The molecular basis for centromere identity and function

Kara L. McKinley and Iain M. Cheeseman

Abstract | The centromere is the region of the chromosome that directs its segregation in mitosis and meiosis. Although the functional importance of the centromere has been appreciated for more than 130 years, elucidating the molecular features and properties that enable centromeres to orchestrate chromosome segregation is an ongoing challenge. Most eukaryotic centromeres are defined epigenetically and require the presence of nucleosomes containing the histone H3 variant centromere protein A (CENP-A; also known as CENH3). Ongoing work is providing important molecular insights into the central requirements for centromere identity and propagation, and the mechanisms by which centromeres recruit kinetochores to connect to spindle microtubules.

The transmission of an intact genome to daughter cells during cell division is a fundamental requirement for the viability of cells and organisms. In eukaryotes, DNA is packaged into chromosomes, which must be faithfully replicated and segregated during cell division. To achieve accurate segregation, chromosomes rely on a specialized region known as the centromere. The centromere recruits the kinetochore, which is a proteinaceous macromolecular structure that forms attachments to the microtubules of the mitotic and meiotic spindles. Together, centromeres and kinetochores are the central players in chromosome segregation. Defects in centromere or kinetochore function can lead to the loss or disruption of genomic information, resulting in developmental defects or disease[1].

The crucial function of the centromere has been appreciated for more than 130 years. The centromere was first observed by light microscopy as the chromosomal attachment site for spindle microtubules in dividing cells[2] (FIG. 1a). As the centromere protects and maintains sister chromatid cohesion during mitosis and meiosis[3–6], this region of the chromosome is also visible in many organisms as the primary constriction on condensed mitotic chromosomes (FIG. 1b). Geneticists subsequently combined these cytological observations with the analysis of recombinant progeny to define the positions of genes relative to the centromere, and thereby translate genetic maps onto physical ones[7,8].

Although the centromere has been described extensively by cytological and genetic approaches, defining the molecular features that confer its functions is a central ongoing pursuit[9]. When first defining the term centromere in 1936, Cyril Darlington commented that "[the centromere must] be considered in terms of function rather than form, since the function is evident and the form elusive" (REF. 10). Elucidating the 'form' of centromeres has remained challenging, because centromeres require numerous molecular features that vary across eukaryotes. Despite this complexity and variation, several common themes have emerged regarding the molecular basis of centromere function. In the vast majority of eukaryotes, centromere specification is primarily epigenetic and depends on the presence of specialized nucleosomes containing the histone H3 variant centromere protein A (CENP-A; also known as CENH3). Centromere function requires the combination of CENP-A-containing nucleosomes, features of the underlying DNA sequence, unique combinations of chromatin marks, and interactions with kinetochore proteins.

In this Review, we highlight recent work on the molecular basis of centromere function, with a focus on the vertebrate centromere. We describe the current understanding of the genetic and epigenetic features that define centromeres, the mechanisms of centromere propagation and the recognition of the centromere by the kinetochore. This work is revealing Darlington's elusive 'form' underlying the crucial functions of the centromere in the propagation of the genome to cells and gametes.

Centromere DNA structure and function

In the majority of eukaryotes analysed to date, the centromere is specified epigenetically (BOX 1), such that specific DNA sequences are neither strictly necessary nor sufficient for centromere function. Instead, the unifying characteristic of most eukaryotic centromeres is the presence of CENP-A. Nonetheless, recent work has

Whitehead Institute and Department of Biology, MIT, Nine Cambridge Center, Cambridge, Massachusetts 02142, USA.
Correspondence to K.L.M. and I.M.C.
kara.l.mckinley@gmail.com; icheese@wi.mit.edu

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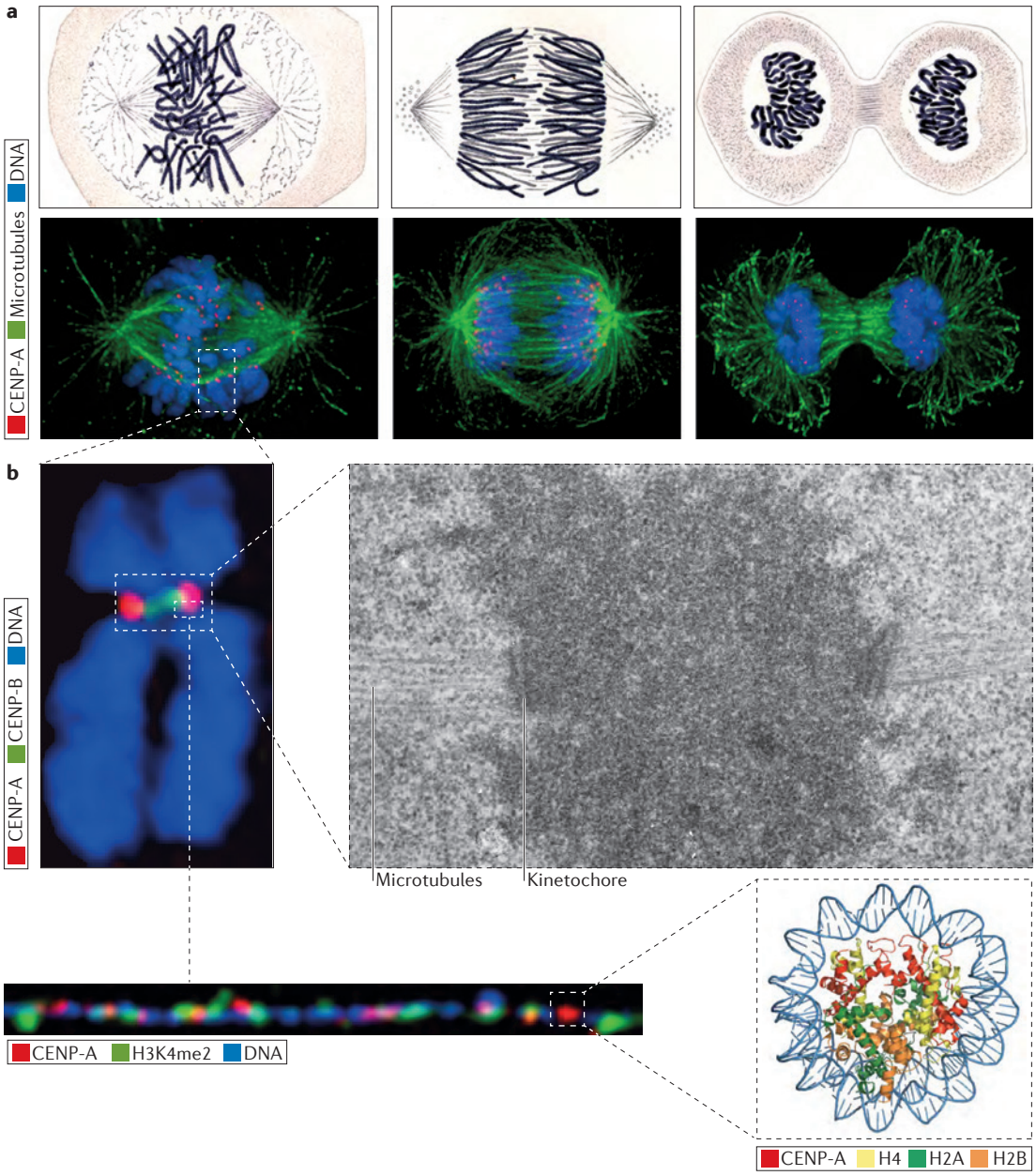
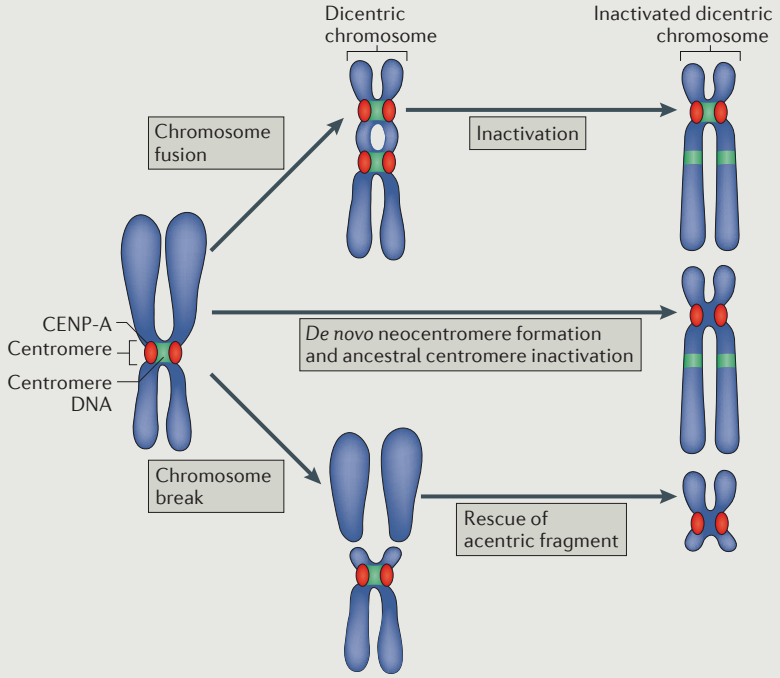


Figure 1 | **Visualization of the centromere. a** | Comparison of images of mitotic Salamander cells hand-drawn by Walther Flemming in 1882 (REF. 2) (top) with immunofluorescence images of human cells (bottom) stained for microtubules, centromere protein A (CENP-A) and DNA. The images show cells at different phases of a mitotic cell cycle: late prometaphase–metaphase (left), anaphase (middle) and telophase (right). **b** | Images of the centromere at increasing resolution. Top left: immunofluorescence image of a mitotic chromosome stained for DNA, CENP-A and CENP-B (a marker for the α-satellite DNA repeats that are present at most human centromeres). Top right: electron micrograph of the centromeric region of a mitotic chromosome showing centromeric chromatin (dark cloud), kinetochore and microtubules. Bottom left: immunofluorescence image of a stretched centromeric chromatin fibre showing patches of CENP-A interspersed with histone H3, in this case specifically H3 dimethylated on Lys 4 (H3K4me2). Image courtesy of Elaine Dunleavy, adapted from REF. 101. Bottom right: crystal structure of the CENP-A nucleosome[90] (RCSB Protein Data Bank (PDB) ID: 3AN2). Part **b**, top right, adapted from REF. 215. Republished with permission of the American Society of Cell Biology, from 'Depletion of centromeric MCAK leads to chromosome congression and segregation defects due to improper kinetochore attachments'; Kline-Smith SL, Khodjakov A, Hergert P, Walczak CE. *Mol. Biol. Cell* **15**:3, 2004 permission conveyed through Copyright Clearance Center, Inc.

highlighted evolutionary and functional preferences for specific DNA structures that strongly indicate that they contribute to centromere function, as we describe in this section.

A common structure for centromeric DNA sequences. Most eukaryotes have monocentric chromosomes, in which a centromere is assembled at a single localized region (FIG. 2a). Notable exceptions are some nematodes

Box 1 | **Evidence for the epigenetic nature of the centromere**



The first evidence that the centromere is specified epigenetically came from human patient samples containing dicentric chromosomes in which one centromere was functionally inactivated without changes to its underlying DNA sequence[70] (see the figure, top). In subsequent work, epigenetic centromere inactivation was observed in dicentric chromosomes in diverse contexts[199–201]. Centromere inactivation is also frequently observed in Robertsonian fusions[202] and in isodicentric Y chromosomes generated by sister chromatid recombination of Y chromosome palindromes[203]. These data indicate that centromere sequences are not sufficient for centromere function.

Compelling evidence that centromere sequences are not necessary for centromere function comes from centromeres at atypical sites, termed neocentromeres (reviewed in REF. 69; see the figure, middle and bottom). For example, routine karyotyping of a human patient in 1993 revealed a chromosome fragment that had lost its centromeric DNA but was nonetheless stably maintained in mitosis, assembled a functional kinetochore and mediated sister chromatid cohesion in the absence of canonical underlying DNA repeats[54]. Subsequent work revealed cases of inherited neocentromeres, demonstrating that these structures are stable in both mitosis and meiosis[204,205]. Neocentromeres have also been generated experimentally in diverse organisms by selecting for their ability to rescue acentric chromosomal fragments[126,206–209]. Neocentromeres have been observed in otherwise normal karyotypes, in which the centromere DNA sequences remain intact but have lost centromere function[205] (see the figure, middle), reinforcing the insufficiency of centromere sequences that was proposed by observation of dicentric chromosome inactivation. CENP, centromere protein.

(including *Caenorhabditis elegans*), and some insects and plants, which assemble a diffuse centromere along the entire length of the chromosome, a phenomenon known as holocentricity[11] (FIG. 2a). Species with monocentric chromosomes can either have point centromeres, containing short DNA sequences, or regional centromeres (FIG. 2a), which contain kilobases to megabases of DNA[12]. Point centromeres are found in some budding yeasts[12], including *Saccharomyces cerevisiae*[13], and are defined as those centromeres in which the precise centromeric DNA sequence is necessary and sufficient for kinetochore assembly and DNA segregation[14–16]. Regional centromeres are typically composed of repetitive DNA sequences that contribute to, but are not sufficient for, centromere function. However, some organisms contain regional centromeres that are non-repetitive, such as the yeast *Candida albicans*[17], or have a mixture of repetitive centromeres and non-repetitive centromeres, such as orang-utan[18], horse[19] and chicken[20]. Repetitive centromeres consist of retrotransposons and/or long arrays of simple tandem repeats, referred to as satellite DNA[21].

The precise DNA sequences found at centromeres vary dramatically across evolution, and it has been proposed that this rapid evolution is a consequence of meiotic drive[22]. Despite the divergence in centromere sequences, regional centromeres possess a modular structure that is shared by many taxa. Regional centromeres typically consist of a central core, which is where the CENP-A nucleosomes reside and is composed of homogenous ordered repeats; and an outer heterochromatic domain, termed the pericentromere, that typically contains less ordered repeats (FIG. 2a,b). For example, centromeres of the fission yeast *Schizosaccharomyces pombe* contain a centromere core of non-repetitive sequences flanked by perfect inner inverted repeats and less ordered outer repeats[23]. Similarly, the *Mus musculus* centromere core is composed of minor satellite arrays containing homogenous 120 bp repeats flanked by less ordered ~234 bp major satellite repeats[24]. Primate centromeres are built on arrays of a 171 bp monomer termed α-satellite DNA[25–28]. In humans and other great apes, monomers are arranged head-to-tail to form higher-order repeats that are themselves reiterated across the centromere core. The human pericentromere contains flanking monomers that lack higher-order repeats and that have reduced identity between monomers (see REF. 29 for a further review of centromeric DNA structure) (FIG. 2b). Thus, centromeres frequently arrange their divergent centromere sequences in a common repetitive structure.

Evolutionary preference for repetitive DNA structures. Cytogenetic comparisons between closely related species have revealed that some centromeres adopt new positions over evolutionary time subsequent to a speciation event without transposing the surrounding genetic markers, a phenomenon known as centromere repositioning[30] (FIG. 2c). These structures are referred to as evolutionary new centromeres (ENCs) and have been observed in primates and other mammals (reviewed in REF. 31) and in birds[32]. A striking property of ENCs is that they typically contain the same molecular features as do the 'old' centromeres within the karyotype, including the species-specific satellite DNAs. For example, all nine ENCs in macaque contain α-satellite arrays and large segmental duplications, making them indistinguishable from 'old' macaque centromeres[33]. Thus, it is postulated that ENCs are seeded upon new, non-repetitive DNA sequences in a manner analogous to neocentromeres (BOX 1) but subsequently acquire their species-specific satellite DNA over time. The recent ENCs on orang-utan chromosome 9 and horse chromosome 11 have not acquired satellite DNA and

Meiotic drive
Preferential transmission of a genetic element during meiosis, such that it is represented in more than 50% of the gametes of a heterozygote.

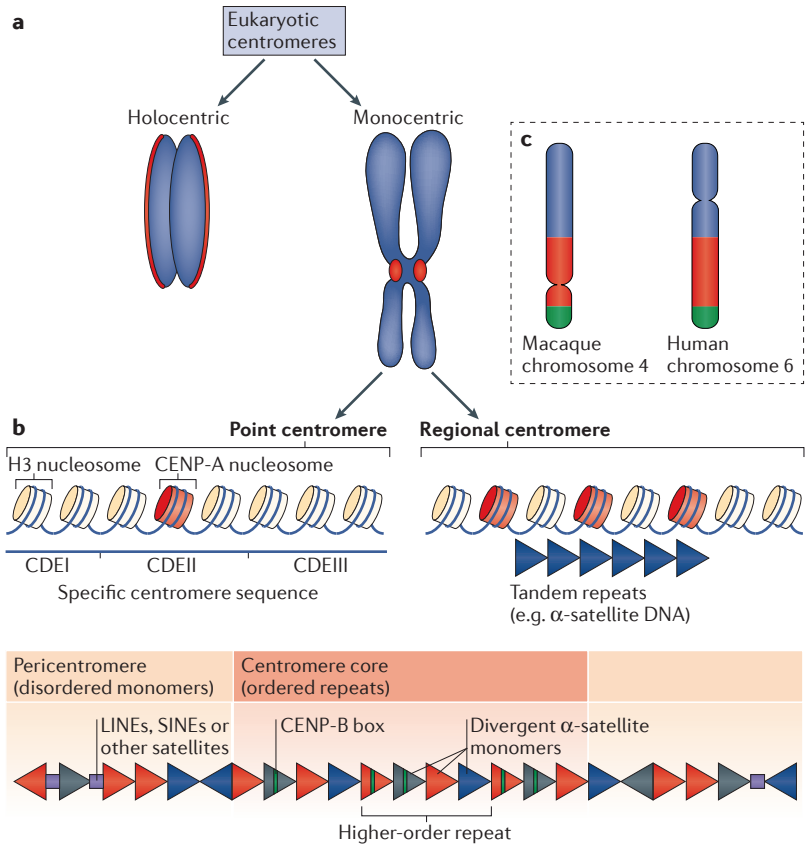


Figure 2 | **Centromere specification. a** | Diverse types of centromeres are found across eukaryotes. Holocentric chromosomes assemble a diffuse centromere across the whole chromosome. Monocentric chromosomes assemble a centromere at a single localized site on the chromosome, which is visible as a constriction between the chromosomes in mitosis (known as the primary constriction). Monocentric chromosomes can be further classified into those with point centromeres and those with regional centromeres. Point centromeres contain a specific DNA sequence that is sufficient for centromere function (here illustrated with the *Saccharomyces cerevisiae* DNA architecture), which assembles a single centromere protein A (CENP-A) nucleosome. Regional centromeres contain large regions of DNA that is often repetitive (such as α-satellite DNA in primates) and assemble numerous CENP-A nucleosomes. **b** | Primate centromeres are built from α-satellite monomers (depicted as triangles), which are largely but not completely identical, as indicated by the different coloured triangles. Patterns of these monomers arranged head-to-tail are reiterated over the centromere core (red) as higher-order repeats. Some monomers within the centromere core contain a sequence termed the CENP-B box (green), which binds to the centromere DNA-binding protein CENP-B. The centromere core is flanked by less ordered monomers, which comprise the pericentromere (orange). Long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs) and other satellites (squares) are found interspersed with α-satellite monomers in the pericentromere[216]. **c** | Schematic showing a comparison between macaque and human orthologous chromosomes that have undergone centromere repositioning such that the position of the centromere has moved but the surrounding markers have not, as indicated by the colour blocks, which represent syntenic regions. CDE, centromere DNA element; H3, histone H3. Part **c** adapted from Ventura, M. *et al.* Evolutionary formation of new centromeres in macaque. *Science* **316**, 243–246 (2007). Reprinted with permission from AAAS.

may be intermediates in this maturation process[18,19]. Chromosomes harbouring ENCs also exhibit a decay of the satellite sequences at the ancestral site[34]. The acquisition of a modular structure of tandem repeats by ENCs further supports a contribution of such DNA structures to centromere function.

Contributions of DNA sequences to centromere function. As described above, centromere function in organisms with point centromeres strictly depends on the centromeric DNA sequence. Thus, these sequences can confer mitotic and meiotic stability when introduced into exogenous minichromosomes in organisms with point centromeres, such as budding yeast[13]. In organisms with regional centromeres, specific sequences are not necessary or sufficient for centromere function in some contexts (BOX 1). However, regional centromere DNA sequences can also confer centromere function on exogenous DNA in some organisms, including *S. pombe*[35] and primates[36], indicating that they can have a role in the *de novo* specification of a centromere.

Extensive work has sought to use α-satellite DNA to build human centromeres *de novo* and generate human artificial chromosomes (HACs). In pioneering work, cloned α-satellite DNA from human chromosomes enabled linear human minichromosomes[37] and yeast artificial chromosomes[38] to be stably inherited in human cells. These systems demonstrated that α-satellite DNA was sufficient to initiate centromere formation. The analysis of HAC formation also permitted structure–function studies of the α-satellite DNA, revealing a key role for the higher-order repeats[39]. The mechanisms by which α-satellite DNA sequences initiate centromere formation are the subject of current investigations. Recently, it was suggested that α-satellite arrays adopt chromatin marks that favour the deposition of CENP-A nucleosomes (see below)[40,41]. Together, this work is beginning to bridge the gap between the centromere DNA sequences and the epigenetic marks that are required for centromere function.

DNA sequence-dependent binding proteins at the centromere. For specific DNA sequences to confer centromere functions, they must be recognized by proteins that recruit the chromosome segregation machinery. This may occur through generating a permissive environment for particular epigenetic marks or through interactions with sequence-specific DNA binding proteins. At the point centromeres of budding yeast, the centromere DNA element III sequence (CDEIII) is recognized by the sequence-specific binding protein Cbf3 (REF. 42), providing a straightforward link between centromere sequence and function. However, potential roles for a sequence-specific DNA-binding protein are more challenging to predict in organisms with regional centromeres, particularly because centromere sequences vary dramatically across species, whereas centromere proteins are largely conserved. The only known centromere sequence element that is conserved between primates and rodents is the CENP-B box[43,44], a 17 bp sequence that binds to the protein CENP-B[45]. The CENP-B box is found in the minor satellite of *M. musculus* and some monomers within the higher-order repeats of human α-satellite arrays. Although *M. musculus* and great apes share the CENP-B box, some primates lack CENP-B boxes[46], and the rodent *Mus caroli* contains a divergent CENP-B box that retains the nine base pairs required for CENP-B binding[47].

Owing in part to its inconsistent conservation, the importance of the CENP-B box and of the protein itself remain poorly understood. CENP-B directly interacts with and stabilizes both CENP-A nucleosomes and the kinetochore protein CENP-C to contribute to centromere function[48–50]. However, *Cenpb*-knockout mice are viable[51–53], and neocentromeres are maintained without acquiring CENP-B-binding capability[54]. Perhaps most intriguingly, the human Y chromosome centromere lacks CENP-B boxes[43] and does not bind to detectable CENP-B protein[55]. Similarly, the Y chromosome of *M. musculus* lacks the minor satellite sequences that contain the CENP-B box[56]. However, Y chromosome sequences are not sufficient to generate HACs without acquiring other centromeric α-satellites from the host cells[37,57], and HAC formation requires the CENP-B box[39,58]. Together, these data indicate that CENP-B, like the centromere sequences it binds, is not strictly required at the centromere but makes functional contributions to maximize mitotic fidelity that contribute particularly to the generation of centromeres *de novo*.

Centromere epigenetics

Despite contributions from the DNA sequences and structures present at centromeres, centromere identity is defined epigenetically in most eukaryotes (BOX 1). Below, we describe the specialized centromeric chromatin that marks this region of the chromosome.

CENP-A is an epigenetic hallmark of centromeres. In most eukaryotes, the defining feature of centromeres is the presence of nucleosomes containing CENP-A. CENP-A was first identified as a centromere-specific antigen recognized by antibodies from human patients with the autoimmune disease CREST syndrome[45]. Concurrent and subsequent work found that CENP-A was a component of chromatin with biochemical similarity to histones[59–62], and that it shared homology with histone H3 (REFS 61,63). CENP-A homologues have been identified in diverse eukaryotes on the basis of their similarity to H3 (REFS 64–66). As a centromere-specific H3 variant, CENP-A is a compelling candidate for an epigenetic mark of centromere identity[67,68]. Consistent with a fundamental requirement for CENP-A in centromere function, CENP-A is found at all identified neocentromeres[69], as well as at the active centromeres of dicentric chromosomes[70], and is essential for the localization of all known kinetochore components[48,71,72]. Importantly, artificial targeting of CENP-A to an ectopic chromosomal locus is also sufficient to generate structures that are capable of directing microtubule attachment and chromosome segregation[73–76].

CENP-A nucleosomes possess unique structural properties. The existence of a centromere-specific histone raises intriguing possibilities regarding how CENP-A is specialized to mark the position of the centromere and recruit downstream kinetochore proteins. At the sequence level, CENP-A contains two important regions: a histone-fold domain that has 62% sequence identity with H3 in humans; and an amino-terminal tail that differs more significantly from H3 (REF. 63) and even between CENP-As from different species[77] (FIG. 3a). Within the histone-fold domain, the first loop and second α-helix (L1–α2) are necessary for targeting CENP-A to the centromere and are sufficient to confer centromere targeting when introduced into chimeras with H3 (REFS 78,79). Therefore, this region is referred to as the CENP-A-targeting domain (CATD) (FIG. 3a). Sequences within CENP-A nucleosomes also confer centromere-specific functions through the direct binding of the core kinetochore proteins CENP-N and CENP-C (FIG. 3a). In particular, CENP-N binds directly to the CATD of CENP-A[76,80,81]. CENP-C makes extensive contacts with the CENP-A nucleosome: with the six residues of the CENP-A carboxy-terminal tail[80,82,83]; with other histones within the CENP-A nucleosome[82]; and with the CENP-A CATD[76,84]. The CENP-A N-terminal tail has also been implicated in the recruitment of kinetochore proteins in various organisms[48,76,85–87]. Thus, variations between CENP-A and H3 at the sequence level confer centromere specificity and kinetochore assembly properties on CENP-A.

CENP-A nucleosomes also have structural distinctions from canonical H3-containing nucleosomes, with the potential to make contributions to centromere function. The structural properties of the CATD make the free $(CENP\text{-}A\text{–}H4)_2$ tetramer more conformationally rigid than the $(H3\text{–}H4)_2$ tetramer, as determined by hydrogen–deuterium exchange, and cause the CENP-A–CENP-A interface to be rotated when compared to the H3–H3 interface in a canonical nucleosome, generating a more compact structure[88,89]. However, in the crystal structure of the octameric nucleosome, the CENP-A–CENP-A axis appears similar to the H3–H3 axis from canonical nucleosomes[90]. Recent work indicates that CENP-A nucleosomes in solution sample both forms, and that binding of CENP-C shifts the nucleosome to the state similar to that of canonical nucleosomes[91]. In addition, there is an extensive, ongoing debate regarding whether the CENP-A nucleosome forms a hemisome (with one molecule each of CENP-A, H4, H2A and H2B) that wraps DNA in a right-handed manner, or an octamer (reviewed in REF. 92). Finally, CENP-A nucleosomes confer structural alterations on centromeric chromatin. For example, CENP-A arrays are more condensed[93,94], but with a DNA entry and exit site that is loose compared to canonical nucleosomes[90,93–96], a property that is enhanced by CENP-C binding[91]. Thus, sequence and structural specializations of CENP-A nucleosomes and CENP-A-containing chromatin generate fundamental distinctions between centromeric chromatin and bulk chromatin.

Centromere propagation

Faithful centromere inheritance is crucial for the transmission of the genome, as failure to propagate the centromere results in the inability of a chromosome to attach to the mitotic spindle, leading to loss of the chromosome and the information that it encodes. On monocentric

Evolutionary new centromeres
(ENCs). Centromeres at a different site from the centromere of the chromosome ancestor, for which the movement of the centromere cannot be parsimoniously explained by a simple chromosome rearrangement.

Neocentromeres
Regions of chromosomes that have the functional characteristics of a centromere, but occur at a site distinct from the site of centromere formation for the chromosome in most organisms of the species, and lack canonical centromere DNA sequences.

Human artificial chromosomes
(HACs). Units of exogenous DNA that segregate autonomously in human cells.

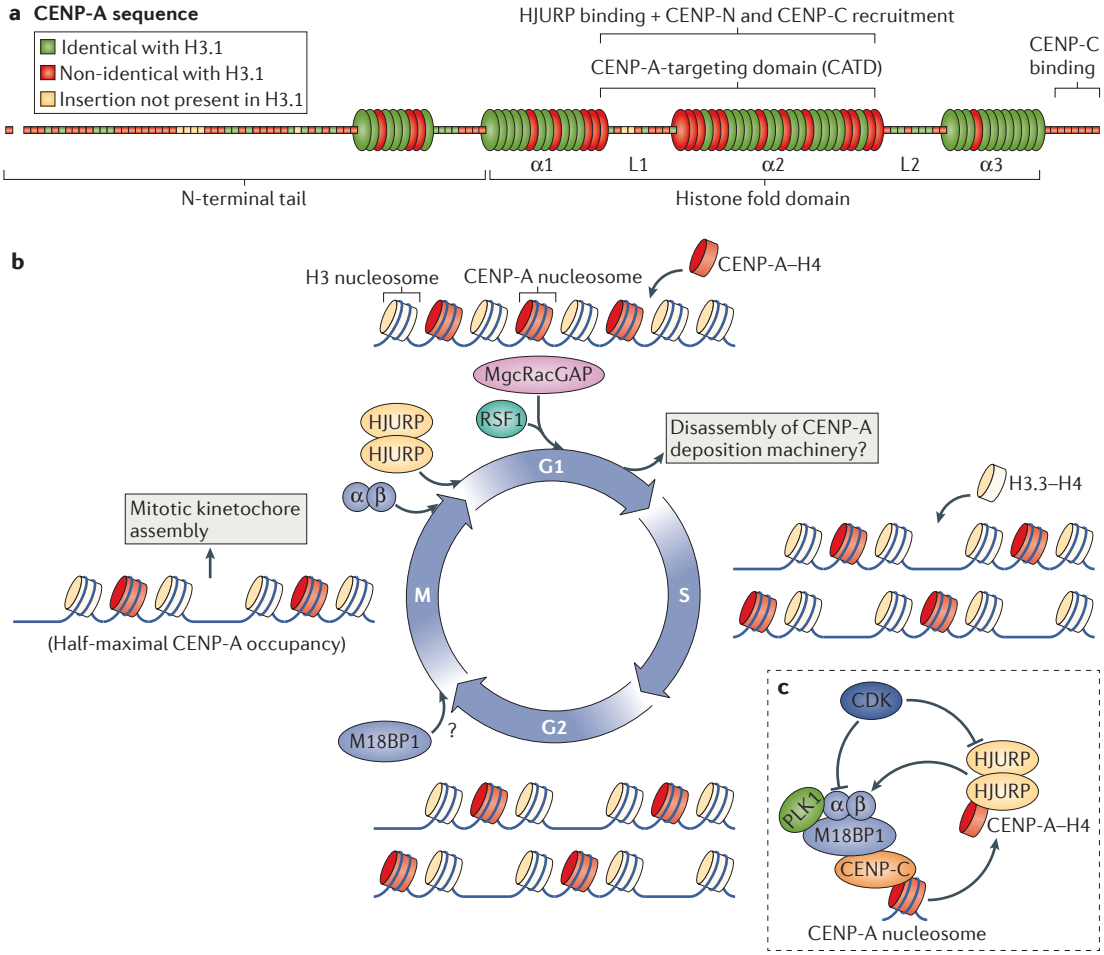


Figure 3 | **Specialization and propagation of centromere protein A (CENP-A). a** | Model of human CENP-A primary and secondary structure, showing conservation with histone H3. Each segment corresponds to a single amino acid and is coloured according to its conservation with human H3.1, as indicated. The first amino-terminal amino acid, shown detached, represents the cleaved initiator Met. Barrels represent α-helices, and rods represent loops. Within the histone-fold domain, the helices are designated α1 to α3, and the loops are designated L1 and L2. L1 and α2 comprise the CENP-A-targeting domain, which is sufficient to target CENP-A to centromeres, owing to its interaction with the CENP-A chaperone HJURP. This region also binds to CENP-N[81] and is important for CENP-C recruitment[76,84]. CENP-C also binds to the carboxy-terminal residues of CENP-A[80,82,83]. **b** | Model for the changes to CENP-A chromatin during the cell cycle. The timing of the localization of the CENP-A deposition factors is indicated. At S phase, existing CENP-A is partitioned between the replicated sisters, and gaps are filled with histone H3.3. Although centromere localization of MIS18-binding protein 1 (M18BP1) precedes recruitment of MIS18α and MIS18β[116], the precise onset of its localization has not been established. By mitosis, M18BP1 localizes to centromeres, followed by MIS18α and MIS18β at mitotic exit. An HJURP dimer[217] is recruited in early G1 phase to direct new CENP-A deposition. New CENP-A is stabilized in late G1 by MgcRacGAP (male germ cell Rac GTPase-activating protein) and RSF1 (remodelling and spacing factor 1). Defining the mechanisms that remove these assembly factors once CENP-A deposition is complete also remains an important open question. **c** | Model for the two-step regulation of CENP-A deposition. Cyclin-dependent kinase (CDK) prevents CENP-A deposition outside G1 phase by inhibiting MIS18 complex localization, MIS18 complex assembly and HJURP recruitment. Polo-like kinase 1 (PLK1) binds to the MIS18 complex to promote CENP-A deposition at centromeres during G1.

chromosomes, the spurious formation of centromeres at two distinct loci allows a single chromatid to attach simultaneously to opposing spindle poles, resulting in mis-segregation or fragmentation of the chromosome by spindle forces. The fragmentation of dicentric chromosomes can result in breakage–fusion–bridge cycles that confer cascading chromosomal instability[97,98]. Therefore, the centromere must be faithfully inherited at a single site on each chromosome through all mitotic and meiotic divisions (BOX 2).

The CENP-A-deposition machinery. In most eukaryotes, centromere inheritance requires the transmission of CENP-A nucleosomes to maintain the epigenetic mark on each sister chromatid. Fundamental to this transmission is the striking stability of CENP-A, which does not exchange once it is incorporated at centromeres[91,99,100] and is conservatively partitioned between the newly replicated sister chromatids during the S phase of the cell cycle[91,99,100]. Unlike canonical histones, the deposition of new CENP-A is uncoupled from DNA replication, such that

Box 2 | **Transmission of the CENP-A nucleosome during meiosis**

In addition to its central role in mediating mitotic divisions, the centromere must also be propagated during meiosis to be transmitted to the progeny. Transmission of Y chromosome neocentromeres between generations[204] demonstrates that the position of the human centromere is heritable through the male germ line, independently of the underlying DNA sequence. Unlike the majority of canonical histones, centromere protein A (CENP-A) is not exchanged for protamines during sperm development in mammals[60], *Xenopus laevis*[210] or *Drosophila melanogaster*[211,212], and it can therefore provide a template for the centromeres in the progeny. Indeed, in *D. melanogaster*, maintenance of CENP-A (also known as Cid) in the sperm is required for centromere propagation and for the faithful segregation of the paternal chromosomes in the embryo, as sperm chromosomes lacking CENP-A are unable to template a centromere *de novo*[212]. By contrast, in *Caenorhabditis elegans*, CENP-A is not continuously maintained throughout meiosis and so does not follow this self-templating pattern, as sperm do not contribute CENP-A following fertilization; CENP-A is instead provided by the oocyte, which removes CENP-A during the pachytene stage of prophase I and reloads it in diplotene[213].

In those organisms that maintain their centromeres through meiosis, the molecular mechanisms that replenish CENP-A following meiotic S phase are poorly understood. Several differences from the mechanisms of CENP-A replenishment during the mitotic cell cycle have been proposed. In *D. melanogaster*, CENP-A is assembled during prophase I of female meiosis, and during both prophase I and after exit from meiosis II in the male[211]. CENP-A deposition is similarly biphasic during the meiotic divisions that produce male gametes in rye[214]. The mechanisms that transmit centromere position and features through the germ line in vertebrates remain a key unanswered question.

the occupancy of CENP-A molecules at the centromere is half-maximal during mitosis, when the centromere recruits the complete kinetochore (FIG. 3b). Determining the nature of centromeric chromatin following CENP-A dilution in S phase remains an area of active investigation, with current models indicating that the gaps left by this dilution are filled by H3.3 (REF. 101). In human cells, new CENP-A molecules are deposited during the subsequent G1 phase[79].

The deposition of new CENP-A requires the coordinated activity of several assembly factors (FIG. 3b,c). CENP-A has a dedicated histone chaperone, HJURP (Holliday junction recognition protein)[102,103], which recognizes CENP-A as distinct from H3 via specific contacts between the CATD and the N-terminal CENP-A-binding domain of HJURP[104–107]. The HJURP CENP-A-binding domain is homologous to the yeast CENP-A chaperone Scm3 (suppressor of chromosome mis-segregation 3)[108] and is sufficient to direct the incorporation of CENP-A at an ectopic locus[75]. HJURP localizes to centromeres only during G1 (REFS 102,103), when new CENP-A deposition occurs. Consistent with this, HJURP does not participate in the partitioning of CENP-A between sister chromatids during S phase[100].

In addition to HJURP, CENP-A deposition in G1 requires the three-subunit MIS18 complex, which is composed of MIS18α, MIS18β and MIS18-binding protein 1 (M18BP1 (REF. 109); also known as KNL2 (REF. 110)). Intriguingly, not all components of the MIS18 complex are conserved across eukaryotes, with a single MIS18 homologue in fungi[111] (which have no identified M18BP1), and an M18BP1 homologue (KNL-2) but no MIS18α or MIS18β homologues in *C. elegans*[110]. In *Drosophila melanogaster,* the functions of the MIS18 complex and HJURP seem to be combined in a single molecule, Chromosome alignment defect 1 (Cal1)[112,113].

Histone chaperone
A protein that binds to histones to facilitate nucleosome assembly.

M18BP1 has been shown to interact with CENP-C in both human cells and *Xenopus laevis*[114,115]. As CENP-C binds directly to CENP-A nucleosomes as described above, this provides a mechanism to ensure that the MIS18 complex and HJURP are recruited only to sites of pre-existing centromeres to locally direct the incorporation of new CENP-A. The interaction between M18BP1 and CENP-C is crucial for the recruitment of the MIS18 complex during CENP-A assembly in the G1 phase in human cells[114,116]. However, *X. laevis* M18BP1 is recruited via CENP-C during mitosis but not during interphase, suggesting that additional M18BP1 recruitment mechanisms exist[84,115]. CENP-C has also been proposed to contribute to CENP-A deposition beyond MIS18 complex recruitment[84], including by binding directly to HJURP[117]. Finally, CENP-C[91], the RSF (remodelling and spacing factor) complex[118], and the centralspindlin component MgcRacGAP (male germ cell Rac GTPase-activating protein)[119] have been implicated in the maintenance of CENP-A once it is incorporated at centromeres. Together, these centromere-specialized assembly factors ensure the specific incorporation of CENP-A at centromeres.

Generation of a CENP-A-permissive chromatin environment. Although CENP-A is an essential component of most centromeres, it is not the sole driver of centromere specification. CENP-A homologues are absent in some organisms, including trypanosomes and some insects with holocentric chromosomes[120,121], raising the possibility that alternative strategies for centromere specification have arisen during evolution. Even in CENP-A-containing organisms, additional molecular features contribute to defining an active centromere, including the properties of the underlying DNA sequence (see above), the composition of the surrounding chromatin and post-translational modifications of CENP-A itself (FIG. 4). Moreover, individual CENP-A nucleosomes are found frequently at non-centromeric sites throughout the chromosomes in human cells[122], indicating that the presence of CENP-A alone is not sufficient for centromere formation.

The core centromere and pericentromere are distinguished not only by the organization of their DNA sequence repeats as described above, but also by distinct chromatin signatures that are crucial for their functions. Early studies associated centromeres with heterochromatin[123], and subsequent work has found that the pericentromere in particular is heterochromatic, containing hypermethylated H3 Lys 9 (H3K9)[124,125], although non-repetitive centromeres and neocentromeres frequently lack surrounding heterochromatin[126,127]. In contrast to the heterochromatic pericentromere, at the core centromere, CENP-A-containing nucleosomes are interspersed with canonical H3-containing nucleosomes with transcriptionally permissive marks, particularly dimethylated H3K4 (H3K4me2)[128–130] and H3K36me2 (REF. 40) in human and *D. melanogaster* cells. Recent analyses of HAC formation and maintenance have revealed that artificially increasing heterochromatin at the α-satellite array is detrimental to CENP-A deposition and centromere function[41,131], whereas H3K4me2 and increased H3K9 acetylation promote CENP-A maintenance[40,41].

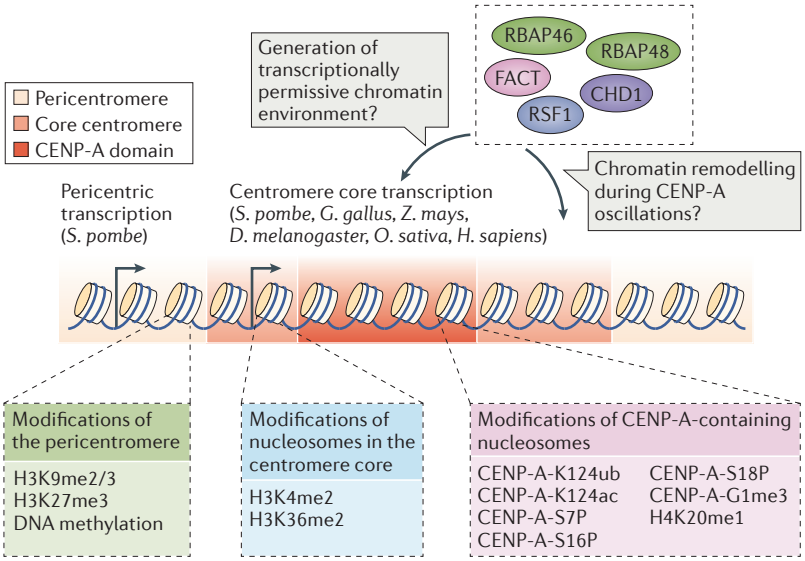


Figure 4 | **Centromeric chromatin.** Model of the epigenetic modifications at the core centromere, the centromere protein A (CENP-A) domain and the pericentromere. In addition to the sequence and structural specializations that differentiate CENP-A-containing chromatin from bulk chromatin, post-translational modifications of CENP-A nucleosomes contribute to centromere function. Human CENP-A is monoubiquitylated at Lys124 (K124ub) within the histone-fold domain by COPS8 (CUL4–RBX1–COP9 signalosome complex subunit 8)[218] to promote its centromere targeting. Acetylation at Lys124 (K124ac) has also been reported[219]. Moreover, diverse other post-translational modifications of CENP-A[219–221] and histone H4 in the CENP-A nucleosome[222] have been described, including methylation (me) and phosphorylation (P). Defining the functional contributions of these modifications remains an important challenge. CHD1, chromodomain helicase DNA-binding protein 1; *D. melanogaster*, *Drosophila melanogaster*; FACT, facilitates chromatin transcription; *G. gallus*, *Gallus gallus*; *H. sapiens*, *Homo sapiens*; *O. sativa*, *Oryza sativa*; RBAP, retinoblastoma-binding protein; RSF1, remodelling and spacing factor 1; *S. pombe*, *Schizosaccharomyces pombe*; *Z. mays*, *Zea mays*.

This indicates that both the presence of transcriptionally permissive marks and the absence of heterochromatin in the centromere core are important for CENP-A localization to centromeres (FIG. 4).

The importance of chromatin marks that are permissive for transcription at the core centromere raises the possibility that transcription of the centromere and pericentromere plays a part in centromere propagation and function. In fission yeast, transcripts from the pericentromeric repeats contribute to the formation of pericentromeric heterochromatin, which in turn is required for *de novo* CENP-A deposition on minichromosomes[132]. In addition, transcripts derived from the centromere core have been reported in diverse organisms[133,134] (FIG. 4). In human cells, RNA polymerase II (Pol II) and several transcription factors localize to mitotic centromeres[135], and transcripts have been detected from the α-satellite sequences of HAC centromeres[40]. Broadly disrupting Pol II results in kinetochore defects[135,136], as well as defects in the deposition of new CENP-A nucleosomes[137]. However, tethering strong transcriptional activators to the centromere is deleterious to centromere function in many organisms[131,138], indicating that the transcriptional requirement for centromere identity and function must be finely tuned.

Chromatin remodellers associated with active transcription have also been implicated in the deposition of new CENP-A (FIG. 4), including RSF1, FACT (facilitates chromatin transcription), chromodomain helicase DNA-binding protein 1 (CHD1), and retinoblastoma-binding protein p46 (RBAP46; also known as RBBP7) and RBAP48 (also known as RBBP4)[103,109,111,118,119,139–141]. These proteins may facilitate new CENP-A deposition through the generation of the necessary transcriptionally permissive centromere core, or they may play a direct part in remodelling centromeric chromatin to accommodate its oscillations between maximal and half-maximal CENP-A occupancy throughout the cell cycle (FIGS 3b,4). For example, if H3.3 replaces CENP-A nucleosomes following DNA replication, this H3.3 must be exchanged for new CENP-A during the following G1. The MIS18 complex has also been proposed to contribute to the chromatin remodelling in anticipation of new CENP-A deposition, including by recruiting factors that regulate DNA methylation[142] and histone acetylation[109,111]. As a result, tethering a histone acetyltransferase to a HAC centromere can partially complement depletion of the MIS18 complex[41]. However, recent work indicates that the MIS18 complex also functions directly in the CENP-A-deposition process by interacting with the HJURP chaperone[143,144].

Regulation of CENP-A deposition. Multiple regulatory safeguards have been identified that ensure the faithful deposition of new CENP-A-containing nucleosomes exclusively at centromeres. In metazoa, CENP-A deposition occurs around mitosis or following mitotic exit[99,115,145–147]. This temporal restriction isolates CENP-A deposition from the deposition of canonical H3, which is coupled to DNA replication in S phase. The cell cycle restriction of CENP-A deposition relies heavily on phosphorylation downstream of cyclin-dependent kinase (CDK)[148] (FIG. 3c). Ongoing work indicates that CDK negatively regulates CENP-A incorporation at numerous steps. In *D. melanogaster*, the degradation of cyclin A has a key role in deposition of CENP-A[112,146]. In human cells, CDKs phosphorylate the MIS18 complex subunit M18BP1 to reduce its centromere localization[148] and to prevent recruitment of the MIS18α and MIS18β subunits[116] outside G1. CDK phosphorylation of HJURP disrupts its localization to centromeres[149], whereas CDK phosphorylation of CENP-A itself on Ser68 has been reported to inhibit the CENP-A–HJURP interaction[150], although the role of Ser68 in CENP-A deposition is controversial[48,76,84,100,105,107].

In addition to this temporal regulation by CDKs, CENP-A deposition requires a licensing step by Polo-like kinase 1 (PLK1)[116] (FIG. 3c). Thus, centromere propagation requires a two-step regulatory paradigm that is analogous to the regulation of DNA replication by CDK and by DBF4-dependent kinase (DDK)[151]. PLK1 binds to and phosphorylates the MIS18 complex to promote MIS18 complex localization and to license the centromere for CENP-A deposition[116]. Bypassing both the CDK regulation of MIS18 complex assembly and PLK1 licensing by constitutively targeting the MIS18α subunit to the centromere results in CENP-A deposition

throughout the cell cycle and severe mitotic defects[116]. This indicates that the temporal isolation of CENP-A deposition is important for centromere function.

Spatial restriction of CENP-A deposition. The regulated deposition of CENP-A nucleosomes ensures the epigenetic propagation of the centromere at a persistent location on each chromosome. Many organisms also have strategies to prevent CENP-A deposition at non-centromeric sites, at which they could make inappropriate attachments to the mitotic spindle. In *S. cerevisiae,* mis-targeted CENP-A is removed by the combined action of the FACT chromatin remodeller and the E3 ubiquitin ligase Psh1 (Pob3/Spt16 histone-associated 1), which targets ectopic CENP-A for degradation[152,153]. In fission yeast, the proteasome subunit Rpt3 (regulatory particle triphosphatase 3) interacts with CENP-A and has been implicated in restricting the size of the CENP-A domain[154]. However, a similar proofreading mechanism to remove ectopic CENP-A has not yet been identified in vertebrates, consistent with the persistence of CENP-A molecules at non-centromeric sites in the genome in human cells[122].

CENP-A deposition is also restricted within the centromere. In humans, mouse and chicken, the CENP-A domain occupies only a small portion of the core centromere sequences[122,155,156]. There is significant variation in the size of the CENP-A domain among human chromosomes (0.4–4.2 Mb for a set of analysed X and Y chromosomes[157]), although an approximately equivalent ratio between the size of the CENP-A domain and that of the α-satellite array is maintained[157]. The CENP-A domain of neocentromeres is restricted to an even smaller region, with reports of between 40 kb and 0.5 Mb[96,126,127,158]. How the CENP-A domain is restricted in size in vertebrates remains an area of active investigation. Exogenous CENP-A expression in human cells leads to downregulation of the endogenous CENP-A protein[99], and CENP-A overexpression far beyond this level results in mis-localization of CENP-A to chromosome arms[63,86,159]. These data indicate that the restriction of the CENP-A domain occurs, at least in part, at the level of modulating total protein in the cell, as recently proposed in human cells[122]. In chicken and *D. melanogaster*, high local concentrations of the CENP-A chaperones HJURP or Cal1, respectively, can also drive centromere expansion[144,160]. Intriguingly, these homeostasis mechanisms maintain CENP-A in large excess of the amount required for kinetochore function, as cells depleted of CENP-A to as little as 10% or even 1% of its initial level recruit kinetochore proteins and at least partially direct chromosome segregation[48,71].

Centromere recognition

The centromere achieves its key function — the segregation of its corresponding chromosome — by recruiting the kinetochore, which is the macromolecular structure that mediates attachment to the microtubules of the mitotic spindle and functions as a signalling hub to ensure accurate chromosome segregation[161]. Thus, understanding how centromere form begets function hinges crucially on defining the network that connects the centromere components to the proteins of the kinetochore.

Components of the centromere–kinetochore interface. Establishing the architecture of the centromere–kinetochore interface has been accelerated by the discovery of several key molecular players over the past ten years[162–165]. The proteins of the centromere–kinetochore interface are collectively referred to as the constitutive centromere-associated network (CCAN; also known as the interphase centromere complex (ICEN)) (FIG. 5). The CCAN is a group of 16 proteins that localize to the centromere throughout the cell cycle[161]. These proteins are designated in vertebrates with alphabetical CENP- names (CENP-C, CENP-H, CENP-I, CENP-K, CENP-L, CENP-M, CENP-N, CENP-O, CENP-P, CENP-Q, CENP-U, CENP-R, CENP-T, CENP-W, CENP-S and CENP-X)[45,162–170], although other CENP-named proteins do not represent constitutive centromere components. Within the CCAN, these proteins can be combined into five groups: CENP-C, the CENP-L-N complex[81,171], the CENP-H-I-K-M complex[162,163,172], the CENP-O-P-Q-U-R complex[173,174] and the CENP-T-W-S-X complex[175] (FIG. 5a). Together, these proteins recognize centromeric chromatin and connect it to the kinetochore.

Dissecting the contributions of the CCAN to centromere recognition presents a particular challenge, owing to the differing functional requirements between organisms. Although the CCAN is largely conserved between yeast and human[176,177], it is dispensable in yeast with the exception of the CENP-U homologue Ame1 and the CENP-Q homologue Okp1 (REFS 178,179). In mammals, CENP-U is essential for early mouse development[180], but eliminating CENP-U and CENP-Q results in relatively mild phenotypes in tissue culture cells[174,180]. In addition, some organisms such as *D. melanogaster* and *C. elegans* have a minimal CCAN, for which the only identified CCAN homologue is CENP-C. In this section, we will review the ongoing work to define the precise molecular roles of the CCAN in kinetochore assembly and faithful chromosome segregation.

Interactions at the CCAN–centromere interface. Ongoing work is seeking to establish how the CCAN proteins interact with one another and with centromeric chromatin to build a robust platform for kinetochore assembly on the centromere[71,80,81,117,162,172,181–183]. Within the CCAN, each subcomplex forms numerous direct physical interactions to generate an extensive meshwork[183]. This network is dynamic, such that some subcomplexes rely on different interactions at different stages of the cell cycle[182–184]. CENP-C is a keystone molecule in this assembly and is required for the recruitment of all other CCAN components during mitosis[80,117,173,181,183], in addition to its role in promoting CENP-A deposition as described above.

The CCAN is anchored to the centromere through its interactions with centromeric chromatin. Although each of the CCAN proteins can be co-immunoprecipitated with

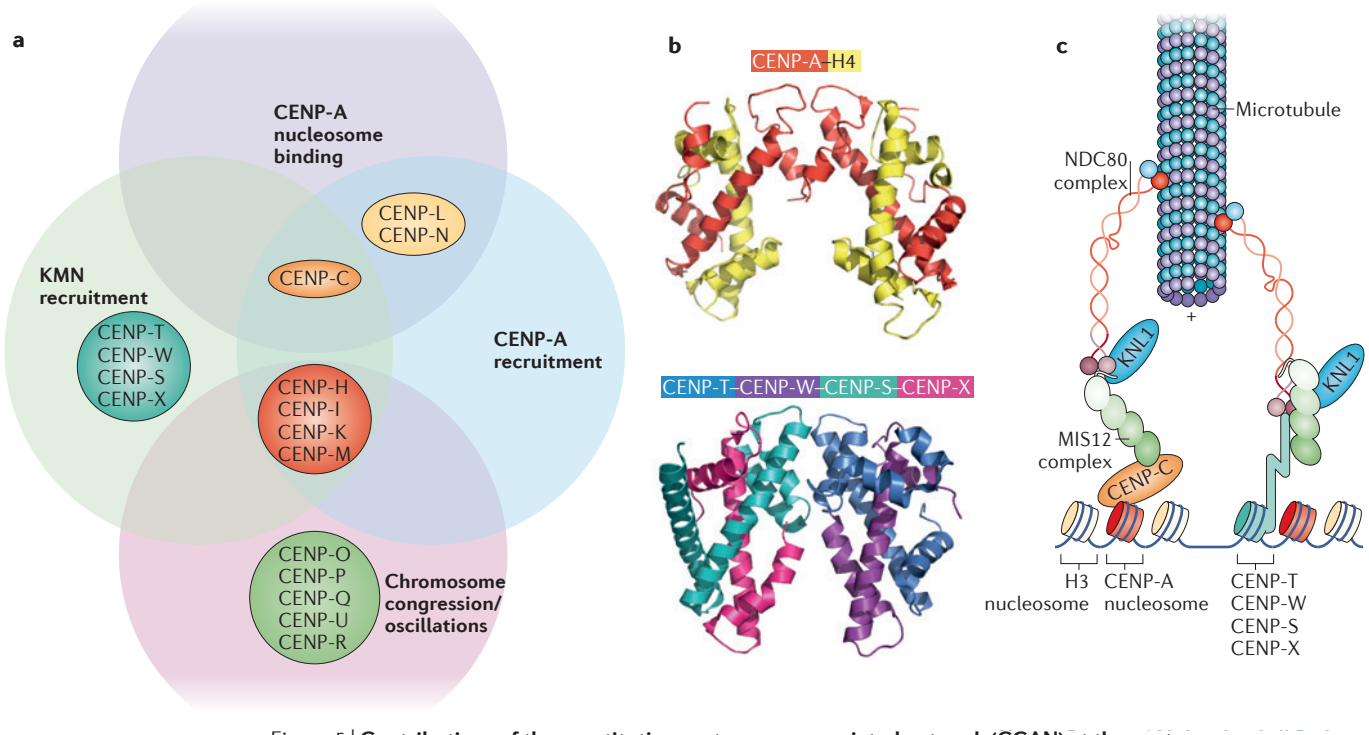


Figure 5 | **Contributions of the constitutive centromere-associated network (CCAN) at the centromere–kinetochore interface. a** | The 16 proteins of the CCAN, designated by CENP- and a letter, can be grouped into subcomplexes as indicated. The subcomplexes are grouped according to functions that have been reported for at least one of their subunits. The KMN comprises KNL1, the MIS12 complex and the NDC80 complex, which together bind to microtubules. **b** | Comparison of the crystal structures of the tetramer composed of the histones CENP-A and H4 in the context of the nucleosome (Protein Data Bank (PDB) ID: 3AN2)[90] — H2A, H2B and DNA are excluded for clarity — with the heterotetramer composed of the histone-fold-containing proteins CENP-T, CENP-W, CENP-S and CENP-X (PDB ID: 3VH5)[175]. **c** | A simplified model of the connectivity from the centromere, to the kinetochore, to the microtubule during mitosis. The contributions of CENP-C and CENP-T to recruiting the microtubule-binding interface of the kinetochore are highlighted, and the other CCAN components are excluded from this model for clarity.

CENP-A nucleosomes, only CENP-C and CENP-N have been reported to bind to nucleosomes directly, by recognizing the key structural distinctions between CENP-A and H3 (REFS 80–82,91) (FIG. 5a) (see above). In addition, several CCAN proteins bind directly to DNA, including CENP-C[185], CENP-Q[173] and the CENP-T-W-S-X complex[175], although the contributions of these activities to CCAN function remain an area of ongoing investigation. The CENP-T-W-S-X complex is particularly intriguing, as it is composed of histone-fold-containing proteins[175,186] and adopts a structure similar to that of canonical nucleosomes (FIG. 5b). In this structure, CENP-T-W and CENP-S-X form dimer pairs that can be combined into a CENP-T-W-S-X heterotetramer[175] or a $(CENP\text{-}T\text{-}W\text{-}S\text{-}X)_2$ octamer[187]. The CENP-T-W-S-X complex wraps DNA, inducing positive supercoils[175,187], and protects a region of ~100 bp from micrococcal nuclease digestion[175], indicating that it may integrate directly into centromeric chromatin. The importance of these nucleosome-like properties for centromere and kinetochore function is still being elucidated, although recent work has revealed that the complex requires both these DNA contacts and a connection to the rest of the CCAN meshwork through the CENP-H-I-K-M complex for its centromere localization[172,175,183].

Recruitment of the outer kinetochore. Once assembled on the centromere, the CCAN provides a platform for the assembly of the outer kinetochore. In particular, CENP-C and CENP-T form parallel but non-redundant pathways that recruit the key microtubule-binding proteins of the kinetochore, the KNL1–MIS12–NDC80 (KMN) network[159,188–191] (FIG. 5c). Indeed, artificial targeting of fragments of CENP-C or CENP-T to an ectopic chromosomal locus is sufficient to recruit the KMN network and generate a kinetochore-like structure that can direct chromosome segregation[159,192]. In budding yeast, CENP-U forms a third pathway to recruit the KMN network[173]. In human cells, CENP-I has also been reported to interact with the microtubule-binding proteins of the kinetochore[193]. These protein interactions are regulated in most eukaryotes, such that the CCAN only recruits a full kinetochore during mitosis[194]. Specifically, phosphorylation by Aurora B kinase promotes interactions between CENP-C and the MIS12 complex during mitosis[193,195]. In addition, the NDC80 complex is sequestered outside the nucleus throughout interphase and is thereby spatially separated from the CCAN until mitosis, when CDK phosphorylation promotes its direct interaction with CENP-T[159,191,194].

Functions of the CCAN. Ultimately, the central challenge that remains regarding the centromere–kinetochore interface is to use the identified physical interactions and functional requirements to define fundamental principles for centromere and kinetochore function. In addition to their roles in recruiting the microtubule-binding interface of the kinetochore, CCAN proteins have been proposed to make several additional contributions to chromosome segregation (FIG. 5a). For example, recent work has suggested that the vertebrate CCAN has a key role in resisting the forces generated by spindle microtubules[130,196], as well as controlling metaphase oscillations[197] and chromosome congression through recruiting the motor protein CENP-E[198]. In addition, several CCAN proteins, including CENP-C, CENP-N and CENP-I, have been shown to have key roles in the deposition of new CENP-A nucleosomes at centromeres[66,80,81,114,115,139,192] (FIG. 5a), presenting an appealing model for the propagation of the centromere via kinetochore proteins. The ongoing advances in elucidating the organization of CCAN components and subcomplexes will provide further insight into the functional contributions of the CCAN.

Conclusions

Research in centromere biology continues to provide important insights into the molecular mechanisms that underlie the specification, propagation and recognition of this epigenetically defined chromosomal locus. However, many important mysteries remain to be solved. For example, continuing to define the contributions of DNA architecture and chromatin marks to CENP-A deposition will be crucial for understanding why some sites of spurious CENP-A deposition result in neocentromere formation, whereas others are maintained in the genome inertly. Other key goals for future work include establishing the mechanisms by which the centromere is disassembled and re-assembled to allow passage of the DNA replication fork during S phase, and understanding the differences in CENP-A transmission during the meiotic cell cycle. Through the development of cytological, biochemical and genetic tools, researchers are defining Cyril Darlington's 'form' of the centromere in increasing molecular detail. Future work faces the challenge of further dissecting endogenous centromeres and building them *de novo* to define exactly how the form imparts the function.

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Competing interests statement
The authors declare no competing interests.

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