Molecular architecture of the kinetochore–microtubule interface

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Abstract | Segregation of the replicated genome during cell division in eukaryotes requires the kinetochore to link centromeric DNA to spindle microtubules. The kinetochore is composed of a number of conserved protein complexes that direct its specification and assembly, bind to spindle microtubules and regulate chromosome segregation. Recent studies have identified more than 80 kinetochore components, and are revealing how these proteins are organized into the higher order kinetochore structure, as well as how they function to achieve proper chromosome segregation.

Transmission electron microscopy
A method that is used to image at high resolution by passing electrons through a sample that has been thinly sectioned and stained with heavy-metal compounds to generate contrast.

High-pressure freezing methods
Methods that are used to prepare samples for electron microscopy in which the sample is quickly cooled to low temperatures under high pressure. Preserves ultrastructure better than chemical fixation techniques.

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The accurate distribution of the replicated genome during cell division is crucial for cellular viability and organismal development. DNA replication and replication-coupled formation of cohesion generates physically connected sister chromatids. The mitotic machinery accurately segregates all sister chromatids, ensuring that each daughter cell inherits a precise complement of the genome. Chromosome segregation occurs on a bipolar spindle-shaped structure that is built from microtubules, 25-nm diameter polymers of α/β-tubulin dimers. Upon entry into mitosis, replicated interphase chromosomes are compacted within the nucleus to facilitate their segregation within the dimensions of a cell (FIG. 1a; prophase). Concomitantly, chromosomes build a special structure to connect with spindle microtubules. In vertebrates, these connections occur at the site of the primary constriction of condensed chromosomes (the vertex of the familiar X shape). This site was initially called the centromere (from the Greek 'centro-', meaning 'central', and '-mere', meaning 'part') and later the kinetochore (from the Greek 'kineto-', meaning 'move', and '-chore', meaning 'means for distribution'). We will refer to the centromere as the region of chromosomal DNA that directs kinetochore assembly and to the kinetochore as the proteinaceous structure that associates with this DNA.

The interactions between the kinetochore and spindle microtubules are central to the alignment and segregation of chromosomes on the spindle (FIG. 1a,b). Following breakdown of the nuclear envelope, kinetochores start to interact both laterally and in an end-on manner with spindle microtubules (FIG. 1b; prometaphase). By metaphase, all chromosomes become bi-oriented, with sister kinetochores exclusively connected to microtubules that emanate from opposite spindle poles (FIG. 1a). However, during the progression from prometaphase to metaphase, some chromosomes may be delayed in connecting to the spindle, whereas others may be inappropriately attached or have only one of their sister kinetochores connected (FIG. 1b). To avoid loss of genomic information, the kinetochore monitors the attachment state and activates signalling pathways to prevent anaphase onset in the presence of incorrectly attached or unattached kinetochores (FIG. 1b). Once bi-orientation occurs for all chromosomes in the cell, the machinery that separates sister chromatids is activated and the separated chromatids move to opposite spindle poles (FIG. 1a; anaphase). Then, during telophase, chromatids decondense, the nuclear envelope re-forms and a cortical actomyosin ring bisects the cell, between the separated chromatid masses, to generate two daughter cells with exact copies of the duplicated genome.

The above summary highlights the central role of the kinetochore in chromosome segregation. In this review we focus on the molecular architecture of the kinetochore–spindle interface, specifically on the different protein groups that comprise the stable core of this structure. We summarize recent advances in the understanding of the activities of these protein groups and of how they are assembled, organized and functionally integrated to achieve accurate chromosome segregation.

Kinetochore ultrastructure

Electron microscopy provided the first insights into the structure of kinetochores[1]. Conventional chemical fixation procedures and thin-section transmission electron microscopy of chromosomes in vertebrate cells revealed that kinetochores have a trilaminar morphology[2] (FIG. 2a). Although such a layered structure is less distinct when analysed by modern high-pressure freezing methods[1] (FIG. 2b), this morphology has historically influenced the

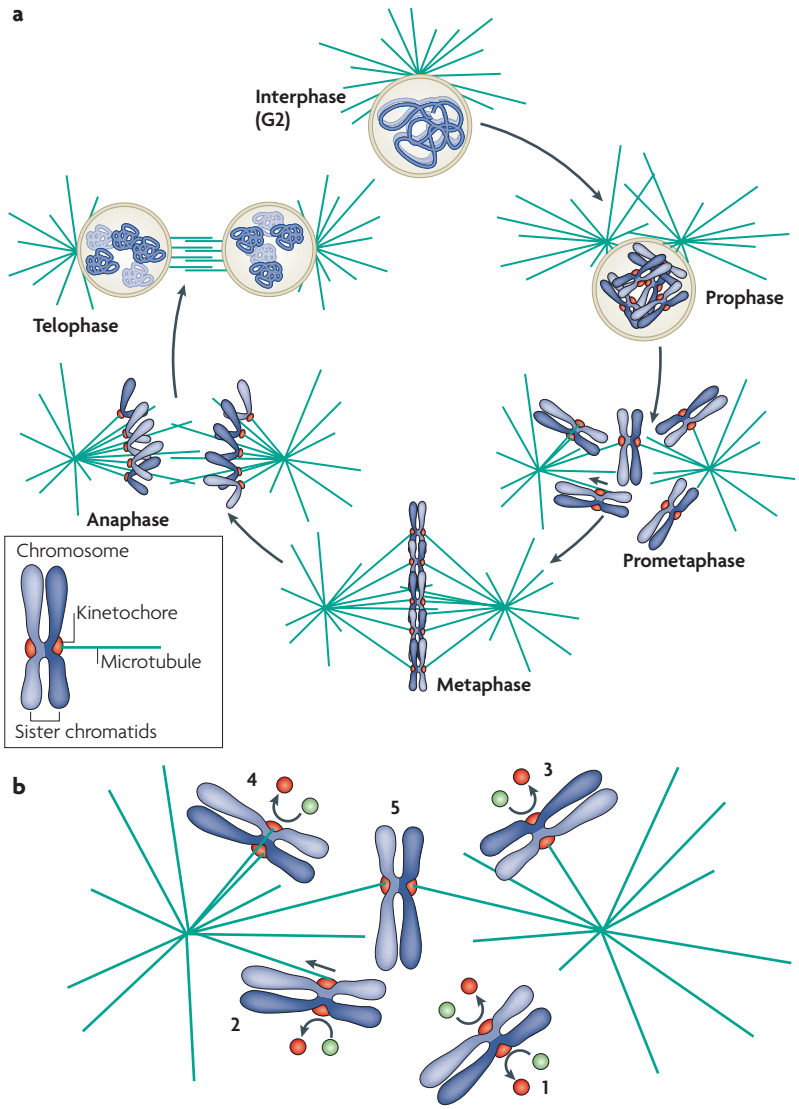


Figure 1 | **Mitotic chromosome segregation. a** | A summary of chromosome–spindle interactions during the M phase of the cell cycle. Replicated interphase chromatin is condensed during prophase; concomitantly, kinetochores assemble on the centromere regions of chromosomes. After nuclear envelope breakdown, during prometaphase, kinetochores interact with spindle microtubules. By metaphase, all chromosomes are bi-oriented and aligned in the middle of the spindle. During anaphase, separated sister chromatids move away from each other to opposite spindle poles. Subsequently, during telophase, the chromatid masses decondense and the nuclear envelope reforms to generate the daughter nuclei. **b** | A detailed view of the prometaphase stage, highlighting key activities of the kinetochore in chromosome segregation. Various intermediates (**1**–**5**) can be detected along the path from unattached (**1**) to bi-oriented (**5**) chromosomes. Lateral associations between kinetochores and spindle microtubules (**2**), which result in poleward chromosome movement, are frequently observed after nuclear envelope breakdown. Lateral attachments mature to end-on attachments, first with one kinetochore (**3**) and subsequently with both (**5**). Unattached kinetochores (as in **1**, **2** and **3**) catalyse the formation of an inhibitor (red circles) that prevents anaphase onset. Attachment errors, such as the one depicted in **4**, are also common and are detected and eliminated to prevent chromosome loss.

division of the kinetochore into distinct regions (FIG. 2): the inner kinetochore, which forms the interface with chromatin; the outer kinetochore, a 50–60-nm-thick region that forms the interaction surface for spindle microtubules; and the central kinetochore, the region between the inner and outer kinetochore that appears less dense by conventional fixation electron microscopy. Electron microscopy analysis carried out in the presence of drugs that prevent microtubule polymerization shows a dense array of fibres, called the fibrous corona, that extend away from the outer kinetochore (FIG. 2a). Finally, the term inner centromere refers to the chromatin that is located between the two sister kinetochores (FIG. 2).

Although budding yeast kinetochores associate with just a single microtubule, kinetochores in all other organisms examined so far bind to multiple spindle microtubules. In humans, 15–20 microtubules are bound to each kinetochore[3]. Electron tomography studies are beginning to probe the higher resolution structure of the kinetochore and have indicated that the outer plate is probably comprised of a loose fibrillar structure[4]. Using immunoelectron microscopy, a subset of proteins has been localized to specific kinetochore domains[1]. Integration of higher resolution tomographic ultrastructural analysis with detailed examination of the components that are required for kinetochore assembly and microtubule attachment is an important area of future research.

Molecular composition of kinetochores

Initial efforts to define kinetochore composition were hampered by the low abundance of constituent proteins and because these proteins are required for cell viability. The first human kinetochore proteins were identified using human autoantibodies that recognized three major antigens, CENP-A, CENP-B and CENP-C (for 'centromere protein')[5]. Particularly important was the discovery that CENP-A was a variant of histone H3 (REFS 6,7), one of the core subunits of nucleosomes. Immunofluorescence-based screening of antibodies that had been prepared against chromosome scaffolds identified additional proteins that localize to the fibrous corona and inner centromere[8,9].

A combination of genetics, RNA interference (RNAi)-based screens and biochemistry was used in model organisms to functionally identify components of the kinetochore that are required for chromosome segregation. These studies were particularly successful in budding yeast, which has an unusually well defined, short centromeric DNA sequence (the kinetochore structure of budding yeast has been reviewed recently[10,11]). Although most kinetochore proteins show limited homology between species (in many cases 20% or less identity), sequence-based searches have identified vertebrate counterparts to many of the proteins that have been identified in model organisms (see REFS 12–14).

In the past decade, mass-spectrometry-based proteomics has greatly accelerated the elucidation of kinetochore composition in model organisms and vertebrates. Purification and mass-spectrometry-based analysis of centromeric chromatin[15,16] and mitotic spindles[17,18] as well as affinity purifications using validated kinetochore proteins as targets[13,19–21] have identified a large number of proteins that function at kinetochores and have grouped this large protein set into defined subcomplexes.

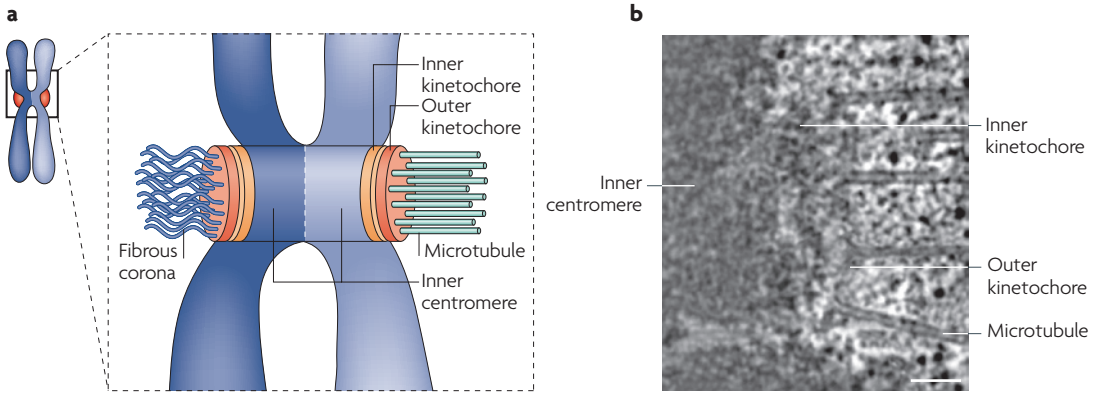


Figure 2 | **Vertebrate kinetochore ultrastructure. a** | A schematic of a mitotic chromosome with paired sister chromatids — the chromatid on the right is attached to microtubules and the chromatid on the left is unattached. The inner kinetochore, the outer kinetochore, the inner centromere and the fibrous corona, which is detectable on the unattached kinetochore, are highlighted. **b** | Electron micrograph of a human kinetochore (image courtesy of Y. Dong and B. McEwen, State University of New York at Albany, USA). The micrograph represents a single slice from a tomographic volume of a high-pressure frozen mitotic cell and has been labelled as in **a** to highlight the key structural features of the kinetochore. Scale bar, 100 nm.

Currently, around 80 kinetochore proteins have been identified in humans. Although there are some organism-specific differences, the major themes in kinetochore composition and organization are conserved throughout eukaryotes. The human kinetochore proteins identified so far, and their likely counterparts in model organisms, are summarized in Supplementary information S1 (table).

The identification of multiple conserved kinetochore subcomplexes has stimulated efforts to elucidate their functions and has focused on their contributions to five major processes: kinetochore specification (determining where to assemble the kinetochore on the chromosome); kinetochore assembly; microtubule binding (including generating a core microtubule-attachment site, microtubule dynamics at kinetochores and kinetochore motility); monitoring kinetochore–microtubule attachments to prevent anaphase entry in the presence of unattached kinetochores; and regulating kinetochore–microtubule attachments to ensure accurate chromosome segregation. Below, we highlight the main players that are currently thought to make important contributions to these processes.

Kinetochore specification

If a chromosome fails to specify a site for kinetochore formation, it will be unable to attach to the spindle and will not be segregated during mitosis. Alternately, if multiple discrete sites of kinetochore assembly occur on a single chromatid, inappropriate attachments can connect that chromatid to both spindle poles, leading to its fragmentation by spindle forces. Restricting kinetochore assembly to one site (monocentric chromosome architecture) or extending kinetochore assembly along the entire length of each sister chromatid (holocentric chromosome architecture[22]) are two solutions to this problem that have been observed in different eukaryotic lineages. Both solutions are based on a similar specialized chromatin foundation that directs kinetochore assembly.

Most organisms lack a precise DNA sequence that determines the site of kinetochore assembly. In humans, centromere regions are enriched in tandemly repeated arrays of a 171-base pair (bp) α-satellite DNA sequence. In this sequence, there is a 17-bp motif called the CENP-B box[23], which can be bound by the inner centromere protein CENP-B. Both α-satellite DNA and CENP-B boxes are required for the generation of artificial chromosomes from transfected DNA in cultured human HT1080 cells[24], which implies a requirement for these regions of DNA during *de novo* centromere formation. However, analysis of stably inherited dicentric chromosomes and neocentromeres that are formed on acentric chromosome fragments has shown that α-satellite DNA is neither necessary nor sufficient for centromere activity[25]. In addition, eliminating CENP-B from mice has no adverse effects on kinetochore function[25]. These findings indicate that established centromeric loci can be stably maintained through mitotic and meiotic divisions in the absence of an interaction between CENP-B and the CENP-B box or α-satellite DNA. Therefore, in most eukaryotes, the site of kinetochore assembly is thought to be controlled primarily by epigenetic, rather than sequence based, mechanisms[26].

The primary candidate for an epigenetic mark of kinetochore specification is the specialized chromatin that is present at centromeres. Centromeric chromatin consists of linearly interspersed regions of CENP-A nucleosomes — in which histone H3 is replaced by the H3 variant CENP-A — and canonical histone H3 nucleosomes[27] (FIG. 3). As CENP-A is a fundamental determinant of kinetochore identity, recent studies have focused on the mechanisms that propagate this specialized centromeric chromatin to maintain centromere identity.

Mechanisms that target CENP-A to centromeres. A combination of mechanisms, including the targeted deposition of new CENP-A nucleosomes to regions of pre-existing CENP-A nucleosomes and the elimination of CENP-A nucleosomes from ectopic sites[28],

Electron tomography
A technique in which sections that are larger than those typically used for transmission electron microscopy are imaged at various angles. Provides increased resolution and some three-dimensional imaging capacity.

α-satellite DNA
Repetitive DNA that is found at the centromeres of human cells.

HT1080 cells
Human cells that are used for the generation of artificial human chromosomes.

Dicentric chromosomes
Chromosomes that have two centromeres.

Neocentromeres
Chromosomal sites that do not contain typical repetitive centromeric DNA, but that do acquire centromeric chromatin, can assemble kinetochores, can recruit other centromeric proteins and are transmitted faithfully during meiosis and mitosis.

Acentric
A chromosome or chromosomal fragment that lacks a centromere.

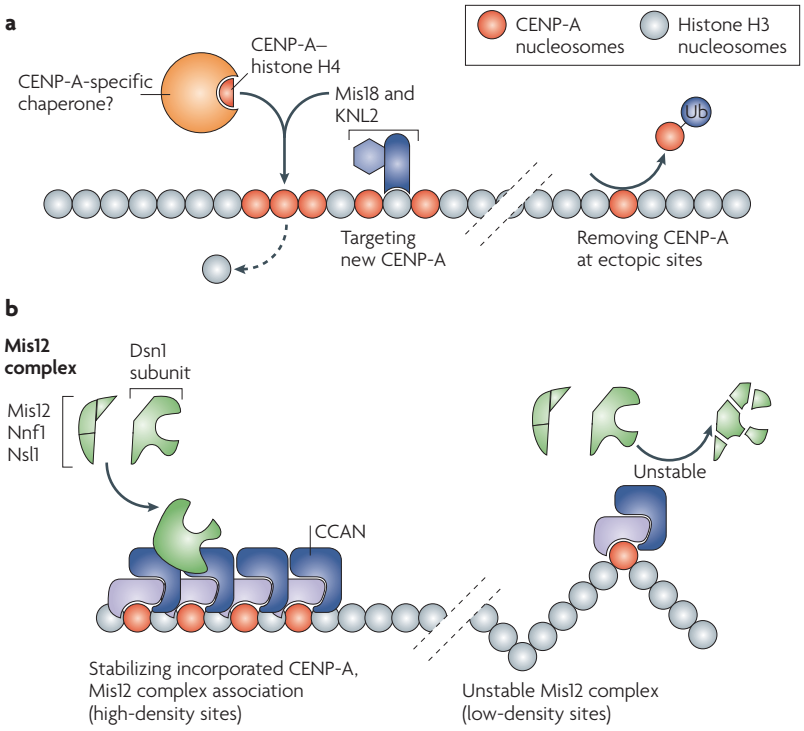


Figure 3 | **Kinetochore specification.** A model showing the proteins and complexes that are implicated in kinetochore specification. Centromeric chromatin is characterized by the presence of specialized nucleosomes that contain the histone H3 variant CENP-A. **a** | Factors that restrict CENP-A to centromeres. CENP-A (which forms a dimer with histone H4) is epigenetically maintained at centromeres by a combination of activities, including targeted deposition and removal at ectopic sites, possibly through ubiquitylation and degradation. The two proteins specifically implicated in CENP-A loading, Mis18 and KNL2 (also known as M18BP1), which form a complex in vertebrates, are depicted as guiding specific CENP-A loading. The mechanism of action of these proteins is currently not known. **b** | Kinetochore proteins downstream of CENP-A that are involved in kinetochore specification. The constitutive centromere-associated network (CCAN), which is closely associated with CENP-A nucleosomes throughout the cell cycle, is shown as stabilizing newly incorporated CENP-A. We suggest that the Mis12 complex (which consists of Dsn1 (known as KNL-3 in *Caenorhabditis elegans*), Mis12, Nnf1 and Nsl1) provides a second layer of specificity by acting as a molecular 'keystone' that licenses kinetochore assembly. This proposal is based on the inherent instability of the Dsn1 subunit of the Mis12 complex from *C. elegans* and humans. We speculate that interactions between an array of centromeric chromatin and multiple Mis12 complexes lead to the local stabilization of Dsn1 and restrict kinetochore assembly to that region of the chromosome.

ensure the maintenance of centromere identity (FIG. 3). Overexpression of CENP-A can overcome this regulation and allow it to be incorporated at non-centromeric loci[29,30], which implies that control of CENP-A levels is important for its specific incorporation at centromeres.

CENP-A itself is important for ensuring its targeting to centromeres. There is a striking structural difference in rigidity between CENP-A and histone H3 nucleosomes, which is mediated by a short CENP-A-targeting domain (CATD) in the histone fold[31,32]. Chimaeras in which the CATD from CENP-A is swapped into histone H3 target to centromeres and maintain cell viability when endogenous CENP-A levels are significantly reduced[31,33]. The increased structural rigidity conferred by the CATD might also be important for kinetochore function and for generating the unique chromatin environment at these regions.

SANT domain
A motif that was identified on the basis of its homology to the DNA-binding domain of c-Myb.

Two extrinsic factors, Mis18 and KNL2 (also known as M18BP1), have been implicated specifically in CENP-A deposition and the maintenance of centromere identity (FIG. 3a)[14,34,35]. KNL2 contains a Myb (also known as SANT; for 'SWI3, ADA2, NCoR, TFIIIB') domain, which is commonly found in DNA-binding proteins and chromatin-remodelling complexes. CENP-A deposition at centromeres is inhibited by disrupting Mis18 function in fission yeast and human cells[14,34] and by disrupting KNL-2 function in *Caenorhabditis elegans* (which have a holocentric chromosome architecture) and human cells[34,35]. A recent large-scale RNAi screen in *Drosophila melanogaster* cells identified a novel protein, CAL1, that is also required for CENP-A loading[36]. Although not visibly conserved, CAL1 might represent a highly divergent version of KNL2 or a functional equivalent.

In humans, two Mis18 isoforms, Mis18α and Mis18β, form a complex with KNL2 that localizes transiently to centromeres during a brief period of the cell cycle, starting in telophase and persisting through early G1 phase[34] (FIG. 4). Importantly, this timing is similar to when new CENP-A deposition occurs[37,38] (FIG. 4). Although this temporal loading pattern is consistent with the appealing hypothesis that a functional mitotic kinetochore licenses new CENP-A incorporation in the subsequent cell cycle[39], preventing kinetochore–microtubule interactions does not affect new CENP-A loading following mitotic exit[37,38].

Understanding the mechanism of action of Mis18 and KNL2-family proteins is an important future goal. So far, a direct association between these proteins and CENP-A has not been reported. One possibility is that the basal cellular machinery that is involved in chromatin assembly, such as the chaperone RbAp46/48, is adapted by Mis18 and KNL2 to direct CENP-A assembly (FIG. 3a). A role for RbAp46/48 family members in CENP-A loading has been implied by genetic studies in fission yeast and biochemical analysis in *Drosophila melanogaster*[14,40], and other chromatin-remodelling activities have also been implicated in CENP-A localization to kinetochores in fungi[41]. Alternatively, Mis18 and KNL2 could function to generate a chromatin environment that is permissive to CENP-A incorporation[14,34].

In budding yeast, Scm3, a non-histone protein that has been isolated as a suppressor of CENP-A^{Cse4} mutants, is required for CENP-A^{Cse4} deposition and stability[42–44]. No counterparts to Scm3 have been identified outside of fungi, but one research group provocatively suggested that Scm3 replaces the two histone H2a–H2b dimers that are present in canonical H3 nucleosomes to form an altered centromeric nucleosome together with CENP-A^{Cse4} (REF. 42). However, a prior proteomics study that isolated centromeric nucleosomes from budding yeast found that histones H2a and H2b co-purified with CENP-A^{Cse4}, but not Scm3 (REF. 45). Thus, a Scm3-containing altered nucleosome may only be transiently formed *in vivo* and may eventually be converted to an H2a–H2b-containing nucleosome. A recent study has also suggested that the centromeric nucleosome may be tetrameric during interphase, with a single copy of CENP-A, H4, H2A and H2B[46], unlike canonical octomeric nucleosomes.

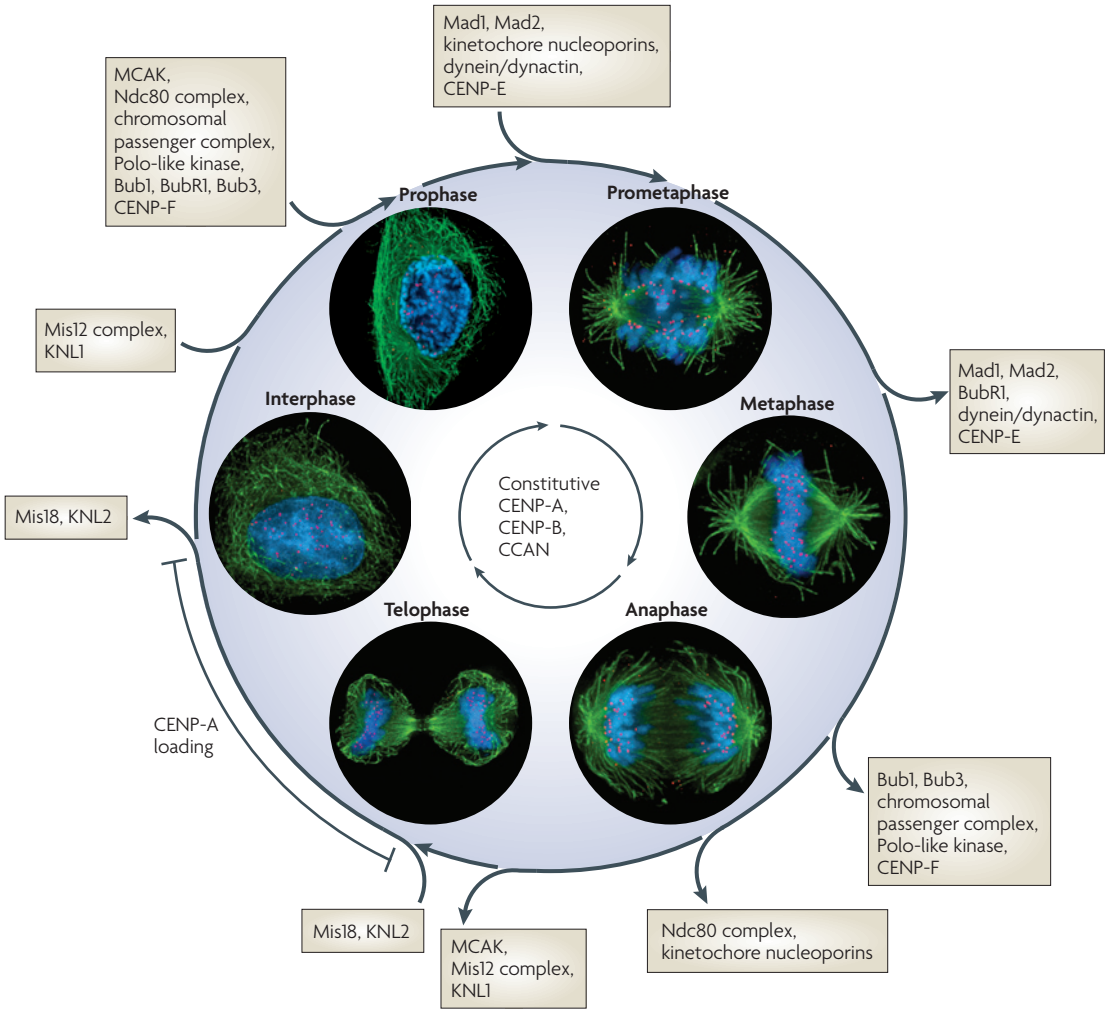
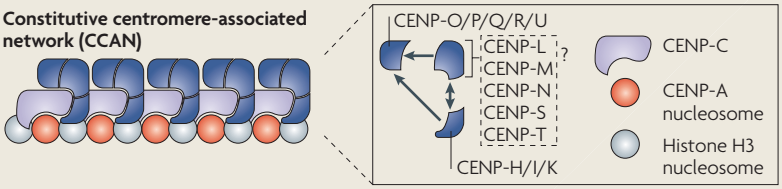


Figure 4 | **Kinetochore composition is dynamically regulated during the cell cycle.** Immunofluorescence images showing DNA (blue), microtubules (green) and kinetochore localization (red) throughout the cell cycle in human cells. Arrows on the periphery of the circle indicate when during the cell cycle the indicated protein (or proteins) begin to associate with, or delocalize from, the kinetochore. Arrows representing delocalization indicate the initial reduction of protein levels, but not necessarily the absolute loss of the components listed. The major themes in kinetochore localization that are highlighted in this figure are: constitutive localization (observed for CENP-A and the constitutive centromere-associated network (CCAN)); late interphase localization (observed for the Mis12 complex and KNL1, both of which are important for kinetochore assembly and microtubule interactions); prophase accumulation (observed for a number of proteins, including the microtubule-binding Ndc80 complex); localization after nuclear envelope breakdown (observed for a number of proteins, including the checkpoint pathway effectors Mad1 and Mad2 and the motors dynein and CENP-E); delocalization following microtubule attachment (observed for certain checkpoint-pathway components and motor complexes); delocalization at the metaphase–anaphase transition (observed for a number of proteins, including the chromosomal passenger complex); delocalization during late anaphase–telophase (observed for the stably bound Ndc80 complex, KNL1 and the Mis12 complex); and localization during late telophase–early G1 (observed for the CENP-A-loading factors Mis18 and KNL2 (also known as M18BP1)). The mechanisms underlying this elaborate choreography of assembly and disassembly are poorly understood.

Purification of CENP-A nucleosomes from human cells has identified a network of proteins that is constitutively present at centromeres and that includes CENP-C and 13 interacting proteins (CENP-H, CENP-I, CENP-K–U)[15,16,21,47]. Subsets of these proteins have been referred to as the CENP-H–I complex, the CENP-A-nucleosome-associated complex (NAC), or the CENP-A-nucleosome distal complex (CAD). However, these nomenclatures do not incorporate the complete range of proteins present in this network. Therefore, we propose referring to this entire group of proteins as the constitutive centromere-associated network (CCAN), on the basis of their colocalization with CENP-A nucleosomes throughout the cell cycle in vertebrates (BOX 1).

Although all tested CCAN subunits require CENP-A for their localization[48,49] and do not affect CENP-A association with centromeres once CENP-A has been deposited, CENP-C[36], the CENP-H/I/K subclass and CENP-M/N might contribute to the targeting and/or

Box 1 | **The constitutive centromere-associated network**



In vertebrates, a subset of kinetochore proteins colocalize with CENP-A throughout the cell cycle and co-purify with CENP-A nucleosomes. We propose referring to this protein set as the constitutive centromere-associated network (CCAN; see figure). This network is comprised of CENP-C and 13 interacting proteins (CENP-H, CENP-I, CENP-K–U). CENP-C is an essential component of the inner kinetochore and is the only protein in this network that has been identified in all eukaryotes[54,159–162]; the non-CENP-C components of the CCAN have not been identified so far in *Drosophila melanogaster*, *Caenorhabditis elegans* or plants. On the basis of *in vivo* and biochemical studies in vertebrates, the CENP-H, CENP-I and CENP-K–U subunits of the CCAN can be divided into two well defined subclasses and five less well characterized subunits. Depletion of components of the first subclass of proteins, comprising CENP-H, CENP-I and CENP-K, causes cell-cycle arrest, kinetochore-assembly defects and severe chromosome mis-segregation[12,16,21,47,64,68,163,164]. The second subclass of proteins, comprising CENP-O, CENP-P, CENP-Q, CENP-R and CENP-U (also known as CENP-50/PBIP)[16,21,47], is non-essential for viability in chicken cells, but is required to maintain viability during recovery from microtubule-depolymerizing conditions[21,165]. In human cells, depletion of individual members of this second subclass causes defects in chromosome segregation that are severe enough to cause cell lethality[16,21,166]. The remaining subunits — CENP-L, CENP-M, CENP-N, CENP-S and CENP-T — are less well characterized, but preliminary studies indicate that these proteins are important for kinetochore function[16,21,47].

stabilization of new CENP-A[21] (FIG. 3b). In budding yeast, Chl4, the CENP-N counterpart, is required for *de novo* kinetochore formation, but not for the duplication of existing kinetochores[50]. In fission yeast, Mis6, the CENP-I homologue, is required for CENP-A localization[51]. The CCAN is also important in mitotic kinetochore assembly in vertebrates and fungi, downstream of CENP-A (see below).

Kinetochore assembly

The layered kinetochore ultrastructure observed by electron microscopy (FIG. 2) is reflected by a step-wise assembly of kinetochore components from the chromatin to the outer kinetochore. A less linear hierarchy with complex feedback occurs at the outer kinetochore and fibrous corona, where assembly of components is transient and is influenced by the microtubule attachment state and local signalling pathways. We focus here primarily on the stably associated components that comprise the structural core of the kinetochore.

CENP-A chromatin forms the foundation for kinetochore assembly in yeast[52], *C. elegans*[53,54], *D. melanogaster*[55] and vertebrates[48,49]. In human cells, the CCAN is constitutively associated with CENP-A chromatin throughout the cell cycle[16,21] (FIG. 4). Additional proteins are recruited to kinetochores during late G2 phase, prophase or specific stages of mitosis, and are either depleted following microtubule attachment or persist until the onset of anaphase or the end of mitosis (FIG. 4). As cells begin to exit mitosis, many proteins delocalize from kinetochores, whereas others, such as Mis18 and KNL2, transiently localize from telophase until G1 phase (FIG. 4). There is currently no mechanistic understanding of the temporal dynamics of kinetochore-protein assembly/disassembly, which presumably involves cell-cycle-controlled synthesis and degradation, as well as post-translational cues from the cell-cycle machinery.

Although centromere specification is primarily directed by regulating CENP-A deposition, subtle overexpression of CENP-A in human cells results in the incorporation of CENP-A throughout the chromosome with no noticeable chromosome-segregation defects[7]. A higher level of CENP-A overexpression in humans[29] or *D. melanogaster*[30] causes the mistargeting of some other kinetochore proteins and, in rare cases, the formation of ectopic microtubule-attachment sites. However, this only occurs at a subset of loci in which CENP-A is inappropriately incorporated, which indicates that there are probably additional mechanisms to ensure that kinetochore assembly downstream of CENP-A occurs at specific sites. A potential component in this process of kinetochore specification is the conserved four-subunit Mis12 complex (FIG. 3b), the inhibition of which causes a reduction in the recruitment of multiple proteins to kinetochores[12,13,56,57]. We suggest that the Mis12 complex functions as a molecular 'keystone' that is required to license the process of kinetochore assembly (FIG. 3b). Recent evidence indicates that the Dsn1 subunit of the Mis12 complex is intrinsically unstable, but is stabilized by binding to the other three subunits[57,58]. If stable Mis12 complex formation is facilitated by a high density of CENP-A chromatin (including CENP-A-associated proteins, such as the CCAN; FIG. 3b), this would provide a mechanism for restricting kinetochore assembly on a chromosome.

Other molecules that are involved in kinetochore assembly include the CENP-C and CENP-H/I/K subclasses of the CCAN, and KNL1 (also known as Spc105). KNL1-family members are required for chromosome segregation and cell viability in all systems that have been examined so far[59–61]. In *C. elegans*, CENP-C and KNL-1 are required for the localization of all known outer kinetochore proteins[13,54,60]. In vertebrates, CENP-C is upstream of most other kinetochore proteins, including the Mis12 complex[49,62]. CENP-H/I/K are not required for the mitotic localization of CENP-A and CENP-C, but contribute to the localization of outer kinetochore proteins, including components of the mitotic checkpoint (also known as the spindle-assembly checkpoint), the microtubule-binding Ndc80 complex and the coiled-coil-domain-containing protein CENP-F[21,49,63,64]. In humans, the Mis12 complex, KNL1 and the Ndc80 complex associate with Zwint[13,56], a coiled-coil protein that functions as a receptor for the transiently associated Rod–ZW10–Zwilch (RZZ) complex[65,66]. The RZZ complex, in turn, recruits the minus-end-directed motor dynein to kinetochores[67].

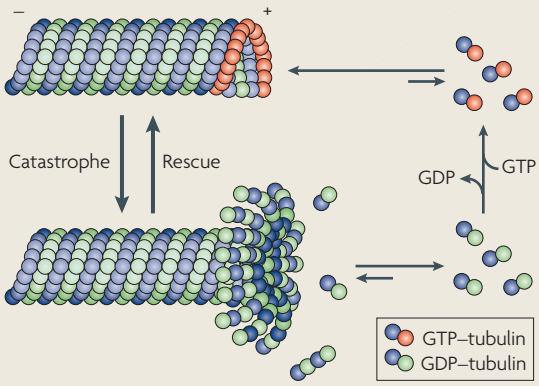
The amount of CCAN, Mis12 complex, KNL1 and Ndc80 complex at kinetochores does not change significantly from late prophase until late anaphase, irrespective of microtubule-attachment status (FIG. 4). In addition, as determined by photobleaching assays, the subunits of the CCAN, the Mis12 complex and the Ndc80 complex

Coiled-coil domain
A protein structural domain that mediates subunit oligomerization or protein–protein interactions. Coiled coils contain between two and five α-helices that twist around each other to form a supercoil.

Minus end
The slower polymerizing end of a microtubule, in which α-tubulin is exposed. In cells, minus ends do not grow — they are either stable or depolymerizing.

Photobleaching assays
Experiments in which a fluorescently labelled protein that is localized to a specific site is exposed to a brief intense pulse of light to bleach the fluorescence, followed by recovery, during which unbleached protein from elsewhere (typically the cytoplasm) replaces the bleached protein. The time required for, and the extent of, the recovery provide information on the local dynamics of the protein at that site.

Box 2 | **Microtubule dynamics**



Microtubules are 25-nm diameter hollow polymers that are comprised of 12–15 protofilaments that are formed by head-to-tail association of αβ-tubulin dimers. The fixed orientation of the tubulin dimers makes the microtubule lattice polar — this polarity is central to the ability of motor proteins to move cargo to specific locations *in vivo*. Microtubules exhibit a specialized non-equilibrium polymerization behaviour, termed dynamic instability, in which polymerizing and rapidly depolymerizing polymers coexist at steady state. The transition from polymerization to depolymerization is referred to as a catastrophe, and the reverse transition is referred to as a rescue. Polymerization-triggered GTP hydrolysis on β-tubulin provides the energy source for this non-equilibrium behaviour; the resulting GDP is locked into the polymer lattice until depolymerization releases the subunit. The energy of GTP hydrolysis is stored as mechanical strain in the GDP–tubulin polymer lattice, resulting in >1,000-fold higher dissociation of GDP–tubulin at a depolymerizing end relative to dissociation of GTP–tubulin at a polymerizing end. A polymerizing microtubule end is thought to persist because of a lag between subunit addition and nucleotide hydrolysis, which results in a stabilizing cap of GTP–tubulin. Loss of this cap, either stochastically or by the action of external factors, triggers release of the stored mechanical strain and a switch to rapid depolymerization. Nucleotide exchange on the released tubulin dimers re-primes these dimers for a new polymerization cycle. Both polymerization and depolymerization generate significant forces and can do productive work *in vivo*. Microtubules exhibit a steep nucleation barrier — it is much more difficult to start a new polymer than it is to elongate an existing one. This feature is important in cells, where localized microtubule-nucleating complexes control the spatial organization of microtubule arrays. For a detailed introduction to microtubule structure and dynamics, see REF. 167.

turnover slowly at the kinetochore[63,68,69]. These properties are consistent with centromeric chromatin, the CCAN and the KNL1–Mis12 complex–Ndc80 complex (KMN) network that comprises the stable kinetochore structure that is observed by electron microscopy.

Microtubule binding at kinetochores

A key function for the kinetochore is to attach chromosomes to spindle microtubules and to either generate or transduce the forces that are required for chromosome segregation. Multiple different microtubule-associated proteins function at kinetochores to generate a core attachment site, couple kinetochore movement to disassembling microtubules, affect the polymerization dynamics of kinetochore-bound microtubules and drive translocation along spindle microtubules.

The core attachment site. Kinetochore–microtubule attachments at the outer kinetochore must be robust enough to transduce forces to drive chromosome motility while also coupling the intrinsic dynamic instability of bound microtubule polymers to chromosome movement (for a primer on microtubule dynamic instability, see BOX 2). A number of proteins with microtubule-directed activities are localized to kinetochores. However, although these microtubule-interacting proteins contribute significantly to the fidelity of segregation, the results of *in vivo* studies are not consistent with these proteins forming the core attachment sites at the outer kinetochore.

Recent work suggests that the core kinetochore–microtubule attachment site is comprised by the KMN network and is likely to be formed by two closely apposed low-affinity microtubule-binding sites — one in the Ndc80 complex and a second in KNL1 (REF. 58; FIG. 5a). Functional analysis of the Ndc80 complex in a number of organisms has demonstrated that it is essential for kinetochore–microtubule interactions[19,60,70,71]. This complex of four proteins forms a rod-like structure with two globular heads at each end separated by a long coiled-coil region[72,73]. One end of the rod, composed of the globular regions of Ndc80 and Nuf2, localizes to the outer regions of the kinetochore[74] and binds directly to microtubules[58,75]. In fact, a conserved region in the N terminus of Ndc80 has a fold that is similar to that of the calponin-homology microtubule-binding domain of the plus-end tracking protein end-binding-1 (EB1)[75]. The other end of the Ndc80 complex rod, composed of the globular regions of Spc24 and Spc25 (REF. 76), is more closely apposed to the inner kinetochore. In *C. elegans*, SPC-24 and SPC-25 are required to associate with a kinetochore-bound receptor formed by the Mis12 complex and KNL-1 (REF. 58). In vertebrates, both the Mis12 complex and the CCAN influence Ndc80 complex localization to kinetochores[21,57], and an association between CENP-H and NDC80 has been reported[68].

Although the Ndc80 complex has weak microtubule-binding affinity on its own, when it associates with the Mis12 complex and KNL1, the microtubule-binding affinity is synergistically increased[58]. Both KNL1 and the Ndc80 complex also display concentration-dependent microtubule binding, which indicates a cooperativity in their association with the polymer lattice[58]. At the outer kinetochore, these two different binding sites are organized in a high-density array, which provides a distributed series of intrinsically weak sites that make multiple contacts to a single microtubule. Measurements in budding yeast have indicated that there are likely to be around eight Ndc80 complexes per microtubule[69], and

Calponin-homology domain
An ~110-residue domain that is found in many cytoskeletal and signal-transduction proteins.

Plus end
The faster polymerizing end of a microtubule, in which β-tubulin is exposed.

electron microscopy analysis of the outer kinetochore in vertebrates has revealed multiple fibrillar structures that interact with individual microtubules at the surface of the outer kinetochore[4]. Such a distributed low-affinity binding-site architecture would allow attachments to remain dynamic in response to growing or shrinking microtubules without resulting in the detachment of a kinetochore from the microtubules. The multiplicity of microtubules bound per kinetochore, which are not necessarily coordinated with each other in terms of their polymerization state[77], could also contribute to the stable, yet dynamic, nature of the attachment.

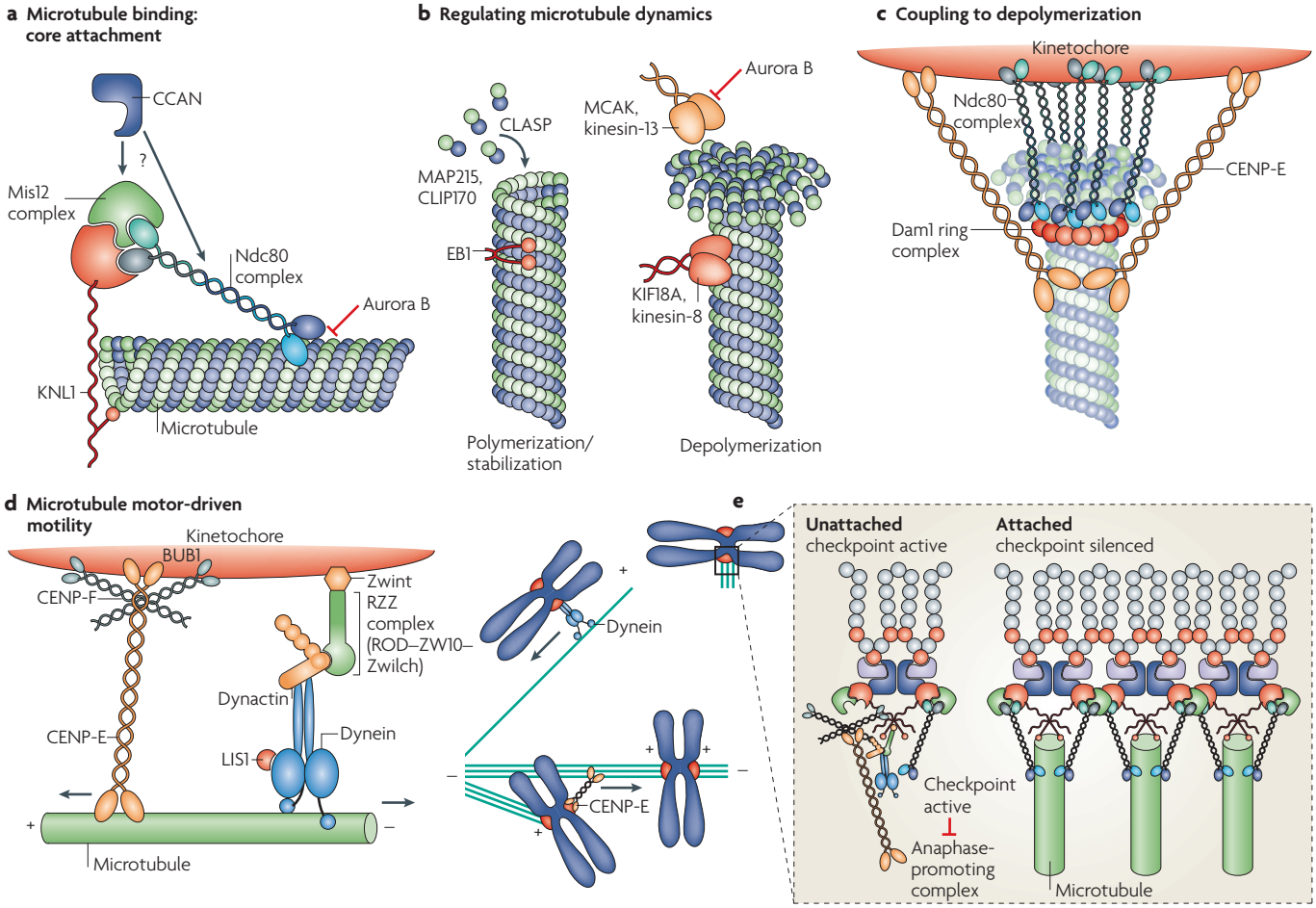


Figure 5 | **Molecular mechanisms underlying specific microtubule-directed activities at the kinetochore.** Models showing the proteins and complexes that are implicated in the different microtubule-directed activities at kinetochores. **a** | The core microtubule-attachment site. The components of the KNL1–Mis12 complex–Ndc80 complex (KMN) network are conserved throughout the eukaryotic kingdom. The association between the Mis12 complex and KNL1 generates a binding site for the Ndc80 complex. Both KNL1 and the Ndc80 complex directly bind to microtubules. In vertebrates and fungi, the constitutive centromere-associated network (CCAN) plays an important role in localizing the Ndc80 complex; however, this protein group is not present in *Caenorhabditis elegans* and may be missing in *Drosophila melanogaster*. **b** | Controlling microtubule dynamics. Cytoplasmic linker protein (CLIP)-associating protein (CLASP), MAP215 (also known as Stu2 in yeast) and CLIP170 are the major microtubule-associated proteins that are implicated in promoting polymerization at kinetochores. EB1 binds and stabilizes the microtubule lattice. The major depolymerization activities are contributed by kinesin-13 (also known as MCAK), which is non-motile, and kinesin-8 (also known as KIF18A), which has both motor and depolymerization activities. **c** | Coupling chromosome movement with microtubule depolymerization. Experimental evidence exists for this activity in kinesins, specifically from CENP-E and the yeast Dam1 complex, which oligomerizes to form a ring around the microtubule. We speculate that multiple Ndc80 complexes interacting with a single microtubule may also provide coupling activity, although this has not been directly demonstrated. **d** | Translocation along microtubules. The two motor proteins that are localized to kinetochores are CENP-E and dynein. Dynein translocates laterally associated kinetochores to the vicinity of spindle poles. CENP-E translocates along the kinetochore fibre of an already bi-oriented chromosome to move a mono-oriented chromosome towards the spindle equator. Dynein and the associated proteins LIS1 and the ROD–ZW10–Zwilch (RZZ) complex also contribute a poleward force at end-on attached kinetochores that contributes to the chromosome alignment and segregation. **e** | Integrated model of the vertebrate kinetochore, which shows differences in the microtubule-binding proteins under different attachment states. Protein complexes are depicted as detailed in FIG. 3 and BOX 1. Kinetochores that are unattached to microtubules recruit additional proteins to facilitate microtubule interactions and signal cell-cycle arrest.

Although the Ndc80 complex is crucial for generating robust attachments, weak kinetochore–microtubule interactions can form in cells that have been depleted of the Ndc80 complex[13,60,70]. Other proteins that might function in parallel to KNL1 and the Ndc80 complex to interact with microtubules include the motor proteins dynein and the kinesin CENP-E. Eliminating CENP-E reduces the number of microtubules bound to each kinetochore[3]. The RZZ complex targets dynein and its activator complex, dynactin, to kinetochores[67]. Perturbing dynein function globally by disrupting the dynactin complex or locally at the kinetochore using RZZ complex depletion does not prevent the formation of kinetochore–microtubule attachments, although perturbation does cause defects in the stability of attachments, chromosome alignment and segregation[78,79].

In addition to the motor proteins, the large coiled-coil protein CENP-F displays weak microtubule-binding activity on its own[80] and associates with the dynein-interacting proteins NDE1 and NDEL1 (REF. 81). The recently identified proteins Ska1 and Ska2 localize to kinetochores and spindle microtubules[82] and might also function at the interface with microtubules. Similarly, the centrosomal protein Cep57 localizes to *Xenopus laevis* kinetochores and might play a role in kinetochore–microtubule attachments[83]. Finally, the regulatory protein Shugoshin (Sgo1; also known as MEI-S332) has been suggested to interact with microtubules[84], in addition to its role in protecting centromeric cohesion (see below), although this protein primarily localizes to the inner centromere and not to the outer kinetochore.

Controlling microtubule dynamics. Movement of chromosomes towards the spindle poles is coupled to depolymerization of microtubules at kinetochores, whereas movement away from the poles is coupled to microtubule polymerization[85]. A number of kinetochore-localized proteins have activities that implicate them in the active control of the polymerization state of kinetochore microtubules. These include microtubule-depolymerizing proteins such as the kinesin-13 family of depolymerases and the dual motor and microtubule-depolymerase kinesin-8 (also known as Kip3 in yeast)[86] (FIG. 5b). Conversely, the widely conserved microtubule-binding protein cytoplasmic linker protein (CLIP)-associating protein (CLASP) localizes to kinetochores, where it promotes the growth of kinetochore-bound microtubules[87–89] (FIG. 5b). The plus ends of microtubules are embedded at kinetochores, and there are also various microtubule-plus-end-associated proteins, including CLIP170, EB1 and MAP215 (also known as Stu2 in yeast), that accumulate in the vicinity of the outer kinetochore and that have been linked to kinetochore function. CLIP170 localizes to kinetochores even after microtubule depolymerization and has been implicated in generating initial interactions between kinetochores and microtubules[90]. EB1 has recently been shown to bind and stabilize the seam of the microtubule lattice[91] (FIG. 5b). MAP215 promotes microtubule polymerization and is essential for spindle assembly and chromosome segregation in all eukaryotes[92].

Coupling binding to polymerization dynamics. Core attachment sites at the outer kinetochore must not only bind to microtubules, but must also possess the ability to couple movement of the chromosome to microtubule-polymerization dynamics. Multiple independently acting Ndc80 complexes bound to a single microtubule might allow the kinetochore to remain bound to a dynamic microtubule (FIG. 5c). In addition, studies nearly a decade ago showed that kinesins can function as couplers to depolymerizing microtubules and indicated that CENP-E could perform such a function at kinetochores in vertebrates[93] (FIG. 5c). However, CENP-E loss-of-function does not cause catastrophic chromosome alignment or segregation defects, although there is consistent mis-segregation of a small number of chromosomes[3,94].

Work in budding yeast has identified the 10-subunit Dam1 complex, which forms an oligomeric ring, comprised of ~16 complexes, around microtubules[95–98]. The Dam1 complex couples movement of cargo to depolymerizing microtubules and also promotes tension-dependent polymerization of microtubules[99–102] (FIG. 5c). The Dam1 complex has not yet been found outside of fungi and is non-essential in fission yeast[103], in which there are several microtubules present at each kinetochore. This raises the possibility that its essential function in budding yeast is related to the requirement to maintain an attachment to the single kinetochore microtubule in this organism. However, it remains possible that an equivalent, as-yet uncharacterized, complex works together with the KMN network to provide robust coupling to growing and shrinking microtubules in metazoans.

Kinetochore motility. Kinetochores also associate laterally with and translocate along microtubules polymers (FIG. 5d). Two microtubule-motor proteins — dynein (minus-end directed) and CENP-E (plus-end directed) — have been implicated in this type of chromosome movement in vertebrates[79,104,105]. During prometaphase, dynein-powered poleward movement along the sides of microtubules is proposed to facilitate end-on interactions at the kinetochore outer plate by moving a chromosome into the proximity of the spindle poles, where the microtubule density is high (FIG. 5d). In yeast, the minus-end directed kinesin Kar3 might perform a similar function[102,106]. Plus-end directed CENP-E motility along the kinetochore–microtubule fibres of already aligned chromosomes is proposed to facilitate movement of chromosomes that are trapped close to a spindle pole towards the spindle equator[105] (FIG. 5d). Consistent with this proposal, the majority of chromosomes in CENP-E-depleted cells can align properly, but a subset remains at the poles and is missegregated[94,107].

Overall, there are therefore a number of different microtubule-directed activities present at the kinetochore, and these function together to capture microtubules and generate robust, yet dynamic, end-on attachments where movement is coupled to changes in the growth and shrinkage of bound microtubules (FIG. 5e). Many questions remain about how these components contribute to kinetochore–microtubule interactions,

Motor proteins
Mechanochemical enzymes that couple ATP hydrolysis to movement along a polymer lattice. The two major microtubule-directed motor protein families are the kinesins and dyneins.

Seam
Microtubules are composed of a series of linear protofilaments, which laterally associate and close to form a hollow cylindrical polymer. The seam is formed at the point of closure and is characterized by a change in the lateral tubulin–tubulin interactions relative to elsewhere in the polymer.

Cargo
Proteins that are carried along the polymer lattice by motor proteins.

and understanding how the distinct activities of these components are coordinated remains a major future challenge.

Maintaining fidelity

Accurate chromosome segregation is crucial for viability and cell division. Multiple regulatory mechanisms function at kinetochores to ensure the fidelity of cell division. These include a set of checkpoint proteins that monitor correct kinetochore–microtubule attachments, as well as signalling proteins that control aspects of kinetochore function and coordinate kinetochore activities with cell-cycle progression.

The mitotic checkpoint. To ensure the fidelity of chromosome segregation, the mitotic checkpoint senses defects in kinetochore–spindle-microtubule attachments and prevents cell-cycle progression until all chromosomes in a cell are correctly connected to the spindle (FIG. 5e). The best studied components of the mitotic checkpoint pathway (Mad1, Mad2, Mad3 (also known as BubR1), Bub1, Bub3 and Mps1) were first identified in budding yeast. All of these proteins transiently localize to kinetochores, and Mad1, Mad2 and Mad3 are progressively depleted by microtubule attachment (FIG. 4). Recent studies have identified additional components that are required for checkpoint signalling in metazoans, including CENP-E[108], the RZZ complex[67], the microtubule-affinity regulating kinase (MARK)-family kinase TAO1 (REF. 109), Polo-like kinase-1-interacting checkpoint helicase (PICH)[110], which localizes to the inner centromere region, and the deubiquitylase USP44, which is proposed to control the cytosolic ubiquitylation status of the spindle-checkpoint target CDC20 (REF. 111). Dynein at kinetochores has been proposed to function in checkpoint inactivation[78], and a protein called Spindly was recently identified as being important in dynein recruitment and checkpoint silencing[112]. The mechanisms underlying spindle-checkpoint activation and inactivation have been reviewed recently[113] and are not discussed further here owing to space constraints.

Regulating kinetochore function. In addition to signalling to the cell cycle in order to halt mitotic progression until sister kinetochores are correctly attached to the spindle, several regulatory pathways control kinetochore assembly and the formation of bipolar kinetochore–microtubule attachments. Chief among these are six mitotic protein kinases: Aurora B, Polo-like kinase-1 (PLK1)[114], cyclin-dependent kinase-1 (CDK1) and the checkpoint kinases MPS1 (REFS 115,116), BUB1 (REFS 117,118) and BUBR1 (REF. 119), which have roles in chromosome segregation in addition to their functions in checkpoint signalling. The development and use of specific small-molecule inhibitors for many of these kinases is facilitating the dissection of the multiple functions of these proteins during mitosis[120].

The four-subunit chromosomal passenger complex, which includes the Aurora B kinase, is required for correcting errors in kinetochore–microtubule attachments[121]. In cases in which both sister kinetochores attach to microtubules from the same spindle pole, these sister kinetochores are not under tension. This lack of tension is proposed to activate the kinase activity of Aurora B, possibly by a mechanism that is intrinsic to the passenger complex subunits inner centromere protein (INCENP) and survivin[122]. Activated Aurora B phosphorylates kinetochore-bound substrates, which results in the destabilization of kinetochore–microtubule attachments[123]. There are several identified substrates for Aurora B at kinetochores, including the Dam1 ring complex[124], the microtubule-depolymerizing kinesin MCAK[125,126] and the Ndc80 complex[58,124,127] (FIG. 5a,b). In particular, Aurora B-dependent phosphorylation of the Ndc80 subunit of the Ndc80 complex decreases the microtubule-binding affinity[58] of this complex, which provides a potential direct mechanism for eliminating incorrect kinetochore–microtubule attachments.

PLK1 also regulates several aspects of kinetochore assembly, chromosome segregation and mitotic checkpoint function[128]. PLK1 is responsible for generating a tension-sensitive phosphoepitope on kinetochores[129,130]. PLK1 associates with several kinetochore proteins, including CENP-U (also called PBIP1)[131] and PICH, through its Polo-box domain[110]. PLK1 phosphorylation of downstream substrates, including both CENP-U[131] and nuclear distribution protein C (NUDC)[132], appears to help to recruit PLK1 to kinetochores.

Protein phosphatases that counteract the mitotic kinases and that are localized to kinetochores and/or inner centromeres have also been identified. Protein phosphatase-1 localizes to kinetochores and opposes Aurora B phosphorylation[133,134]. Sgo1 is present in a complex with protein phosphatase-2A (PP2A) and is required to localize PP2A to centromeres[135–137], where PP2A prevents the proteolytic cleavage of centromeric cohesin complexes (and probably has additional functions). A recent study has shown that SGO2 (also known as Tripin), which shares a small region of homology with Sgo1 and also associates with PP2A[135], is required to localize MCAK to the inner centromere, potentially by modulating a local kinase–phosphatase balance[138].

Other post-translational modifications also regulate kinetochore proteins and their activities. The methyltransferase Set1 interacts genetically with Aurora B kinase[139] and might directly methylate kinetochore proteins in yeast. The small ubiquitin-like modifier protein SUMO modifies and affects the localization and function of inner kinetochore proteins in budding yeast[140] and the GTPase-activating protein RanGAP1 in vertebrates[141], which controls the GTP hydrolysis of the regulatory protein Ran[142] (see below).

Nuclear pores at kinetochores

Although the majority of kinetochore proteins described above localize exclusively to kinetochores, some localize to other cellular structures during interphase. The ZW10 subunit of the RZZ complex localizes to the endoplasmic reticulum and functions in membrane trafficking[143], CLASP localizes to the Golgi where it contributes to microtubule organization[144], and CENP-F localizes to the nuclear envelope by farnesylation of its C terminus[145].

Mitotic checkpoint pathway
The signal transduction pathway that is responsible for detecting chromosomes that are improperly attached to the mitotic spindle and arresting the cell cycle during metaphase until these errors have been corrected.

Helicase
An enzyme that unwinds double-stranded nucleic acids in an energy-dependent manner.

Polo-box domain
The portion of the polo kinase that is responsible for binding to substrates.

GTPase-activating protein
A protein that inactivates small GTP-binding proteins, such as Ras-family members, by increasing their rate of GTP hydrolysis.

AT-hook domain
A nine-amino-acid protein domain that binds to the minor groove of A and T rich DNA.

Nucleoporins
Protein subunits of the nuclear pore complex.

Aneuploidy
The ploidy of a cell refers to the number of chromosome sets that it contains. Aneuploid karyotypes are chromosome complements that are not a simple multiple of the haploid set.

In addition, a number of nuclear pore proteins relocalize to kinetochores following nuclear envelope breakdown. This group includes the ten-subunit Nup107–160 complex[146,147] and the associated AT-hook domain protein ELYS (also known as MEL-28 in *C. elegans*) (REFS 148–150). Kinetochore-localized nucleoporins have important roles in chromosome segregation that are unrelated to their function at nuclear pores[146]. One potential role for the Nup107–160 complex might be in the recruitment of components of the Ran-GTPase signalling pathway, including the nuclear export factor CRM1 and a complex of RanGAP1 and Ran binding protein-2 (RanBP2), to kinetochores[146]. The Ran pathway has been suggested to have several functions at kinetochores during chromosome segregation[151]. A distinct function of relocalizing nuclear pore proteins to kinetochores might be in the reassembly of the nuclear envelope around the segregated chromosomes at the end of mitosis[149]. Finally, many mitotic checkpoint proteins are localized to the nuclear pore during G2–prophase, which indicates a possible connection between nucleoporins and checkpoint signalling[151].

Conclusions and perspectives

The kinetochore has a fundamental role in facilitating chromosome segregation during cell division. Here, we have highlighted the stable components of this structure and their functions as defined from *in vivo* and *in vitro* studies. The nature of the interactions between the different components that help make the kinetochore a suitable 'handle' for pulling and moving chromosomes, the mechanism of action of individual proteins and complexes, the means for integrating the actions of different proteins, the regulation of this entire structural ensemble, and the interplay between the mechanics and regulatory pathways all remain major topics for future investigation.

In addition to elucidating the basic mechanisms of chromosome segregation, studies of the kinetochore are proving relevant to cancer aetiology and treatment. Severe problems in chromosome segregation cause cell death, whereas minor errors result in aneuploidy, which occurs in many tumour cells and which has been suggested for more than a century to promote tumorigenesis[152]. Multiple components of the kinetochore have been implicated in tumour progression or are correlated with tumour prognosis, including the mitotic checkpoint[153] and the structural components that we have focused on above[154–158]. As the kinetochore is required specifically in actively dividing cells, it represents an attractive target for anti-mitotic chemotherapy. Indeed, inhibitors for Aurora kinases, PLK1 and CENP-E are currently in early-stage clinical trials. Future work will continue to define the basic molecular mechanisms by which kinetochore proteins function together to facilitate chromosome segregation, and will hopefully also contribute new diagnostic and therapeutic approaches to cancer.

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DATABASES

UniProtKB: http://ca.expasy.org/sprot
CENP-A | CENP-B | CENP-C | CENP-E | CENP-H | CENP-I | MCAK | PLK1 | Sgo1 | SGO2

FURTHER INFORMATION

Arshad Desai's homepage:
http://cancer.ucsd.edu/summaries/abdesai.asp
Iain Cheeseman's homepage: http://mit.edu/biology/www/facultyareas/facresearch/cheeseman.html

SUPPLEMENTARY INFORMATION

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