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CENP-C unwraps the human CENP-A nucleosome through the H2A C-terminal tail

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Abstract

Centromeres are defined epigenetically by nucleosomes containing the histone H3 variant CENP-A, upon which the constitutive centromere-associated network of proteins (CCAN) is built. CENP-C is considered to be a central organizer of the CCAN. We provide new molecular insights into the structure of human CENP-A nucleosomes, in isolation and in complex with the CENP-C central region (CENP-C^{CR}), the main CENP-A binding module of human CENP-C. We establish that the short αN helix of CENP-A promotes DNA flexibility at the nucleosome ends, independently of the sequence it wraps. Furthermore, we show that, *in vitro*, two regions of human CENP-C (CENP-C^{CR} and CENP-C^{motif}) both bind exclusively to the CENP-A nucleosome. We find CENP-C^{CR} to bind with high affinity due to an extended hydrophobic area made up of CENP-A^{V532} and CENP-A^{V533}. Importantly, we identify two key conformational changes within the CENP-A nucleosome upon CENP-C binding. First, the loose DNA wrapping of CENP-A nucleosomes is further exacerbated, through destabilization of the H2A C-terminal tail. Second, CENP-C^{CR} rigidifies the N-terminal tail of H4 in the conformation favoring H4^{K20} monomethylation, essential for a functional centromere.

Keywords CENP-A; CENP-C; centromere; cryo-EM; nucleosome
Subject Categories Chromatin, Transcription & Genomics; Structural Biology
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Introduction

The centromere is a chromosomal locus that directs accurate segregation of chromosomes during cell division [1]. Defects in chromosome segregation lead to aneuploidy, a hallmark of cancer [2].

DNA sequences underlining human centromeres are composed of AT-rich repeats (termed α-satellites), but these are neither necessary nor sufficient for centromere function. Instead, centromeres are specified epigenetically by the presence of the histone H3 variant, CENP-A [reviewed in 3]. Chromatin containing CENP-A nucleosomes must have unique structural properties to organize the constitutive centromere-associated network (CCAN). It is therefore critical to gain a full structural understanding of the CENP-A nucleosome alone and in complex with the two key components of the CCAN with which it interacts directly and specifically—CENP-C and CENP-N [4]. Initial clues on CENP-A nucleosome-specific features came from crystallographic studies of the $(CENP\text{-}A/H4)_2$ tetramer [5] and of the CENP-A nucleosome [6]. These studies implied that the CENP-A nucleosome has an octameric histone core, similar to canonical nucleosomes in composition and structure. DNA ends in the crystal structure of CENP-A nucleosome [6] are disordered, indicating increased DNA flexibility. Recently, cryo-EM structures of the human CENP-A nucleosome in complex with human CENP-N have been reported by several groups [7–9], revealing high-resolution molecular determinants for the CENP-A/CENP-N interaction.

CENP-C is a central component of the CCAN, responsible for interactions both with the CENP-A nucleosome on the chromatin-side and with subunits of the Mis12 complex on the kinetochore side [10]. Human CENP-C is a 934 amino acid long disordered protein, depletion of which leads to cell division defects and chromosome mis-segregation [11,12]. Two regions in human CENP-C have been identified as nucleosome binding regions: (i) the central region (aa 426–537), CENP-C^{CR}, that is necessary and sufficient to promote CENP-A nucleosome binding *in vitro* and kinetochore targeting *in vivo* and (ii) the CENP-C motif (aa 736–758), CENP-C^{motif}, that is conserved across species, but is not sufficient for centromere targeting in the absence of endogenous CENP-C as it requires the CENP-C dimerization domain. The CENP-C^{motif} is dispensable for epigenetic stability of the CENP-A nucleosomes [10,11,13].

The current molecular understanding of the CENP-A nucleosome/CENP-C interactions is based on the crystal structure of the canonical *D. melanogaster* nucleosome in which the C-terminal tail of histone H3 is replaced by the C-terminal tail of rat CENP-A, in complex with the rat CENP-C motif [14].

Here, we report a 3.8 Å cryo-EM structure of the CENP-A nucleosome that confirms flexibility of DNA ends as an intrinsic property of CENP-A nucleosomes. We find that terminal DNA flexibility is

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independent of the nature of the underlining DNA sequence and is instead dictated primarily by the N-terminal tail of CENP-A. Furthermore, we find both nucleosome binding domains of CENP-C, CENP-C^{CR} and CENP-C^{motif}, to be specific for CENP-A nucleosomes, where CENP-C^{CR} shows stronger binding. We also determined the cryo-EM structure of the human CENP-A nucleosome in complex with human CENP-C^{CR} at 3.1 Å resolution and identified CENP-A^{V532} and CENP-A^{V533} as the key determinants for strong affinity of the CENP-A/CENP-C interaction. We notice conformational changes within the CENP-A nucleosome upon binding of CENP-C^{CR}. The enhanced DNA unwrapping is facilitated by destabilization of the H2A C-terminal tail while the H4 N-terminal tail is stabilized in the conformation that favors centromere-specific $H4^{K20}$ monomethylation.

In summary, our work provides a high-resolution, integrated view of the human CENP-A nucleosome with its key CCAN partner, human CENP-C. We establish CENP-A nucleosomes as the sole CENP-C binder, and we provide a molecular understanding for the higher specificity of the CENP-C^{CR} compared to the CENP-C^{motif}. Finally, our study identifies conformational changes in the nucleosome, taking place upon binding.

Results

CENP-A nucleosome has flexible DNA ends, irrespective of DNA sequence

Ever since CENP-A has been identified as the key epigenetic mark of the centromere, a central question has been how it is distinguished from canonical nucleosomes [15]. Initial *in vitro* studies [5,6] together with recent research in cells strongly support an octameric nucleosome, similar to the canonical one [16,17]. In the last 10 years, several studies both *in vivo* [16,18] and *in vitro* [6,18–20] have identified flexible DNA ends as a unique feature of CENP-A nucleosomes but to which degree DNA sequence and/or crystal packing contributed to unwrapping remained unclear. To determine this directly, we MNase digested CENP-A nucleosomes assembled both on synthetic super-positioning DNA "601" [21] and on two natural α-satellite DNA constructs [16], with and without the CENP-B box, a 17 bp sequence recognized by CENP-B [22], respectively. Since the exact nucleosome positioning on the sequence that contains the CENP-B box is not precisely mapped, we used a full-length α-satellite repeat (171 bp) with a CENP-B box at one of the ends. For all three DNA sequences, we observed faster DNA digestion when assembled on CENP-A nucleosomes in comparison with H3 nucleosomes (Figs 1A and EV1A). The DNA unwrapping of the CENP-A nucleosome has been linked to properties of the N-terminal sequence of CENP-A [18,23]. Indeed, when we substitute residues 1–49 of CENP-A with 1–50 of H3, we completely loose the DNA flexibility (Fig EV1B). The converse is also true where H3 nucleosomes bearing the N-terminal tail of CENP-A undergo DNA unwrapping to a similar extent as CENP-A nucleosomes (Fig EV1B). We therefore conclude that flexibility of the DNA ends is an intrinsic property of the CENP-A nucleosome that is regulated by its N-terminal tail and is independent of DNA sequence.

Next, we used cryo-EM to obtain a high-resolution structure of CENP-A nucleosomes on 601 DNA at 3.8 Å (Figs 1B and EV1C and EV2, Appendix Table S1). In contrast to the crystal structure [6] where electron density for the terminal 13 DNA bp is missing, probably due to its flexible nature, our cryo-EM structure of CENP-A nucleosomes reveals density for the entire 145 bp of DNA used in nucleosome reconstitutions. However, the map is less defined and has a lower local resolution for the terminal DNA (Figs 1C and EV2E), indicating local flexibility. The modeled DNA is shifted by 4 Å in comparison with the one in the H3 nucleosome [24] (Fig 1D and E). These results are in agreement with our MNase experiments and with a recent antibody-stabilized structure of the CENP-A nucleosome [20]. Interestingly, despite the low resolution for the DNA, we can clearly model the N-terminus of CENP-A all the way to CENP-A^{R42}, including the αN helix (Fig 1F). The αN helix of CENP-A is shorter, disrupted by CENP-A^{G46} and the bulky CENP-A^{W47}, while H3 continues with one extra turn (Fig 1E). CENP-A^{R42}, CENP-A^{R43}, and CENP-A^{R44} are all involved in DNA binding, although the interactions are slightly different on the two sides of the nucleosome (Figs 1F and EV1D). In our structure, we can also clearly see other CENP-A-specific features of the nucleosome (Fig EV1D): the C-terminal tail (-LEEGLG) that specifically binds CENP-C, and L1 loop containing CENP-A^{R80} ("RG-loop") that specifically binds CENP-N.

CENP-C^{CR} competes out the CENP-C^{motif} on CENP-A nucleosomes

Initial efforts to identify human CCAN proteins that directly and specifically bind CENP-A nucleosomes uncovered the CENP-C and CENP-N proteins [4,25]. In those studies, only the central region of human CENP-C (CENP-C^{CR}, aa 426–537) was characterized for CENP-A binding (Fig 2A). In 2013, Kato *et al* [14] reported a crystal structure of the canonical fruit fly H3 nucleosome in which the C-terminal tail of H3 was replaced by a mutated rat CENP-A

Figure 1. CENP-A nucleosome has flexible DNA ends independently of the wrapped DNA sequence.

A Graphs showing the relative abundance of undigested DNA as a function of time during digestion with micrococcal nuclease (MNase) for three types of DNA wrapped around the CENP-A nucleosome (red) and the H3 nucleosome (green). For α-satellite DNA with initial size of 171 bp, size ranges (141–171) corresponding to DNA lengths above NCP (nucleosome core particle) is presented. Data are presented as mean (SD) for each time point based on three independent experiments. Corresponding virtual gels from Bioanalyzer are in Fig EV1A.

B Cryo-EM density map of the human CENP-A nucleosome, color-coded for histones and DNA.

C Cryo-EM maps of the H3 nucleosome (PDB 6ESF) and the CENP-A nucleosome, colored based on local resolution.

D Overlay of cryo-EM maps of the H3 nucleosome (green; PDB 6ESF) and the CENP-A nucleosome (red). Note shorter and moved density for DNA on the CENP-A nucleosome indicated by the arrow.

E Overlay of the N-terminal tail of CENP-A (red) and H3 (green; PDB 6ESF), illustrating shorter αN helix of CENP-A (obstructed by the presence of bulky CENP-A^{W47}) and terminal nucleosomal DNA moved by 4 Å.

F The N-terminal tail of CENP-A (red) makes contacts with the DNA (cyan) at the entry/exit sites.

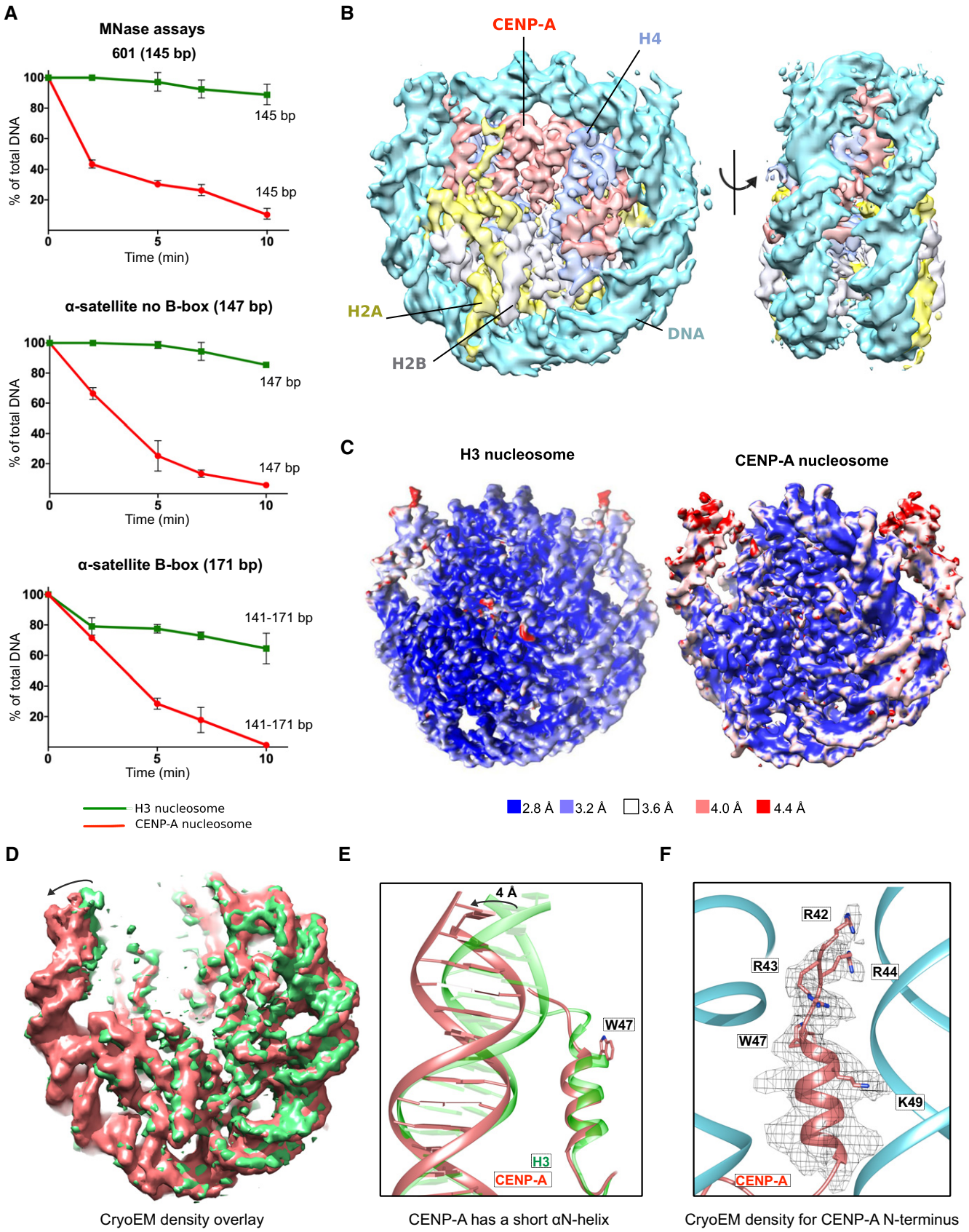
A
MNase assays
601 (145 bp)
% of total DNA
Time (min)
145 bp
145 bp
α-satellite no B-box (147 bp)
147 bp
147 bp
α-satellite B-box (171 bp)
141-171 bp
141-171 bp
H3 nucleosome
CENP-A nucleosome
B
CENP-A
H4
H2A
H2B
DNA
C
H3 nucleosome
CENP-A nucleosome
2.8 Å
3.2 Å
3.6 Å
4.0 Å
4.4 Å
D
CryoEM density overlay
E
4 Å
W47
H3
CENP-A
CENP-A has a short αN-helix
F
R42
R43
R44
W47
K49
CENP-A
CryoEM density for CENP-A N-terminus

Figure 1.

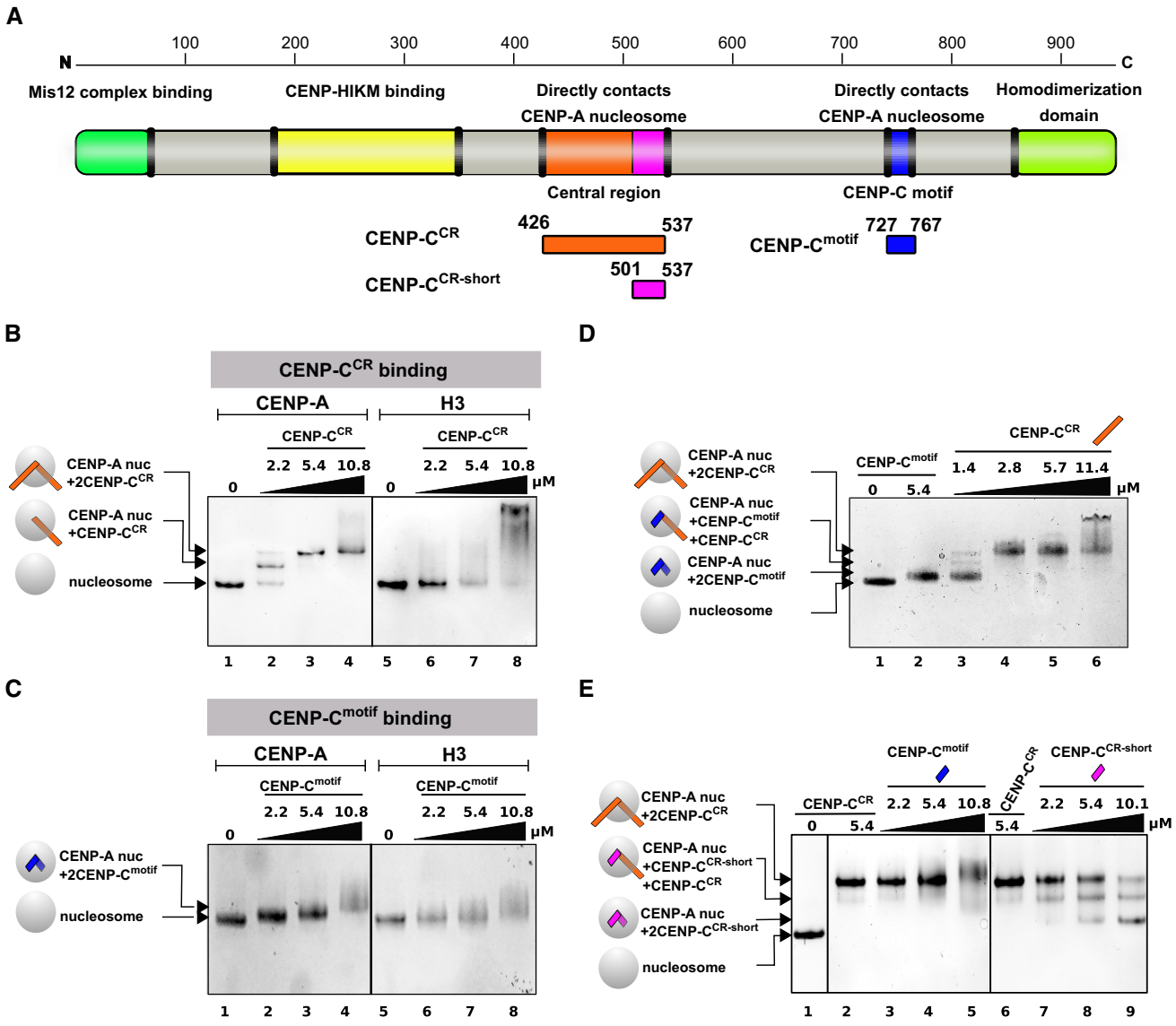

Figure 2. Both CENP-C^{CR} and CENP-C^{motif} bind specifically to the CENP-A nucleosome, and CENP-C^{CR} easily competes out CENP-C^{motif} bound to CENP-A.

A Schematic diagram of the full-length CENP-C protein, indicating parts involved in interactions with other proteins or homo-dimerization. Constructs used in this study are depicted below the diagram.

B Native PAGE gel stained with Coomassie blue showing complexes formed between CENP-A or H3 nucleosome and CENP-C^{CR}. Lane 1: CENP-A nucleosome, Lanes 2–4: Increasing amounts of CENP-C^{CR} are added to CENP-A nucleosome. Generation of a sharp band with slower mobility indicates formation of a specific CENP-A/CENP-C^{CR} complex. Lane 5: H3 nucleosome. Lanes 6–8: Increasing amounts of CENP-C^{CR} are added to H3 nucleosome. Smear on the gel indicates formation of non-specific H3/CENP-C^{CR} complexes.

C Same experiment as in (B) using CENP-C^{motif}. Lane 1: CENP-A nucleosome. Lanes 2–4: Increasing amounts of CENP-C^{motif} are added to CENP-A nucleosome. Upon binding CENP-C^{motif}, CENP-A nucleosome migrates slower through the gel. Note only modest change in mobility due to small size of CENP-C^{motif}, comparing to CENP-C^{CR} in (B). Lane 5: H3 nucleosome. Lanes 6–8: Increasing amounts of CENP-C^{motif} are added to H3 nucleosome. Smear on the gel indicates formation of non-specific H3/CENP-C^{motif} complexes.

D Native gel showing CENP-C^{CR} competing out CENP-C^{motif} bound to CENP-A nucleosome. Lane 1: CENP-A nucleosome. Lane 2: CENP-A/CENP-C^{motif} complex. Lane 3–6: Increasing amounts of CENP-C^{CR} are added to the pre-formed CENP-A/CENP-C^{motif} complex. Formation of slower migrating bands indicates that longer CENP-C^{CR} is replacing shorter CENP-C^{motif} bound to the CENP-A nucleosome.

E Native gel showing the inability of CENP-C^{motif} to compete out CENP-C^{CR} bound to CENP-A nucleosome. Lane 1: CENP-A nucleosome. Lanes 2 and 6: CENP-C^{CR}/CENP-A nucleosome complex. Lanes 3–5: Increasing amounts of CENP-C^{motif} are added to the pre-formed CENP-A/CENP-C^{CR} complex. Formation of smear at high amounts of CENP-C^{motif} added indicates that CENP-C^{motif}, at high concentrations, non-specifically binds CENP-A/CENP-C^{CR} complex rather than replacing bound CENP-C^{CR}. Lanes 7–9: Increasing amounts of CENP-C$^{CR\text{-}short}$ are added to the pre-formed CENP-A/CENP-C^{CR} complex. Formation of bands with higher mobility indicates that smaller CENP-C$^{CR\text{-}short}$ is effectively replacing bigger CENP-C^{CR} bound to CENP-A nucleosome.

Data information: For (B–E), 2.4 μM nucleosomes are used in all experiments.

C-terminus in complex with the rat CENP-C residues 710–734, a region known as the CENP-C motif (CENP-C^{motif}). Analysis of the structure identified the hydrophobic interactions between the CENP-A C-terminal tail and the CENP-C^{motif}, and the authors proposed that both CENP-C^{CR} and CENP-C^{motif} bind nucleosomes, with 5–10 times higher affinity for CENP-A then for H3 nucleosomes. To determine whether CENP-C^{CR} binds the nucleosome with a different affinity than the CENP-C^{motif}, we prepared complexes of both and analyzed their mobility on native PAGE. We found that both the CENP-C^{CR} and the CENP-C^{motif} make complexes with CENP-A nucleosomes in a ~2:1 ratio. The complexes travel as sharp bands on the native gel, indicating their uniform nature (Fig 2B and C). Surprisingly, when mixed with H3 nucleosomes, both CENP-C constructs result in a smear on the native gel, indicating non-specific binding (Fig 2B and C). We conclude that *in vitro* CENP-C^{CR} and CENP-C^{motif} bind only CENP-A nucleosomes specifically.

Furthermore, we performed a competition experiment to determine which CENP-C region has higher affinity for CENP-A nucleosomes. When we preassemble the human CENP-A/CENP-C^{motif} complex and titrate in CENP-C^{CR}, we observe formation of the CENP-A/CENP-C^{CR} complex with almost full saturation at 2 × molar access of CENP-C^{CR} (Fig 2D). In contrast, titrating in the CENP-C^{motif} to a pre-formed CENP-A/CENP-C^{CR} complex results in a smear on the native PAGE, indicative of non-specific binding of CENP-C^{motif} to CENP-A/CENP-C^{CR} complexes (Fig 2E). The CENP-C^{motif} is 71 residues shorter than CENP-C^{CR} (40 versus 111 residues), and it is possible that extra residues beyond those directly interacting with the CENP-A C-terminus are providing additional affinity. To test this, we made a truncated CENP-C^{CR} that has only residues predicted to interact with the CENP-A C-terminal tail (501–537; CENP-$C^{CR\text{-}short}$) and is comparable in size to the CENP-A^{motif}. First, we confirmed specificity of CENP-$C^{CR\text{-}short}$ for CENP-A nucleosomes (Appendix Fig S1). Interestingly, we also observed that CENP-$C^{CR\text{-}short}$ completely loses its ability to bind H3 nucleosomes. This suggests that the additional 71 residues present in the CENP-C^{CR} are responsible for the non-specific H3 nucleosome binding, likely through interactions with DNA. Next, we tested if CENP-$C^{CR\text{-}short}$ can compete out CENP-C^{CR} from pre-assembled CENP-A/CENP-C^{CR} complexes. We find that, in contrast to the CENP-C^{motif}, CENP-$C^{CR\text{-}short}$ efficiently replaces CENP-C^{CR} bound to CENP-A nucleosomes (Fig 2E), demonstrating that residues within the nucleosome binding region of CENP-C^{CR} contribute to high affinity binding.

In summary, we conclude that CENP-C binds only CENP-A nucleosomes specifically and that CENP-C^{CR} provides the major interactions between CENP-A and CENP-C. Our findings are consistent with studies in cells that found CENP-C^{CR} to be essential for epigenetic propagation of the centromere [13].

Strong hydrophobic interactions are contributing specificity and high affinity between the CENP-A nucleosome and CENP-C^{CR}

Next, we aimed to define how the high affinity CENP-C^{CR} interaction is achieved. The crystal structure of the H3-GIEGGL/rat CENP-C^{motif} has identified two types of interactions involved in complex formation: (i) electrostatic interactions of rat CENP-$C^{R717/R719}$ with the acidic patch on H2A/H2B, and (ii) hydrophobic interactions between rat CENP-$C^{Y725,\ W726}$ and the hydrophobic C-terminal tail of CENP-A [14]. A sequence comparison of the rat CENP-C^{motif} with the human CENP-C^{motif} and human CENP-C^{CR} reveals the existence of analogous residues in the human protein but does not explain the higher affinity of the CENP-C^{CR} (Fig 3A). Also, since crystallographic studies [14] used a canonical nucleosome with a grafted C-terminus of CENP-A, it failed to capture possible conformational changes that could occur within the CENP-A nucleosome upon CENP-C binding. To resolve this, we solved the 3.1 Å cryo-EM structure of the human CENP-A nucleosome in complex with human CENP-C^{CR} (Fig 3B, Appendix Table S2, Figs EV3 and EV4). Our maps show CENP-C bound to both sides of the nucleosome via the CENP-$A^{R521,\ R522}$ anchoring residues, while the remaining residues in CENP-C^{CR} show fuzzy interactions. In order to visualize a larger CENP-C fragment, we have further sorted particles and increased CENP-C occupancy on one of the sides, so that we can trace residues 519-536. We observe strong electrostatic interactions between human CENP-$C^{R521,\ R522}$ and the acidic patch formed by H2A/H2B (Figs 3C and EV3A). Neutralization of a negative surface contributed by H2A/H2B on the nucleosome is a feature common to a handful of other nucleosome binding molecules (reviewed in [26]) that was also seen in the chimeric H3 nucleosome/CENP-C^{motif} structure [14]. We also observe human CENP-$C^{W530,\ W531}$ tightly fitting the hydrophobic cleft formed by the C-terminal tail of CENP-A (Figs 3C and EV3B). Furthermore, the conformation of the C-terminal tail of CENP-A changes slightly upon binding (Fig 3D), and we observe more extended hydrophobic interactions in comparison with those reported in the crystal structure (Fig 3E) [14]. Two bulky tryptophans (CENP-$C^{W530,\ W531}$) are clamped between CENP-A^{R131} and CENP-$A^{L135,\ L139}$ while CENP-C^{V532} and CENP-C^{V533} stabilize the hydrophobic patch on H4 formed by $H4^{V58}$ and $H4^{V61}$. These interactions explain the perturbations observed by NMR in H4 residues within the nucleosome upon CENP-C^{CR} binding [14]. To test whether

Figure 3. Strong hydrophobic interactions are contributing specificity and high affinity between the CENP-A nucleosome and CENP-C^{CR}.

A Sequence alignment of CENP-C^{CR} and CENP-C^{motif} regions from different mammals. Conserved residues involved in CENP-A binding are highlighted in blue (electrostatic interactions) and pink (hydrophobic interactions). Residues identified in this study to contribute higher affinity of CENP-C^{CR} comparing to CENP-C^{motif} are boxed.

B Cryo-EM density map of the human CENP-A nucleosome, color-coded for histones, in complex with CENP-C^{CR} (magenta). CENP-C^{CR} binds CENP-A nucleosome through hydrophobic region (big box) and arginine anchor (small box). Interacting residues in each of the regions are shown in (C).

C Ribbon diagram showing interactions of the CENP-C^{CR} hydrophobic region (left) and CENP-C^{CR} arginine anchor (right) with the CENP-A nucleosome.

D Overlay of the CENP-A C-terminal tail before (gray) and after (red) binding of CENP-C^{CR}.

E Interactions between H3-GIEGGL and rat CENP-C^{motif} as observed in PDB 4X23 [14].

F Schematic diagram of mutated CENP-C^{CR} sequences used to test importance of CENP-C^{V532} and CENP-C^{V533} for generation of CENP-A/CENP-C^{CR} complexes.

G Native gels showing the impaired ability of mutated CENP-C^{CR} to form complexes with the CENP-A nucleosome. The molar ratio of CENP-C^{CR}/CENP-A nucleosomes is shown above each lane.

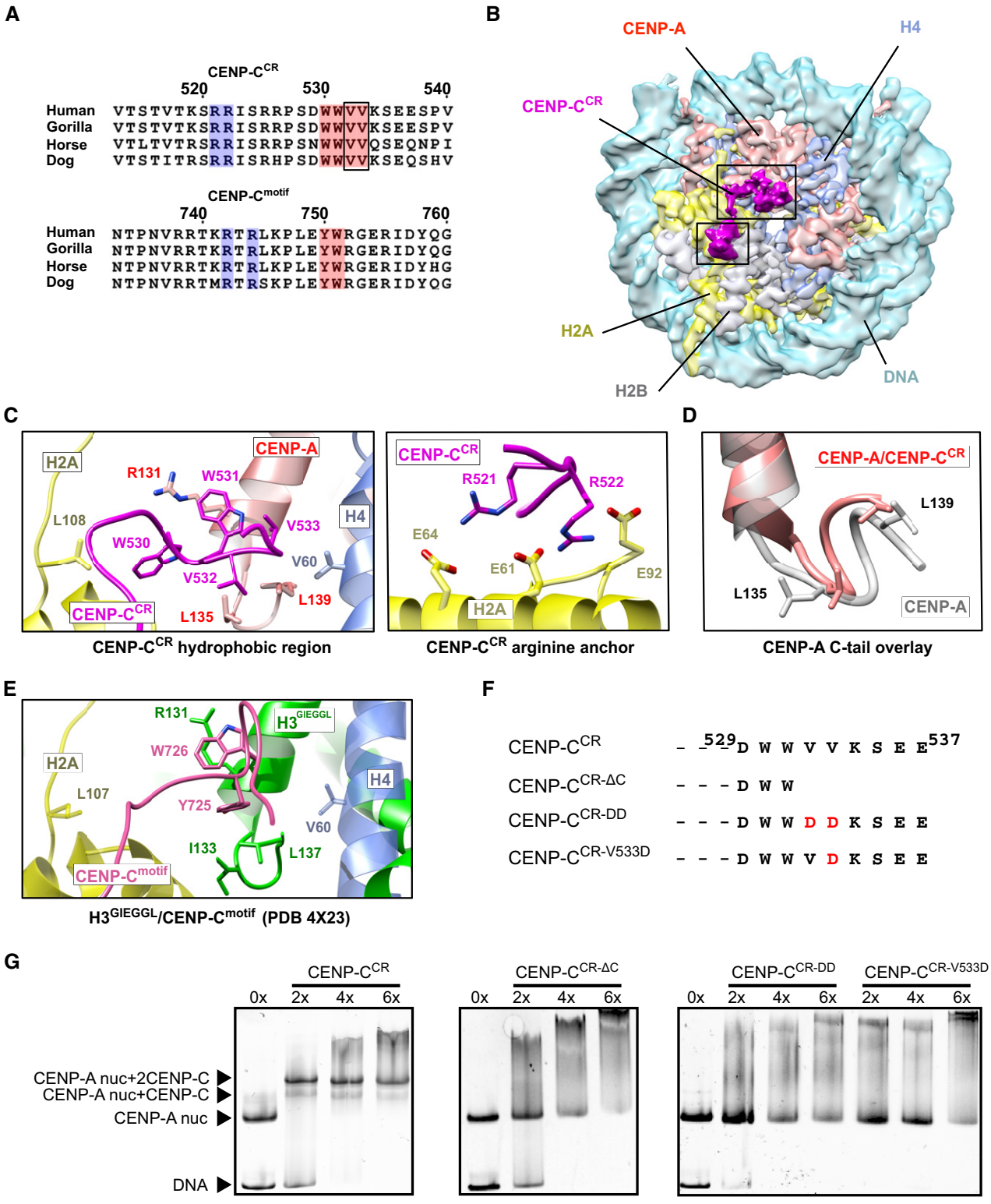

Figure 3.

extended hydrophobicity contributed by CENP-C$^{V532,\ V533}$ plays a role in stronger CENP-A nucleosome/CENP-C^{CR} interactions, we generated a CENP-C mutant devoid of residues 530–537, CENP-C$^{CR\text{-}\Delta C}$ (Fig 3F). This mutant fails to form a complex with the CENP-A nucleosome (Fig 3G), indicating that the residues following CENP-C$^{W530,\ W531}$ are important for binding. Point mutations further revealed that replacement of CENP-C^{V533} with aspartic acid is sufficient to eliminate binding to the CENP-A nucleosome (Fig 3F and G).

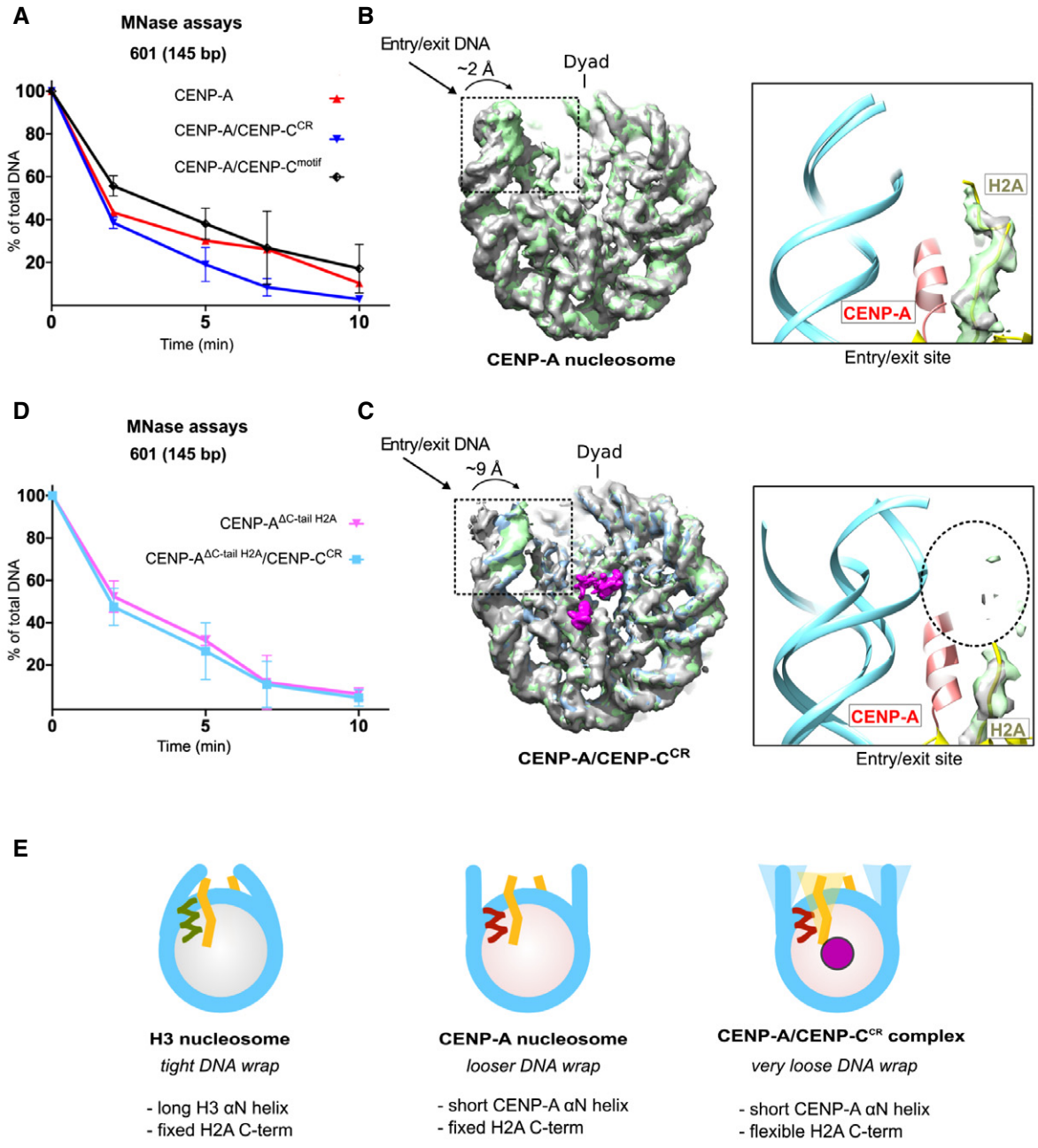

Figure 4. The C-terminus of H2A is destabilized in the CENP-A/CENP-C^{CR} complex.

A Graph showing the relative abundance of undigested DNA (145 bp) as a function of time during digestion with micrococcal nuclease (MNase) for the CENP-A nucleosome (red), the CENP-A/CENP-C^{CR} complex (blue), and the CENP-A/CENP-C^{motif} complex (black).

B Overlay of three cryo-EM maps (gray, blue, and green) of the CENP-A nucleosome obtained by sorting on the DNA entry/exit site (Fig EV2F). Distance between most open (gray) and most closed (green) map is 2 Å. The entry/exit site is boxed, and the corresponding model is shown on the right. Note that the density of the H2A C-terminus is well defined.

C Overlay of three cryo-EM maps (gray, blue, and green) of the CENP-A/CENP-C^{CR} complex obtained by sorting on the DNA entry/exit site (Fig EV4F). The distance between the most open (gray) and most closed (green) maps is 9 Å. Map assigned to CENP-C^{CR} is shown in magenta. The entry/exit site is boxed, and the corresponding model is shown on the right. Note the absence of a clearly defined density of the H2A C-tail (indicated by the dotted circle).

D Same type of data as in (A) for the CENP-A nucleosome assembled with tailless H2A (H2A, 1–109) alone (pink) and in complex with CENP-C^{CR} (light blue).

E Schematic representation of the interplay between the N-terminus of H3 or CENP-A and the C-terminus of H2A in regulating flexibility of nucleosomal DNA wrap. DNA (cyan); longer H3 αN (green); shorter CENP-A αN (red); H2A C-terminus (yellow); CENP-C (magenta).

Data information: For (A, D), data are presented as mean (SD) for each time point based on three independent experiments and virtual gels are available in Fig EV5.

We conclude that human CENP-C^{CR} binds the human CENP-A nucleosome through electrostatic interactions, involving CENP-C$^{R521,\ R522}$ (neutralizing the acidic patch on the nucleosome) as well as via an extensive hydrophobic network formed between CENP-C$^{W530,\ W531,\ V532,\ V533}$ and the C-terminal tail of CENP-A, aided by the hydrophobic patch of H4. Our data show that CENP-C^{CR} makes additional hydrophobic contacts with the CENP-A nucleosome that have not been observed for CENP-C^{motif}. These interactions are required for the higher affinity of CENP-C^{CR} versus the CENP-C^{motif}.

Conformational changes within the human CENP-A nucleosome upon CENP-C binding

The nucleosome in our experiments bears the full-length human CENP-A, which enables us to assess conformational changes upon CENP-C binding. It has previously been proposed that CENP-C binding rigidifies the histone core, rendering it more histone H3-like, while at the same time further enhancing DNA unwrapping [27]. The MNase experiments on CENP-A nucleosomes in complex with CENP-C^{CR} or CENP-C^{motif} show increased DNA digestion in both cases (Figs 4A and EV5A). Comparison of the local resolution maps between CENP-A and CENP-A/CENP-C^{CR} complex (Figs EV2E and EV4E) indicates more extensive DNA unwrapping in samples with bound CENP-C^{CR}. To further confirm this, we performed careful cryo-EM classification of particles based on the conformations at DNA entry/exit sites for each of the two samples (Fig EV4F). We find that, although flexible, the DNA at the entry/exit site in CENP-A nucleosome samples only a limited space (distance between most extreme DNA conformation is 2 Å) (Fig 4B). In contrast, in the CENP-A/CENP-C^{CR} complex the most extreme distance between different subpopulations is 9 Å (Fig 4C). Concomitantly, we observe no density for the C-terminal tail of H2A (Fig 4C), indicating disorder in this part of the nucleosome upon CENP-C binding. This is in striking contrast to the cryo-EM structure of the canonical nucleosome with tightly wrapped DNA, where the density for the C-terminal tail of H2A is one of the most well-resolved parts of the structure [24]. It has been reported that removal of the C-terminal tail of H2A leads to decreased stability of the nucleosome and alters nucleosome positioning and interactions with H1 [28,29]. Molecular dynamic studies have identified residues H2A^{K118} and H2A^{K119} to interact with DNA, most likely securing the DNA wrap [30]. Indeed, if we remove residues 110–130 in H2A from the CENP-A

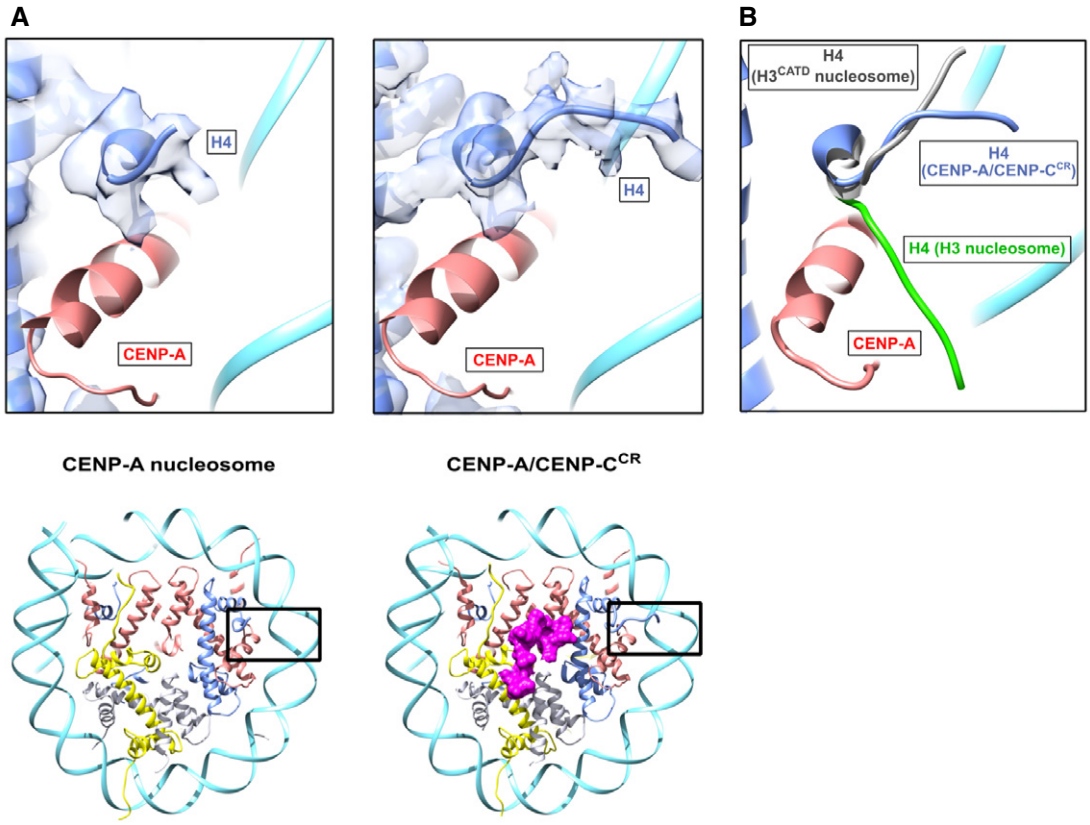

Figure 5. The N-terminal tail of H4 is stabilized in upwards conformation in the CENP-A/CENP-C^{CR} complex.

A Structure of the H4 N-terminal tail with corresponding cryo-EM density in the CENP-A nucleosome (left) and CENP-A/CENP-C^{CR} complex (right). Position of the H4 N-terminal tail within the nucleosome is boxed on ribbon diagram below. Histones and DNA are color-coded as in Fig 1, and cryo-EM density assigned to CENP-C is shown in magenta.

B Overlay of the H4 N-tail from the CENP-A/CENP-C^{CR} complex (blue) with the H4 N-terminus from the H3 nucleosome (green; PDB 6ESF) and H3^{CATD} nucleosome (gray; PDB 5Z23).

nucleosome (CENP-A$^{\Delta C\text{-tail H2A}}$), we observe increased DNA digestion (Figs 4D and EV5B, top), and addition of CENP-C^{CR} to these nucleosomes does not change the nuclease digestion profile (Figs 4D and EV5B, bottom). In contrast, DNA digestion of H3 nucleosomes was only mildly affected by removal of the C-terminus of H2A (Fig EV5C, top). We next hypothesized that the different MNase pattern of CENP-A versus H3 nucleosomes (in the background of ΔC-tail H2A) must be contributed by the residues in the N-terminal tail of CENP-A/H3, since this region of the nucleosome is in close contact with the C-terminal tail of H2A. Indeed, in the context of ΔC-tail H2A, MNase digestion of nucleosomes with an H3 core and CENP-A tail is highly similar to CENP-A nucleosomes while digestion of nucleosomes with a CENP-A core and H3 tail is similar to that of the H3 nucleosome (Fig EV5C, middle). These results demonstrate a contribution of the C-terminal tail of H2A to nucleosome DNA wrapping that synergizes with the N-terminal tail of CENP-A. In the context of the CENP-A nucleosome that has a shorter αN helix, DNA wrapping is already compromised and removal of C-terminal H2A results in further unwrapping (Fig 4E). Consistently, we find that in CENP-A nucleosomes with an H3 N-tail, binding of CENP-C^{CR} alone cannot induce DNA unwrapping (Fig EV5C, bottom). From this, we conclude that the N-terminal tail of CENP-A favors DNA unwrapping which is counteracted by the C-terminus of H2A. CENP-C binding destabilizes the C-terminus of H2A, leading to increased DNA unwinding. We find that this alternative, destabilized, conformation of the H2A C-terminal tail is most likely caused by an interaction between the bulky hydrophobic CENP-C$^{W530,\ W531}$ and H2A^{L108}. In addition, CENP-C$^{W530,\ W531}$ is orienting CENP-A^{R131} to establish a salt bridge with H2A^{Q112} (Fig EV5D).

Furthermore, we noticed that the N-terminal tail of H4 clearly adopts an upwards conformation on both sides of the CENP-A nucleosome relative to canonical nucleosomes (Fig 5A, left), but in the presence of CENP-C^{CR}, this conformation is more rigidified (Fig 5A, right). Monomethylation of H4 is enriched on CENP-A nucleosomes, and it is necessary for epigenetic establishment of the kinetochore [31]. A recent structural study [32] proposed an upward conformation of the H4 tail to be required for establishment and/or maintenance of H4^{K20} monomethylation. In our cryo-EM maps, we observe the N-terminus of H4 in a slightly different but still upwards conformation in comparison with the crystal structure of the H3^{CATD} nucleosome (Fig 5B) [32]. A very recent study [33] indicates that the conformation of the N-terminus of H4 is further modified upon CENP-N binding.

In summary, we find that binding of CENP-C^{CR} to the CENP-A nucleosome enhances DNA unwrapping by destabilizing the C-terminal tail of H2A while, at the same time, stabilizing the H4 N-terminal tail in the upward conformation that may be important for centromere-specific monomethylation of H4^{K20}.

Discussion

CENP-A is a key epigenetic mark to maintain centromere identity, a chromosome locus essential for genome integrity. CENP-A is a histone H3 variant with a histone core bearing 64% identity to H3 while featuring completely divergent tails. As CENP-A is a central epigenetic determinant of the centromere, an important question is how CENP-A containing nucleosomes are distinguished from bulk chromatin. Over the years, a number of models have been proposed, including different histone stoichiometries, presence of non-histone proteins, and alternative DNA wrapping [summarized in 15]. Finally, an octameric nucleosome with a right-handed DNA wrap, much like canonical nucleosomes, emerged as the favorite model for the human CENP-A nucleosome, based on both *in vitro* and *in vivo* studies [5,6,16,17], leaving open the question of what is making CENP-A nucleosomes so unique. A crystal structure of the CENP-A nucleosome [6], *in vivo* MNase experiments [16], and initial cryo-EM studies [18] all pointed toward enhanced DNA unwrapping as a

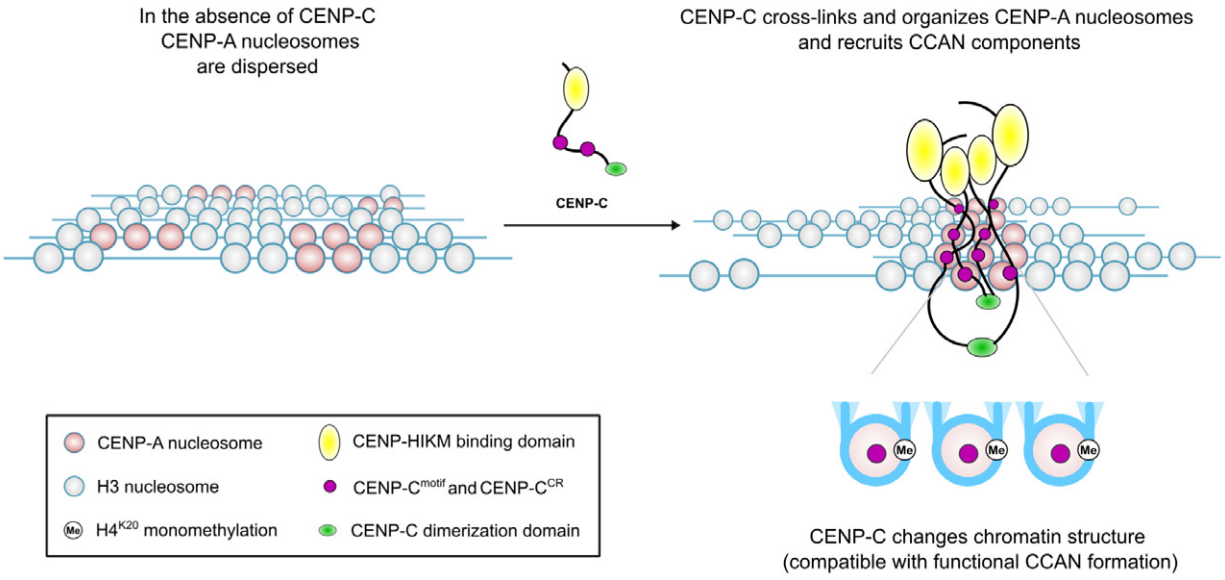

Figure 6. Model illustrating putative role of CENP-C in centromeric chromatin.

CENP-C crosslinks CENP-A nucleosomes, recruits other CCAN components, and pre-conditions chromatin for formation of a functional centromere.

CENP-A-specific feature where unwrapped DNA results in a different chromatin architecture, potentially important for accommodating CCAN components. Indeed, the experiments presented here, together with the structure of human CENP-A, confirm high flexibility of the DNA ends as an intrinsic feature of CENP-A nucleosomes encoded in the N-terminal tail of CENP-A, independent on the DNA sequence.

A key question is how the CENP-A nucleosome directly binds two CCAN components, CENP-N and CENP-C, and how their binding is changing the nucleosome. Recent work [7–9] has provided a view of the CENP-A/CENP-N interaction at atomic resolution, which involves recognition of the solvent-exposed, positively charged L1 loop on CENP-A and an interaction with DNA, with minimal changes to the rest of the nucleosome. For the CENP-C interactions with the CENP-A nucleosome, 2 different parts of CENP-C are proposed to bind: CENP-C^{CR} and CENP-C^{motif}. CENP-C^{CR} is necessary and sufficient for CENP-A nucleosome binding *in vitro* and centromere targeting and stability *in vivo* while the CENP-C^{motif} can be recruited to kinetochores only in the presence of a homo-dimerization domain [10,11,13]. Having two independent CENP-C modules able to bind nucleosome at centromeres with different affinities, led to the proposal [10] of CENP-C, as a direct mediator of CENP-A loading during the cell cycle. The model assumes that the CENP-C module with weaker nucleosome binding, CENP-C^{motif}, alternates between binding of an H3.3 nucleosome and a CENP-A nucleosome while the stronger module, CENP-C^{CR}, remains stably associated with the CENP-A nucleosome in all phases of the cell cycle. We show that, indeed, CENP-C^{CR} binds the CENP-A nucleosome with higher affinity than CENP-C^{motif}, but neither of the CENP-C modules can make uniform complexes with H3 nucleosome.

Furthermore, structural insights of the CENP-A/CENP-C interactions are based on the crystal structure of a chimeric fruit fly H3 nucleosome with the C-terminal tail of rat CENP-A and interaction of this tail with the rat CENP-C^{motif} [14]. We here report a 3.1 Å structure of a complete human CENP-A nucleosome in complex with human CENP-C^{CR} which is essential for epigenetic stability of centromeres [13]. We find a longer hydrophobic stretch on CENP-C^{CR}, formed by CENP-$C^{V532,\ V533}$, to be essential for robustness of the CENP-A nucleosome/CENP-C^{CR} interactions, and we re-define the minimal fragment of CENP-C^{CR} necessary for productive CENP-A binding (residues 501–537).

Previous experiments [27] and those reported here confirm enhanced DNA unwrapping of the CENP-A nucleosome, induced by CENP-C^{CR} binding. In our structure of the CENP-A/CENP-C^{CR} complex, we observe a disordered H2A C-terminal tail and using a combination of MNase experiments and mutagenesis we establish a role for the H2A histone tail in regulating the extent of DNA wrapping on nucleosomes. We find that the C-terminal tail of H2A secures nucleosomal DNA wrapping that counteracts the unwrapping of CENP-A nucleosomes promoted by the short CENP-A αN helix. Upon CENP-C binding, the hydrophobic interaction between H2A and CENP-C is displacing the H2A C-terminal tail, resulting in a very loose DNA wrapping in the CENP-A/CENP-C^{CR} complex. Regulation of the DNA wrap by the C-terminal tail of H2A is likely exploited in general chromatin. For example, H2A variants with different C-terminal tails are known to regulate various biological processes, conferring special properties to the chromatin [34].

Furthermore, in both our structures of the CENP-A nucleosome alone and the CENP-A/CENP-C^{CR} complex, we see an upwards conformation of the N-terminal tail of H4. The conformation is additionally stabilized by CENP-C^{CR} binding, most likely through hydrophobic interactions between CENP-C and H4. $H4^{K20}$ monomethylation is essential for kinetochore assembly [31,32], and binding of CENP-C^{CR} could be enforcing this centromere-specific epigenetic chromatin modification.

Combined, our structures provide an essential and long anticipated high-resolution view of the fundamental building block of the centromere, the human CENP-A nucleosome alone and in complex with CENP-C, a protein that forms the backbone of the constitute centromere complex. Our biochemical and structural analysis establishes CENP-C as an exclusive and multivalent binder of CENP-A nucleosomes that employs two independent modules and homodimerization to crosslink sparse CENP-A domains in centromeric chromatin [35,36] and provides framework for functional CCAN (Fig 6). Binding of CENP-C to CENP-A nucleosomes not only spatially organizes CENP-A nucleosomes and recruits other CCAN components, it also induces conformational changes (DNA unwrapping, neutralization of the acidic patch on H2A and facilitation of $H4^{K20}$ monomethylation) that might be required for establishment and maintenance of functional centromeres (Fig 6).

Materials and Methods

Protein purification

Human histones, CENP-A and the CENP-C central domain, were expressed and purified as previously described in [37]. Briefly, the CENP-A/H4 hetero-tetramer was expressed from a bicistronic plasmid in *E. coli* pLysS under soluble conditions and purified on a hydroxyapatite column followed by cation exchange chromatography. H3, H4, H2A, and H2B were expressed in inclusion bodies and purified under denaturing conditions using a Sephacryl size exclusion column followed by cation exchange chromatography. H2A/H2B and H3/H4 histones were subsequently co-refolded to form hetero-dimers and hetero-tetramers, respectively, and purified with size exclusion chromatography.

GST-tagged recombinant human CENP-C central region (CENP-C^{CR}, aa 426–537), CENP-C motif (CENP-C^{motif}, aa 727–767), and short CENP-C central region (CENP-$C^{CR\text{-}short}$, aa 501–537) were expressed and affinity-purified on a glutathione column. GST was subsequently cleaved overnight by PreScission protease and separated from CENP-C, using cation exchange and size exclusion chromatography.

PCR site-directed mutagenesis was performed to generate CENP-$C^{V532D/V533D}$, CENP-C^{V533D}, CENP-$C^{426–531}$, and CENP-$C^{426–533}$. All mutants were expressed and purified as described for the central CENP-C region.

The H3 histone with a CENP-A N-$tail^{1–49}$ ($H3^{CENP\text{-}A\ (N\text{-}tail)}$), CENP-A histone with a H3 N-$tail^{1–50}$ (CENP-$A^{H3(N\text{-}tail)}$) (Appendix Fig S1B), and a H2A histone lacking the C-terminal residues 110–130 were cloned using In-Fusion® HD cloning strategy (Takara Bio).

145 bp 601 super-positioning DNA and 147 bp α-satellite DNA were purified as described in [38]. Briefly, XL10 cells transformed

with pUC57 plasmids containing 6 × 147 bp α-satellite DNA and 8 × 145 bp 601 super-positioning DNA (gift from Ben Black, UPenn) were cultivated, and DNA was extracted with phenol/chloroform, digested using EcoRV restriction enzyme and further purified using anion-exchange chromatography. 171 bp α-satellite DNA with a CENP-B box was amplified from plasmid using PCR and further purified using anion-exchange chromatography.

601 (145 bp): ATCAGAATCCCGGTGCCGAGGCCGCTCAATTGGT CGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGCGCTGTCCCC CGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGT GTCAGATATATACATCGAT

α-satellite no B-box (147 bp): ATCAAATATCCACCTGCAGATTC TACCAAAAGTGTATTTGGAAACTGCTCCATCAAAAGGCATGTTCAG CTCTGTGAGTGAAACTCCATCATCACAAAGAATATTCTGAGAATGC TTCCGTTTGCCTTTTATATGAACTTCCTCGAT

α-satellite **B-box** (171 bp): GGAGGA**TTTCGTTGGAAACGGGA** TCAACTTCCCATAACTGAACGGAAGCAAACTCAGAACATTCTTTGT GATGTTTGTATTCAACTCACAGAGTTGAACCTTCCTTTGATAGT TCAGGTTTGCAACACCCTTGTAGTAGAATCTGCAAGTGTATATT TTGACCACTTTGGA

Assembly of nucleosomes and nucleosome complexes

CENP-A and H3 nucleosomes were assembled using 601 (145 bp), α-satellite no B-box (147 bp), or α-satellite with B-box (171 bp) DNA. H2A/H2B hetero-dimers, $(CENP\text{-}A/H4)_2$ hetero-tetramers, and DNA were mixed with a molar ratio of 2:1:1 at high salt concentration (2 M NaCl). A gradient dialysis to low salt was performed overnight with a flow rate of 1.5 ml/min using a two-channel peristaltic pump as described in [39]. Assembled nucleosomes were then uniquely positioned on the DNA by a thermal shift for 2 h at 55°C. CENP-A nucleosome and CENP-C^{CR} were complexed by adding 2.2 moles of CENP-C^{CR} to each mole of CENP-A nucleosome. The complex quality was controlled on a 5% native PAGE gel.

Binding experiments

2.4 μM of CENP-A and H3 nucleosomes assembled on 601 (145 bp) DNA were mixed with different amounts of CENP-C^{CR}, CENP-C^{motif}, or CENP-$C^{CR\text{-}short}$ and incubated for 1 h at 4°C. Complex formation was verified on a 5% native PAGE gel.

Competition experiments

2.4 μM of the CENP-A/CENP-C^{motif} complex was mixed with different amounts of CENP-C^{CR}, and the competition between the two domains was tested using 5% native PAGE gel. 2.4 μM of CENP-A/CENP-C^{CR} complex was mixed with different amounts of CENP-C^{motif} and CENP-$C^{CR\text{-}short}$. The competition was then followed using 5% native PAGE gel.

Micrococcal nuclease digestion

2 μg of nucleosomes was incubated with 2 Kunitz units of micrococcal nuclease (NEB) in buffer containing 10 mM Tris HCl pH 7.5, 3 mM $CaCl_2$, and 1 mM DTT at room temperature. Reactions were quenched at different time points (2, 5, 7, 10, and 20 min) by the addition of 250 μl of PB buffer (Qiagen QIAquick PCR Purification Kit) supplemented with 10 mM of EGTA. DNA from each sample was purified with the QIAquick PCR purification kit, and the extent of DNA digestion was quantified by 2100 Bioanalyzer (Agilent). All experiments were done in triplicates.

Cryo-EM grid preparation and data collection and processing

CENP-A nucleosome and CENP-A/CENP-C^{CR} complex were prepared as described above. 3 μl of the sample (1–1.2 mg/ml) was applied to freshly glow-discharged Quantifoil R2/1 holey carbon grid. After 3 s blotting time, grids were plunge-frozen in liquid ethane using a FEI Vitrobot automatic plunge freezer. Humidity in the chamber was kept at 95%.

Electron micrographs were recorded on FEI Titan Krios at 300 kV with a Gatan Summit K2 electron detector (~4,700 micrographs) (Cryo-EM facility at MPI for Biochemistry Martinsried, Germany). The image pixel size was 0.65 Å per pixel on the object scale. Data were collected in a defocus range of 7,000–30,000 Å with a total exposure of 100 $e/Å^2$. Fifty frames were collected and aligned with the Unblur software package using a dose filter [40].

Several thousand particles were manually picked and carefully cleaned in Relion to remove inconsistent particles. The resulting useful particles were then used for semi-automatic and automatic particle picking in Relion. The contrast transfer function parameters were determined using CTFFIND4 [41]. The 2D class averages were generated with the Relion software package [42]. Inconsistent class averages were removed from further data analysis. The 3D refinements and classifications were subsequently done in Relion. All final refinements were done using the auto refine option (Relion). The initial reference was filtered to 60 Å, and C1 symmetry was applied during refinements for all classes. Particles were split into two datasets and refined independently, and the resolution was determined using the 0.143 cut-off (Relion auto refine option). Local resolution was determined with Resmap. All maps were filtered to local resolution using Relion with a B-factor determined by Relion.

Model building

The model was built in Coot [43] and refined using Phenix real_space_refine [44]. Figures are prepared with Chimera [45].

Data availability

The datasets produced in this study are available in the following databases:

CENP-A nucleosome and CENP-A/CENP-C^{CR} complex cryo-EM maps: Electron Microscopy Data Bank (EMDB, www.ebi.ac.uk/pdbe/emdb), accession codes:

- EMD-10155 (CENP-A, Class 1), www.emdb-empiar.org/emd-10155
- EMD-10152 (CENP-A, Class 2), www.emdb-empiar.org/emd-10152
- EMD-10153 (CENP-A, Class 2A), www.emdb-empiar.org/emd-10153
- EMD-10156 (CENP-A, Class 2B), www.emdb-empiar.org/emd-10156

- EMD-10154 (CENP-A, Class 2C), www.emdb-empiar.org/emd-10154
- EMD-10151 (CENP-A/CENP-C^{CR}, Class 1), www.emdb-empiar.org/emd-10151
- EMD-10157 (CENP-A/CENP-C^{CR}, Class 1A), www.emdb-empiar.org/emd-10157
- EMD-10158 (CENP-A/CENP-C^{CR}, Class 1B), www.emdb-empiar.org/emd-10158
- EMD-10159 (CENP-A/CENP-C^{CR}, Class 1C), www.emdb-empiar.org/emd-10159

CENP-A nucleosome and CENP-A/CENP-C^{CR} complex molecular models: Protein Data Bank (PDB, www.rcsb.org), accession codes:

- 6SE0 (CENP-A, Class 1), www.pdbe.org/6se0
- 6SEG (CENP-A/CENP-C^{CR}, Class 1), www.pdbe.org/6seg
- 6SE6 (CENP-A/CENP-C^{CR}, Class 2), www.pdbe.org/6se6
- 6SEE (CENP-A/CENP-C^{CR}, Class 2A), www.pdbe.org/6see
- 6SEF (CENP-A/CENP-C^{CR}, Class 2C), www.pdbe.org/6sef

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Author contributions
NS and MH conceived and supervised the project. AA-A carried out mutagenesis, protein expression, purification and assembled nucleosomes and CENP-C complexes and did MNase experiments and analysis. SB prepared grids for cryo-EM. SB, IBS, and MH collected cryoEM data. SB and MH processed cryo-EM data. AA, NS, SB, and MH analyzed data and built the structure, and AA refined the final models. AA and NS wrote the manuscript, and all authors commented on it.

Conflict of interest
The authors declare that they have no conflict of interest.

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