

The kinetochore and spindle checkpoint in vertebrate cells

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1. ABSTRACT

The centromere is a specialized region of eukaryotic chromosomes that is essential for faithful chromosome segregation during mitosis and meiosis. A kinetochore is assembled at the centromere of each chromatid of a replicated chromosome and forms a dynamic interface with microtubules of the mitotic spindle. In recent years, many kinetochore proteins have been identified in vertebrate cells. After identification of kinetochore components, the process of kinetochore assembly has been studied. Herein, recent advances in our understanding of the kinetochore and spindle checkpoint in vertebrate cells are reviewed. I also review our recent contributions to this field and discuss their implications using chicken DT40 system.

2. INTRODUCTION

Faithful chromosome segregation during mitosis is essential for accurate transmission of genetic material. For accurate chromosome segregation, centromere functions, including sister chromatid adhesion and separation, microtubule attachment, chromosome movement, establishment of heterochromatin, and mitotic checkpoint control are necessary. Because centromere function is important for chromosome segregation, the centromere has been widely studied and reviewed (1-10). A kinetochore is assembled from multiple protein complexes at the centromere of each chromatid of a replicated chromosome. The kinetochore is the structure responsible for microtubule attachment and chromosome movement. The mechanisms that underlie kinetochore assembly have

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been studied extensively in budding yeast; more than 60 kinetochore proteins have been identified, and the protein-protein interactions have been characterized (11-13). Although the basic principles of chromosome segregation are thought to be common to all eukaryotes, it has been difficult to identify vertebrate kinetochore proteins by sequence homology with budding yeast kinetochore proteins. To understand the molecular mechanism of kinetochore assembly in vertebrate cells, components of vertebrate kinetochores must be identified. In the past decade, mass spectrometry-based proteomics approaches have been used to identify additional kinetochore proteins in vertebrate cells (14-18). After identification of kinetochore components, several strategies, including RNA interference (RNAi) in cultured cells, knockout mice, and immunodepletion have been used to study the process of kinetochore assembly (6, 7).

We have used a conditional knockout approach in the chicken B lymphocyte cell line DT40 to study the process of kinetochore assembly and functions of spindle checkpoint proteins (6, 7). The high level of homologous recombination in DT40 cells allows efficient targeted disruption of genes of interest. Several conditional knockout systems with DT40 cells have been established (19), and structures such as the mitotic spindle, centrosome, and centromere in DT40 cells are much larger and more elaborate than the analogous structures in yeast. In addition, the stages of mitosis (prometaphase chromosome congression, metaphase, and transition to anaphase) in DT40 cells are easily distinguished under a light microscope. Therefore, we believe that the DT40 conditional knockout approach is a powerful and reliable tool for studies of the kinetochore and spindle checkpoint. Here, I review our recent contributions to this field and discuss their implications.

3. DNA SEQUENCES FOR KINETOCHORE ASSEMBLY IN VERTEBRATE CELLS

The mechanisms involved in the selection of a specific chromosomal site where the kinetochore structure is formed are not fully understood. Studies of centromere DNAs of many species revealed that the primary DNA sequences are not conserved. There are active discussions regarding the possibility that specific DNA sequences are involved in recruitment of centromere proteins to mediate kinetochore assembly (4,5). The centromere DNA of the budding yeast *Saccharomyces cerevisiae* consists of a 125-bp sequence that specifies the site of kinetochore assembly. However, in other organisms, there does not appear to be a specific DNA sequence required for kinetochore assembly. Human centromere DNA consists of highly repetitive tandem sequence repeats of a 171-bp alpha-satellite DNA sequence (5). The alpha-satellite DNA sequence contains a 17-bp motif called the CENP-B box that binds directly to the centromere protein CENP-B (20). Human artificial chromosomes (HACs) can be created efficiently by *de novo* kinetochore assembly with the CENP-B box-containing alpha-satellite tandem repeat DNA (21-23). However, CENP-B knockout mice are viable without an adverse effect on chromosome segregation (24). The available data

concerning the role of primary DNA sequences in kinetochore formation in vertebrate cells indicate that satellite DNA sequences are preferred substrates for kinetochore formation; however, kinetochore formation can occur without satellite DNA. In fact, a "neocentromere," which functions as a normal centromere but does not contain alpha-satellite DNA, has been identified in human cells (3). After first identification of chromosome with a neocentromere, many examples of chromosomes with neocentromere have now been described, several of which have been characterized in detail (3). Further characterization of neocentromeres and satellite sequences is needed to understand involvements of DNA sequences for kinetochore formation.

4. CENP-A AS AN EPIGENETIC MARKER FOR KINETOCHORE SPECIFICATION

Despite variations in the primary centromere DNA sequences, all centromere sequences, including neocentromeres incorporate CENP-A, a 17-kDa centromere-specific histone H3 variant, suggesting that incorporation of CENP-A is an evolutionarily conserved process (25-30). This process is thought to be essential for specification of the site of kinetochore assembly. Our analysis of DT40 cells with a knockout of CENP-A showed that kinetochore localization of all tested constitutive centromere components is dependent on CENP-A, consistent with the idea that CENP-A is the primary candidate for an epigenetic marker of kinetochore specification (30). Similar results with RNAi experiments in human cells have been reported (28, 31). Although Goshima *et al.* (28) originally reported that hMis12 localizes to kinetochores in a manner partially independent on CENP-A, Liu *et al.* (31) reported that kinetochore localization of hMis12 is dependent on CENP-A like localization of constitutive centromere components. CENP-A is found only at active centromeres (32) and co-purifies with nucleosomes (33). In fact, regions of canonical histone H3 nucleosomes and CENP-A-containing nucleosomes are interspersed in human centromeric chromatin (34). Protein structure studies revealed that CENP-A contains a short CENP-A-targeting domain (CATD) within the histone-fold region (35, 36). Interestingly, ordinary histone H3 with the CATD can target to the centromere and function as a centromere specific histone (35, 36).

Although CENP-A itself may be able to target to the centromere region, several factors appear to be involved in CENP-A deposition in human cells (29, 37, 38). Hayashi *et al.* (29) identified Mis16 and Mis18, which mutated when cause defects in CENP-A deposition in fission yeast. They also identified vertebrate homologues of these proteins. The human homologue of Mis16 is known as RbAP46/48; it is a histone chaperone and is involved in chromatin assembly (39). RNAi analysis of RbAP46/48 in human cells revealed that CENP-A is not incorporated into centromeres under conditions of knockdown of RbAP46/48 expression. There are two isoforms (Mis18 α and Mis18 β) of the human homologue of Mis18. Mis18 α and Mis18 β form a complex with M18BP1 (Mis18-binding protein 1),

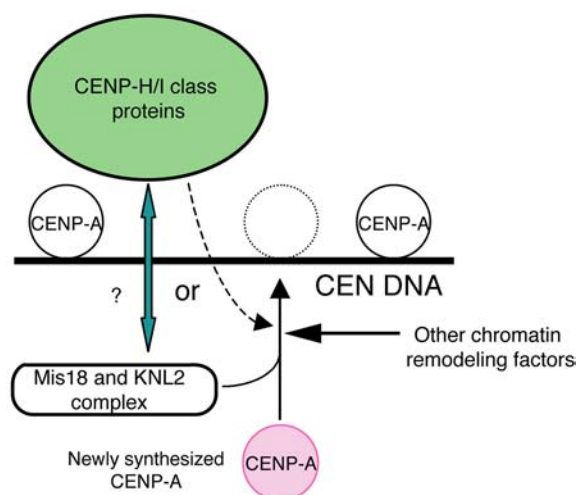


Figure 1. Proposed mechanism for the loading of newly synthesized CENP-A into centromeres. After chromosome replication, half of the existing CENP-A nucleosomes remain at the centromere of each newly replicated sister chromatid together with associated CENP-H/I complex. For nascent CENP-A deposition, the CENP-H/I complex recruits putative CENP-A-loading factors, such as Mis18 and KNL2, and facilitates targeting of CENP-A into the centromeric chromatin. Thus, the CENP-H/I complex may function as a marker for CENP-A incorporation. It is also possible that the CENP-H/I complex itself functions as a CENP-A-loading factor.

which contains a Myb/SANT domain (37). Maddox *et al.* (38) independently found that depletion of KNL2, which is the *C. elegans* homologue of M18BP1, causes defects in deposition of CENP-A into centromeres in *C. elegans*. Interestingly, KNL2/M18BP1 localizes transiently to kinetochores during the telophase to early G1 phase period. This period corresponds to the time during which CENP-A deposition occurs (40).

The molecular mechanism of CENP-A deposition must be complicated. In addition to Mis16, Mis18, and KNL2, other proteins may be involved in CENP-A deposition. To identify additional molecules required for CENP-A deposition, CENP-A nucleosomes have been purified from human cells, and several proteins involved in chromatin remodeling, such as FACT, have been identified (14, 17, 41). It would be interesting to see if these chromatin-remodeling factors are involved in deposition of CENP-A. The purification also identified many constitutive centromere proteins, including CENP-C, CENP-H, and CENP-I and additional kinetochore components CENP-K, -L, -M, -N, -O, -P, -Q, -R, -S, -T, and -50 (U). At the same time, our group has independently purified a CENP-H/I complex that contains CENP-H, -I, -K, -L, -M, -N, -O, -P, -Q, -R, and -50 (U) (18). Because the characteristics of the CENP-H/I complex are described in the next section, I will focus here on the relation of this complex to CENP-A. CENP-A localization is not altered in cells with knockouts of several CENP-H/I complex proteins

(18). Because localization of CENP-H and CENP-I is abolished in CENP-A mutant cells (30), we believe that the CENP-H/I-associated complex functions downstream of CENP-A (Figure 1). Nevertheless, we observed that the rate of incorporation of newly synthesized CENP-A is reduced in cells with knockouts of CENP-H, -I, -K, and -M, indicating that some CENP-H/I complex proteins play important roles in the efficient incorporation of newly synthesized CENP-A into centromeres (18). In our model, members of the CENP-H/I complex act as a marker to direct deposition of newly synthesized CENP-A into centromeric chromatin during interphase or function to stabilize CENP-A (Figure 1). Because localization of CENP-H and CENP-I requires CENP-A, this mechanism in effect makes loading of new CENP-A dependent on pre-existing CENP-A nucleosomes, with the CENP-H/I complex acting as an intermediate. Whereas it is possible that a CENP-H/I-associated protein itself functions as a CENP-A-loading factor, CENP-H/I-associated proteins also play a distinct structural role at mitotic kinetochores and lack similarity to known nucleosome assembly factors. Consequently, we favor the idea that this constitutively centromere-localized complex either directly or indirectly recruits factors that load newly synthesized CENP-A into the kinetochores (Figure 1). A recent study of Chl4, a budding yeast homologue of CENP-N that is a member of the CENP-H/I complex, revealed that Chl4 is essential for *de novo* formation of centromeres but not for maintenance of established centromeres (42). In addition, work in fission yeast has suggested that Mis6, a fission yeast homologue of CENP-I, is involved in CENP-A deposition (43).

5. CENP-H/I COMPLEX IS A MEMBER OF THE CONSTITUTIVE CENTROMERE-ASSOCIATED NETWORK

After CENP-A is deposited into centromere chromatin, many proteins are targeted to the CENP-A chromatin to form a functional kinetochore. In vertebrate cells, constitutive centromere proteins, which were recently termed the constitutive centromere-associated network (CCAN), are associated with the CENP-A chromatin throughout the cell cycle (44). Additional proteins are recruited to the pre-kinetochore structure during late G2 or specific stages of mitosis. CENP-H and CENP-I are constitutive centromere proteins in vertebrate cells (45-48). As mentioned above, to identify additional kinetochore proteins, we performed co-purification with CENP-H or CENP-I in DT40 and HeLa cells. The purified CENP-H/I complex contains 11 constitutive centromere proteins, including CENP-H and CENP-I (18). We characterized these proteins by knockout analyses in DT40 cells. Nine subunits of the CENP-H/I complex can be currently divided into three classes based on phenotypes of knockout cells. The CENP-H class includes CENP-H, -I, and -K; the CENP-M class includes CENP-M; and the CENP-O class includes CENP-O, -P, -Q, -R, and -50 (U). The other two subunits CENP-L and CENP-N require further characterization. DT40 cells with knockouts of all of

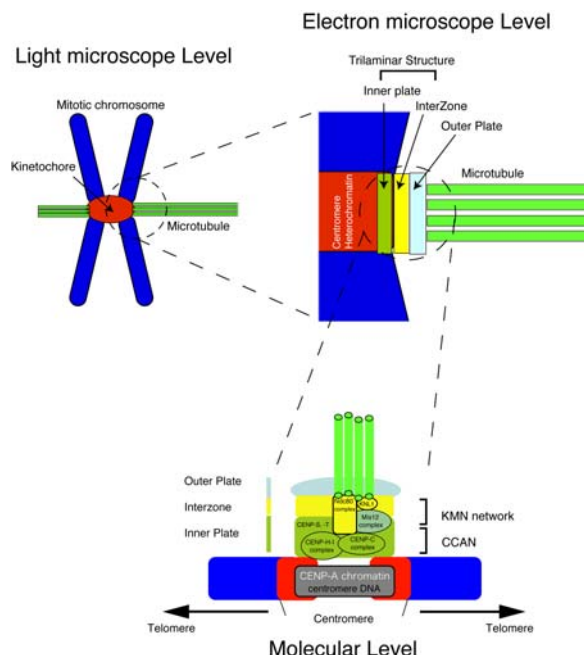


Figure 2. A chromosome is able to be observed by a light microscope. A trilaminar structure of kinetochore is able to be observed by an electron microscope. Molecular biological studies revealed structure of the kinetochore. Current model of kinetochore assembly in vertebrate cells is shown. In chicken DT40 cells, CCAN, which consists of the CENP-H/I complex, CENP-C, CENP-S, and CENP-T, associates with CENP-A throughout the cell cycle. KMN network, which is composed of the Mis12 complex, KNL1, and the Ndc80 complex is targeted to CCAN during late G2. Localization of Mis12 is dependent on that of CENP-C.

CENP-H-class proteins show severe mitotic defects, and kinetochore localization of all CENP-H/I complex proteins and many outer kinetochore proteins is abolished, suggesting that members of the CENP-H class form the core unit for kinetochore assembly (18).

6. DT40 CELLS WITH KNOCKOUTS OF CENP-O CLASS PROTEINS ARE VIABLE

Knockout DT40 cells of all of CENP-O-class proteins are viable and do not show mitotic defects as strong as those in cells deficient for CENP-H-class proteins. Depletion of CENP-O-class proteins does not lead to mislocalization of other kinetochore proteins. One clue for the function of the CENP-O-class proteins is that CENP-50 is essential for recovery from spindle damage (49). We also observed that CENP-50-deficient cells frequently show premature-sister chromatid separation under microtubule depolymerizing conditions. We propose that CENP-50 plays a backup role in sister chromatid adhesion during activation of the mitotic checkpoint pathway. We believe that this CENP-50 function occurs in concert with other CENP-O class proteins, because these proteins form a stable complex throughout the cell cycle (unpublished observation).

7. FUNCTION OF CENP-M CLASS PROTEINS

CENP-M is essential for viability in DT40 cells, and CENP-M-deficient cells show mitotic defects (18). However, the mitotic phenotype of CENP-M-deficient cells is not severe, when compared with that of CENP-H-class-deficient cells. Consistent with the knockout phenotype, our unpublished biochemical data suggest that the CENP-M class differs from the CENP-H and CENP-O classes. However, localization of CENP-M is dependent on both CENP-H and CENP-O class proteins. Although CENP-M still exists on kinetochores, intensities of kinetochore signals of CENP-M are reduced in cells with knockouts of CENP-O class proteins. The relationship of CENP-M to the other less-characterized proteins, CENP-L and CENP-N, is currently unclear. There may be additional components that associate with CENP-M. Further experiments are needed to clarify the structures and functions of CENP-M.

8. OTHER MEMBERS OF THE CCAN

There exist several constitutive centromere proteins including CENP-C, CENP-S, and CENP-T that we did not detect during purification of the CENP-H/I complex. CENP-C, a kinetochore component in higher vertebrate cells, was originally identified as an antigen for anti-centromere antibodies in patients with autoimmune diseases (50, 51). CENP-C localizes to the inner kinetochore plate adjacent to the centromeric DNA (52) and has DNA-binding activity (53). A conditional knockout of CENP-C in chicken DT40 cells (54) and disruption of the CENP-C gene in mice (55) revealed that CENP-C is essential for cell proliferation. Analysis of a conditional knockout of CENP-C in DT40 cells revealed that inactivation of CENP-C causes mitotic delay, chromosome missegregation, and apoptosis (54, 56, 57). CENP-C homologues have been identified from several species, including yeasts, nematode, and fly (27, 58-61). The *C. elegans* homologue of CENP-C, HCP4, is involved in sister kinetochore resolution (62). Recently, a *Drosophila* CENP-C homologue was isolated as a genetic interactant with Separase, which is essential for sister chromatid separation (60). However, the role of CENP-C in sister chromatid separation and resolution in higher vertebrate cells remains unclear. Our recent analysis of CENP-C-deficient DT40 cells showed that CENP-C is essential for localization of Mis12 complex proteins (see below) at centromeres (57) (Figure 2). Similar results were obtained with RNAi experiments in HeLa cells (31). We found that CENP-C signals are weak in interphase nuclei but not in mitotic chromosomes of cells with a knockout of CENP-K, a CENP-H-class protein. These results suggest that centromere localization of CENP-C in interphase nuclei occurs upstream of localization of the Mis12 complex and downstream of localization of the CENP-H-class proteins. However, CENP-C localization is not dependent on the presence of the CENP-H-class proteins at mitotic kinetochores (Figure 2).

CENP-S and CENP-T were identified as constitutive centromere proteins during purification of

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Table1. Vertebrate kinetochore proteins and their counterparts in the yeasts *S. cerevisiae* and *S. pombe*

Vertebrate proteins	Yeast Homologues	Comments
CENP-A	<i>Cse4p</i> (<i>S. cerevisiae</i>) <i>spCENP-A</i> as <i>Cnp1/Sim2</i> (<i>S. pombe</i>)	Centromere specific histone H3
CENP-B	? (<i>S. cerevisiae</i>) <i>Abp1</i> (<i>S. pombe</i>) <i>Cdh1</i> (<i>S. pombe</i>) <i>Cbh2</i> (<i>S. pombe</i>)	Heterochromatin protein
CENP-C	<i>Mif2p</i> (<i>S. cerevisiae</i>) <i>spCENP-C</i> as <i>Cnp3</i> (<i>S. pombe</i>)	CCAN
CENP-H	? (<i>S. cerevisiae</i>) <i>Fta3</i> ? (<i>S. pombe</i>)	CENP-H class of CENP-H/I complex in CCAN
CENP-I/hMis6	<i>Ctf3p</i> (<i>S. cerevisiae</i>) <i>Mis6</i> (<i>S. pombe</i>)	CENP-H class of CENP-H/I complex in CCAN
CENP-K/Solt	? (<i>S. cerevisiae</i>) <i>Sim4</i> ? (<i>S. pombe</i>)	CENP-H class of CENP-H/I complex in CCAN
CENP-L/dJ383J4.3	? (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CENP-H/I complex in CCAN
CENP-M/PANE-1	? (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CENP-M class in CCAN
CENP-N/BM039	<i>Chl4p</i> (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CENP-H/I complex in CCAN
CENP-O/MGC11266	<i>Mcm21p</i> (<i>S. cerevisiae</i>) <i>Mal2</i> (<i>S. pombe</i>)	CENP-O class in CCAN
CENP-P/LOC401541	? (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CENP-O class in CCAN
CENP-Q/FLJ10545	? (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CENP-O class in CCAN
CENP-R/EN3/ITGB3	? (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CENP-O class in CCAN
CENP-S	? (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CCAN
CENP-T	? (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CCAN
CENP-U (50)/KLIP1/MLF1IP	? (<i>S. cerevisiae</i>) ? (<i>S. pombe</i>)	CENP-O class in CCAN
hMis12	<i>Mtw1p</i> (<i>S. cerevisiae</i>) <i>Mis12</i> (<i>S. Pombe</i>)	Mis12 complex in KMN network
hNsl1	<i>Nsl1p</i> (<i>S. cerevisiae</i>) <i>Mis14</i> (<i>S. pombe</i>)	Mis12 complex in KMN network
hDsn1	<i>Dsn1p</i> (<i>S. cerevisiae</i>) <i>Mis13</i> (<i>S. Pombe</i>)	Mis12 complex in KMN network
hNnf1	<i>Nnf1p</i> (<i>S. cerevisiae</i>) <i>Nnf1</i> (<i>S. pombe</i>)	Mis12 complex in KMN network
hKNL1	<i>Spc105</i> (<i>S. cerevisiae</i>) <i>Spc7</i> (<i>S. Pombe</i>)	KMN network
hNdc80/Hec1	<i>Ndc80p</i> (<i>S. cerevisiae</i>) <i>Ndc80</i> (<i>S. Pombe</i>)	Ndc80 complex in KMN network
hNuf2	<i>Nuf2p</i> (<i>S. cerevisiae</i>) <i>Nuf2</i> (<i>S. Pombe</i>)	Ndc80 complex in KMN network
hSpc24	<i>Spc24p</i> (<i>S. cerevisiae</i>) <i>Spc24</i> (<i>S. Pombe</i>)	Ndc80 complex in KMN network
hSpc25	<i>Spc25p</i> (<i>S. cerevisiae</i>) <i>Spc 25</i> (<i>S. Pombe</i>)	Ndc80 complex in KMN network

ENP-A nucleosomes in human cells (14, 17, 41). Although these proteins were not detected during purification of the CENP-H/I complex from chicken DT40 cells or human HeLa cells, it is possible that these proteins are associated with the CENP-H/I complex. Further studies will clarify the functions of these proteins in kinetochore assembly.

9. KMN NETWORK PROTEINS ARE REQUIRED FOR THE KINETOCHORE-MICROTUBULE INTERACTION

During late G2 phase, several proteins are targeted to the CCAN. This step is essential for establishment of a functional kinetochore. The first player in this step is the Mis12 complex (15, 16, 28, 63). Mis12 was originally identified as a fission yeast centromere protein (64), but ordinary BLAST searches did not identify

homologous sequences in the genomes of higher vertebrates. hMis12, a human homologue of Mis12, was identified through a novel bioinformatics approach (28). Mis12-associated proteins were then identified from human HeLa cells through proteomics approaches (15, 16, 63). Further biochemical studies showed that hMis12 forms a stable complex (Mis12 complex) with hNnf1, hNsl1, and hDsn1 (Table 1) (63). The Mis12 complex is recruited to the constitutive centromere structure during late G2 phase, and kinetochore localization is dependent on inner kinetochore protein CENP-C (31, 57). Interestingly, the Mis12 complex co-purifies with outer kinetochore components Ndc80 complex, Zwint1, and KNL-1 (15, 16), suggesting that the Mis12 complex acts as an important connector between the outer and inner kinetochores.

Many studies of the Ndc80 complex have suggested that the complex, which contains Ndc80, Nuf2,

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Spc24, and Spc25, is important for the kinetochore-microtubule interaction (65-69). Recent studies indicated that one end of the complex binds directly to microtubules (70-72) and that the other end of the complex localizes more closely to the inner kinetochore, where the Ndc80 complex associates with the Mis12 complex and KNL-1. This network of KNL-1, the Mis12 complex, and the Ndc80 complex is critical for the kinetochore-microtubule interaction and is called the KMN network (70). Biochemical analyses revealed that the Ndc80 complex has weak microtubule binding affinity; however, when the Mis12 complex and KNL-1 associate with the Ndc80 complex, the microtubule-binding affinity of the Ndc80 complex increases, indicating that the KMN network is an essential part of the kinetochore for interaction with microtubules (70). We also made DT40 conditional-knockout cell lines for Mis12, Ndc80, Nuf2, and KNL1 (63, 67, 73). Phenotype analyses of these mutants indicated that Ndc80 is critical for the kinetochore-microtubule interaction; microtubules did not attach to the kinetochore properly in Ndc80- or Nuf2-knockout cells (67). Analyses of Mis12- or KNL1-knockout cells indicated that both proteins are linked closely with Ndc80. These findings are consistent with the biochemical data (63, 73). Taken together, the results of our analyses of DT40 knockouts support the idea that the KMN network is essential for the kinetochore-microtubule interaction (63, 67, 73). Biochemical analyses also suggested that phosphorylation of Ndc80 by Aurora B kinase is important for regulation of attachment of KMN to microtubules (70, 71). We can use DT40 cells with various mutations of Ndc80 to clarify the role of phosphorylation of Ndc80 in microtubule attachment. Such genetic experiments will be useful to confirm the biochemical data.

After attachment of kinetochores to microtubules, chromosomes must be moved and segregated to each daughter cell. For this process, several microtubule-binding proteins and motor proteins, including CLIP170, EB1, XMAP215, MCAK, kinesin, and dynein complex, are required. Although there are few reports regarding these proteins in the DT40 system, they have been characterized in human cells (44).

10. SPINDLE CHECKPOINT ACTIVATION IN DT40 CELLS WITH KNOCKOUTS OF VARIOUS KINETOCHORE COMPONENTS

In eukaryotic cells, chromosome segregation is regulated by a spindle checkpoint that prevents onset of anaphase until all chromosomes have aligned at the metaphase plate (74). The checkpoint proteins sense defects in kinetochore-microtubule attachment or microtubule tension. When the functions of inner kinetochore proteins are disrupted in DT40 cells, checkpoint proteins still localize to the kinetochores, and progression of the cell cycle is delayed during mitosis. We observed that mitotic checkpoint proteins, including members of the Mad and Bub families, localize to the outer kinetochore. These observations do not make sense, if the inner kinetochore structure is disrupted completely by knockout of inner kinetochore proteins, including CENP-H/I complex

proteins, CENP-A, and CENP-C. We actually observed attachment of kinetochores to microtubules in CENP-I-deficient cells (47). The fact that microtubule binding and mitotic checkpoint signaling proceed even when a number of components of the inner kinetochore plate are absent indicates that kinetochore assembly does not follow a simple linear pathway in vertebrates. In knockouts of inner kinetochore proteins or CCAN components, the checkpoint response is subsequently overcome, and cells proceed to the next cell cycle without normal chromosome segregation. Because checkpoint proteins, which first localized at kinetochores in cells with knockout of several CCAN proteins, finally detach from kinetochores during mitotic delay, we concluded that many CCAN inner kinetochore proteins are involved in maintenance of the mitotic checkpoint. For checkpoint proteins to function, the CCAN must provide signals to them. Transmission of such signals may require phosphorylation of CCAN components, and we have observed such phosphorylation of several CCAN components in response to treatment of cells with nocodazole, a spindle poison in DT40 cells (our unpublished data).

11. CONCLUSION

We and other groups have identified new centromere proteins in recent years, and studies of the molecular mechanisms of proper kinetochore assembly in vertebrate cells are presently underway. In addition, RNA molecules may be involved in kinetochore assembly and function in vertebrate cells (75, 76). We must clarify the mechanisms that determine when and how a kinetochore assembly site is chosen and transmitted. It is likely that there are common principles by which centromeres function in all eukaryotes. As mentioned above, the chicken DT40 cell line is a powerful tool for studies of kinetochore assembly. At present, the RNAi knockdown system is being used widely to examine cellular functions in cultured vertebrate cells. However, there are several problems with the RNAi technique, including incomplete knockdown or non-specific knockdown of expression. In contrast, phenotype analyses of DT40 cells are relatively accurate. Because the entire chicken genome has been sequenced, it is easy to generate mutants and to conduct genome-wide studies. We are confident that the common principles by which centromeres function in all eukaryotic cells will be clarified by studies of DT40 cells. We predict that approximately 100 proteins are localized at the kinetochore. After identification and characterization of many kinetochore proteins in DT40 system, it will be necessary to reconstitute the kinetochore structure *in vitro* to understand kinetochore assembly. It will also be necessary to show kinetochore movement with purified kinetochore proteins and microtubules *in vitro*. A combination of genetics studies and biochemical reconstitution studies is needed to complete our understanding of kinetochore structure and function.

In addition to understanding the fundamental mechanisms of kinetochore assembly, we must understand the relation of kinetochores to cancer, which may be caused by chromosome instability. A point mutation of BubR1,

which is essential for mitotic checkpoint control, was found in patients with colon cancer (77). CENP-A and CENP-H are expressed at high levels in some types of cancer cells (78, 79). These reports suggest that chromosome instability due to a defect in centromere function is related directly to cancer. Therefore, further knowledge of the mechanism of kinetochore assembly may increase our understanding of the processes involved in carcinogenesis.

12. ACKNOWLEDGMENTS

I thank members of the Fukagawa laboratory for discussion and comments. I also thank Iain Cheeseman for useful discussion about kinetochore assembly. Experiments in the Fukagawa laboratory are supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (MEXT).

13. REFERENCES

- Pluta, A.F., Mackay, A.M., Ainsztein, A.M., Goldberg, I.G., and Earnshaw, W.C. The centromere: hub of chromosomal activities. *Science*, 270, 1591-1594 (1995)
- Craig, J.M., Earnshaw, W.C., and Vagnarelli, P. Mammalian centromeres: DNA sequence, protein composition, and role in cell cycle progression. *Exp. Cell Res.*, 246, 249-262 (1999)
- Choo, K.H. Domain organization at centromere and neocentromere. *Dev. Cell*, 1, 165-177 (2001)
- Mellone, B.G., and Allshire, R.C. Stretching it: putting the CEN(P-A) in centromere. *Curr. Opin. Cell Biol.*, 13, 191-198 (2003)
- Cleveland, D.W., Mao, Y., and Sullivan, K.F. Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell*, 112, 407-421 (2003)
- Fukagawa, T. Assembly of kinetochores in vertebrate cells. *Exp. Cell Res.*, 296, 21-27 (2004)
- Fukagawa, T. Centromere DNA, proteins and kinetochore assembly in vertebrate cells. *Chromosome Res.*, 12, 557-567 (2004)
- Chan, G.K., Liu, S.T., and Yen, T.J. Kinetochore structure and function. *Trends Cell Biol.*, 15, 589-598 (2005)
- Kline-Smith, S.L., Sandall, S., and Desai, A. Kinetochore-spindle microtubule interactions during mitosis. *Curr. Opin. Cell Biol.*, 17, 35-46 (2005)
- Carroll, C.W., and Straight, A.F. Centromere formation: from epigenetics to self-assembly. *Trends Cell Biol.*, 16, 70-78 (2006)
- Cheeseman, I.M., Anderson, S., Jwa, M., Green, E.M., Kang, J., Yates, J.R. III, Chan, C.S., Drubin, D.G., and Barnes, G. Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell*, 111, 163-172 (2002)
- De Wulf, P., McAinsh, A.D., and Sorger, P.K. Hierarchical assembly of the budding yeast kinetochore from multiple subcomplexes. *Genes Dev.*, 17, 2902-2921 (2003)
- Westermann, S., Cheeseman, I.M., Anderson, S., Yates, J.R. III, Drubin, D.G., and Barnes, G. Architecture of the budding yeast kinetochore reveals a conserved molecular core. *J. Cell Biol.*, 163, 215-222 (2003)
- Obuse, C., Yang, H., Nozaki, N., Goto, S., Okazaki, T., and Yoda, K. Proteomics analysis of the centromere complex from HeLa interphase cells: UV-damaged DNA binding protein 1 (DDB-1) is a component of the CEN-complex, while BMI-1 is transiently co-localized with the centromeric region in interphase. *Genes Cells*, 9, 105-120 (2004)
- Cheeseman, I.M., Niessen, S., Anderson, S., Hyndman, F., Yates, J.R. III, Oegema, K., and Desai, A. A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes Dev.*, 18, 2255-2268 (2004)
- Obuse, C., Iwasaki, O., Kiyomitsu, T., Goshima, G., Toyoda, Y., and Yanagida, M. A conserved Mis12 centromere complex is linked to heterochromatic HP1 and outer kinetochore protein Zwint-1. *Nature Cell Biol.*, 6, 1135-1141 (2004)
- Folts, D., Jansen, L.E., Black, B.E., Bailey, A.O., Yates, J.R. III, and Cleveland, D.W. The human CENP-A centromeric nucleosome-associated complex. *Nature Cell Biol.*, 8, 458-469 (2006)
- Okada, M., Cheeseman, I.M., Hori, T., Okawa, K., McLeod, I.X., Yates, J.R. III, Desai, A., and Fukagawa, T. The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nature Cell Biol.*, 8, 446-457 (2006)
- Brown, W.R., Hubbard, S.J., Tickle, C., and Wilson, S.A. The chicken as a model for large-scale analysis of vertebrate gene function. *Nature Rev. Genet.*, 4, 87-98 (2003)
- Masumoto, H., Masukata, H., Muro, Y., Nozaki, N., and Okazaki, T. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J. Cell Biol.*, 109, 1963-1973 (1989)
- Harrington, J.J., Van Bokkelen, G., Mays, R.W., Gustashaw, K., and Willard, H.F. Formation of de novo centromeres and construction of first-generation human artificial microchromosomes. *Nature Genet.*, 15, 345-355 (1997)
- Ikeno, M., Grimes, B., Okazaki, T., Nakano, M., Saitoh, K., Hoshino, H., McGill, N.I., Cooke, H., and Masumoto, H. Construction of YAC-based mammalian artificial chromosomes. *Nature Biotechnol.*, 16, 431-439 (1998)
- Ohzeki, J., Nakano, M., Okada, T., and Masumoto, H. CENP-B box is required for de novo centromere chromatin assembly on human alphoid DNA. *J. Cell Biol.*, 159, 765-775 (2002)
- Hudson, D.F., Fowler, K.J., Earle, E., Saffery, R., Kalitsis, P., Trowell, H., Hill, J., Wreford, N.G., de Kretser, D.M., Cancilla, M.R., Howman, E., Hii, L., Cutts, S.M., Irvine, D.V., and Choo, K.H. Centromere protein B null mice are mitotically and meiotically normal but have lower body and testis weights. *J. Cell Biol.*, 309-319 (1998)
- Howman, E.V., Fowler, K.J., Newson, A.J., Redward, S., MacDonald, A.C., Kalitsis, P., and Choo, K.H.A. Early disruption of centromeric chromatin organization in centromere protein A (Cenpa) null mice. *Proc. Natl. Acad. Sci. USA*, 97, 1148-1153 (2000)
- Blower, M.D., and Karpen, G.H. The role of *Drosophila* CID in kinetochore formation, cell-cycle

The kinetochore and spindle checkpoint

- progression and heterochromatin interactions. *Nature Cell Biol.*, 3, 730-739 (2001)
27. Oegema, K., Desai, A., Rybina, S., Kirkham, M., and Hyman, A.A. Functional analysis of kinetochore assembly in *Caenorhabditis elegans*. *J. Cell Biol.*, 153, 1209-1225 (2001)
28. Goshima, G., Kiyomitsu, T., Yoda, K., and Yanagida, M. Human centromere chromatin protein hMis12, essential for equal segregation, is independent of CENP-A loading pathway. *J. Cell Biol.*, 160, 25-39 (2003)
29. Hayashi, T., Fujita, Y., Iwasaki, O., Adachi, Y., Takahashi, K., and Yanagida, M. Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres. *Cell*, 118, 715-729 (2004)
30. Regnier, V., Vagnarelli, P., Fukagawa, T., Zerjal, T., Burns, E., Trouche, D., Earnshaw, W., and Brown, W. CENP-A is required for accurate chromosome segregation and sustained kinetochore association of BubR1. *Mol. Cell Biol.*, 25, 3967-3981 (2005)
31. Liu, S.T., Rattner, J.B., Jablonski, S.A., and Yen, T.J. Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells. *J. Cell Biol.*, 175, 41-53 (2006)
32. Warburton, P.E., Cooke, C.A., Bourassa, S., Vafa, O., Sullivan, B.A., Stetten, G., Gimelli, G., Warburton, D., Tyler-Smith, C., Sullivan, K.F., Poirier, G.G., and Earnshaw, W.C. Immunolocalization of CENP-A suggests a novel nucleosome structure at the inner kinetochore plate of active centromeres. *Curr. Biol.*, 7, 901-904 (1997)
33. Ando, S., Yang, H., Nozaki, N., Okazaki, T., and Yoda, K., CENP-A, -B, and -C chromatin complex that contains the I-type alpha-satellite array constitutes the prekinetochore in HeLa cells. *Mol. Cell Biol.*, 22, 2229-2241 (2002)
34. Blower, M.D., Sullivan, B.A., and Karpen, G.H. Conserved organization of centromeric chromatin in flies and humans. *Dev. Cell*, 2, 319-330 (2002)
35. Black, B.E., Foltz, D.R., Chakravarthy, S., Luger, K., Woods, V.L. Jr., and Cleveland, D. Structural determinants for generating centromeric chromatin. *Nature*, 430, 578-582 (2004)
36. Black, B.E., Jansen, L.E., Maddox, P.S., Foltz, D.R., Desai, A.B., Shah, J.V., and Cleveland, D.W. Centromere identity maintained by nucleosomes assembled with histone H3 containing the CENP-A targeting domain. *Mol. Cell*, 25, 309-322 (2007)
37. Fujita, Y., Hayashi, T., Kiyomitsu, T., Toyoda, Y., Kokubu, A., Obuse, C., and Yanagida, M. Priming of centromere for CENP-A recruitment by human hMis18alpha, hMis18beta, and M18BP1. *Dev Cell*, 12, 17-30 (2007)
38. Maddox, P.S., Hyndman, F., Monen, J., Oegema, K., and Desai, A. Functional genomics identifies a Myb domain-containing protein family required for assembly of CENP-A chromatin. *J. Cell Biol.*, 176, 757-763 (2007)
39. Verreault, A., Kaufman, P.D., Kobayashi, R., and Stillman, B. Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell*, 87, 95-104 (1996)
40. Jansen, L.E., Black, B.E., Foltz, D.R., and Cleveland, D.W. Propagation of centromeric chromatin requires exit from mitosis. *J. Cell Biol.*, 176, 795-805 (2007)
41. Izuta, H., Ikeno, M., Suzuki, N., Tomonaga, T., Nozaki, N., Obuse, C., Kisu, Y., Goshima, N., Nomura, F., Nomura, N., and Yoda, K. Comprehensive analysis of the ICEN (Interphase Centromere Complex) components enriched in the CENP-A chromatin of human cells. *Genes Cells*, 11, 673-684 (2006)
42. Myhre, K., and Bloom, K.S. Differential kinetochore protein requirements for establishment versus propagation of centromere activity in *Saccharomyces cerevisiae*. *J. Cell Biol.*, 160, 833-843 (2003)
43. Takahashi, K., Chen, E.S., and Yanagida, M. Requirement of Mis6 centromere connector for localizing a CENP-A-like protein in fission yeast. *Science*, 288, 2215-2219 (2000)
44. Cheeseman, I.M., and Desai, A. Molecular Architecture of the Kinetochore-Microtubule Interface. submitted.
45. Sugata, N., Munekata, E., and Todokoro, K. Characterization of a novel kinetochore protein, CENP-H. *J. Biol. Chem.*, 274, 27343-27346 (1999)
46. Fukagawa, T., Mikami, Y., Nishihashi, A., Regnier, V., Haraguchi, T., Hiraoka, Y., Sugata, N., Todokoro, K., Brown, W., and Ikemura, T. CENP-H, a constitutive centromere component, is required for centromere targeting of CENP-C in vertebrate cells. *EMBO J.*, 20, 4603-4617 (2001)
47. Nishihashi, A., Haraguchi, T., Hiraoka, Y., Ikemura, T., Regnier, V., Dodson, H., Earnshaw, W.C., and Fukagawa, T. CENP-I is essential for centromere function in vertebrate cells. *Dev. Cell*, 2, 463-476 (2002)
48. Liu, S.T., Hittle, J.C., Jablonski, S.A., Campbell, M.S., Yoda, K., and Yen, T.J. Human CENP-I specifies localization of CENP-F, MAD1 and MAD2 to kinetochores and is essential for mitosis. *Nature Cell Biol.*, 5, 341-345 (2003)
49. Minoshima, Y., Hori, T., Okada, M., Kimura, H., Haraguchi, T., Hiraoka, Y., Bao, Y.C., Kawashima, T., Kitamura, T., and Fukagawa, T. The constitutive centromere component CENP-50 is required for recovery from spindle damage. *Mol. Cell Biol.*, 25, 10315-10328 (2005)
50. Moroi, Y., Peebles, C., Fritzler, M.J., Steigerwald, J., and Tan, E.M. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. USA*, 77, 1627-1631 (1980)
51. Earnshaw, W.C., and Rothfield, N. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma*, 91, 313-321 (1985)
52. Saitoh, H., Tomkiel, J., Cooke, C.A., Ratrie, H., Maure, M., Rothfield, N.F., and Earnshaw, W.C. CENP-C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell*, 70, 115-125 (1992)
53. Yang, C.H., Tomkiel, J., Saitoh, H., Johnson, D.H., and Earnshaw, W.C. Identification of overlapping DNA-binding and centromere-targeting domains in the human kinetochore protein CENP-C. *Mol. Cell Biol.*, 16, 3576-3586 (1996)
54. Fukagawa, T., and Brown, W.R.A. Efficient conditional mutation of the vertebrate CENP-C gene. *Hum. Mol. Genet.*, 6, 2301-2308 (1997)

55. Kalitsis, P., Fowler, K.J., Earle, E., Hill, J., and Choo, K.H.A. Targeted disruption of mouse centromere protein C gene leads to mitotic disarray and early embryo death. *Proc. Natl. Acad. Sci. USA*, 95, 576-582 (1998)
56. Fukagawa, T., Pendon, C., Morris, J., and Brown, W. CENP-C is necessary but not sufficient to induce formation of functional centromere. *EMBO J.*, 18, 4196-4209 (1999)
57. Kwon, M.S., Hori, T., Okada, M., and Fukagawa, T. CENP-C is involved in chromosome segregation, mitotic checkpoint function, and kinetochore assembly. *Mol. Biol. Cell*, 18, 2155-2168 (2007)
58. Brown, M.T., Goetsch, L., and Hartwell, L.H. MIF2 is required for mitotic spindle integrity during anaphase spindle elongation in *Saccharomyces cerevisiae*. *J. Cell Biol.*, 123, 387-403 (1993)
59. Moore, L.L., and Roth, M.B. HCP-4, a CENP-C-like protein in *Caenorhabditis elegans*, is required for resolution of sister centromeres. *J. Cell Biol.*, 153, 1199-1208 (2001)
60. Heeger, S., Leismann, O., Schittenhelm, R., Schraidt, O., Heidmann, S., and Lehner, C.F. Genetic interactions of separase regulatory subunits reveal the diverged *Drosophila* Cenp-C homolog. *Genes Dev.*, 19, 2041-2053 (2005)
61. Holland, S., Ioannou, D., Haines, S., and Brown, W.R. Comparison of Dam tagging and chromatin immunoprecipitation as tools for the identification of the binding sites for *S. pombe* CENP-C. *Chromosome Res.*, 13, 73-83 (2005)
62. Moore, L.L., Stanvitch, G., Roth, M.B., and Rosen, D. HCP-4/CENP-C promotes the prophase timing of centromere resolution by enabling the centromere association of HCP-6 in *Caenorhabditis elegans*. *Mol. Cell Biol.*, 25, 2583-2592 (2005)
63. Kline, S.L., Cheeseman, I.M., Hori, T., Fukagawa, T., and Desai, A. The human Mis12 complex is required for kinetochore assembly and proper chromosome segregation. *J. Cell Biol.*, 173, 9-17 (2006)
64. Goshima, G., Saitoh, S., and Yanagida, M. Proper metaphase spindle length is determined by centromere proteins Mis12 and Mis6 required for faithful chromosome segregation. *Genes Dev.*, 13, 1664-1677 (1999)
65. Wigge, P.A., and Kilmartin, J.V. The Ndc80p complex from *Saccharomyces cerevisiae* contains conserved centromere components and has a function in chromosome segregation. *J. Cell Biol.*, 152, 349-360 (2001)
66. DeLuca, J.G., Moree, B., Hickey, J.M., Kilmartin, J.V., and Salmon, E.D. hNuf2 inhibition blocks stable kinetochore-microtubule attachment and induces mitotic cell death in HeLa cells. *J. Cell Biol.*, 159, 549-555 (2002)
67. Hori, T., Haraguchi, T., Hiraoka, Y., Kimura, H., and Fukagawa, T. Dynamic behavior of Nuf2-Hec1 complex that localizes to the centrosome and centromere and is essential for mitotic progression in vertebrate cells. *J. Cell Sci.*, 116, 3347-3362 (2003)
68. McClelland, M.L., Gardner, R.D., Kallio, M.J., Daum, J.R., Gorbsky, G.J., Burke, D.J., and Stukenberg, P.T. The highly conserved Ndc80 complex is required for kinetochore assembly, chromosome congression, and spindle checkpoint activity. *Genes Dev.*, 17, 101-114 (2003)
69. Mikami, Y., Hori, T., Kimura, H., and Fukagawa, T. The functional region of CENP-H interacts with the Nuf2 complex that localizes to centromere during mitosis. *Mol. Cell Biol.*, 25, 1958-1970 (2005)
70. Cheeseman, I.M., Chappie, J.S., Wilson-Kubalek, E.M., and Desai, A. The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell*, 127, 983-997 (2006)
71. DeLuca, J.G., Gall, W.E., Ciferri, C., Cimini, D., Musacchio, A., and Salmon, E.D. Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. *Cell*, 127, 969-982 (2006)
72. Wei, R.R., Al-Bassam, J., and Harrison, S.C. The Ndc80/HEC1 complex is a contact point for kinetochore-microtubule attachment. *Nature Struct. Mol. Biol.*, 14, 54-59 (2007)
73. Cheeseman, I.M., Hori, T., Fukagawa, T., and Desai, A. hKNL1 and the CENP-H/I/K Complex Function in Parallel Pathways for Human Kinetochore Assembly. Submitted.
74. Musacchio, A., and Hardwick, K.G. The spindle checkpoint: structural insights into dynamic signalling. *Nature Rev. Mol. Cell Biol.*, 3, 731-741 (2002)
75. Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Nakayama, T., and Oshimura, M. Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nature Cell Biol.*, 6, 784-791 (2004)
76. Wong, L.H., Brettingham-Moore, K.H., Chan, L., Quach, J.M., Anderson, M.A., Northrop, E.L., Hannan, R., Saffery, R., Shaw, M.L., Williams, E., and Choo, K.H. Centromere RNA is a key component for the assembly of nucleoproteins at the nucleolus and centromere. *Genome Res.*, 17, 1146-1160 (2007)
77. Cahill, D.P., Lengauer, C., Yu, J., Riggins, G.J., Willson, J.K., Markowitz, S.D., Kinzler, K.W., and Vogelstein, B. Mutations of mitotic checkpoint genes in human cancers. *Nature*, 392, 300-303 (1998)
78. Tomonaga, T., Matsushita, K., Yamaguchi, S., Oohashi, T., Shimada, H., Ochiai, T., Yoda, K., and Nomura, F. Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. *Cancer Res.*, 63, 3511-3516 (2003)
79. Tomonaga, T., Matsushita, K., Ishibashi, M., Nezu, M., Shimada, H., Ochiai, T., Yoda, K., and Nomura, F. Centromere protein H is up-regulated in primary human colorectal cancer and its overexpression induces aneuploidy. *Cancer Res.*, 65, 4683-4689 (2005)

Key Words: Kinetochore, Centromere, Mitosis, DT40, Review

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