mice are viable (Hudson et al. 1998; Kapoor et al. 1998; Perez-Castro et al. 1998). Furthermore, discovery of neocentromeres where a functional kinetochore is established in the absence of alpha-satellite DNA demonstrates that alpha-satellite DNA sequences are not absolutely essential for kinetochore formation (du Sart et al. 1997; Marshall et al. 2008). Analysis of histone modifications at some human neocentromeres by chromatin immunoprecipitation (ChIP) using antibodies against tri-methylated K9 of histone H3 have revealed a lack of heterochromatin enrichment around each neocentromere, suggesting that large domains of heterochromatin are not required for centromere formation (Alonso et al. 2010). This is in contrast to the case of fission yeast or *Drosophila*, in

which heterochromatin facilitates kinetochore formation (Folco et al. 2008; Ishii et al. 2008; Olszak et al. 2011). However, sister chromatid cohesion is weaker than normal at human neocentromeres (Alonso et al. 2010). In addition, Aurora B does not localize at the proper position in human neocentromeres, potentially causing defects in error correction of kinetochore-microtubule attachments (Bassett et al. 2010). These reports suggest that heterochromatin formed on a highly repetitive array is dispensable for kinetochore formation but provides additional activities for a fully functional kinetochore to mediate faithful chromosome segregation in human cells.

Recent deep-sequencing or ChIP-chip analyses for centromere-associated DNA in various organisms revealed that some chromosomes do not contain tandem repetitive sequences. These include chicken chromosomes 5, 27, and Z or horse chromosome 11 (Fig. 2) (Shang et al. 2010; Wade et al. 2009). These findings, together with the discovery of human neocentromeres, indicate that tandem-repetitive DNA is not essential for kinetochore formation. The position of neocentromeres

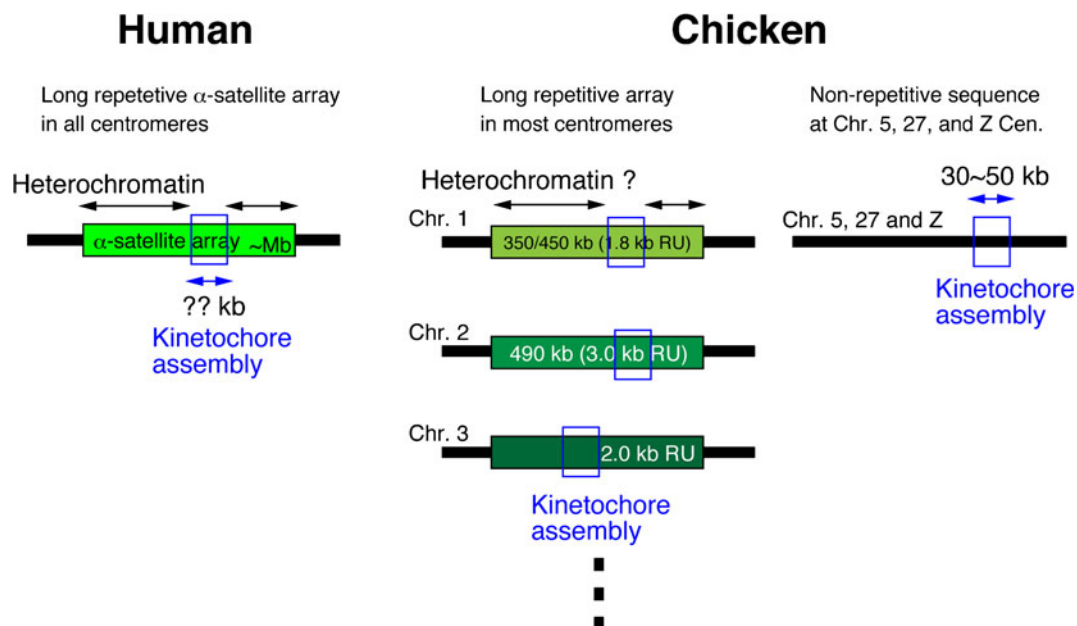


Fig. 2 Organization of centromeric DNA in human and chicken cells. Human centromeres typically contain large tandem-repeated alpha-satellite sequences. Such large repeated sequences may not be essential for centromere formation but may be responsible for the generation of a robust centromere, which contains heterochromatin near the core centromere region. It is hard to determine the exact size of the core region of centromeres on which kinetochores are formed. Whereas most chicken centromeres possess large

tandem-repeated sequences analogous to those in humans, three chromosomes (5, 27, and Z) possess non-tandem-repeated sequences as centromeric DNA. The sizes of non-repetitive centromeres of chickens occupied by CENP-A are 30–50 kb long. As the amount of CCAN proteins on non-repetitive centromeres is similar to that on centromeres with repetitive arrays (Johnston et al. 2010), the core region for centromeres may also be as little as 30–50 kb in centromeres with long repetitive arrays

and native centromeres without repetitive sequences is inherited by the next generation, suggesting that the kinetochore is specified by sequence-independent epigenetic mechanisms. Many previous studies have supported this idea and centromere-specific histone H3 variant CENP-A serves as an epigenetic mark for kinetochore specification (Black and Cleveland 2011; Vafa and Sullivan 1997; Warburton et al. 1997). However, considering the fact that most chromosomes usually have a centromere with a repetitive DNA sequence in vertebrate cells, we would like to propose that centromeres with non-repetitive sequences may be immature centromeres that are created by centromere repositioning, and that tandem-repetitive sequences were subsequently added during the process of evolution to form stable centromeres (Shang et al. 2010).

CCAN serves as a core structure on centromeric chromatin

As centromeres are specified by sequence-independent epigenetic mechanisms, it is essential to understand how centromeric chromatin is established and how centromeric chromatin is linked to spindle microtubules. To address these questions, it is critical to identify a complete list of kinetochore components and characterize them. Initially, three kinetochore components were identified from sera of patients with autoimmune diseases and referred to as CENP-A, -B, and -C (Earnshaw and Rothfield 1985; Moroi et al. 1980). CENP-A is a centromere-specific histone H3 variant (Palmer et al. 1987) and is incorporated into centromeric DNA as a nucleosome (Tachiwana et al. 2011), which is an important epigenetic mark for kinetochore specification (Black and Cleveland 2011). Analyses of dicentric chromosomes have revealed that CENP-A is incorporated only at active centromeres, but not at inactive ones, which supports the idea that CENP-A provides an epigenetic marker for kinetochore specification. CENP-B is a DNA-binding protein that specifically recognizes the 17-bp sequence called a CENP-B box in alpha-satellite DNA (Masumoto et al. 1989). CENP-B is not essential for kinetochore formation. However, CENP-B enhances the efficient de novo formation of human artificial chromosomes through binding to the CENP-B box, suggesting that CENP-B binding to DNA may play some role in establishing the kinetochore (Okada et al. 2007). In addition to CENP-A and CENP-B, CENP-

C is also a DNA-binding protein (Saitoh et al. 1992; Yang et al. 1996) and, therefore, these three proteins should contribute to the establishment of centromeric chromatin. Importantly, CENP-A and CENP-C are found only at active centromeres, whereas CENP-B is also found at inactive centromeres on stable dicentric chromosomes (Earnshaw et al. 1989; Sullivan and Schwartz 1995). Following the identification of CENP-A, -B, and -C, CENP-H and -I were then identified as proteins that localize to kinetochores throughout the cell cycle (Fukagawa et al. 2001; Nishihashi et al. 2002; Sugata et al. 1999).

Since the identification of CENP-A, -B, -C, -H, and -I, three laboratories have independently performed a proteomic approach and identified an additional 13 proteins that are associated with centromeric chromatin in both chicken and human cells (Perpelescu and Fukagawa 2011; Takeuchi and Fukagawa 2012). These proteins are referred to as CCAN (Fig. 1). Based on gene knock-outs in chicken DT40 cells and knockdown experiments with RNAi in human cells, the CENP-A-containing nucleosome stands the furthest upstream in the localization hierarchy, because CENP-A disruption causes mislocalization of all CCAN proteins (Liu et al. 2006; Regnier et al. 2005). Based on biochemical, functional, and structural analyses, CCAN proteins are divided into at least four sub-complexes: CENP-C, CENP-T-W-S-X, CENP-H-I-K-L-M-N, and CENP-O-P-Q-R-U (Fig. 3) (Cheeseman and Desai 2008; Hori et al. 2008a; 2008b; Nishino et al. 2012; Okada et al. 2006).

CENP-C is a kinetochore-specific protein with DNA binding activity (Saitoh et al. 1992; Yang et al. 1996). As CENP-C binds to DNA, it is possible that CENP-C associates with CENP-A-containing nucleosomes. However, ChIP experiments revealed that CENP-C strongly associates with canonical histone H3-containing nucleosomes when chromatin is completely digested with micrococcal nuclease (MNase) into mono-nucleosomes (Ando et al. 2002; Hori et al. 2008a; Obuse et al. 2004b). When polynucleosomes with partial MNase digestion were used, CENP-A was co-precipitated with CENP-C, suggesting that CENP-C binds to H3-containing nucleosome adjacent to CENP-A-containing nucleosomes. On the other hand, in vitro biochemical experiments demonstrated that the six amino acids on the C terminus of CENP-A interact with the middle region of CENP-C, and these residues are essential for kinetochore assembly in *Xenopus* egg extracts (Carroll et al. 2010; Guse et al. 2011). Considering these results, we

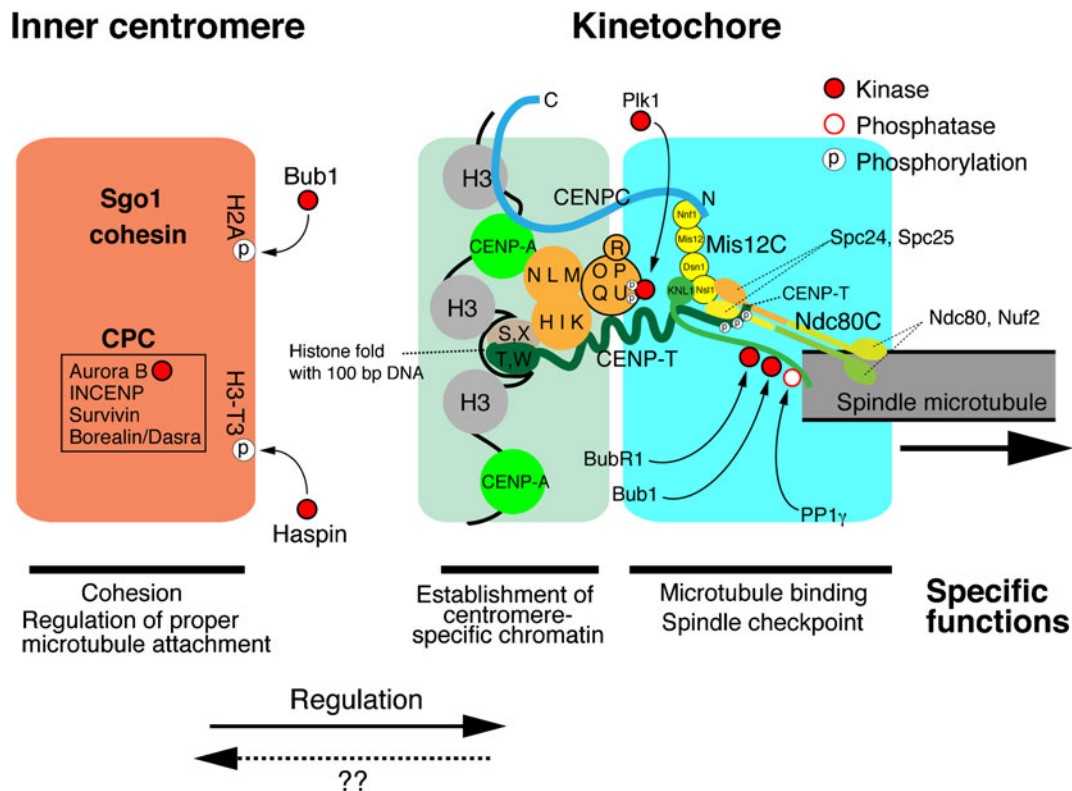


Fig. 3 Molecular organization of centromere and kinetochore in vertebrate cells. The inner centromere structure underlies the kinetochore. The inner centromere is responsible for the centromeric cohesion between sister chromatids established by cohesion complex, Shugoshin (Sgo1) and the chromosome passenger complex (CPC). Localization of both Sgo1 and CPC is related with phosphorylation of H2A by Bub1 kinase and histone H3 T3 by haspin kinase, respectively (Kelly et al. 2010; Wang et al. 2010; 2011; Yamagishi et al. 2010). CENP-A-containing nucleosomes function as epigenetic marks for kinetochore specification. In addition to CENP-A, CENP-C, and the CENP-T-W-S-X complex, other

suggest that CENP-C interacts with CENP-A, leading to kinetochore assembly, but also binds adjacent the H3-containing nucleosomes to establish strong contacts with centromeric chromatin. Ribeiro et al. (2010) performed a kinetochore unfolding assay and demonstrated that CENP-C is required for the structural integrity of mitotic kinetochore chromatin. Disruption of CENP-C causes a strong reduction in kinetochore localization of the Mis12 complex, which associates with the microtubule-binding Ndc80 complex (Kwon et al. 2007; Liu et al. 2006; Milks et al. 2009). Biochemical experiments demonstrated that a conserved N-terminal region of CENP-C binds directly to the Mis12 complex and this interaction appears to be stoichiometric (Screpanti et al. 2011). In addition, a GST pull-down experiment revealed that the

CCAN components are involved in the establishment of centromeric chromatin. Both CENP-C and CENP-T connect the chromatin to the KMN components. Phosphorylation of the CENP-TN terminus is essential for its stable interaction with the Ndc80 complex that directly binds spindle microtubules. Nsl1 of the Mis12 complex and KNL1 also contribute to stabilizing the interaction of the Ndc80 complex with the inner kinetochore. Several kinases and phosphatases, including BubR1, Bub1, Plk1, and PP1γ, are recruited to kinetochores. Phosphorylation of kinetochore proteins is involved in the spindle-checkpoint pathway or the establishment of proper kinetochore microtubule attachments

CENP-C N terminus binds Nnf1, a subunit of the Mis12 complex (Przewlaka et al. 2011). Furthermore, ectopic localization of the CENP-C N terminus into the centrosomes induced an accumulation of KMN on centrosomes in *Drosophila* cells (Przewlaka et al. 2011). Based on this evidence, we propose that CENP-C is a strong candidate for a linker molecule between centromeric chromatin and the microtubule binding components of the outer kinetochore.

The CENP-T-W-S-X complex is also a DNA-binding complex at kinetochores. CENP-T and CENP-S were originally identified by mass spectrometry analysis in CENP-A-containing polynucleosomes (Foltz et al. 2006). Following identification of these proteins, we identified CENP-W as a CENP-T-associated protein

(Hori et al. 2008a) and CENP-X as a CENP-S-associated protein in both human and chicken cells (Amano et al. 2009). Both CENP-T and CENP-W possess histone-fold domains, and these two proteins form a tight complex that shows DNA binding activity. To test whether the CENP-T-W complex directly binds to the CENP-A-containing nucleosome, we performed ChIP experiments and detected only histone H3 with the mononucleosomal chromatin fraction associated with CENP-T-W but detected both H3 and CENP-A with the polynucleosomal chromatin fraction. This result suggests that the CENP-T-W complex associates with canonical H3-containing nucleosomes, but not with CENP-A-containing nucleosomes (Hori et al. 2008a). Observation of centromeric chromatin by a super-resolution microscope supports this conclusion (Ribeiro et al. 2010).

Both CENP-S and CENP-X also have histone-fold domains and form a tight complex similar to the CENP-T-W complex (Nishino et al. 2012). These two complexes possess similar structural features and CENP-T or -W was detected as a CENP-S-interacting protein based on high-sensitivity mass spectrometry analysis (Amano et al. 2009). Nevertheless, the phenotype of CENP-T-W depletion is different from that of the CENP-S-X depletion. Whereas CENP-T-W depletion results in severe mitotic defects, the phenotype of CENP-S-X depletion is relatively mild (Amano et al. 2009). Thus, the relationship between these two complexes was unclear. However, our structural analysis on these two complexes has clearly shown that CENP-T, -W, -S, and -X form a heterotetramer that exhibits DNA supercoiling activity. We proposed that this complex forms a nucleosome-like structure (Nishino et al. 2012). Based on the structural analysis of the CENP-T-W-S-X complex, we identified amino acid residues responsible for the tetramer formation of CENP-T-W-S-X and examined the significance of this tetramer formation *in vitro* and *in vivo*. We created mutant CENP-T and CENP-S proteins that do not form a heterotetramer *in vitro* and used them to replace wild-type CENP-T or CENP-S with mutant CENP-T or CENP-S, respectively, in DT40 cells. We found that kinetochore localization of CENP-T or CENP-S was reduced and a functional kinetochore was not formed in these cells (Nishino et al. 2012). These data suggest that tetramer formation of the complex is essential for the establishment of centromeric chromatin to form a functional kinetochore.

CENP-T is a ~600-amino-acid protein whose C-terminal 100-amino-acid region contains a histone-

fold domain. The 500-amino-acid N-terminal region is predicted to be an unstructured domain whose exact function is unclear. We demonstrated that the extreme N-terminal 100-amino-acid region of CENP-T is essential for the localization of outer kinetochore proteins including the Ndc80 complex (Gascoigne et al. 2011). In addition, biochemical experiments demonstrated that the recombinant human CENP-T-W complex associated with an engineered Ndc80^{Bonsai} complex (Gascoigne et al. 2011). These data suggest that CENP-T connects centromeric chromatin with outer kinetochores, as CENP-C does. As CENP-T localization is distinct from CENP-C, we proposed that there are two parallel pathways to connect centromeric chromatin with outer kinetochores (Hori et al. 2008a). Based on EM observations, we found that the distribution of centromeric chromatin is deformed by the tension from spindle microtubules during mitosis (Suzuki et al. 2011). Our idea is that the unstructured N-terminal 500-amino-acid region of CENP-T is responsible for this structural deformation of centromeric chromatin. We demonstrated that the N-terminal CENP-T region is flexible, based on high-speed atomic-force microscopy (AFM) analysis, and that CENP-T was stretched under tension in cells expressing GFP (N-terminus) and RFP (C-terminus) double-fused CENP-T. These results support our idea that a flexible CENP-T region is responsible for kinetochore stretching caused by tension from spindle microtubules (Suzuki et al. 2011).

CENP-H, -I, -K, -L, -M, and -N proteins were purified from CENP-A-containing polynucleosomes in human HeLa cells (Foltz et al. 2006; Izuta et al. 2006; Obuse et al. 2004b) or identified as CENP-H- and CENP-I-associated proteins in chicken DT40 cells (Okada et al. 2006). DT40 knockout cells for these proteins showed strong mitotic defects. As we observed that kinetochore localization of these six proteins is interdependent, we classified these proteins as a functional group. However, it is possible that this group can be divided into subgroups. CENP-H was originally identified as a constitutive centromeric coiled-coil protein from mouse and human cells (Sugata et al. 1999). CENP-I was identified as a vertebrate homologue of fission yeast Mis6 protein (Nishihashi et al. 2002; Saitoh et al. 1997). CENP-K was reported as Solt, which is a binding partner of transcriptional regulator SoxLZ/Sox6 (Yamashita et al. 2000). CENP-M was also reported as proliferation-associated nuclear element 1 (PANE1), which is highly expressed in proliferating cells such as

activated lymphoid cells and tumors (Bierie et al. 2004; Renou et al. 2003). Although CENP-K or -M was originally identified in the context of transcription or cell proliferation, the relationship between kinetochore function and these biological events remains unclear.

Kinetochore localization of CENP-H group proteins is dependent on CENP-A, indicating that localization of these proteins into kinetochores occurs downstream of CENP-A (Okada et al. 2006; Regnier et al. 2005). Whereas CENP-A localization at the kinetochore is not altered in CENP-H-deficient cells, incorporation of newly synthesized GFP-CENP-A into kinetochores is impaired in CENP-H-, -I-, -K- and CENP-M-deficient cells (Okada et al. 2006). This suggests that the CENP-H group proteins are required for optimal deposition of newly synthesized CENP-A into centromeres. We proposed that the chromatin-remodeling factor FACT (facilitates chromatin transcription) might facilitate CENP-A deposition coordinately with CENP-H group proteins (Okada et al. 2009). We demonstrated that kinetochore localization of FACT was dependent on CENP-H group proteins and CENP-A incorporation was abolished in FACT-deficient cells.

Carroll et al. (2009) demonstrated that CENP-N could directly bind CENP-A-containing nucleosomes in vitro. They also showed that the C-terminal region of CENP-N interacts with CENP-L. This interaction may facilitate kinetochore assembly. In addition, the CENP-H group proteins may be required for spindle-checkpoint function, as Mad1 or Mad2 kinetochore localization is reduced in cells depleted for CENP-I (Liu et al. 2003; Matson et al. 2012). Furthermore, Amaro et al. (2010) demonstrated that the CENP-H group proteins were involved in molecular control of kinetochore-microtubule dynamics and chromosome oscillations. Although there have been some studies on the functional roles of the CENP-H group in kinetochores, the precise function of these proteins is still unclear. Whereas kinetochore localization of the CENP-H group occurs downstream of CENP-T, CENP-T signals are also reduced in CENP-H-deficient cells, suggesting that the CENP-H group is also involved in the formation of centromeric chromatin.

CENP-O, -P, -Q, -R, and -U are not essential for the growth of DT40 cells because DT40 knockout cells

for these proteins are viable (Hori et al. 2008b). CENP-U (50) was originally reported as MLF1IP or KLIP because this was isolated in the context of the development of erythroleukemias or transcriptional regulation (Hanissian et al. 2004; Pan et al. 2003). We identified this protein as an MgcRac-GAP-interacting protein and referred to it as CENP-50, as this 50-kDa protein localizes to kinetochores throughout the cell cycle in chicken DT40 cells (Minoshima et al. 2005). After that, this was also referred as CENP-U (Foltz et al. 2006; Okada et al. 2006). DT40 cells with a depletion of CENP-U (50) are viable but show a slight mitotic delay. Whereas kinetochore localization of CENP-U (50) depends on CENP-H or CENP-I, these proteins clearly localize to kinetochores in cells with CENP-U (50) depletion, suggesting that localization of CENP-U (50) occurs downstream of CENP-H group proteins. Based on iFRAP (inverse fluorescence recovery after photobleaching) experiments, CENP-U (50) stably associates with centromeres, like CENP-C and CENP-H (Minoshima et al. 2005). A proteomic approach identified CENP-O, -P, -Q, -R, and -U (50) in CENP-A-associated polynucleosomes from human cells (Foltz et al. 2006; Izuta et al. 2006) or CENP-H-I-associated proteins from chicken DT40 cells (Okada et al. 2006). As the phenotype of DT40 cells with depletion of each knockout cell line (CENP-O-, -P, -Q, -R, and -U-knockout) is similar, we classified these proteins as a functional subgroup (Hori et al. 2008b). We also performed co-expression of these five proteins simultaneously in *Escherichia coli* and demonstrated that these proteins form a stable complex (Hori et al. 2008b). Localization of CENP-O, -P, -Q, and -U is interdependent and CENP-R localization occurs downstream of these four proteins, suggesting that CENP-O, -P, -Q, and -U form a tight complex and that CENP-R associates with this complex (Hori et al. 2008b).

To uncover the roles of CENP-O complex proteins in mitosis, we examined mitotic progression following release from nocodazole block of cells depleted for CENP-O complex proteins. Interestingly, when CENP-U (50)-deficient cells are treated with nocodazole and the drug is subsequently washed out, these cells are arrested at prometaphase by activation of the spindle-assembly checkpoint, suggesting that CENP-O complex proteins are essential for recovery from spindle damage (Hori et al. 2008b; Minoshima et al. 2005). Kang et al. (2006) showed that human CENP-U (50) (they referred this protein as PBIP1) is phosphorylated at S77 and S78, and phosphorylation at these sites is

essential for localization of polo-like kinase 1 (Plk1) in kinetochores. We confirmed that mutation of the corresponding residues in chicken CENP-U (50) (S62 and T63) caused a reduction of centromere localization of Plk1, particularly in the G2 phase (Hori et al. 2008b). In addition, cells in which expression of CENP-U (50) is replaced with that of the CENP-U (50) mutant show defects in recovering from spindle damage similar to the CENP-U (50) knockout cells, suggesting that phosphorylation of CENP-U (50) and Plk1 are involved in CENP-U (50) function during the recovery from spindle damage. Although the recruitment of Plk1 to kinetochores requires the CENP-O complex in the G2 phase, there are several pathways localizing Plk1 to kinetochores during prometaphase through BubR1 and INCENP (Elowe et al. 2007; Goto et al. 2006). We propose that one pathway may be dependent on phosphorylation of S62 and T63 of chicken CENP-U (50) in G2 or prometaphase. In this process, other CCAN proteins and/or CCAN-associated proteins might be phosphorylated particularly by Plk1 to establish proper kinetochores after spindle damage (Fig. 3) (Santamaria et al. 2011). Consistent with this hypothesis, Lenart et al. (2007) demonstrated that the number of microtubules attached to kinetochores is reduced in human cells treated with the Plk1 inhibitor BI2536, based on EM observation. In addition, phosphorylation of BubR1 by Plk1 contributes to stable kinetochore–microtubule interactions in human cells (Elowe et al. 2007).

The KMN functions as an interface with spindle microtubules

Once a centromere is specified by CENP-A and centromeric chromatin is established by the CCAN, the kinetochore must make robust interactions with spindle microtubules during mitosis to achieve accurate chromosome segregation. A key interface with spindle microtubules is the KMN network. KMN network components start to localize to kinetochores around the G2 phase and dissociate at the end of mitosis in vertebrate cells. The KMN network can be reconstituted using recombinant proteins and binds to tubulin in vitro (Cheeseman et al. 2006). Functional kinetochores during mitosis must possess multiple activities to undergo accurate chromosome segregation, including microtubule binding, spindle-assembly-checkpoint and sister chromatid cohesion in the inner centromere. As the

KMN network is essential for establishment of a functional kinetochore, components of the KMN network must possess critical functional properties (Fig. 3).

KNL1 was originally identified by an RNAi-based functional genomic screen in *Caenorhabditis elegans* (Desai et al. 2003) and later homologues were identified in other organisms (Cheeseman et al. 2004; Kerres et al. 2004; Nekrasov et al. 2003; Obuse et al. 2004a; Przewloka et al. 2007). KNL1 is a large protein (~300 kDa in human cells) and binds to other proteins at multiple KNL1-binding sites. KNL1 possesses microtubule-binding activity on its N-terminal basic residues (Cheeseman et al. 2006; Pagliuca et al. 2009). Analysis of *C. elegans* KNL1 suggests that microtubule binding by KNL1 is involved in spindle-checkpoint silencing (Espeut et al. 2012). However, microtubule binding by KNL1 may not contribute to a major pulling force for chromosome segregation.

The N-terminal region of KNL1 binds to protein phosphatase 1 gamma (PP1 γ) (Liu et al. 2010). PP1 γ dephosphorylates substrates of Aurora B kinase such as Ndc80, and dephosphorylated Ndc80 binds tightly to spindle microtubules (Liu et al. 2010). Kinase activity of Aurora B is required for error correction of kinetochore–microtubule attachments, as phosphorylated KMN proteins do not interact with microtubules (Cheeseman et al. 2006; Ciferri et al. 2008; DeLuca et al. 2006; Welburn et al. 2010). Thus, kinetochore substrates for Aurora B must be dephosphorylated to generate proper kinetochore–microtubule attachments, and PP1 γ –KNL1 contributes to this process (Liu et al. 2010).

KNL1 also interacts with spindle-checkpoint kinases Bub1 and BubR1 through its N-terminal KI motif (Fig. 3) (Bolanos-Garcia et al. 2011; Kiyomitsu et al. 2007; 2011; Krenn et al. 2012). Although depletion of KNL1 causes mislocalization of both Bub1 and BubR1 in human cells (Kiyomitsu et al. 2007), mutant Bub1, which does not bind to KNL1 still localizes to kinetochores. This suggests that KNL1 is a major receptor for such checkpoint proteins, but that these checkpoint proteins have multiple binding sites in kinetochores (Krenn et al. 2012). Bub1 was originally identified as a conserved spindle-checkpoint protein (Hoyt et al. 1991; Taylor and McKeon 1997). A recent study suggested that Bub1 is also involved in the phosphorylation of histone H2A in the centromere region (Fig. 3) (Kawashima et al. 2010). Phosphorylated histone H2A recruits Shugoshin (Sgo1) and Aurora B to the inner centromeres to establish sister-chromatid cohesion at

the centromere region (Fernius and Hardwick 2005; Kitajima et al. 2005; Perera et al. 2007; Tang et al. 2004; Vaur et al. 2005; Wang et al. 2011; Yamagishi et al. 2010). Thus, KNL1 may become involved in establishment of the inner centromere through its interactions with Bub1.

The C terminus of KNL1 binds to Zwint-1 of the RZZ complex or Nsl1 and Dsn1 of the Mis12 complex (Kiyomitsu et al. 2007; 2011; Petrovic et al. 2010). Thus, KNL1 interacts directly with the Mis12 complex. However, it is unclear whether KNL1 directly binds to the Ndc80 complex. Kinetochore localization of the Ndc80 complex is not completely abolished in KNL1-depleted human or chicken cells, but is completely lost by simultaneous depletion of KNL1 and CENP-K (Cheeseman et al. 2008), suggesting that Ndc80 localization depends on both KNL1-Mis12 and the CCAN. The next major challenge is to address how KNL1 and the CCAN coordinately contribute to the localization of the Ndc80 complex on kinetochores in vertebrate cells.

The Mis12 complex contains four proteins (Nnf1, Mis12, Dsn1, and Nsl1) and was identified using a proteomic approach (Cheeseman et al. 2004; Obuse et al. 2004a). Further biochemical analysis confirmed that these four proteins form a stable complex (Kline et al. 2006). The Mis12 complex acts as a hub to connect other KMN components with kinetochores. Recent structural and biochemical analyses showed that the four subunits of the Mis12 complex form a linear rod-shaped unit with the order: Nnf1, Mis12, Dsn1, and Nsl1. The Nnf1-side faces toward the inner kinetochore and Nnf1 directly interacts with CENP-C (Przewlaka et al. 2011). Nsl1 faces toward the outer kinetochore and directly binds to both the Ndc80 complex and KNL1 (Kiyomitsu et al. 2010; Petrovic et al. 2010). The PXVXL motif in the Nsl1 C terminus is responsible for interactions with Spc24-Spc25 of the Ndc80 complex (Petrovic et al. 2010). Interestingly, this PXVXL motif also binds to HP1 proteins. Kiyomitsu et al. (2010) proposed that the mitotic function of Nsl1 requires HP1–Nsl1 association during interphase. However, the biological significance of the proposed HP1–Nsl1 interaction is still unclear.

The Ndc80 complex contains Ndc80, Nuf2, Spc24, and Spc25 and is well conserved (DeLuca et al. 2005; Janke et al. 2001; Martin-Lluesma et al. 2002; McClelland et al. 2003; Hori et al. 2003; Wigge et al. 1998; Wigge and Kilmartin 2001). Structural analysis on the engineered Ndc80^{Bonsai} complex revealed that the Ndc80 complex has a dumbbell-like shape with a long coiled-

coil structure and globular domains at both ends (Ciferri et al. 2008). The globular domains of Spc24 and Spc25 tightly interact with the Mis12 complex, and the Ndc80-Nuf2 side of this complex directly binds to the microtubule lattice (Ciferri et al. 2008; DeLuca et al. 2006; Wei et al. 2007). Microtubule interactions of the Ndc80 complex are electrostatic and is regulated by phosphorylation of the Ndc80 N terminus by Aurora B (Cheeseman et al. 2006; DeLuca et al. 2006; Welburn et al. 2010). However, the detail N terminus structure of Ndc80 is still unclear, because the N-terminal tail (1–79aa) is disordered (Ciferri et al. 2008). As the Ndc80 complex is a key factor in ensuring robust interaction of kinetochores with microtubules, major questions in this field include determining how the Ndc80 complex is recruited to a kinetochore and organizes the kinetochore structure, and how the Ndc80 complex regulates microtubule interaction for accurate chromosome segregation during mitosis and even anaphase. To address these questions, additional structural, biochemical, and cell biological analyses continue.

Creation of an engineered kinetochore

Following the accumulation of knowledge about kinetochore architecture (Fig. 3), artificial kinetochores may possibly be created to directly understand the molecular assembly of kinetochores. In fact, various groups have tried to create an artificial kinetochore by genetic engineering at a non-centromere region of a chromosome in recent years. This approach was first performed in budding yeast using plasmid DNA (Kiermaier et al. 2009; Lacefield et al. 2009). These experiments revealed that tethering of outer-kinetochore protein Dam1 is sufficient for generation of a functional kinetochore.

Following these experiments, a similar approach was taken in vertebrate or *Drosophila* cultured cells. In such cases, the bacterial LacO–LacI system has frequently been used. Firstly, a cell line in which an array of LacO (Lac operator) repeats is integrated into the non-centromere region of a chromosome is established. Then, a LacI (Lac repressor)-fused kinetochore protein is expressed in the cell line with the LacO array, and the LacI fusion protein is expected to be targeted to the LacO array at the non-centromere region. Finally, kinetochore formation in this region is characterized. As CENP-A is a candidate epigenetic marker for kinetochore specification, Barnhart et al. (2011) expressed HJURP, a CENP-A-specific histone chaperone fused to

LacI (Dunleavy et al. 2009; Foltz et al. 2009) in human U2OS cells with a long LacO array (~2 Mb) on the arm of chromosome 1. They observed that CENP-A was recruited to the LacO locus and kinetochore assembly was induced at the LacO locus in LacI-HJURP-expressing cells (Barnhart et al. 2011). Similarly, Mendiburo et al. (2011) tethered CENP-A-GFP-LacI to a non-centromere region in *Drosophila* S2 cells by the LacO–LacI system and found induction of kinetochore assembly at the non-centromere locus. In addition to formation of these engineered kinetochores, Guse et al. (2011) demonstrated that various kinetochore proteins in *Xenopus* egg extracts are assembled on CENP-A-containing polynucleosomes, assembled in vitro. They also demonstrated that kinetochore assembly did not occur on histone H3-containing polynucleosomes and concluded that six amino acids at the C terminus of CENP-A are essential for kinetochore assembly. These recent results in genetic engineering studies confirm earlier suggestions that CENP-A provides a mark for kinetochore assembly.

Although CENP-A may work as a mark for kinetochore assembly, it is still unclear which molecules serve as a structural core for kinetochore assembly. We postulate that CENP-T and CENP-C are candidates for this function, because both proteins connect centromeric chromatin located in the inner kinetochore with outer kinetochore proteins. To directly test this possibility, we targeted CENP-T or CENP-C to a LacO array at arm region in both human and chicken cells. Simultaneous tethering of CENP-T and CENP-C into the non-centromere region recruited most kinetochore proteins except for CENP-A at the non-centromere region in human cells (Gascoigne et al. 2011). In addition, we showed that targeting CENP-T-LacI to the LacO locus produced a degree of kinetochore function following removal of the endogenous centromere region using the Cre-loxP system (Gascoigne et al. 2011; Shang et al. 2010). This is the best evidence to date that the induced kinetochore-like structure partially rescued endogenous kinetochore function. As we did not detect CENP-A in the CENP-T- or CENP-C-induced kinetochores, the engineered kinetochore bypasses the requirement for CENP-A nucleosomes. This result is consistent with our model in which CENP-A provides a mark for kinetochore assembly and CENP-T and/or CENP-C functions as a structural core for kinetochore formation. Prendergast et al. (2011) reached a similar conclusion based on FRAP analysis of CENP-A and CENP-T.

Although we showed that an artificial kinetochore could be created on a non-centromeric chromosome arm region, a centromere is usually specified at a single locus of a chromosome and a kinetochore is formed on that centromere. In addition to CENP-A, other CCAN proteins must be involved in the establishment of centromere-specific chromatin, which apparently serves as an epigenetic mark for kinetochore specification. It is thus essential to better define the precise function of each CCAN component. An experimental system based on the engineered kinetochore could facilitate understanding of the functional roles of CCAN.

Conclusion

The kinetochore is an essential interface with microtubules from the mitotic spindle. Based on EM observations, the kinetochore was visualized as a tri-layered structure in 1960s, but it was unclear at that time which molecules were involved in the formation of such a structure. Recent studies have revealed that more than 100 proteins are localized to kinetochores and clarified the molecular organization of kinetochores (Fig. 3). This is excellent progress, and we are confident that continued studies will provide us with further knowledge of kinetochore structure and function. The ultimate goal of this field of research will be the creation of artificial functional kinetochores in vitro and their application in vivo. Such a goal was only a kind of dream 50 years ago, but we are now confident that this is achievable in the near future, based on the knowledge being accumulated in this field.

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