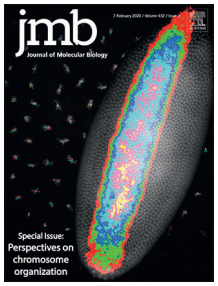
Review Article



The Elusive Structure of Centro-Chromatin: Molecular Order or Dynamic Heterogenetity?

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Abstract

The centromere is an essential chromatin domain required for kinetochore recruitment and chromosome segregation in eukaryotes. To perform this role, centro-chromatin adopts a unique structure that provides access to kinetochore proteins and maintains stability under tension during mitosis. This is achieved by the presence of nucleosomes containing the H3 variant CENP-A, which also acts as the epigenetic mark defining the centromere. In this review, we discuss the role of CENP-A on the structure and dynamics of centromeric chromatin. We further discuss the impact of the CENP-A binding proteins CENP-C, CENP-N, and CENP-B on modulating centro-chromatin structure. Based on these findings we provide an overview of the higher order structure of the centromere.

Introduction

Chromatin is a structurally heterogeneous and inherently dynamic nucleoprotein complex, and is critically important for regulating processes such as gene expression or DNA replication, but also for local or large-scale genome organization.[1] One particular chromatin state, the centromere, is the primary point of constriction in human chromosomes[2,3] and acts as an essential region for proper chromosome segregation. The main function of the centromere is the recruitment of the kinetochore; a large protein complex responsible for connecting the sister chromatids to the spindle microtubules (Figure 1(a)). The underlying DNA of the centromere varies widely across species,[4] ranging from 125 bp "point" centromeres in budding yeast,[5] and 5–10 Mb repetitive "regional" centromeres in mammals[6] to chromosome spanning holocentromeres[7] in some insects, worms, and plants. In most cases, this underlying DNA is not essential for centromere function[8] and its exact role is not well understood.

The molecular composition of the kinetochore remained a mystery until it was observed that antibodies from patients suffering from scleroderma spectrum disease (CREST syndrome) recognized human centromeres.[9,10] This realization allowed the identification of three kinetochore components, namely CENP-A, CENP-B, and CENP-C.[11] Of these, CENP-A, a histone H3 variant, emerged as the key marker for a functional centromere in the vast majority of eukaryotes.[12–14] CENP-A is considered to be an epigenetic marker for centromere specification and is essential for the formation of active centromeres in most eukaryotes, including in dicentric and neocentromeres,[15–17] and it acts as a platform for kinetochore recruitment.[18–21] The overall molecular-level architecture of the centromere, and the underlying chromatin structure, termed centro-chromatin[22] (Figure 1(b)), remains an area of active study. Centro-chromatin can be separated into two major regions: the centromere core which is interspersed with CENP-A nucleosomes and forms the hub for recruitment of the kinetochore,

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and the pericentromeric region which associates with heterochromatin. In this review, we will focus on the centromere core and summarize recent advancements in the understanding of centrochromatin with a focus on its hierarchical organization, heterogeneous structure and dynamics.

Centromeric DNA

The centromeres of most organisms vary widely between species in both size and organization. In *Saccharomyces cerevisiae* the centromere consists of a single ~125 bp region, a so-called "point" centromere, which consists of three centromere-determining DNA elements (CDEI – CDEIII). These short sequence elements collectively specify a functional centromere by recruiting the centromere binding factor three (CBF3) complex, the budding yeast protein complex which directly engages the CDEIII element to deposit CENP-A (Cse4).[5,23]

In contrast, most organisms exhibit "regional" centromeres which are formed from repetitive DNA sequences. These repeat regions are themselves poorly conserved across species in both size and underlying DNA. In *Schizosaccharomyces pombe* centromeres form on a 40–100 kb region containing a core "cnt" region for CENP-A recruitment flanked by an "otr" region that form heterochromatin.[24] In humans, these repeats are made up of ~4 Mb long α-satellite repeats with a base repeating unit of ~171 bp.[6,25] Similarly, in mice, they are made up of the 'major and minor satellite repeats' which associate with heterochromatin and assemble CENP-A respectively.[26,27]

Like the CBF3 complex in budding yeast, regional centromeres in humans and other mammals encompass a partially conserved DNA-binding factor, called CENP-B. In humans, CENP-B is recruited by the CENP-B box,[28,29] a 17-bp motif found within some of the α-satellite repeats.[30] However, unlike CBF3, CENP-B is not essential for centromere function.[31–33] Together, α-satellite repeats and the CENP-B box have been shown to be required for first generation centromere formation in human artificial chromosomes (HACs).[34–36] However, this function can be bypassed by assembling CENP-A onto pre primed HACs.[37] Similarly, studies on human dicentric chromosomes containing α-satellite repeats[38] and functional neocentromeres lacking α-satellite DNA revealed that α-satellite DNA is neither necessary

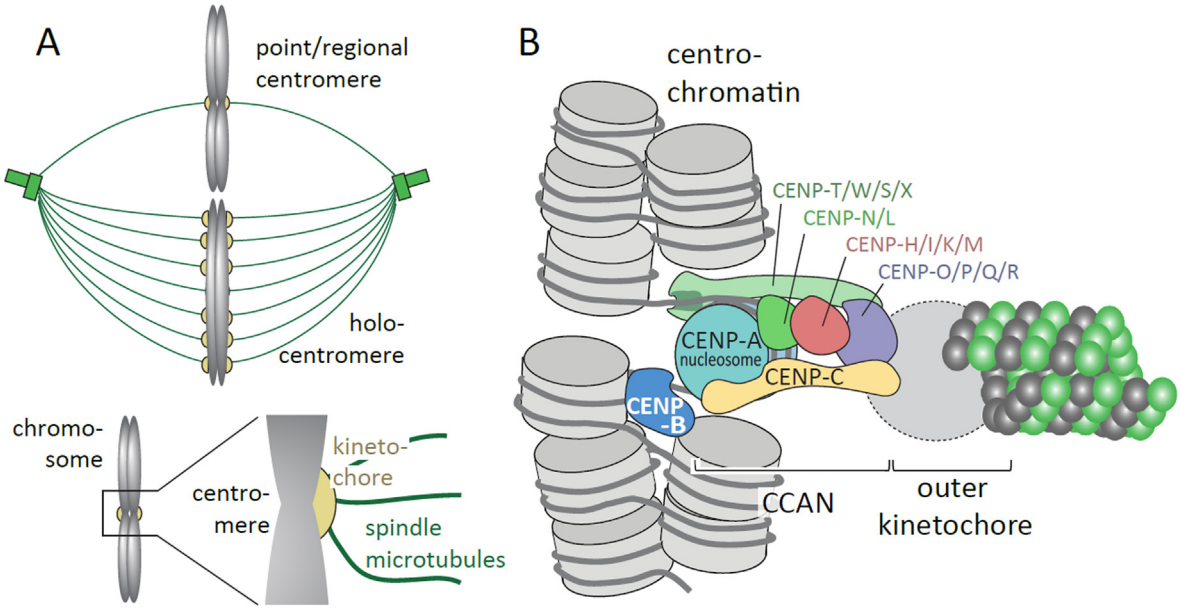

Figure 1. Organization of the centromere and kinetochore: (a) Schematic of the mitotic chromosome marking the various types of centromeres i.e point centromeres which are marked by a specific DNA sequence essential for centromere function, Regional centromeres contain repetitive DNA sequences, and holocentromeres consist of centromeres dispersed along the entire length of the chromosome. The kinetochore structure recruits onto the centromere (yellow). The spindle microtubules originate from the centrosomes (green) and attach to the kinetochore. (b) Schematic of the mitotic kinetochore showing simplified protein–protein interactions. Canonical nucleosomes are shown to forming next-neighbor face-to-face stacking interactions similar to 30 nm fibers and the CENP-A nucleosome exhibiting an untwisted and open conformation as shown by Takizawa *et al.*[100] CCAN components are shown based on their simplified positions with CENP-N-L, CENP-C, and CENP-B directly binding to CENP-A. CENP-B and CENP-T-W-S-X interacting with nucleosomal DNA and CENP-H-I-K-M and CENP-O-P-Q-R linking between the various components. Finally, the outer kinetochore is recruited to the CCAN and binds to the microtubules.

nor sufficient for centromere activity. These findings provide evidence that centromeres are regulated epigenetically rather than by the underlying sequence. This does leave the question of the importance of CENP-B, whether it acts purely as a structural factor or assists in recruitment or stabilization of CENP-A and the kinetochore. We discuss the various functions of CENP-B in greater detail further in this review.

The CENP-A nucleosome at the centromere core

Centromeric DNA, like most other domains of the genome, is organized into arrays of nucleosomes, of which a subset contains CENP-A instead of canonical H3. At the sequence level CENP-A shares only about a ~50% amino acid identity with canonical H3. Most importantly, it differs in its histone fold domain, containing a region called the CENP-A targeting domain (CATD) composed of its L1 and α2 region (Figure 2(a)). The CATD is both necessary and sufficient for the deposition of CENP-A at centromeres.[39,40] CENP-A serves to recruit and maintain the components of the constitutive centromere associated network (CCAN). The overall function of the CCAN is to recruit the outer kinetochore components to which spindle microtubules will attach. It can be sub divided into several functional groups, namely; CENP-C, CENP-N-L, CENP-H-I-K-M, CENP-T-W-S-X, and CENP-O-P-Q-R-U (Figure 1(b)). The CATD of CENP-A directly contacts CENP-C,[41] and CENP-N[42] and localizes them to the centromere. Importantly, H3 chimeras, containing a CATD, are able in the short term to rescue cells from which CENP-A was depleted, as they are able to assemble a functioning centromere.[39,40] CENP-B, CENP-N-L, and CENP-T-W-S-X further bind directly to DNA and help license the centromere.[30,43,44] Of note, a more detailed discussion on the recruitment and function of the CCAN can be summarized in some recent reviews.[20,21,45,46]

Interestingly, the recruitment of new human CENP-A depends on the presence of pre-existing CENP-A.[41,47,48] Unlike canonical H3, CENP-A deposition in humans is decoupled from replication and occurs after mitotic exit in G1 phase.[49–51] The CENP-A/H4 heterodimer is deposited by its dedicated histone chaperone HJURP *via* interaction with the CATD.[52–55] The regulation of CENP-A deposition is due to interaction of the Mis18 complex with CENP-C and CENP-I at the centromere[56,57] and subsequent recruitment of the cytosolic HJURP-CENP-A complex.[58,59] Deposition is further regulated by post-translational modifications (PTMs) of CENP-A, including phosphorylation of S68 and ubiquitination of K124 of CENP-A which have been reported to be important for regulatory purposes. The latter PTM, in particular, might be a critical regulator, as it could prevent stable binding to HJURP[60–63] and diminishing centromeric CENP-C.[64] However the importance of these modification is currently disputed.[65,66] Other factors such as the chromatin remodelling factor RSF1,[67] the Rac GTPase activating protein MgcRacGAP,[68] and RNA pol II mediated α-satellite transcripts[69,70] have been found to also play a regulatory role in CENP-A deposition though their exact mechanisms are unclear. Thus, a complex regulatory mechanism exists to ensure accurate deposition of CENP-A.

The structure and dynamics of centromeric nucleosomes

The presence of CENP-A is responsible for establishing a unique chromatin state at the centromere. While comparing the crystal structure of nucleosomes formed with CENP-A to nucleosomes containing canonical H3 shows a largely similar structure, two major differences emerge (Figure 2(b)): First, the CENP-A loop one protrudes from the nucleosome exposing R80 and G81. This exposed RG-loop forms a point of recruitment for CENP-N.[41,61,71,72] Second, the αN helix of CENP-A is one turn shorter than in canonical H3, reducing DNA contacts and thus increasing the flexibility of DNA at the nucleosome entry and exit sites.[73–75] In consequence, CENP-A containing nucleosomes only organize around ~120 bp of DNA,[12,73,76] as opposed to the ~147 bp DNA in canonical nucleosomes.[77] The loss in DNA organization at the DNA entry/exit sites of the nucleosome further destabilizes binding of linker histones H1, which, in canonical nucleosomes can extend the amount of DNA protected by ~20 bp beyond 147 bp.[78,79] In centromeric nucleosomes, the DNA is thus much more flexible, allowing a multitude of entry/exit angles,[74] and much increased structural heterogeneity.[73]

This increase in DNA flexibility also most likely results in increased dynamics within CENP-A nucleosomes. Even canonical nucleosomes exhibit a range of internal dynamics, including transient DNA unwrapping events (which are generally rare and short-lived) or translational shifts along the genomic DNA.[80] Locally, DNA unwrapping events expose sites which are generally protected within the nucleosome, providing transient access sites for sequence-specific DNA binding factors, such as transcription factors.[80–84] Such unwrapping dynamics have been observed to be increased in CENP-A nucleosomes, e.g. by high-speed AFM.[85] In agreement, the nucleosomal DNA in CENP-A nucleosomes is more susceptible to nucleases,[86] indicating increased site exposure. Does increased DNA flexibility translate into more dynamic nucleosome cores as well? Initial hydrogen–deuterium exchange experiments showed rigidified particles as a function of the CATD.[87] However, recent mechanic measurements indicate that CENP-A

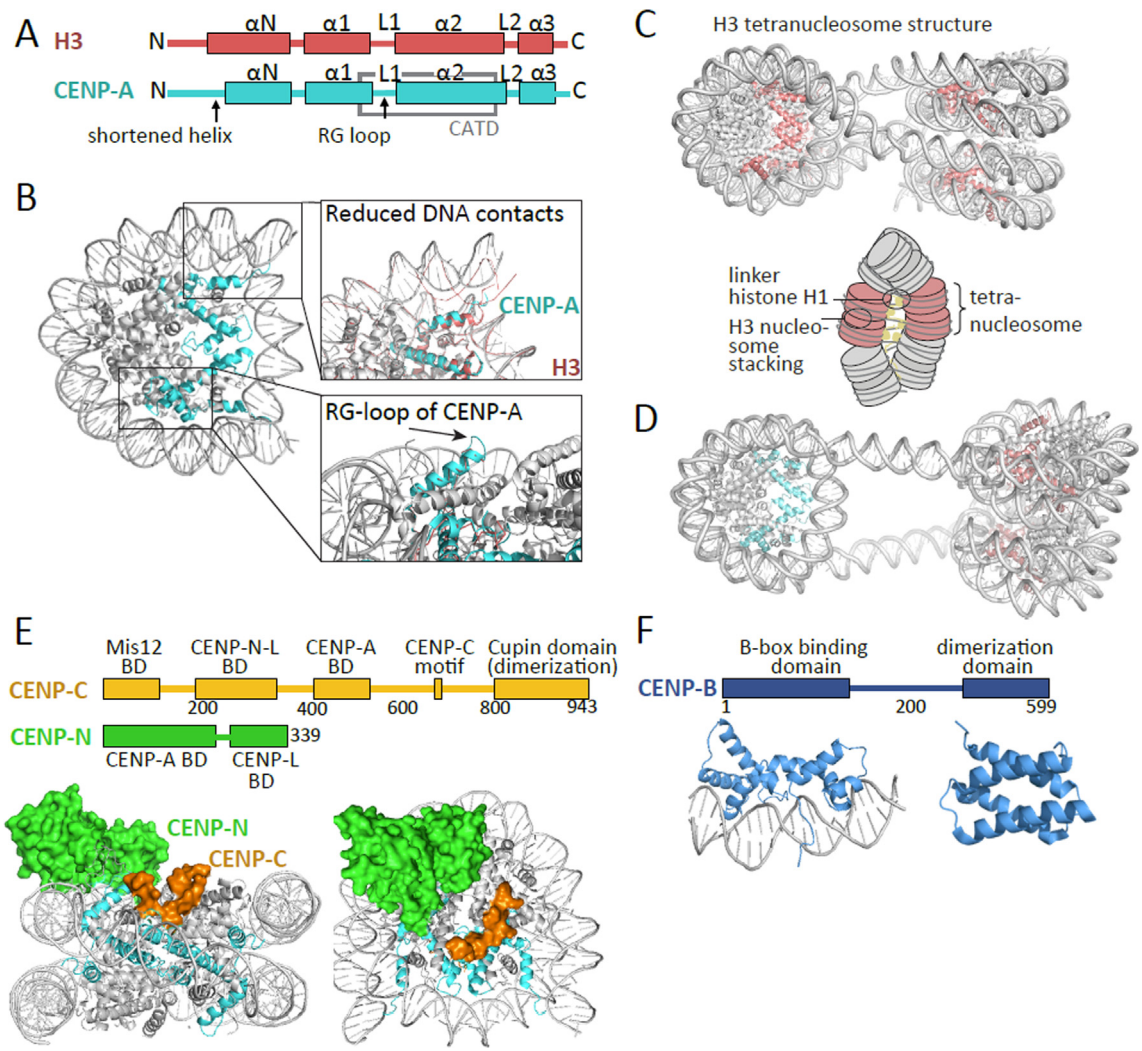

Figure 2. The core centromeric nucleosome complex: (a) Schematic representation of H3 and CENP-A. The location of the shortened αN helix, RG loop which binds CENP-N, C-terminal tail which binds CENP-C and CENP-A targeting domain (CATD) of CENP-A are highlighted. (b) Cryo-EM structure of the CENP-A nucleosome (pdb: 6DZT for H3 and 6EOC for CENP-A). Insets show the two major structural differences between H3 and CENP-A. (Top) shortened N terminal helix of CENP-A (cyan) vs H3 (red) resulting in more flexible entry/exit DNA. (Bottom) exposed RG loop (L1, see arrow) of CENP-A (cyan) vs H3 (red) for CENP-N recruitment. (c) Structure of a H3-containing tetranucleosome unit (pdb: 1ZBB), schematic view of a H3-containing chromatin fiber. (d) Structure of H3-CA-H3 trinucleosome (pdb: 6L49). (e) Schematic of the CENP-A binding proteins CENP-C and CENP-N and structure of CENP-A bound to CENP-N$^{1–200}$ and CENP-C$^{519–537}$ (pdb: 6MUO). CENP-N is shown to interact with both the RG loop of CENP-A as well as nucleosomal DNA while CENP-C sits in the acidic patch of the CENP-A nucleosome. BD: binding domain. (f) Schematic of CENP-B and structure of the CENP-B DNA binding domain bound to the CENP-B box (pdb: 1HLV) and the CENP-B dimerization domain (pdb: 1UFI). The CENP-B B-Box binding domain can be seen to induce a kink into the CENP-B box.

nucleosomes are more elastic than canonical nucleosome core particles.[88] Importantly, the increased flexibility of CENP-A nucleosomes has a direct impact on its physiological function. Elegant experiments in immortalized mouse embryonic fibroblasts using chimeric CENP-A with an H3 N-terminus (thus with reduced DNA flexibility) revealed an aberrant mitosis phenotype.[74] Local flexibility at centromeres is thus important for proper centromere function, but the molecular origins are unclear.

Local organization of centro-chromatin

One intriguing possibility why flexible CENP-A nucleosomes are required for centromere function

is that they can accommodate alternative local chromatin structures. Canonical chromatin can exist in multiple states: Under physiological conditions nucleosomes can engage in next-neighbor face-to-face stacking interactions, as observed in the crystal structure of a tetranucleosomal unit[89] (Figure 2(c)). Such face-to-face interactions are stabilized by a 4-helix bundle (H2A/H2B) and a contact between the H4-tail and the acidic patch of the neighboring nucleosome. Importantly, due to constraints of the DNA path, such tetranucleosome contacts are most stable for linker lengths of 10*n* (where *n* is the number of bp separating the nucleosomes).[89–91] Multiple tetranucleosome units can then form higher order structure, i.e. 30 nm fibers, exhibiting two intertwined stacks of nucleosomes (Figure 2(c)).[1,92,93] Integration of H1 further stabilizes these conformations.[93,94] However, alternative structures, e.g. involving nearest-neighbor solenoidal interactions or interdigitated structures,[1] as well as fluid-like organization[95] have also been observed, indicating that local chromatin is very malleable. Time-resolved measurements further revealed that chromatin structure is highly dynamic, exhibiting local structural fluctuations – in particular opening and reformation of tetranucleosomal units – on the microsecond to second timescale.[96] Together with local unwrapping events, these motions enable proteins, e.g. TFs, to access buried DNA sites, and subsequently remodel chromatin structure by stabilizing open states.[97]

In centromeric chromatin, the increased flexibility of CENP-A nucleosomes has implications for local chromatin structure and dynamics. First, the DNA linker length in centromeric regions does not obey the 10*n* register, but is around 24 bp in average (assuming fully wrapped nucleosomes). This destabilizes the formation of regular tetranucleosome-like stacking and impairs higher order structure formation.[98]

Moreover, the terminal 13 bp of DNA in CENP-A nucleosomes are much more flexible[73] resulting in much higher degrees of freedom in this structure. This is also seen by hydrogen–deuterium exchange coupled to mass spectrometry (H/DX-MS) analysis of arrays of CENP-A nucleosomes. These studies show that the DNA super helical termini within each nucleosome are loosely connected to CENP-A.[99] The highly flexible ends of CENP-A interfere with binding of the linker histone H1, allowing for a more open chromatin structure.[74] Direct cryoEM investigations by Takizawa *et al.* of reconstituted tri-nucleosome arrays, containing a CENP-A nucleosome in the center, revealed a highly heterogeneous structural ensemble, including untwisted conformations (Figure 2(d)).[100] On the other hand, somewhat counterintuitively, sedimentation experiments show CENP-A arrays to more readily form compact structures compared to canonical H3 arrays.[99] It is thus conceivable that CENP-A containing nucleosomes are able to engage in canonical stacking interactions (also because these interactions are mediated by H2A, H2B and H4), but that CENP-A locally increases the flexibility and dynamics to a large extent. Importantly, the increased flexibility and dynamics of CENP-A are indeed required for proper centromere function.[74] On a chromatin fiber level, these structural and dynamic changes are however still not understood and a subject of current research.

Structure of the core centromeric nucleosome complex and kinetochore

The structure, dynamics and function of centromeric chromatin are further controlled by CCAN proteins. Once deposited, CENP-A recruits the two CENP-A binding proteins CENP-C and CENP-N, which together form the core centromeric nucleosome complex (CCNC). Of these, CENP-C acts as an anchor for kinetochore assembly by subsequently binding to several kinetochore components. Structurally, human CENP-C is a long intrinsically disordered protein containing an N-terminal Mis12 (outer kinetochore) binding domain, a middle CENP-N-L binding domain, two CENP-A binding motifs; the central domain, and CENP-C motif, as well as a structured C-terminal homodimerization domain[101–104] (Figure 2(e)). The nature of CENP-C binding to CENP-A is of two types, with the CENP-C motif being the primary mode of binding in many species,[102,105] but with the central domain having higher affinity in mammals.[41,103] Both regions bind to the surface of the nucleosome through its acidic patch and through CENP-A specific hydrophobic contacts. Moreover, the central domain can displace the CENP-C motif, as shown for *in-vitro* reconstituted CENP-A nucleosomes.[106] Recent studies have further indicated that CENP-C binding affinity may change with the cell cycle, rendering CENP-C binding an essential regulatory mechanism of centromere structure between cell stages.[102,107] CENP-N, the other member of the CCNC, forms part of the CENP-N-L subcomplex. It binds to the CENP-A nucleosome through its N terminal globular domain which interacts directly with the CATD (Figure 2(e)). CENP-N interaction with CENP-A is further stabilized through interactions with the nucleosomal DNA.[43,71,72] Finally, CENP-N also interacts directly with CENP-C through its C-terminal domain.[20,43,103]

Recent biophysical and cryo-EM studies have begun to shed light on the impact of CENP-C and CENP-N binding on the CENP-A nucleosome. Binding of the CENP-C central domain has been shown to flatten and rigidify CENP-A nucleosomes[88,108,109] yet further enhance DNA unwrapping[106] possibly creating a more permissive

chromatin environment. *In vivo* studies further determined, that CENP-C binding is required for CENP-A retention at the centromere[110] though there is some debate on this issue.[111] Similarly CENP-N also stabilizes CENP-A nucleosomes by fastening CENP-A to nucleosomal DNA.[43,71,72] Additionally, while CENP-C and CENP-N both bind to CENP-A through the CATD, they do so through complementary interfaces allowing for an additive stabilizing affect to the CCNC.[112] Together CENP-N and CENP-C provide significant stability to the CENP-A nucleosome *in vitro*, although their effect on stability *in vivo* is less clear.[111]

Interestingly, in mammals, simple ectopic deposition of CENP-A and CCNC formation is insufficient for kinetochore assembly and neocentromere formation. Overexpression of CENP-A in HeLa cells has been shown to increase its mislocalisation to non-centromeric sites. Ectopic CENP-A is then able to recruit CENP-C, CENP-N, and Mis12, but the formation of ectopic kinetochores is prevented.[113,114] This regulation can however be overcome by "seeding" of CENP-A, i.e. through artificially tethering HJURP or CENP-I to an ectopic locus, resulting in the induction of functional neocentromeres.[37] In contrast, in yeast and *Drosophila*, simple ectopic CENP-A deposition does lead to the assembly of functional kinetochores.[115]

CENP-B is the only kinetochore component to bind specifically to a conserved DNA sequence called the CENP-B box within the satellite repeat sequence.[30] As CENP-B requires DNA access, the increased dynamic flexibility of DNA at centromeric nucleosomes might be important for CENP-B binding. While CENP-B is not essential for centromere function,[31–33] CENP-B boxes are generally sandwiched between two satellite repeats and appear to be a standard part of the dominant functional centromere.[116] Structural analysis of CENP-B binding to the CENP-B box shows an ability to induce kinks in DNA[117] (Figure 2(f)). Moreover, CENP-B is able to homodimerize through it C-terminal domain and may serve to bridge two satellite monomers.[118,119] CENP-B has also been shown to bind and stabilize the CCNC and enhance centromere function[120,121] with depletion of CENP-B greatly enhancing cell lethality on loss of CENP-A.[122] In addition to its role in CENP-A stabilization, CENP-B can promote heterochromatinization by recruiting Suv39h1 and its binding partner HP1[123,124] and simultaneously induce open chromatin by recruiting ASH1L, a H3K36 methyltransferase. These combined functions render it a key regulator of the centromere chromatin state.[124] Thus, while CENP-B is not essential for centromere function, it may play an important in the structure and stability of the centromere.

The mammalian CENP-T-W-S-X complex is essential for centromere function, acting alongside CENP-C as a pillar linking the centromeric DNA and the outer kinetochore.[125] The exact recruitment mechanism of the CENP-T-W-S-X complex is currently unknown but seems to be dependent on the CENP-H-I-K-M complex[104,126] and microtubule contacts.[127] Structural studies on *Gallus gallus* CENP-T-W-S-X complex show that it contains histone fold domains and is able to form a tetrameric structure, recruiting DNA into a nucleosome like organization.[128] However, though unlike canonical nucleosomes it induces positive rather than negative supercoiling.[129] On the other hand, the crystal structures of CENP-T^{cnn1} binding to the *Saccharomyces cerevisiae* CCAN/Ctf19 complex show that it is oriented in a manner that supports binding to the DNA gyre of CENP-A[130,131] rather than linker DNA (Figure 3 (a)). The exact role of the CENP-T-W-S-X complex on the centromere structure remains unclear and is an interesting area for further study.

Structure of the core centromere

For proper chromosome segregation, the centromere requires many unique properties. It needs to provide a substrate for recruitment of the kinetochore and a rigid connection to the microtubules, while also providing elasticity and resistance to tension. To do this CENP-A needs to exposed to the surface of the chromosome, sufficiently open to allow kinetochore loading, and the underlying chromatin of the core- and pericentromere must be rigid enough to bear mitotic tension. The exact higher order structure of CENP-A containing chromatin, based on its high flexibility, dynamics or inability to form stable tetranucleosomal units, might be important but remains a subject of active research. Early models proposed the formation of an amphipathic, solenoidal superhelix or of radial loops which worked to expose CENP-A to the outer surface of chromatin.[132] Alternatively, super resolution microscopy of other CCAN components such as CENP-T alongside CENP-A were compatible with a structure similar to a layered boustrophedon.[133] It must be noted that, from a physical perspective, the ability of chromatin to form such highly ordered structures over long time and length scales is however unlikely, due to the flexible and dynamic nature of CENP-A nucleosomes. Currently there is no consensus on which of these models is most likely.

Examining the exact distribution of CENP-A at native centromere is complicated by the presence of repeat DNA preventing sequencing-based techniques such as ChIP-Seq. Examination by biochemical analysis and immunofluorescence,[132] or by super-resolution microscopy[133] on stretched chromatin fibers suggest that the centromere exists as islets of CENP-A nucleosomes interspersed

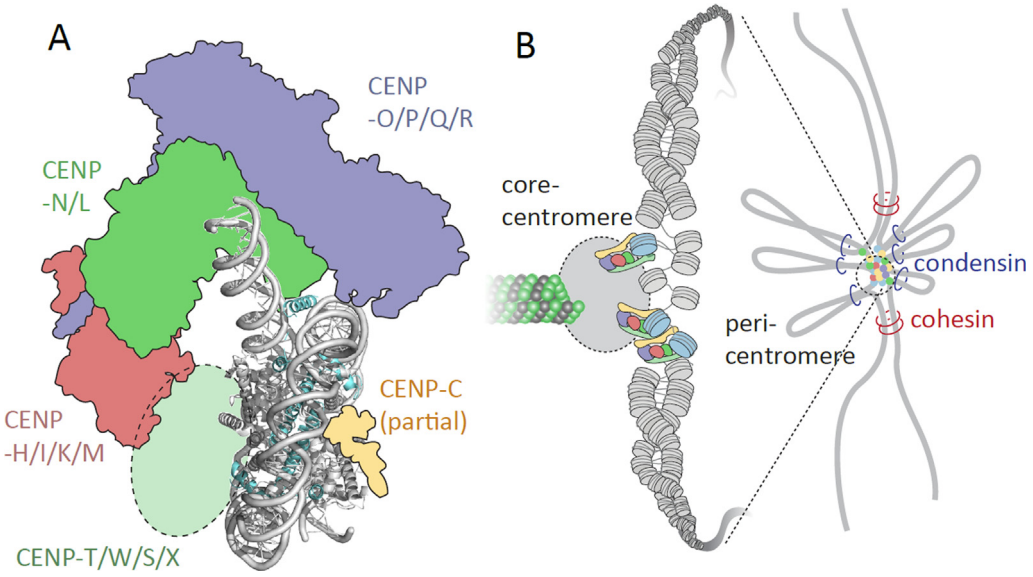

Figure 3. Structure of the CCAN and its role in centromere structure. (a) Organization of the CCAN-CENP-A complex (based of the structure from *S. cerevisiae*, pdb: 6QLD) illustrating the relative positions and size of the CCAN and its components colored as in Figure 1. (b) Schematic of higher order centromeric structure. The model shows the sister chromatids bound by centromeric cohesin with condensin forming DNA extrusions and loops. The centromere contains compacted H3 containing chromatin interspersed with untwisted CENP-A nucleosomes able to form open regions for recruitment of one or two copies of the CCAN. This is aided by CENP-B which can induce kinks into the nucleosomal DNA. The core centromere is exposed to the surface by clustering of core centromeric CENP-A nucleosomes either across multiple chromosomes as in point centromeres or intra chromosomal clustering of CENP-A in regional centromeres possibly by the CCAN and by association with the flanking pericentromeric heterochromatin.

between regions containing H3 nucleosomes. Further quantification of chromosomal CENP-A provides a limit of ~200 CENP-A nucleosomes per mitotic centromere.[134] This creates the question as to how these CENP-A nucleosomes are able to coordinate to form attachments to the spindle microtubules.

The first glimpse of the 3-D structure of a core centromere came recently from circular chromatin conformation capture (4C-seq) measurements of *Gallus gallus* neocentromeres.[135] Though unable to show direct CENP-A – CENP-A interactions within the core centromere due to the resolution of the 4C method, Nishimura *et al.* show frequent interaction within a centromeric region, suggesting clustering of CENP-A nucleosomes.

As described above, the presence of CENP-A alone already alters local chromatin structure drastically, increasing DNA exposure and dynamics. Such local increase in accessibility and dynamics might benefit local recruitment of CCAN proteins, as they can invade centromeric chromatin.[96,97] Indeed, centromeric chromatin structure may have an effect on CCAN recruitment, with the work of Allu *et al.* providing evidence that chromatin compaction at mitosis may cause changes in CCAN stoichiometry and resulting in only one face of the CENP-A nucleosome being able to recruit the full kinetochore.[112]

The presence of CCAN members is also likely to affect the higher order structure of CENP-A islets, with dimeric CENP-B and CENP-C possibly being able to cross-link or cluster chromatin fibers.[118,119,136] Indeed, observations by atomic force microscopy demonstrated the ability of CENP-C to induce clustering of CENP-A containing chromatin arrays.[88] Conversely, cryo-EM studies revealed ladder like assemblies of CENP-A nucleosomes bridged by CENP-N,[43] which stabilized nucleosomes in an unstacked conformation. CENP-B, on the other hand, might bias chromatin towards open states by further bending nucleosomal DNA outwards.[117]

Beyond the various centromeric proteins, many conserved epigenetic marks play a significant role in maintaining the structure of regional centromeres. Studies using stretched chromatin fibers and high resolution microscopy show that the CENP-A-containing “core centromere” region is unique in that it bears histone modifications associated with both euchromatin and heterochromatin.[22] It is characterized by the presence of H3K4me2/H3K36me2 and transient H3K9ac modification but simultaneously lack of H4K5/8/16ac marks,[22,124,137] both usually required for active transcribed chromatin. This suggests the core centromere possesses an open structure coupled with low or transient transcription.

With regards to methyl marks, early studies showed a lack of H3K9me2/3, which is associated with heterochromatin. In contrast, more recent observations, using super resolution microscopy, found areas enriched in H3K9me3 at active centromeres.[133] This was confirmed by affinity purification and mass spectrometry of human centromeric chromatin, showing the presence of dual modified H3K9me2/K27me2.[138] Finally, in repetitive centromeres, the centromere-boundary is marked by pericentromeric regions that are highly enriched in H3K9me2/3 and H3K27me3, and contain HP1.[22,124,138]

The presence of chromatin marks associated with transcription and open chromatin is most likely necessary to create a permissive structure necessary for CENP-A incorporation but at the same time also allow for the transcription of centromeric RNA by RNA pol II.[70,139–141] Studies on these cenRNA using knockdowns or overexpression show that they complex with CENP-A and CENP-C. Reducing their levels further affects CENP-A deposition.[137,141] The exact role of these centromeric transcripts is beyond the scope of this review. More detailed discussion on centromeric transcription can be found in some recent reviews.[139,142–144]

Taken together, these studies paint a much clearer picture of the core centromeric chromatin, as a relatively open structure with interspersed flexible CENP-A nucleosomes which are able to cluster spatially through interactions within the CCAN. Whether this results in a regular higher order structure (i.e. loop or sheet)[19] or forms *via* a mechanism similar to liquid–liquid phase separation or gelation as seen for the chromosomal passenger complex (CPC)[145,146] remains unknown and an interesting area for future study.

Structure of the pericentromeric chromatin in vivo

The pericentromeric regions flank the core centromere and act as a boundary for the CENP-A containing core.[147] In regional centromeres, the pericentromeric is rich in heterochromatin, H3K9me2/3 and H3K27me3 and HP1.[22,124,138] In fission yeast and *Drosophila*, artificial formation of heterochromatin by tethering of the H3K9 methyltransferase Clr4 or HP1 respectively, even induces CENP-A deposition.[148,149] The only exceptions to the requirement for heterochromatin-containing pericentromeric regions appears to be in neocentromeres of vertebrate cells.[150,151] This contradiction was recently resolved by the work by Nishimura *et al.*, who used 4C-seq to show long range interactions between neocentromeres in chicken DT40 cells and heterochromatin containing regions, thus confirming the importance of flanking heterochromatin on centromere structure and function.[135] Interestingly these long range interaction are dependent on the CCAN and more specifically on the CENP-H group of protein.[135] Thus the presence and maintenance of the pericentromeric heterochromatin state appears to be essential for proper chromosome segregation[152] and acts with the CCAN to maintain centromere stability and constrain the spread of the core centromere into adjacent euchromatin regions.[153,154]

The main structural role of the pericentromeric chromatin is the recruitment of the SMC (structural maintenance of chromosomes) complex proteins cohesin and condensin.[155–157] At the centromere, these proteins play an important role in cohesion between sister chromatids and DNA loop formation. The exact mechanism of cohesin recruitment to the centromeres is not completely understood but is suspected to occur through pericentromeric HP1/Suv39 in mammals,[158] and through the Scc2-Scc4 cohesin loaders in budding yeast.[159–161] The SMC proteins are in turn regulated by additional factors, such as shugoshin, and the chromosome passenger complex (CPC).[162–167] The mechanism by which shugoshin and the CPC are recruited to the centromere consists of various feedback loops among the CPC components, phosphorylation of histones H3 and H2A, and multiple mitotic kinases and phosphatases, including PP2A, Haspin, Aurora B, Plk1, Bub1, and CDK1. Recent reviews discuss this topic in detail.[146,168–170]

The non-repetitive point centromeres of *Saccharomyces cerevisiae* provide the most extensive snapshot of a looped pericentromeric structure. Studies using chromatin conformation capture (3C) in yeast showed the clear presence of centromere loops.[171,172] Further observations using LacO/LacI-GFP arrays placed in yeast pericentromers showed that these arrays were radial when they appeared as foci, but were axial when they appeared extended, also indicating a looped structure.[156] Localization of SMC proteins during mitosis provided evidence for a barrel-like structure, that extends between the kinetochores and overlaps with pericentromeric chromatin[171] with cohesin at the periphery and condensin localizing around the center of the barrel.[156] This data allowed Bloom and colleagues to propose a cruciform "bottle-brush" structure for centromeric chromatin, with intra chromosomal cohesin trapping the core centromere and extruding it perpendicular to the chromosomal axis. More recent work by Paldi *et al.* modifies this model to propose that cohesin forms loops on either side of the centromere, until halted by convergent genes at the pericentromere border. These convergent genes act as traps for inter-sister chromatid cohesion.[173]

How these models extend to regional centromeres in currently unknown. SMC proteins have been shown to localize to the pericentromeres of most higher-eukaryotes[174,175] and likely play a similar role in maintaining its higher order structure (Figure 3

(b)). Regional centromeres do not show inter chromosomal centromere clustering in mitosis unlike point centromeres but may use a similar mechanism to cluster intra centromere CENP-A loci. Work by Trivedi *et al.* also allows for the possibility for liquid–liquid phase separation at the centromere. They show that the CPC is able to form liquid like droplets at physiological centromeric concentrations and could associate with alpha satellite transcripts.[145,146] Similarly, heterochromatin associated proteins such as HP1 have also been shown to phase separate.[176,177] Thus, while recent work has begun to unveil the higher order structure of regional pericentromeres it remains very much an open question. Recent work on determining the sequence of repetitive DNA[178,179] at centromeres will likely be key in extending powerful techniques like Hi-C and CHIP-Seq to help determine the centromere structure.

Conclusion

The centromere and kinetochore have emerged to as a truly elaborate structure which plays a fundamental role in mitosis. Recent advances in cryo-electron microscopy, chromosome capture techniques, human artificial chromosome formation, and super resolution microscopy have delivered key insights into the structure of the mitotic centromere. Yet we are only beginning to fully understand the overall architecture, intricate dynamics and regulation of the centromeric chromatin. Key questions remain with respect to the structural organization of centro-chromatin beyond single CENP-A nucleosomes. How are CENP-A nucleosomes embedded in centro-chromatin and how do they shape overall chromatin organization over short and long distances? How do complex, multivalent and potentially transient macromolecular interactions control recruitment and positioning of CENP-A and CCAN proteins within the centromere? Moreover, what is the nature of the nucleosome like structure of CENP-T/W/S/X? What is the role of CENP-B in shaping the structure and function of centro-chromatin? And finally, what are the distinct roles of various histone PTMs markers as well as the underlying DNA structure in centromere organization? Future studies, combining structural biology and dynamic biophysics of individual reconstituted or isolated segments centro-chromatin, as well as super-resolution imaging in living cells and functional assays are required to solve these questions, and reveal the fine structure of this intriguing chromatin compartment.

CRediT authorship contribution statement

Harsh Nagpal: Conceptualization, Writing - original draft, Writing - review & editing. **Beat Fierz:** Writing - review & editing, Project administration, Funding acquisition.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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1 Employers could choose their customized charging demand to be served by WPC. For example, additional miles could be added to allow additional mileages for other travel activity purposes. Also, the actual charging demand could be adjusted if different vehicle types with various fuel economies are considered.

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