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Structure, Assembly and Reading of Centromeric Chromatin

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Abstract

Centromeres are epigenetically defined chromatin domains marked by the presence of the histone H3 variant CENP-A. Here we review recent structural and biochemical work on CENP-A, and advances in understanding the mechanisms that propagate and read centromeric chromatin domains.

In the majority of eukaryotes, centromeres are not defined by a specific DNA sequence but instead by the presence of the centromeric histone CENP-A/CenH3 (CENP-A in humans, Cse4 in budding yeast, CID in *Drosophila*, HCP-3/CeCENP-A in *C. elegans*; referred to generally as CENP-A hereafter except where the specific species identity is important). CENP-A chromatin functions as both part of an epigenetic mark for centromere identity and a structural foundation for assembly of the kinetochore, the multi-protein machine that forms the primary attachment site on chromosomes for spindle microtubules during cell division.

The lack of a specific conserved centromeric DNA sequence had been long-suggested by the diversity of DNA sequences found at centromeres throughout evolution [1], as well as by the presence of monocentric and holocentric chromosome architecture in extant eukaryotes. The latter, documented to date in nematodes and several lower plant as well as insect species, is characterized by the presence of centromere activity along the length of the chromosome during mitosis [2]. The strongest functional evidence for sequence independence of centromere activity has been derived from studies of neocentromeres, which form rarely at non-centromeric sites on chromosomal loci that bear no sequence identity to the endogenous centromeric DNA in the same species [3]. A well-studied exception to the sequence-independence of centromeres is the budding yeast *S. cerevisiae*, where a specific 125 bp DNA sequence is sufficient for centromere activity—however, this centromere also assembles a single CENP-A nucleosome that is well-positioned on the CEN DNA sequence [4]. Interestingly, in the sleeping sickness pathogen *Trypanosoma brucei* and during meiosis in *C. elegans,* chromosome segregation may occur without CENP-A [5,6] but the mechanisms directing kinetochore assembly in these cases are not known.

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Here, we will focus on the predominant CENP-A dependent centromere definition mechanism, reviewing recent work that has advanced our understanding of the structure and assembly of this specialized chromatin domain. In particular, we review recent structural efforts that have provided an atomic view of CENP-A-containing nucleosomes and pre-nucleosomal complexes, and biochemical/cell biological studies that have revealed how this specialized chromatin builds a kinetochore.

(Resolving) The Debate on DNA-Bound Structures Built from CENP-A

While the importance of chromatin containing CENP-A at centromeres is widely accepted, the same is not true for the architecture of the DNA-CENP-A assembly that is functionally relevant at centromeres. The initial discovery that CENP-A is a histone H3 variant and the mapping of its centromeric identity to its histone fold [7,8], led to the parsimonious hypothesis that CENP-A forms a structure similar to H3-containing nucleosomes and that differences in the CENP-A histone-fold underlie recognition by specialized trans-acting machinery. Initial *in vitro* reconstitution experiments in the presence of a DNA substrate were consistent with this view [9]. In subsequent years, reconstitution efforts in multiple species have consistently revealed that CENP-A forms robust octameric nucleosomes *in vitro* [10]. A recent experiment employing mixtures of histone H3 and the budding yeast CENP-A (Cse4) in the same assembly reactions showed that assembly of homotypic octamers containing only CENP-A or H3 is strongly favored [11] this result is consistent with relative paucity of H3 in CENP-A chromatin immunoisolations following extended micrococcal nuclease digestion [12]. The major (and consistent) difference observed between H3 nucleosomes and CENP-A nucleosomes *in vitro*, starting from the first study of CENP-A nucleosomes, has been the extent of DNA protection. CENP-A nucleosomes protect less DNA than H3 nucleosomes (~120 bp vs 145 bp) in nuclease digestion experiments [9].

The view that CENP-A forms octameric nucleosomes *in vitro* has been challenged in recent years by two alternative proposals: i) CENP-A assembles into half-nucleosomes (hemisomes) with right-handed DNA winding topology [13]; this proposal was based on reconstitutions with *Drosophila* CENP-A (CID), atomic force microscopy of *Drosophila* interphase CENP-A chromatin [14] and topological analysis of minichromosomes isolated from yeast; ii) Budding yeast CENP-A forms a distinct non-nucleosomal structure that lacks histones H2A and H2B; this proposal was based on formation of a hexameric complex of CENP-A/H4 and the CENP-A binding protein Scm3 in high salt, and the inability to detect H2A/H2B using chromatin immunoprecipitation *in vivo* at budding yeast centromeres [15]; for an in depth discussion of alternative proposals, see [16]. Subsequent *in vitro* efforts from multiple groups have argued against these views and provided additional evidence for an octameric CENP-A nucleosome with left-handed DNA winding topology regardless of whether the assembly is performed using dialysis from high salt or using a chaperone at physiological salt [11,17–22]. Thus, at least in vitro, it appears that CENP-A can form octameric nucleosomes closely resembling H3 nucleosomes. Whether an octameric nucleosome is assembled on budding yeast centromeric DNA in vitro is less clear; the highly AT-rich CEN DNA sequence disfavors octameric nucleosome assembly [22,23], but Cse4 is nonetheless able to form robust octamers on other DNA sequences [22]. A definitive answer in this system will likely require better understanding of the architectural contributions of other budding yeast-specific CEN DNA-binding proteins such as the CBF3 complex [24].

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Atomic Structures of the CENP-A Nucleosome and CENP-A:H4 Pre-Nucleosomal Complex

In the past two years, structural studies have shed much-needed light on CENP-A and CENP-A-DNA complexes, with the most significant advance being the crystal structure of a full human CENP-A nucleosome [20]. Comparison of CENP-A and H3 nucleosome structures that were determined with the same DNA sequence reveals almost perfect overlap, with a root mean square deviation of 0.6 Å comparing 663 of 706 carbon-α atoms in the octamer (Figure 1A, B). The primary difference between the two structures is that while the entire 147 bp of DNA is well-ordered in H3 nucleosome, only 121 bp of the same DNA fragment are ordered in the CENP-A nucleosome, with 13 bp on either end disordered (Figure 1B). This finding is consistent with biochemical experiments and may explain early observations that centromeric chromatin exhibits unusual nuclease sensitivity [25]. Another structural difference was that the Loop 1 region of CENP-A adopts a more extended and flexible conformation than in the canonical H3 nucleosome (Figure 1C) [20,21]. Overall, the CENP-A nucleosome structure has revealed remarkable similarity to H3 nucleosomes, with the major structural difference being less strong binding of DNA at the entry and exit sites.

The nucleosome structure of CENP-A was preceded by structures of the human and budding yeast (*Kluyveromyces lactis*) CENP-A:H4 tetramers without bound DNA [21,26]. Both structures showed significant compaction (by 10–14°) of the tetramer relative to $H3_2$:$H4_2$ in the canonical H3 nucleosome structure (Figure 2; an atomic structure of the isolated H3:H4 tetramer has not been determined). This compaction was proposed to be a distinguishing property of CENP-A [21]. However, within the structure of the full centromeric nucleosome no difference is observed from H3, suggesting that the observed compaction may be due to the lack of bound DNA and/or histones H2A/H2B. The human CENP-A:H4 tetramer structure revealed an extensive network of hydrophobic residues anchoring the ends of the CENP-A:H4 interaction more tightly than the H3:H4 interaction [21]. This "hydrophobic stitching" likely explains the lower exchange in hydrogen-deuterium exchange assays for CENP-A nucleosomes compared to H3 nucleosomes [10]. As the rigid region includes the CENP-A α2 helix, which comprises part of the centromere targeting domain, this stitching may confer physical properties that are important for CENP-A function at centromeres *in vivo*.

Does the similarity of H3 and CENP-A nucleosome structures close the door on native CENP-A chromatin structures in cells adopting alternative states? Mutations in the budding yeast CENP-A (Cse4) based on interfaces in the canonical H3 nucleosome structure and suppression of the lethality of an Scm3 deletion by overexpression of Cse4 argue for an octamer in *vivo* in this organism [19]; however, the absence of H2A/H2b at CEN DNA by chromatin immunoprecipitation remains unexplained. In *Drosophila* and human cells, atomic force microscopy of digested CENP-A chromatin from asynchronous nuclei has revealed the presence of particles precisely half the height of H3 nucleosomes that were isolated in the same manner, and a single CENP-A molecule was detected by immunoEM in these particles [14,27]. One possible explanation for structural variation is assembly transition states representing pre and/or post DNA replication. Alternatively, *in vivo* factors, such as the CENP-A associated proteins known as the CCAN [28], may stabilize an intermediate state that is not favored *in vitro*. Additional means to directly assess the structure of CENP-A-DNA assemblies in cells, including imaging methods that do not require isolation and nuclease digestion, will hopefully resolve this debate.

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Structural Analysis of the CENP-A Chaperone Scm3/HJURP

The question of how CENP-A is recognized and specifically deposited at centromeres has focused on the histone fold domain since early work [7]. Analysis of chimeric proteins revealed that Loop 1 and the α2 helix of CENP-A, termed the CENP-A targeting domain (CATD), are responsible for targeting CENP-A to centromeres [10,29]. While the importance of the CATD in centromere definition by CENP-A is well established, whether the H3-CATD chimera is sufficient for centromere function is less clear. In human cells, the H3-CATD chimera complements RNAi-based partial depletions of CENP-A [30], but genetic studies in yeast and plants suggest that additional parts of the CENP-A molecule are essential [30–32]. In particular, essential elements have been defined in the tail and the extreme C-terminus of budding yeast CENP-A; in *Arabidopsis,* introduction of a tailswap mutant (CENP-A histone-fold domain with the H3 tail) into a CENP-A null mutant does not inhibit mitotic divisions but leads to meiotic defects and the formation of haploid plants[32,33].

Understanding how the CATD is critical for centromeric targeting has been greatly advanced by the discovery of a conserved CENP-A specific chaperone family (HJURP in human cells and Scm3 in budding yeast) recognizing this region. HJURP/Scm3 chaperones are not ubiquitous – both *Drosophila* and *C. elegans* appear to lack them [34]. HJURP and Scm3 share a short region of homology that is sufficient for CENP-A recognition (Figure 3A) [15,17,35]. Three recent studies have now revealed the structural basis for CATD recognition by these chaperones: an x-ray crystal structure of HJURP bound to CENP-A:H4 (Figure 3B) [36], and both NMR and x-ray structures of budding yeast (NMR: *S. cerevisiae*, x-ray: *K. lactis*) Scm3 bound to Cse4:H4 (Figure 3C)[26,37]. The two x-ray structures, despite the extreme sequence divergence of Scm3 and HJURP, look similar (0.6 Å root mean square deviation between 148 common α-carbon atoms in the three chains) while the NMR structure is significantly different (Figure 3B, C)—the reasons for this are unclear but may reflect the different protein regions used in the NMR study or the fact that the three proteins were fused into a single chain (see [26,37,38] for debate on this issue). Here, for brevity, we will restrict our interpretations to the two structures that agree.

The most striking feature revealed by the x-ray structures is that the region of homology between the two histone chaperones forms a long α-helix that binds antiparallel to the CENP-A/Cse4 α2 helix (Figure 3B, C)—this configuration of the complex is incompatible with both CENP-A:CENP-A tetramerization and with DNA binding [26,36]. Thus, both structures are of a heterotrimer containing one copy each of HJURP/Scm3, CENP-A/Cse4, and H4. The formation of a heterotrimer was confirmed by analytical ultracentrifugation using full-length proteins from both *K. lactis* and *S. cerevisiae* [26]. Scm3 is not observed to associate with the Cse4 loop 1 region, and while HJURP associates with this region of CENP-A, it does not contact residues that would provide significant specificity over H3 [36]. Thus, the critical recognition of CENP-A/Cse4 occurs in the α2 helix. Both structures show specific interactions between chaperone residues and CENP-A/Cse4-specific residues in this helix; mutating these residues disrupts *in vitro* binding [26,36].

Overall, the structures of Scm3/HJURP bound to CENP-A:H4 together with biochemical analysis have revealed a trimeric complex with the key recognition occurring via the α2 helix of the CATD. The CATD is also the recognition site for the E3 ubiquitin ligase Psh1 that functions to reduce CENP-A misincorporation at non-centromeric sites in budding yeast [39,40]. While there is no obvious sequence homology between Psh1 and Scm3, structural analysis of Psh1 may help reveal whether the recognition properties observed with Scm3 are also utilized in other contexts to recognize CENP-A.

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Recent biochemical studies have shown that HJURP/Scm3 exhibits chaperone activity, promoting the formation of CENP-A:H4 nucleosomes *in vitro* [17,18,35]. Thus, an Scm3 molecule likely delivers a CENP-A:H4 dimer to the DNA for assembly. This result raises the question whether the CENP-A_2:$H4_2$ tetramer purified following expression in bacteria is functionally relevant during assembly; the fact that the H3:H4 chaperone Asf1 also binds a dimer (albeit at different elements than $\alpha 2$; [41]), suggests that chaperone-bound physiologically relevant cargo during both CENP-A and H3 nucleosome assembly is a dimer. Once the CENP-A:H4 dimers are recruited to centromeric regions, they likely assemble first into CENP-A_2:$H4_2$ tetramers bound to DNA. In the process of tetramerization and DNA binding, HJURP/Scm3 is ejected from the complex [22,23]. A higher-molecular weight complex of *S. cerevisiae* Scm3:Cse4:H4, potentially corresponding to a 2:2:2 heterohexamer, has been observed *in vitro* at high salt (2.0M NaCl) but not at lower salt (0.5M NaCl) [15,37]. This finding suggests the possibility of a transient hexameric complex existing as an assembly intermediate at centromeres.

Steps in Propagation of CENP-A Domains *in Vivo*

Centromeric CENP-A is proposed to be partitioned to sister chromatids during DNA replication and replenished later in the cell cycle. Intervening “gaps” between CENP-A nucleosomes following replication are likely filled by nucleosomes containing histone H3.1 and H3.3, a variant normally associated with non-replicative chromatin assembly. New CENP-A loading occurs soon after anaphase in mammalian cells and *Drosophila* embryos ([42,43]; see [44] for evidence that loading occurs in metaphase in cultured *Drosophila* cells); in plants, loading occurs in G2, whereas in fission yeast it occurs during both S phase and G2 [45,46]. A reduction in H3.3 levels at centromeres in G1 of human cells suggests that H3.3 acts as a “placeholder” and is replaced by CENP-A nucleosomes during replenishment [47].

The loading of new CENP-A nucleosomes must require CENP-A:H4-HJURP complexes to target specifically to centromeres through protein-protein/DNA interactions. In the case of budding yeast, Scm3 is recognized by the CBF3 complex, which specifically binds to CEN DNA [48]. As CBF3 is not conserved outside of budding yeasts, targeting of CENP-A:H4-HJURP likely occurs by an interaction with other proteins that recognize existing centromeric chromatin domains. The Mis18 complex (comprised of Mis18 and Knl2/MI8BP1 [49,50]) is the primary candidate for this function, but the mechanism by which this complex recognizes centromeric chromatin and recruits pre-nucleosomal CENP-A complexes is currently unclear (only a weak Scm3-Mis18 interaction has been observed in fission yeast [51]. However, consistent with this view, ectopically targeting HJURP to non-centromeric DNA promotes *de novo* CENP-A incorporation and kinetochore assembly without a requirement for Mis18/Knl2 [18]. Work in *Xenopus* egg extracts has shown that the centromere protein CENP-C, which is closely associated with CENP-A nucleosomes, can bind to and recruit Knl2 (and thus Mis18 [52]); however, this does not appear to be the case in *C. elegans* embryos [49]. Knl2 itself contains a conserved Myb-type DNA binding domain, raising the possibility that its ability to directly bind DNA plays a critical role [49]. Interestingly, the Mis18 and Knl2 family proteins are not always present together—Mis18 is not found in *C. elegans*, while Knl2 appears to be absent from fission yeast, and both proteins are absent in *Drosophila*, which instead relies on a protein named Cal1 for CENP-A assembly [53,54].

Analysis of Knl2 in human cells has revealed an additional maintenance step during centromere propagation, where the small GTPase Cdc42 helps ensure that newly incorporated CENP-A is retained at centromeres [55]. A recent study of H3 nucleosome assembly *in vitro* has identified a non-nucleosomal histone-DNA intermediate with the

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dimensions of a nucleosome that is converted by an ATPase chromatin motor into nucleosomes [56]—whether such an intermediate exists for CENP-A nucleosomes and whether specific mechanisms, such as the Cdc42 GTPase cycle, are involved in stabilizing such an intermediate are avenues for future investigation.

The rules by which CENP-A incorporation are guided to a specific chromosomal site are likely to involve both recognition of pre-existing CENP-A nucleosomes and other cues that reflect specific chromatin states [57]. In support of this, *de novo* CENP-A incorporation is observed at non-centromeric sites in *Drosophila* cells following ectopic targeting of the heterochromatin protein HP1 [58]. *De novo* CENP-A incorporation and neocentromerization is also rapidly observed on naked DNA injected into the germline of the holocentric nematode *C. elegans* [59]; however, this reaction appears to be independent of HP1 family proteins. In mammals, the alpha-satellite sequence binding CENP-B protein controls *de novo* CENP-A chromatin formation depending on the chromatin context [60]. Mechanistic insights into *de novo* CENP-A chromatin domain formation are likely to reveal principles that are utilized in the context of pre-existing CENP-A nucleosomes to propagate centromere identity [61,62] and enable artificial centromere engineering.

Reading CENP-A Chromatin to Build a Kinetochore

Two major advances in understanding how CENP-A chromatin templates assembly of the kinetochore have emerged from biochemical work in *Xenopus* egg extracts and through ectopic targeting of proteins to non-centromeric chromosomal loci in mammalian cells [63,64]. *In vitro* analysis had revealed that CENP-C, a widely conserved centromeric protein, specifically recognizes a short C-terminal tail sequence that is specific to CENP-A [65]. CENP-A nucleosome arrays built on DNA attached to beads recruit kinetochore proteins in *Xenopus* egg extracts. Remarkably, a chimeric H3 with the C-terminal CENP-C binding motif is sufficient for this recrutiment activity. This result points to a critical role for the C-terminal CENP-A tail in kinetochore assembly, via its recognition by CENP-C [63]. Surprisingly, this tail sequence is not conserved outside of vertebrates, although the central importance of CENP-C in kinetochore assembly is widely conserved. Understanding how CENP-C recognizes CENP-A nucleosomes outside of vertebrates will be important to address in future work.

Proteomic analysis of CENP-A nucleosomes identified an extensive set of proteins (collectively referred to as the CCAN) specifically associated with centromeres throughout the cell cycle [12,28,66]. Of these components, CENP-N specifically recognizes the CATD of CENP-A; however, the contribution of this recognition to kinetochore assembly is currently unclear [63,67]. The CENP-T/W complex has been proposed to bind H3 nucleosomes adjacent to CENP-A nucleosomes to direct kinetochore assembly [68] and CENP-T changes its conformation when kinetochores are attached and under tension [69]. Ectopic targeting experiments in human cells suggest that kinetochore assembly is directed by a cooperative mechanism, involving CENP-C recognition of the C-terminal tail of CENP-A, and CENP-T recognition of H3 nucleosomes adjacent to CENP-A [64]. Some species, such as *D. melanogaster* and *C. elegans*, appear to lack the CCAN and rely exclusively on CENP-C for kinetochore assembly [70–72]. How CENP-T/W recognize H3 nucleosomes in proximity to CENP-A nucleosomes but not elsewhere in the genome is an important question to address in future work.

In addition to the molecular mechanisms underlying recognition of CENP-A chromatin, the organization of this chromatin to form a surface for kinetochore assembly is critical for chromosome segregation. Superresolution imaging has suggested a complex layered

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organization of CENP-A and H3 nucleosomes, with binding of CENP-C forming a platform for kinetochore assembly [73].

Conclusion

Here, we have summarized recent advances in centromere biology. This is an exciting time when the convergence of structural/biochemical studies and *in vivo* work is providing a glimpse of how centromeric chromatin is defined, propagated and read to direct chromosome segregation. Many questions remain in each area and the future holds great promise for converting initial insights into deeper mechanistic understanding.

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37) illustrate the structural basis for how the HJURP/Scm3 chaperone specifically recognizes CENP-A/Cse4. The papers also illustrate that CENP-A recognition is conserved despite the sequence divergence between budding yeast Scm3 and metazoan HJURP. Note that the structure in Ref 37, which employs NMR, differs significantly from Ref 26. [PubMed: 21606327]

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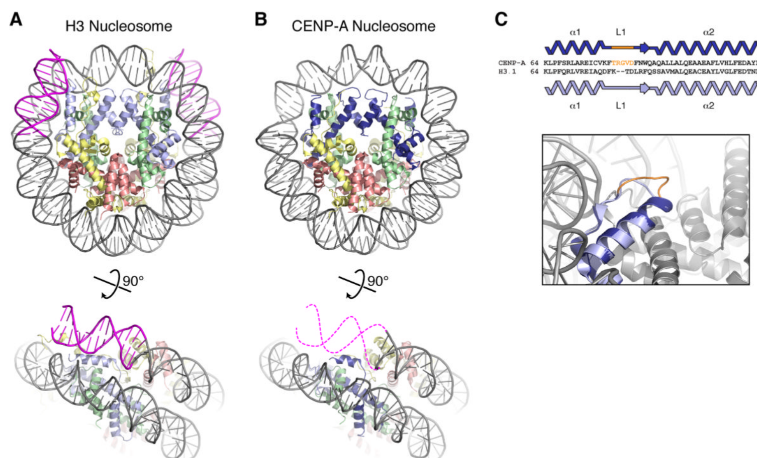

Figure 1.

Canonical and centromeric nucleosomes. **(A)** Canonical H3-containing nucleosome (PBD ID 1KX5) [74], with histone H3 light blue, H4 green, H2A yellow, H2B red, and DNA gray (13 bp on each end colored magenta). **(B)** CENP-A-containing nucleosome (PDB ID 3AN2) [20], with CENP-A colored dark blue. *Bottom:* view of one DNA end, with the 13 bp of disordered DNA indicated by dashed magenta lines (figure adapted from [20]). **(C)** Loop 1 differences in CENP-A and H3 (adapted from [20]). *Top:* Sequence alignment showing the two-residue insertion in CENP-A loop 1. *Bottom:* closeup view of the loop 1 region, with CENP-A from [20] in dark blue and H3 from [74] in light blue. Shown in orange are CENP-A residues 79–83, which are ordered in the CENP-A monomer shown (potentially due to crystal packing interactions) and disordered in the second CENP-A monomer. An earlier CENP-A_2:$H4_2$ tetramer structure also showed a distinct conformation of CENP-A loop 1 with high crystallographic B-factors indicating flexibility [21]. While mutation or truncation of loop 1 mildly affects centromere targeting of CENP-A *in vivo* [20], the mechanism of this defect remains unknown.

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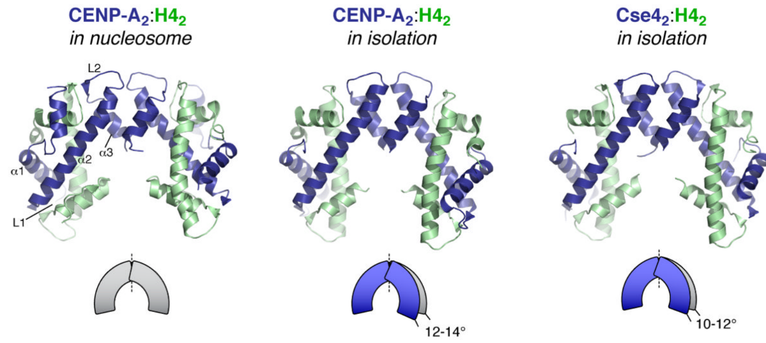

Figure 2.

CENP-A_2/Cse4_2:$H4_2$ tetramer structures, in the context of the full nucleosome (left) (PDB ID 3AN2) [20], and in isolation (middle: human CENP-A_2:$H4_2$ (PDB ID 3NQJ) [21], right: budding yeast (*K. lactis*) Cse4_2:$H4_2$ (PDB ID 2YFW) [26]), colored as in Figure 1. Secondary structure elements of one CENP-A monomer are labeled (left panel). Both tetramer structures determined without bound H2A:H2B and DNA show compaction of the tetramer by 10–14°, relative to the conformation of both the $H3_2$:$H4_2$ and CENP-A_2:$H4_2$ tetramers in the context of the full nucleosome (illustration adapted from [21]).

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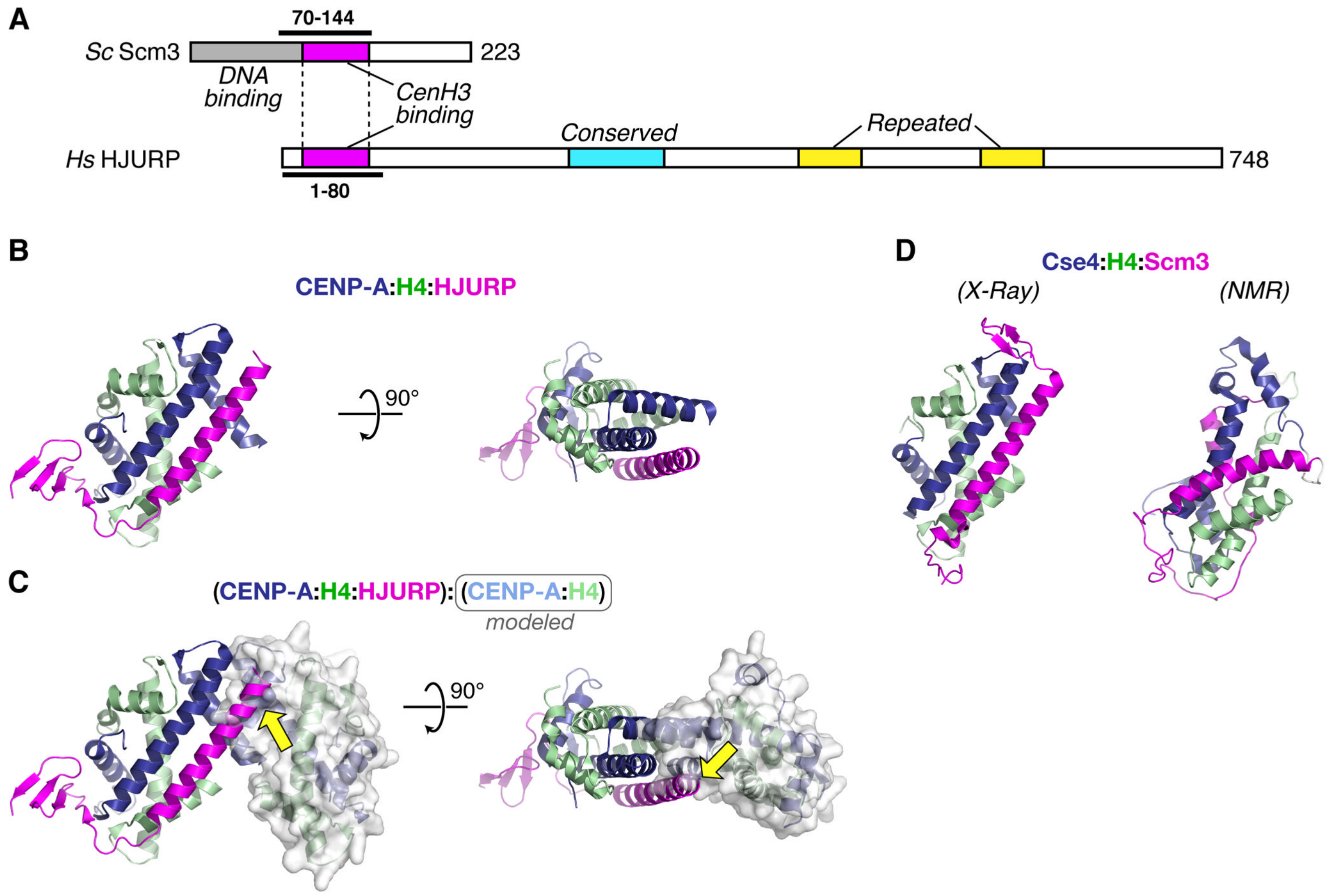

Figure 3.

Structural analysis of the CENP-A chaperone HJURP/Scm3. **(A)** Diagram of *S. cerevisiae* Scm3 and *H. sapiens* HJURP. Conserved domains identified in [34] are shown: CENP-A/Cse4 binding region (Scm3 residues 90–142, HJURP residues 16–68) magenta, HJURP conserved domain (228–304) cyan, HJURP repeated regions (411–462 and 556–608) yellow. The DNA-binding region of Scm3 (1–113) identified by Xiao et al [23] is shown in gray. Constructs used for x-ray crystallography are indicated by thick lines. **(B)** Side and top views of the CENP-A:H4:HJURP trimer structure (PDB ID 3R45) [36], with HJURP shown in magenta. **(C)** The CENP-A:H4:HJURP trimer aligned with a second CENP-A:H4 dimer (molecular surface displayed), showing how HJURP partially occludes the CENP-A:CENP-A interface in the tetramer (yellow arrows). **(D)** Two structures of the budding-yeast Cse4:H4:Scm3 trimer. *Left*: structure obtained by X-ray crystallography of the proteins from *K. lactis* (PDB ID 2YFV) [26]. *Right*: structure obtained by NMR of the *S. cerevisiae* proteins (PDB ID 2L5A) [37]. All structures are oriented the same relative to the left-hand copy of CENP-A/Cse4 in Figure 2.