CENP-C directs a structural transition of CENP-A nucleosomes mainly through sliding of DNA gyres

Samantha J Falk1,4, Jaehyoun Lee2,4, Nikolina Sekulic1,3, Michael A Sennett2, Tae-Hee Lee2 & Ben E Black1

In diverse eukaryotes, centromere location is specified by a unique chromatin domain containing CENP-A nucleosomes1–3. CENP-A and other constitutive centromere proteins track with newly formed centromeres (i.e., neocentromeres, which lack the repetitive α-satellite DNA found at typical human centromeres)4–6 but are absent from the silenced centromeres in pseudodicentric chromosomes4–7. The notion that CENP-A, a histone variant, carries epigenetic information specifying centromere location has been bolstered by many lines of evidence (reviewed in refs. 1–3), including key experiments using CENP-A–nucleosome assembly (through direct or indirect targeting or reconstitution) to seed new centromeres at ectopic chromosomal loci8–12, on a plasmid13 or in Xenopus extracts14.

Although CENP-A is thought to be the key molecule specifying centromere location, it does not act alone when performing its essential centromere function. For example, we have recently found that CENP-A maintains centromere identity through collaborating with the constitutive centromere protein CENP-C15. In the absence of CENP-C, CENP-A confers an altered shape on the octameric histone core, and when CENP-A is directed to chromosome locations lacking a high local concentration of CENP-C, it is destabilized15. The nucleosome shape deviation originates from rotation at the CENP-A–CENP-C interface16, which requires the movement of H2A–H2B dimers away from each other to avoid steric clashing. Indeed, H2A–H2B dimers are 5 Å further away from each other in CENP-A nucleosomes than in canonical H3-containing nucleosomes15, but the nature of the structural rearrangement—which is central to understanding the altered path of CENP-A nucleosomal DNA—remains unclear (Fig. 1a). The central domain of CENP-C (CENP-CCD) contacts the C-terminal tail of CENP-A as well as discrete surfaces on histones H2A and H4 (refs. 17,18) and reshapes the CENP-A nucleosome15 (Fig. 1a), thus providing a prime example demonstrating that nucleosome shape and function can be modulated in a manner analogous to the allosteric regulation of enzymes.

Here we set out to define the CENP-A–nucleosome structural transition resulting in an altered path of nucleosomal DNA, because of its importance for understanding both the epigenetic maintenance of centromere identity and the possible ways in which nucleosome structure and function can be modulated.

RESULTS
DNA gyre sliding in the CENP-A nucleosome

The 147 bp of DNA that wraps a canonical nucleosome makes ~1.7 turns around the histone core, thus resulting in two DNA gyres that contact the H2A–H2B dimers in the histone core19. H2A–H2B dimers moving away from each other in CENP-A nucleosomes may cause the two gyres to move away from each other, thus resulting in a separation of the DNA gyres (Fig. 1a). Alternatively, the movement of the dimers may result in the DNA gyres moving laterally past each other, thus resulting in a tightening of the DNA wrapping at the point of contact of the dimers (Fig. 1a). To measure the relative contribution of these two types of DNA-gyre movement, we designed a FRET-based scheme using two nucleosomal DNAs (DNA1 and DNA2; Fig. 1b), each derived from a human α-satellite sequence20 in which the dyad position precisely matches the location where CENP-A nucleosomes map at native centromeres21. In the case of DNA1 (Fig. 1c), gyre separation would result in the fluorophores moving away from each other, and FRET efficiency (ΦFRET) would decrease. For lateral DNA gyre passing, the donor fluorophore would move closer to the acceptor fluorophore, thus resulting in higher ΦFRET. In the case of DNA2 (Fig. 1c), gyre separation would also result in

1Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA. 2Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania, USA. 3Present address: Biotechnology Centre of Oslo, Department of Chemistry, University of Oslo, Oslo, Norway. 4These authors contributed equally to this work. Correspondence should be addressed to T.-H.L. (txl18@psu.edu) or B.E.B. (blackbe@mail.med.upenn.edu).

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Figure 1 A single-molecule FRET approach to assess the DNA wrapping of nucleosomes. (a) Cartoon schematic of H3, CENP-A and CENP-A nucleosomes bound by CENP-C\textsuperscript{CD}. The schematic for CENP-A nucleosomes indicates an altered histone-core structure in which the H2A-H2B dimers rotate away from each other, thus suggesting altered DNA wrapping that could lead to the DNA gyres either separating or sliding past each other. (b) Diagram of the two DNAs used in single-molecule FRET experiments to investigate differences in the DNA wrapping of H3, CENP-A, and CENP-A nucleosomes bound by CENP-C\textsuperscript{CD}. The DNA sequence is derived from human \(\alpha\)-satellite DNA, and the donor and acceptor fluorophores are represented by green and red lollipops, respectively. Base-pair numbering corresponds to the 5’ (−) to 3’ (+) direction relative to the dyad. (c) Predictions of the change in \(\Phi_{\text{FRET}}\) between CENP-A and H3 nucleosomes for the two fluorophore pairs if the DNA gyres separate or slide past each other, or if both movements occur. (d) Native PAGE of the indicated samples visualized by Cy3 fluorescence. Uncropped gel image is shown in Supplementary Data Set 1.

The fluorophores moving away from each other, thus leading to a decrease in \(\Phi_{\text{FRET}}\). However, if lateral DNA gyre passing were to occur, the donor fluorophore would move further away from the acceptor fluorophore, thus resulting in a decrease in \(\Phi_{\text{FRET}}\) for DNA2. An important aspect of our design is that we expected the absolute structural change, because the FRET fluorophores are separated by the same distance from each other on each DNA. Therefore, if gyre separation were to dominate, \(\Phi_{\text{FRET}}\) would decrease by a similar magnitude for both DNA1 and DNA2 (Fig. 1c). If DNA gyre lateral passing were to dominate, then \(\Phi_{\text{FRET}}\) would increase for DNA1 and decrease for DNA2, but the magnitude of the change would be approximately equal (Fig. 1c). If both types of movement

Figure 2 CENP-A nucleosomes adopt altered DNA wrapping that reverts to canonical wrapping upon CENP-C\textsuperscript{CD} binding. (a) \(\Phi_{\text{FRET}}\) values for H3, CENP-A and CENP-A nucleosomes bound by CENP-C\textsuperscript{CD}, plotted as histograms and fitted to a Gaussian distribution for DNA1 and DNA2. (b) Summary of \(\Phi_{\text{FRET}}\) values for the indicated nucleosomes and DNA. Efficiency values are the centers of the Gaussian curve fittings in a. (c) Summary of changes in \(\Phi_{\text{FRET}}\) values between the indicated nucleosomes. The differences in \(\Phi_{\text{FRET}}\) are calculated for three different nucleosome comparisons (indicated in columns A and B) on both DNA1 and DNA2. Error is reported as the uncertainty in the average FRET at a 95% confidence interval. Total numbers of individual nucleosome measurements taken from three separate slides: DNA1, 249 (H3), 157 (CENP-A), 355 (CENP-A + CENP-C\textsuperscript{CD}); DNA2, 128 (H3), 312 (CENP-A), 145 (CENP-A + CENP-C\textsuperscript{CD}).
were to substantially contribute to altering the DNA path, then the absolute $\Phi_{\text{FRET}}$ measured for DNA1 would be different from that for DNA2, because DNA gyre separation and lateral DNA gyre passing would have opposite effects on $\Phi_{\text{FRET}}$ for DNA1, but both would result in decreased $\Phi_{\text{FRET}}$ for DNA2 (Fig. 1c).

Our measurement setup for single-molecule FRET was based on one that we have used to address diverse issues in nucleosome structure and dynamics. Nucleosomes (and nucleosomal complexes; native PAGE in Fig. 1d) exhibited a range of $\Phi_{\text{FRET}}$ values, and we focused our analysis on the high-FRET group with nucleosomes positioned on the DNA as designed. We performed separate analyses on nucleosomes in low- and medium-FRET groups, in which positioning on the DNA template varied slightly (Online Methods, Supplementary Fig. 1 and Supplementary Table 1). In all bins, CENP-A nucleosomes reconstituted with DNA1 exhibited a significantly increased $\Phi_{\text{FRET}}$ relative to that of H3 nucleosomes (Fig. 2a–c). Further, the absolute change in $\Phi_{\text{FRET}}$ was almost identical among these comparisons (Fig. 2a–c).

The low- and medium-FRET subgroups that we observed may represent subpopulations of nucleosomes with varying gaps between the two nucleosomal DNA gyres. Changes in $\Phi_{\text{FRET}}$ can originate from changes in physical and photophysical properties of the fluorophores, such as rotational freedom and fluorescence quantum yield. Because CENP-A is highly unlikely to be in direct contact with the fluorophores (approximate locations of CENP-A and fluorophores in Fig. 1b), CENP-A in the nucleosomes was unlikely to affect the rotational freedom or the quantum yield of the fluorophores. Together, these data indicated that DNA alteration in CENP-A nucleosomes relative to its conventional counterparts containing canonical H3 is heavily dominated by the gyres laterally passing each other, and there is only a small contribution from the DNA gyre separation.

**CENP-A reverts the altered DNA wrapping of CENP-A nucleosomes**

We predicted that CENP-C-DD binding to the CENP-A nucleosome would cause the gyres of the DNA to slide back to a conventional nucleosome orientation. This conclusion was based on our earlier findings and the present findings that the major form of structural alteration in unbound CENP-A nucleosomes occurred via DNA gyre sliding (Figs. 1 and 2). Indeed, CENP-A nucleosomes bound by CENP-C-DD had essentially the same $\Phi_{\text{FRET}}$ as those of isolated canonical nucleosomes containing conventional H3, thus suggesting that the internal DNA wrapping of these two nucleosome complexes was nearly identical (Fig. 2a–c). It is unlikely that the $\Phi_{\text{FRET}}$ changes upon CENP-C binding were due to altered fluorophore photophysics, given that CENP-C binding exerted opposing effects on $\Phi_{\text{FRET}}$ in the two different nucleosomes. Therefore, the $\Phi_{\text{FRET}}$ changes that we observed were mainly due to the changes in the distance between the fluorophores. Together, our findings provide a view of the starting and ending points of the protein and DNA (Figs. 1 and 2) components of CENP-A nucleosomes during the structural transition directed by CENP-C-DD, a process that rigidifies and stabilizes nucleosomes and facilitates the maintenance of centromere identity through cell generations.

**A model of CENP-A structural transitions**

To visualize the structural transition of CENP-A nucleosomes, we constructed a model of its favored state in solution before binding to CENP-C (Fig. 3a). Our model of the CENP-A nucleosome in the absence of CENP-C (Fig. 3a) integrates compaction of the (CENP-A–H4) heterotetramer at the CENP-A–CENP-A interface (Fig. 3b), movement of the H2A–H2B dimers away from each other (Fig. 3c) and lateral passing of the DNA gyres (Fig. 3d) relative to the crystallized form of the CENP-A nucleosome.

The rotation and compaction that initiates at the CENP-A–CENP-A four-helix bundle is simply propagated through the H2A–H2B dimer and leads to DNA gyre sliding. Interestingly, we note that even in the crystallized form of the CENP-A nucleosome the CENP-A–CENP-A four-helix bundle is rotated but not compacted relative to the H3–H3 four-helix bundle in canonical nucleosomes (Supplementary Fig. 2).
is a decreased radius of curvature around the histone core in CENP-A nucleosomes before CENP-C binding. We adjusted the path of DNA by using the FRET measurements (Fig. 2) on a high-resolution CENP-A–nucleosome structural model25 (PDB 3AN2). One chain of each histone (CENP-A, H4, H2A and H2B) on one half of the dyad axis of symmetry was fixed, and the other chains (CENP-A ′, H4′, H2A′ and H2B′) were rotated. After simple model minimization, we observed that the DNA contacts to each half of the nucleosome were maintained, thus causing unrealistic bond distances at the dyad site of the nucleosomal DNA (Fig. 3a, break in the continuity of the DNA ribbon diagram marked with an asterisk). DNA compression at the dyad is expected to accommodate the reduced radius of gyration. In addition to compression, kinking and stretching at histone contact points throughout the nucleosome would also distribute the changes required by the altered CENP-A–nucleosome structural state, as has been well noted in analysis of canonical nucleosomes crystallized on various DNA sequences26–28. Indeed, we found independent evidence of altered DNA conformation in CENP-A nucleosomes, as demonstrated by the increased intercalation and reactivity of N-(2,3-epoxypropyl)-1,8-naphthalimide (ENA) at a GG dinucleotide located 1.5 turns from the dyad (Supplementary Fig. 3). Thus, the DNA near the dyad is substantially altered, whereas the gyres simply pass by each other on the opposite side of the nucleosome without any other large bends forming in the DNA. CENP-A–C2D binds near the dyad15,18 and stabilizes the form of the CENP-A nucleosome where the CENP-A–CENP-A four-helix bundle is rotated into a conventional shape, and the gyres of the DNA slide back to the path found in canonical nucleosomes (Fig. 3 and Supplementary Video 1).

DISCUSSION

The structural variations in CENP-A nucleosomes and the subsequent changes in CENP-C binding provide insight into the basis of epigenetic inheritance of the centromere. Here, we identified the major change in the path of nucleosomal DNA and generated a molecular model to integrate past and current data sets from solution and crystal studies.

CENP-A is found at a high local concentration at the centromere, where it recruits CENP-C; the direct binding of CENP-C to the CENP-A nucleosome then changes the nucleosome’s shape. We suggest that when CENP-A is distributed in the genome—as may be the case in its overexpression in some cancers29—CENP-C molecules can still bind, but limiting amounts of CENP-C or other essential nonhistone centromere proteins30,31 do not support the formation of a stable centromere chromatin domain29,32–34. New centromere formation can be stimulated by artificially generating a high local concentration of nascent CENP-A nucleosomes8–13, and rare neocentromeres occurring in the human population4–6,35 may arise at chromosome-arm sites where clusters of CENP-A nucleosomes recruit CENP-C molecules and together initiate the formation of heritable centromeres. By altering the nucleosome in this way, CENP-C binding stabilizes CENP-A nucleosomes at the centromeres and helps to solidify the foundation of centromeric chromatin. When CENP-C is removed from centromeres, the stability is compromised15. Thus, the CENP-A nucleosome structural transition is intimately linked to CENP-A’s function in epigenetically marking centromere location over long timescales that are biologically relevant. Further, our study provides an important example of how nucleosome shape alteration may be coupled to function for other types of nucleosomes—containing either canonical or variant histones—in diverse chromatin contexts.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.J.F., J.L., N.S., T.-H.L. and B.E.B. designed experiments. M.A.S. performed preliminary experiments that informed the design of the study. S.J.F. and J.L. performed experiments. S.J.F., J.L., N.S., T.-H.L. and B.E.B. analyzed data. N.S. performed modeling. S.J.F., J.L., N.S., T.-H.L. and B.E.B. wrote and edited the paper. T.-H.L. and B.E.B. directed the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

DNA preparation. Nucleosomal DNA was prepared by ligating oligonucleotides as described elsewhere\textsuperscript{36}. For both DNA1 and DNA2, a 20-base single-stranded DNA linker with biotin at one end was added to a 147-bp human \(\alpha\)-satellite sequence\textsuperscript{36}. Each DNA construct was prepared by ligating six oligonucleotides (Integrated DNA Technologies), and both fluorophores were on the forward strand and attached via a six-carbon linker (Integrated DNA Technologies). The FRET donor (Cy3) was attached at the –33 base in DNA1 and the –43 base in DNA2, and the FRET acceptor (Cy5) was attached at the +38 base in both DNA constructs.

Protein preparation. Human histones and CENP-A were prepared as described elsewhere\textsuperscript{16}. Recombinant human CENP-C\textsuperscript{3D} consisting of the central domain only (amino acids 426–537) was GST-tagged and purified on a GST column, cleaved by PreScission protease (GE Healthcare), purified by ion-exchange chromatography and prepared in a buffer containing 20 mM Tris, pH 7.5, 200 mM NaCl, 0.5 mM EDTA, and 1 mM DTT.

Nucleosome reconstitutions. Nucleosomes were reconstituted as described previously\textsuperscript{36}. Briefly, labeled DNA was mixed with H2A–H2B dimers and (H3–H4)\textsubscript{2} or (CENP–A–H4)\textsubscript{2} tetramers in TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) with 2 M NaCl. The mixture was dialyzed stepwise against TE buffers with 850, 650, 500 and 2.5 mM NaCl for 1 h each step, at 4 °C. Nucleosome assembly was assessed with native PAGE and fluorescence imaging (Typhoon 9410, GE Healthcare). CENP-C\textsuperscript{3D} was incubated with CENP-A nucleosomes for 1 h at room temperature, and complex formation was confirmed with native PAGE.

Single-molecule FRET measurements. Single-molecule FRET measurements were carried out as described elsewhere\textsuperscript{22,23}. A quartz microscope slide was coated with a 99:1 mixture of polyethylene glycol (PEG) and biotin-PEG-silane (Laysan Bio); this was followed by incubation with a 100 pM streptavidin-biotin conjugation, and the measurements were completed within 30 min after immobilization to avoid nucleosome disassembly. Fluorescence signals were collected with an electron-multiplying CCD camera (EMCCD; iXon DU-897, Andor Technology) from a home-built prism-coupled total internal reflection fluorescence microscope based on a commercial microscope (TE2000; Nikon). The FRET donor was excited with a 532-nm laser (CrystalLaser, GCL-150-L). The fluorescence signal was separated into two spectral regions, 550–650 and 650–750 nm for Cy3 and Cy5, respectively, with a dichroic mirror (650DCXR, Chroma Technology) and a filter (HQ660/200m, Chroma Technology). The signals from the two fluorophores were collected simultaneously at a rate of 250 ms/frame. FRET efficiency (\(\Phi_{\text{FRET}}\)) at each time point was calculated with the formula

\[
\Phi_{\text{FRET}} = \frac{I_{\text{Cy5}}}{I_{\text{Cy3}}} + I_{\text{Cy5}}
\]

where \(I\) is the signal intensity of the corresponding fluorophore. Measurements were taken from a minimum of three separate slides.

Single-molecule FRET analysis. For each nucleosome class (H3, CENP-A, and CENP-A bound by CENP-C\textsuperscript{3D} on either DNA1 or DNA2), the average \(\Phi_{\text{FRET}}\) per trace was calculated for individual nucleosomes and binned into three groups (low, medium or high FRET). The FRET efficiency distribution in each group was plotted as a histogram, which was normalized to the sample size. The distribution of FRET efficiencies in a histogram is due to the Poissonian fluorescence photon emission statistics (fluorescence intensity fluctuation over time) inherent to any fluorescent signal. The reported uncertainties in the average FRET values are reported at a 95% c.i. Nucleosomes that contained a malfunctioning Cy5 fluorophore or were aggregates (6–21% of the total fluorescent particles in each experiment) on the basis of multiple photobleaching events were not included in the analysis.

Nucleosome modeling. The molecular model of the CENP-A nucleosome in solution (without CENP-C bound) was generated from a high-resolution CENP-A–nucleosome structure (PDB 3AN2)\textsuperscript{25} as a starting model. The DNA sequence was modified to match the DNA sequence that was used in the smFRET experiments, and chains A, B, C, D, I (residues –60 to -1) and J (residues 0 to 60) were moved as a rigid body to satisfy the rotation between chains A and E observed in the (CENP-A–H4), crystal structure\textsuperscript{16} (PDB 3NQJ) and the DNA-gyre sliding movements observed in smFRET between DNA J –33 and +38 (DNA1) and J –43 and +38 (DNA2). Chains E, F, G, H, I (residues 0 to 60) and J (residues –60 to –1) were kept fixed. The energy of the final model was minimized through an annealing procedure in CNS\textsuperscript{37}.

DNA intercalation and footprinting with ENA. Reactions were carried out as previously described\textsuperscript{38}. Briefly, ENA was added from a 4 mM stock (dissolved in DMSO) at 2:1 or 20:1 molar excess over free DNA or nucleosome complexes, respectively. All DNA and nucleosome samples were reconstituted with a 147-bp nonpalindromic form of \(\alpha\)-satellite DNA sequence (the same sequence used in our smFRET experiments) that was hexachlorofluorescein (HEX) labeled at the 5′ end of the top strand only. Samples were incubated overnight at room temperature, shielded from light. After incubation, addition of 4 M NaCl, and subsequent phenol-chloroform and chloroform-only extractions were carried out to remove unreacted ENA, proteins, and excess phenol. DNA was then ethanol precipitated and resuspended in 20 μL of TE buffer (10 mM Tris–Cl and 0.1 mM EDTA). Samples were heated at 95 °C for 30 min; this was followed by the addition of 20 μL 1 M piperridine and an additional 30 min incubation at 95 °C to induce chemical cleavage of alkylated guanines. 1.2 mL of butanol was added to each sample; this was followed by brief vortexing and centrifugation at 12,000g for 2 min at 4 °C. The supernatant was removed, and pellets were resuspended in 150 μL 1% SDS and 1 mL butanol, vortexed briefly, and centrifuged at 12,000g for 2 min at room temperature. The supernatant was discarded, and samples were lyophilized for 20 min to remove excess liquid. Samples were resuspended in 10 μL loading buffer (1x TBE (88 mM Tris–borate, 2 mM EDTA), 90% formamide, and 0.1% bromophenol blue) boiled at 95 °C, and separated by denaturing PAGE (10% polyacrylamide, 7 M urea, 88 mM Tris–borate, and 2 mM EDTA, pH 8.3). Maxam-Gilbert purine-sequencing standards were prepared as previously described\textsuperscript{39}. Gels were imaged on a Typhoon 9200 imager (GE Healthcare).

Movie construction. Movie segments were made in PyMOL (version 1.7.4; http://www.pymol.org/) and assembled in QuickTime Pro (version 7.6.6).

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