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Yoshimasa Takizawa[1,2,7], Cheng-Han Ho[1,7], Hiroaki Tachiwana[3,4,7], Hideyuki Matsunami[2], Wataru Kobayashi[1,3], Midori Suzuki[3], Yasuhiro Arimura[1], Tetsuya Hori[5], Tatsuo Fukagawa[5], Melanie D. Ohi[6], Matthias Wolf [2*] and Hitoshi Kurumizaka [1,3,8*]

[1] Laboratory of Chromatin Structure and Function, Institute for Quantitative Biosciences, The University of Tokyo, Bunkyo-ku, Yayoi, 1-1-1, Tokyo 113-0032, Japan.

[2] Molecular Cryo-Electron Microscopy Unit, Okinawa Institute of Science and Technology Graduate University, 1919-1 Tancha, Onna-son, Kunigami-gun, Okinawa 904-0495, Japan.

[3] Graduate School of Advanced Science & Engineering, Waseda University, Shinjuku, Tokyo 162-8480, Japan.

[4] Department of Cancer Biology, The Cancer Institute of Japanese Foundation of Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan.

[5] Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871, Japan.

[6] Life Science Institute, Cell & Developmental Biology, University of Michigan Medical School, 210 Washtenaw, Ann Arbor, MI 48109-2200, USA.

[7] These authors equally contributed to this work.

[8]Lead Contact

*To whom correspondence should be addressed.

E-mail: kurumizaka@iam.u-tokyo.ac.jp (H. Kurumizaka) or matthias.wolf@oist.jp (M. Wolf)

SUMMARY

The histone H3 variant CENP-A is a crucial epigenetic marker for centromere specification. CENP-A forms a characteristic nucleosome and dictates the higher-order configuration of centromeric chromatin. However, little is known about how the CENP-A nucleosome affects the architecture of centromeric chromatin. In the present study, we reconstituted tri-nucleosomes mimicking a centromeric nucleosome arrangement containing the CENP-A nucleosome, and determined their three-dimensional structures by cryo-electron microscopy. The H3-CENP-A-H3 tri-nucleosomes adopt an untwisted architecture, with an outward-facing linker DNA path between nucleosomes. This is distinct from the H3-H3-H3 tri-nucleosome architecture, with an inward-facing DNA path. Intriguingly, the untwisted architecture may allow the CENP-A nucleosome to be exposed to the solvent in the condensed chromatin model. These results provide a structural basis for understanding the three-dimensional configuration of CENP-A-containing chromatin, and may explain how centromeric proteins can specifically target the CENP-A nucleosomes buried in the robust amounts of H3 nucleosomes in centromeres.

Keywords (up to 10): centromere; CENP-A; chromatin; cryo-EM; nucleosome; histone variant

INTRODUCTION

Centromeres are chromosome regions for kinetochore assembly, and their positions are epigenetically defined and maintained through cell division (Cheeseman and Desai, 2008; Santaguida and Musacchio, 2009; Fukagawa and Earnshaw, 2014). Many proteins participate in the formation and maintenance of the kinetochore structure on centromeres (Obuse et al., 2004; Cheeseman et al., 2004; Minoshima et al., 2005; Foltz et al., 2006; Meraldi et al., 2006; Okada et al., 2006; Hori et al., 2008; Amano et al., 2009). CENP-A (also named CenH3) is a highly conserved centromere-specific histone H3 variant (Palmer et al., 1987) that is essential for designating centromeres as sites for kinetochore assembly (Fukagawa and Earnshaw, 2014; McKinley and Cheeseman, 2016; Müller and Almouzni, 2017; Musacchio and Desai, 2017; Hara and Fukagawa, 2018). In fact, CENP-A functions as an epigenetic mark for centromere specification (Black and Cleveland, 2011).

If the CENP-A level is decreased in cells, then kinetochore assembly is severely impaired (Howman et al., 2000; Régnier et al., 2005; Fachinetti et al., 2013). On the other hand, the overproduction of CENP-A has been found in several types of cancer (Tomonaga et al., 2003; Li et al., 2011; Rajput et al., 2011; McGovern et al., 2012; Stangeland et al., 2015; Sun et al., 2016). In fact, the inappropriate assembly of the CENP-A nucleosome at ectopic loci may lead to a higher incidence of cancer (Lacoste et al., 2014; Shrestha et al., 2017). These findings suggest that proper CENP-A assembly in centromeres ensures cellular homeostasis.

The CENP-A nucleosome recruits additional centromeric proteins to its assembly sites. This occurs even if CENP-A is artificially positioned at a non-centromere locus on a chromosome (Mendiburo et al., 2011; Logsdon et al., 2015; Tachiwana et al., 2015). Thus, the CENP-A nucleosome functions as a structural hub for the assembly of other centromeric proteins. Consistent with these cell biological findings, biochemical and structural analyses demonstrated that some centromeric proteins, including CENP-B, CENP-C, and CENP-N, directly bind to the CENP-A nucleosome (Tanaka et al., 2005; Carroll et al., 2009; 2010; Guse et al., 2011; Tachiwana et al., 2013; Kato et al., 2013; Arimura et al., 2014; Fujita et al., 2015; Fachinetti et al., 2015; Falk et al., 2015; Chittori et al., 2018; Pentakota et al., 2017; Tian et al., 2018). To ensure the specific binding of centromeric proteins to the CENP-A

nucleosome, it must have distinct features as compared to the canonical H3 nucleosome in centromeric chromatin.

The CENP-A nucleosome contains several unique structural attributes, such as the L1 loop (also called RG loop) protrusion (Sekulic et al., 2010; Tachiwana et al., 2011) and flexible DNA ends (Conde e Silva et al., 2007; Sekulic et al., 2010; Tachiwana et al., 2011; Kingston et al., 2011; Dechassa et al., 2011; Panchenko et al., 2011; Arimura et al., 2014; Kono et al., 2015; Arimura et al., 2019). Cryo-electron microscopy (cryo-EM) structures revealed that the RG loop directly binds to CENP-N (Chittori et al., 2018; Pentakota et al., 2018; Tian et al., 2018). The flexible DNA ends of the CENP-A nucleosome may have a strong impact on the higher-order chromatin architecture. The structural organization of the DNA ends could be the mechanism that enhances the accessibility of the CENP-A nucleosome to its binding partners, as compared to the H3 nucleosome. Consistent with this model, the enhanced flexibility of the DNA ends in the CENP-A nucleosome has been observed in human cells, based on native chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-seq) (Hasson et al., 2013; Lacoste et al., 2014). In addition, the loss of the flexibility of the DNA ends in the CENP-A nucleosome abrogates kinetochore assembly (Roulland et al., 2016). These observations strongly suggest that the flexible nature of the DNA segments in the CENP-A nucleosome may play important roles to facilitate the assembly of centromeric proteins. However, it remains unclear how the flexible DNA segments of the CENP-A nucleosome contribute to the establishment of the higher-order structure of the centromeric chromatin.

To address this question, we prepared tri-nucleosomes mimicking a centromeric nucleosome arrangement, in which the CENP-A nucleosome is flanked by two H3 nucleosomes (H3-CA-H3). We also prepared tri-nucleosomes without CENP-A (H3-H3-H3). We then determined the structures of these tri-nucleosomes by cryo-EM. The higher-order structures of the H3-CA-H3 tri-nucleosomes provide a structural model for the role of CENP-A as a platform for kinetochore assembly sites on chromosomes.

RESULTS

Reconstitution of the H3-CA-H3 Tri-nucleosome

In higher eukaryotes, the CENP-A nucleosomes are interspersed throughout the

centromeric chromatin and primarily have H3 nucleosomes as neighbors (Ribeiro et al., 2010; Fukagawa and Earnshaw, 2014; Müller and Almouzni, 2017; Musacchio and Desai, 2017; Nishimura et al., 2019). To study the higher-order structure of centromeric chromatin containing CENP-A nucleosomes, we reconstituted a tri-nucleosome mimicking a centromeric nucleosome arrangement, in which one CENP-A nucleosome is flanked by two H3 nucleosomes (Figure 1A). We prepared the CENP-A nucleosome with cohesive sites at both DNA ends (Figure 1A-b, 1B). H3 nucleosomes with a single cohesive end were also reconstituted, and the CENP-A nucleosome was then connected to two H3 nucleosomes with linker DNAs by T4 DNA ligase (Figure 1A-a,c, 1B, 1C). The resulting H3-CA-H3 tri-nucleosome was purified by native polyacrylamide gel electrophoresis (Figure 1A-right, 1D, 1E). For comparison with non-centromeric chromatin, the conventional H3-H3-H3 tri-nucleosome was also generated by the same procedure (Figure 1A-right, 1D, 1E). In these tri-nucleosomes, the nucleosomes were connected by 22 base-pair linker DNAs, which correspond to the predicted linker DNA length in human centromeric alphoid DNA repeats (Masumoto et al., 1989a; Masumoto et al., 1989b). An SDS-PAGE analysis confirmed that both the CENP-A and H3 molecules were properly incorporated into the tri-nucleosomes with the appropriate stoichiometry (Figure 1D).

Imaging of the Tri-nucleosomes by Cryo-EM

The reconstituted H3-CA-H3 and H3-H3-H3 tri-nucleosomes were visualized by cryo-EM, using a Volta phase plate for in-focus phase contrast. As expected, the images showed the three nucleosomes connected by linker DNAs, and the tri-nucleosome conformation appeared flexible in both the H3-CA-H3 and H3-H3-H3 nucleosomes in the absence of Mg^{2+} ions (Figure 2A). Interestingly, the addition of a physiological concentration (1mM) of Mg^{2+} ion drastically changed the conformations of both the H3-CA-H3 and H3-H3-H3 tri-nucleosomes, resulting in face-to-face stacking of the peripheral H3 nucleosomes against each other on their histone surfaces (Figure 2B).

The Linker DNA Paths of the Tri-nucleosomes

We processed the cryo-EM images of the H3-CA-H3 and H3-H3-H3 tri-nucleosomes in the presence of Mg^{2+}, and obtained 2D class average images (Figure 3 and Figure S1).

Interestingly, in the H3-H3-H3 tri-nucleosome with the 22 base-pair linker DNAs, two different linker DNA conformations were observed. In the inward-path conformation, the linker DNAs of the central nucleosome were connected to the inward-facing strands of the stacked nucleosomes (Figure 3A, blue circles), while another population of H3-H3-H3 tri-nucleosomes adopted a conformation in which the linker DNAs were connected to the outward-facing strands of the stacked nucleosomes in the outward-path conformation (Figure 3A, red circles). Interestingly, the outward-path conformation of the H3-H3-H3 tri-nucleosome was not observed when the linker DNA length was elongated to 30 base pairs (Figure 3B). Therefore, the outward-path structural conformation may not be favorable in H3-H3-H3 tri-nucleosomes when the linker DNA length extends beyond a certain length.

In contrast, we found that the H3-CA-H3 tri-nucleosome only formed the outward-path conformation with the 22 base-pair linker DNAs (Figure 3C), a linker length that closely corresponds to the predicted linker DNA length in human centromeric chromatin (Masumoto et al., 1989a; Masumoto et al., 1989b). This outward-path conformation was still observed even in H3-CA-H3 tri-nucleosomes with 30 base-pair linker DNAs (Figure 3D). These results indicate that the CENP-A nucleosome has the potential to form the outward-path conformation in chromatin.

The adoption of the outward-path conformation may be enhanced by the DNA end flexibility observed in the CENP-A nucleosome located at the center of the tri-nucleosome (Tachiwana et al., 2011; Arimura et al., 2014). The DNA end flexibility is induced by the αN helix of CENP-A (Roulland et al., 2016; Arimura et al., 2019). Interestingly, the CENP-A nucleosome did not adopt the outward-path conformation when it was located on both sides of the central H3 nucleosome in the CA-H3-CA tri-nucleosome with 30 base-pair linker DNAs (Figure S2). In addition, the CENP-A nucleosomes were stacked in the CA-H3-CA tri-nucleosome, as in the H3 nucleosome. Therefore, the proper localization of the CENP-A nucleosome may be important for the outward-path conformation in chromatin.

Cryo-EM Structures of Tri-nucleosomes with 22 base-pair Linker DNAs

We reconstructed the 3D structures of the H3-CA-H3 and H3-H3-H3 tri-nucleosomes with the 22 base-pair linker DNAs in the outward path conformation. For comparison,

the H3-H3-H3 tri-nucleosome structure (outward path) with the 22 base-pair linker DNAs was reconstructed (Figure 4A). An H3-H3-H3 tri-nucleosome structure, in which the two stacked H3 nucleosomes were slightly shifted, was also obtained (Figure S6). This could reflect the dynamic nature of the stacked di-nucleosome within the tri-nucleosome. However, the orientation of the central H3 nucleosome relative to the stacked di-nucleosome was quite similar in both H3-H3-H3 tri-nucleosome structures.

Interestingly, in the H3-CA-H3 tri-nucleosome, the orientation of the central CENP-A nucleosome relative to the stacked H3 di-nucleosome was essentially untwisted, as compared to that of the central H3 nucleosome (Figure 4B). This may be a consequence of the flexible DNA ends of the CENP-A nucleosome. In fact, the linker DNAs of the CENP-A nucleosome did not fit the linker DNA orientation of the H3 nucleosome model (Figure 4C). Therefore, the CENP-A nucleosome with the linker DNA length corresponding to human centromeric alphoid DNA repeats has a strong propensity to change the nucleosome alignment in chromatin.

The Linker DNA Length May Not Affect the CENP-A Configuration in the Tri-nucleosome

We next tested whether this characteristic orientation of the CENP-A nucleosome relative to the stacked H3 di-nucleosome is observed in a situation with a longer linker DNA. We reconstructed the 3D structure of the outward-path conformation of the H3-CA-H3 tri-nucleosome with 30 base-pair linker DNAs, which correspond to the predicted linker DNA length of centromeric alphoid DNA repeats in plants (Henikoff et al., 2001). In this structure, the CENP-A nucleosome orientation relative to the stacked H3 di-nucleosome was quite similar to that in the tri-nucleosome with 22 base-pair linker DNAs (Figures 3D and 5A). In contrast, the 3D structure of the H3-H3-H3 tri-nucleosome with 30 base-pair linker DNAs formed the inward-linker DNA path, consistent with the 2D class averages (Figures 3B and 5B). This structure is essentially the same as the cryo-EM structure of the poly-nucleosome containing the linker histone H1 (Song et al., 2014), although our structure did not contain histone H1 (Figure S7). These results suggest that the untwisted chromatin conformation induced by the CENP-A nucleosome is a common feature that may define the platform chromatin architecture for subsequent assembly of centromeric proteins.

DISCUSSION

CENP-A is a crucial epigenetic marker for centromere specification and kinetochore assembly. The CENP-A nucleosomes buried in centromeric chromatin must specifically bind to centromeric proteins, such as CENP-B, CENP-C, and CENP-N (Cheeseman and Desai, 2008; Santaguida and Musacchio, 2009; Fukagawa and Earnshaw, 2014; Müller and Almouzni, 2017; Musacchio and Desai, 2017). CENP-B enhances CENP-A stability on human artificial chromosomes (Fujita et al., 2015). CENP-N and CENP-C also stabilize the CENP-A nucleosome *in vitro*, although they have minimal effects on centromeric CENP-A stability *in vivo* (Cao et al., 2018). The CENP-A nucleosome bound with CENP-B, CENP-C, and CENP-N leads to the subsequent assembly of additional centromeric proteins, such as the components of the constitutive centromere-associated network (CCAN), followed by the formation of functional kinetochores (McAinsh and Meraldi, 2011; Fukagawa and Earnshaw, 2014; McKinley and Cheeseman, 2015; Müller and Almouzni, 2017; Musacchio and Desai, 2017; Hara and Fukagawa, 2018).

CENP-B binds to the specific DNA sequence (CENP-B box) of alphoid DNA within the CENP-A nucleosome, and also directly interacts with the nucleosomal CENP-A protein (Tanaka et al., 2005; Tachiwana et al., 2013; Fujita et al., 2015; Fachinetti et al., 2015). CENP-C is a key protein for kinetochore assembly (Saitoh et al., 1992; Kwon et al., 2007; Hori et al., 2008; Klare at al., 2015), and binds directly to the CENP-A C-terminal tail and the acidic patch formed with H2A and H2B in the CENP-A nucleosome (Carroll et al., 2010; Guse et al., 2011; Kato et al., 2013; Arimura et al., 2014). CENP-N binds to the CENP-A specific L1 (RG) loop region, together with DNA, in the CENP-A nucleosome (Carroll et al., 2009; Fang et al., 2015; Chittori et al., 2018; Pentakota et al., 2018; Tian et al., 2018). Although structural analyses have revealed the binding modes of CENP-B, CENP-C, and CENP-N to the CENP-A nucleosome, the molecular basis how these centromeric proteins specifically recognize the CENP-A nucleosomes buried in the large amounts of H3 nucleosomes within the centromeric chromatin have remained enigmatic.

It has been suggested that the CENP-A nucleosomes become clustered in the centromeric chromatin (Allshire and Karpen, 2008). This allows the centromeric

proteins to find their target CENP-A nucleosome in the centromeric chromatin. On the other hand, it is difficult to target the single CENP-A nucleosome buried in the H3 nucleosome cluster in the centromeric chromatin. Therefore, in the present study, we focused on understanding the structural consequences when a single CENP-A nucleosome is embedded in the H3 nucleosome array. Supporting this idea, recent reports have suggested that the CENP-A nucleosomes are interspersed with the H3 nucleosomes as neighbors in higher eukaryotes (Ribeiro et al., 2010; Fukagawa and Earnshaw, 2014; Müller and Almouzni, 2017; Musacchio and Desai, 2017; Nishimura et al., 2019).

One plausible model is that the CENP-A nucleosome is targeted by the centromeric proteins, if it protrudes and becomes more accessible within the centromeric chromatin. This could be accomplished if the CENP-A nucleosome favors a specific chromatin conformation. Consistent with this idea, previous studies revealed that the CENP-A nucleosome has distinct structural properties *in vitro* and *in vivo*, as compared with the H3 nucleosome (Conde e Silva et al., 2007; Sekulic et al., 2010; Tachiwana et al., 2011; Kingston et al., 2011; Dechassa et al., 2011; Panchenko et al., 2011; Hasson et al., 2013; Lacoste et al., 2014; Arimura et al., 2014; Kono et al., 2015; Arimura et al., 2019).

In addition to these previous studies, the present study clearly demonstrates how the CENP-A nucleosome adopts a centromere-specific chromatin conformation, using the H3-CA-H3 tri-nucleosomes mimicking the centromeric nucleosome array. In the absence of Mg^{2+} ions, the tri-nucleosome conformation was quite flexible, and no obvious difference was found between the H3-CA-H3 and H3-H3-H3 tri-nucleosomes (Figure 2A). The physiological Mg^{2+} ion concentration reportedly compacts poly-nucleosome fibers (Schwarz and Hansen, 1994). Therefore, we added physiological amounts of Mg^{2+} ion to the tri-nucleosome samples. We surprisingly found that the peripheral two H3 nucleosomes in the tri-nucleosomes were stacked and formed the characteristic tri-nucleosome architecture under the conditions with a physiological Mg^{2+} concentration (Figure 2B). This allowed us to perform the 3D reconstruction of the tri-nucleosomes (Figure 4), which revealed that the CENP-A nucleosome induces the characteristic conformation in tri-nucleosomes.

In the present study, we employed the Widom601 DNA sequence for the tri-

nucleosome reconstitution (Lowary and Widom, 1998). The Widom601 DNA fixes the nucleosome in a single position, thus facilitating the structural analysis. Since the centromeric α-satellite DNA sequence also possesses the propensity for fixing the nucleosome position (Tanaka et al., 2005; Fujita et al., 2015), the overall architectures of the tri-nucleosomes presented here may mimic the centromeric chromatin architecture.

We built chromatin fiber models with the H3-H3-H3 and H3-CA-H3 tri-nucleosome structures (outward path) with 22 base-pair linker DNAs (Figure 6). In the model with the H3-H3-H3 tri-nucleosome structure, the nucleosomes are aligned and form a condensed chromatin fiber without steric clashes (Figure 6A, top panel). Intriguingly, when the H3-CA-H3 tri-nucleosome structure is embedded in the H3-H3-H3 chromatin fiber model, the CENP-A nucleosome is clearly exposed (Figure 6A, bottom panel). In this model, the untwisted CENP-A nucleosome conformation deforms the continuous alignment of the H3 nucleosomes and becomes more accessible than the H3 nucleosome (Figure 6B). Actually, the CENP-A-binding domains of CENP-C and CENP-N and the DNA-binding domain of CENP-B are accessible to the CENP-A nucleosome in the fiber model (Figure 6B). The present chromatin fiber models provide a vision for how the CENP-A nucleosome is exposed for recognition by centromeric proteins in centromeric chromatin, and insights into the mechanisms by which centromere proteins are efficiently assembled in CENP-A containing chromatin. It should be noted that the chromatin fiber model presented in this study was created with the tri-nucleosome structures containing the Widom601 DNA that stably fixes the nucleosome position, and thus the CENP-A nucleosome becomes more accessible on the chromatin fiber with the native centromeric α-satellite DNA sequence. Further studies are awaited.

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Supplemental Information includes seven figures.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.T., H.T., M.W., and H.K.; Methodology, Y.T., H.T., M.W., and H.K.; Investigation, Y.T., C.-H.H., H.T., H.M., W.K., M.S., Y.A., T.H., T.F., M.D.O., and M.W.; Writing – Original Draft, H.K.; Writing – Review & Editing, Y.T., C.-H.H., H.T., T.F., M.D.O., and M.W.; Funding Acquisition, H.K., H.T., T.F., H.M., Y.T., and M.W.; Resources, M.W. and H.K.; Supervision, H.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. **Tri-Nucleosome Reconstitutions.**

(A) Scheme for preparing specific tri-nucleosomes (H3-H3-H3 and H3-CA-H3), using the ligation method. Three kinds of DNA fragments were prepared: two had one cohesive end and one blunt end (a and c), and one had two cohesive ends (b). Mono-nucleosomes were reconstituted on histone octamers containing either histone H3 (blue) or CENP-A (CA) (red). After purification of the mono-nucleosomes, the tri-nucleosomes were prepared by connecting their linker DNAs with DNA ligase and purified. (B) Reconstituted mono-nucleosomes for the tri-nucleosome preparation were analyzed by 18% SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1, H3 nucleosome(a); Lane 2, H3 nucleosome(c); Lane 3, H3 nucleosome(b); Lane 4, CENP-A nucleosome(b). (C) 4% non-denaturing polyacrylamide gel electrophoresis with ethidium bromide staining for the reconstituted mono-nucleosomes. (D) Reconstituted tri-nucleosomes were analyzed by 18% SDS- polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining. Lane 1, H3-H3- H3 tri-nucleosome (H3-H3-H3); Lane 2, H3-CA-H3 tri-nucleosome (H3-CA-H3). (E) 4% non-denaturing polyacrylamide gel electrophoresis of the reconstituted tri-nucleosomes with ethidium bromide staining.

Figure 2. **Mg^{2+}-Dependent Compaction of Tri-nucleosomes.**

(A and B) Representative cryo-EM images of the tri-nucleosomes in the absence (A) or presence (B) of Mg^{2+}. Raw digital micrographs of the tri-nucleosomes (upper) were obtained with a Titan Krios cryo-electron microscope using a Volta phase plate (VPP). Scale bar, 50 nm. Particles of the tri-nucleosomes (lower) were boxed out from the VPP images. Box size, 40 nm.

Figure 3. Two-Dimensional Class Averages of the Tri-nucleosomes containing Different Linker DNA Length.

(A-B) Representative 2D class averages of the H3-H3-H3 tri-nucleosomes containing 22 base-pair (A), and 30 base-pair (B) linker DNAs. (C-D) Representative 2D class averages of the H3-CA-H3 tri-nucleosomes containing 22 base-pair (C) and 30 base-

pair (D) linker DNAs. Red and blue circles indicate the classes with the DNA linker in the outward and inward paths, respectively. 3D-distributions of particle orientations for each class are shown in Figure S1.

Figure 4. Three-Dimensional Structures of the Tri-nucleosomes containing 22 base-pair Linker DNA.

(A) Semi-transparent iso-surface representation of the reconstructed electron potential of the H3-H3-H3 tri-nucleosome, contoured at 6.1 sigma above mean density. Models of the H3-H3-H3 tri-nucleosome, with the crystal structure of the H3 mono-nucleosome (PDB: 3LZ0) colored light blue, were placed into the cryo-EM map. Modeled linker DNAs are shown in light green. (B) Semi-transparent iso-surface representation of the reconstructed electron potential of the H3-CA-H3 tri-nucleosome, contoured at 4.0 sigma above mean density. Models of the H3-CA-H3 tri-nucleosome with the crystal structures of H3 (PDB: 3LZ0) colored light blue and the CENP-A mono-nucleosomes (PDB: 3AN2) colored red were placed into the cryo-EM map. Modeled linker DNAs are shown in light green. Scale bar, 50 Å for both panels. (C) Close-up views of the central nucleosomes of the H3-H3-H3 (left) and the H3-CA-H3 tri-nucleosomes (right).

Figure 5. Three-Dimensional Structures of the Tri-nucleosomes containing the 30 base-pair Linker DNA.

(A) Semi-transparent iso-surface representation of the reconstructed electron potential of the H3-CA-H3 tri-nucleosome, contoured at 2.6 sigma above mean density. Models of the crystal structures of the CENP-A nucleosome (PDB: 3AN2) and the H3 nucleosome (PDB: 3LZ0) were placed into the cryo-EM map. (B) Iso-surface representation of the reconstructed electron potential of the H3-H3-H3 tri-nucleosome, contoured at 3.6 sigma above mean density, placed in the cryo-EM map. A model of the crystal structure of the H3 nucleosome (PDB: 3LZ0) was placed in the cryo-EM map. Scale bar, 50 Å.

Figure 6. A model of the Centromere Chromatin Configuration.

(A) Poly-nucleosome model containing the 22 base-pair DNA, based on the H3-H3-H3 and the H3-CA-H3 tri-nucleosome structures. The CENP-A nucleosome is shown in

pink. The number indicates the assembly order of the nucleosomes. (B) A cartoon model for the centromeric chromatin, based on the cryo-EM structures of the H3-H3-H3 and H3-CA-H3 tri-nucleosomes. The DNA-binding domain of CENP-B (yellow, PDB: 1HLV), the CENP-A nucleosome binding domain of CENP-C (blue, PDB: 4X23), and the CENP-A nucleosome binding domain of CENP-N (green, EMD-7326) are superimposed on the CENP-A nucleosome (pink, PDB: 6C0W). A close-up view of the CENP-A nucleosome model with these CENP-A nucleosome binding domains is presented in the right panel.

Table 1. DNA Sequences for Reconstitution of the Tri-nucleosomes

Name	DNA sequences
DNA (a)	ATCAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGCGC TGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATATACATCGATT GGATAGGCCC
DNA (b) 22 bp	GGACGGCCTGGATAATCAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAA ACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGA TATATACATCGATTGGATAGGCCCCAA
DNA (b) 30 bp	GGACGGCCATCTATATTGGATAATCAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGCTCTAGCA CCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCAC GTGTCAGATATATACATCGATTGGATATATATCTAGGCCCCAA
DNA (c)	CGGCCTGGATAATCAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACG CACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATAT ATACATCGAT

Table 2. Cryo-EM Data Collection and Image Processing for the Tri-nucleosomes.

Sample	H3-CA-H3 with 22 bp (EMD-0768)	H3-H3-H3 with 22 bp (EMD-0770) (EMD-0771)	H3-CA-H3 with 30 bp (EMD-0769)	H3-H3-H3 with 30 bp (EMD-0772)	CA-H3-CA with 30 bp
Electron microscope	Talos Arctica	Talos Arctica	Talos Arctica	Talos Arctica	Titan Krios
Camera	Falcon2	Falcon3	Falcon3	Falcon3	Falcon3
Pixel size (A/pix)	1.40	1.40	1.40	1.40	1.39
Exposure time (second)	1	2	2	2	2
Total dose (e/A^2)	25	80	80	80	68
Movie frames (no.)	17	79	79	79	40
Micrographs (no.)	4,515	4,512	10,351	8,035	808
Particles for 2D class (no.)	92,674	118,395	63,365	109,525	39,060
Particles for 3D class (no.)	43,008	100,725	22,515	20,953	-
Particles in the final map (no.)	4,999	7,312 (class1) 3,777 (class2)	4,574	4,534	-
Symmetry	C2	C2	C1	C2	-
Final resolution (Å)	18.7 Å	Class1: 12.3Å Class2: 15.1Å	19.6 Å	15.7 Å	-

STAR METHODS

CONTACT FOR REAGENT AND RESOUCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Hitoshi Kurumizaka (kurumizaka@iam.u-tokyo.ac.jp).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human histones H2A, H2B, and H3 were expressed in the *E. coli* BL21 (DE3) strain. Human histones H4 and CENP-A were expressed in the *E. coli* JM109 (DE3), and DH5α strains.

METHOD DETAILS

Reconstitution of Tri-nucleosomes

Histones H2A, H2B, H3.1, H4, and CENP-A were prepared as described previously (Tanaka et al., 2004; Kujirai et al., 2018). Three different DNA fragments (DNA(a)-(c) in Fig. 1A) were used for the reconstitution of mono-nucleosomes. The DNA sequences for the tri-nucleosome reconstitution, which were based on the Widom601 sequence, are listed in Supplementary Table 1 (Lowary and Widom, 1998). The DNA(a)-(c) fragments were tandemly inserted into the pGEM-T Easy vector (Promega). After amplification and purification of the plasmids, each fragment was excised with *Eco*RV, and the fragment was separated from the linearized plasmid by the PEG precipitation method. The purified DNA(a) and DNA(c) fragments were dephosphorylated with calf intestine alkaline phosphatase and then digested by *Bgl*I. The purified DNA(b) fragments (for the 22 and 30 base-pair linker DNAs) were digested by *Bgl*I. After the restriction enzyme reactions, the fragments were purified by chromatography on a TSKgel DEAE-5PW column (Tosoh Bioscience).

The ligated tri-nucleosomes were reconstituted by the method described previously (Kobayashi et al., 2016). Three mono-nucleosomes with different cohesive ends were reconstituted by salt dialysis, and were purified by 6% polyacrylamide gel electrophoresis, using a Prep Cell apparatus (Bio-Rad). These mono-nucleosomes

contained DNA(a), DNA(b), and DNA(c), and are named nucleosome(a), nucleosome(b), and nucleosome(c), respectively. The tri-nucleosome was prepared by the ligation of nucleosome(a), nucleosome(b) (22 or 30 base-pair), and nucleosome(c) (0.1 mg DNA/ml) in the ratio of 1:0.9:1, with T4 DNA ligase in 0.12-0.2× ligation buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM DTT, and 1 mM ATP) for 18 hr at 16°C. The resulting tri-nucleosomes were further purified by 4% polyacrylamide gel electrophoresis, using a Prep Cell apparatus. The H3-H3-H3, H3-CA-H3, and CA-H3-CA tri-nucleosomes were stored at 4°C in 20 mM HEPES-KOH (pH 7.8) buffer. The DNA sequences are listed in Supplementary Table 1.

Sample Preparation for Cryo-electron Microscopy

For the tri-nucleosomes formed in the absence of 1 mM $MgCl_2$, the tri-nucleosomes (2.5 µL of 1 mg/mL) were applied to C-flat 2/1 400-mesh grids (Protochips, USA), which were plasma cleaned for 10 seconds at 20W in a 23% H_2, 77% O_2 gas mix, using a Solarus Plasma Cleaner (Gatan, Pleasanton, CA, USA). The grids were blotted for 3 seconds at 16°C at 100% humidity in a Vitrobot Mark IV (Thermo Fisher Scientific (TFS), USA), and immediately plunged into liquid ethane. For the tri-nucleosomes formed in the presence of 1 mM $MgCl_2$, the tri-nucleosomes (0.06 mg/mL, in 20 mM HEPES-KOH buffer (pH 7.8)) were mixed with 20 mM HEPES-KOH buffer (pH 7.8) containing 2 mM $MgCl_2$ at a 1:1 ratio, resulting in 0.03 mg/mL of the tri-nucleosome in 20 mM HEPES-KOH buffer (pH 7.8) containing 1 mM $MgCl_2$. The tri-nucleosome sample was applied to the same grids with the tri-nucleosomes in the absence of 1 mM $MgCl_2$. The sample was removed and applied to the grids again, and then the grids were blotted for 3 seconds at 16°C at 100% humidity in a Vitrobot Mark IV and immediately plunged into liquid ethane.

Cryo-Electron Microscopy

For in-focus phase plate imaging, vitrified samples of the tri-nucleosomes, H3-H3-H3 with 22 base-pair linker DNAs, H3-CA-H3 with 22 base-pair linker DNAs, and CA-H3-CA with 30 base-pair linker DNAs, in the absence or the presence of 1 mM $MgCl_2$, were observed at liquid nitrogen temperature with a Titan Krios cryo-transmission electron microscope (TFS) operated at 300 kV and equipped with a Quantum GIF

imaging filter (Gatan) in the energy-filtered transmission electron microscopy (EFTEM) mode, using a slit width of 20 eV at a calibrated magnification of 35,971x. Digital micrographs were captured with a K2 Summit direct electron detector in the counting mode, at a pixel size of 1.39 Å/pixel at the specimen level. Images were manually collected with a Volta phase plate (VPP) in-focus, as described previously (Machida et al., 2018). Images of CA-H3-CA with 30 base-pair linker DNAs were automatically collected using the EPU software (TFS). A 50 μm C2 condenser aperture was used, and the illumination was set up in the nanoprobe mode within the parallel beam range. For defocused data collection without the phase plate, the vitrified tri-nucleosome samples, the H3-H3-H3 with the 22 base-pair linker DNA, the H3-H3-H3 with the 30 base-pair linker DNA, the H3-CA-H3 with the 22 base-pair linker DNA, and the H3-CA-H3 with the 30 base-pair linker DNA, were observed in the presence of 1 mM $MgCl_2$ at liquid nitrogen temperature with a Talos Arctica cryo-transmission electron microscope (TFS), operated at 200 kV and equipped with a Falcon 2 direct electron detector for the H3-CA-H3 with the 22 base-pair linker DNA or a Falcon 3 detector in the linear mode for the others. Cryo-EM images were automatically collected using the EPU software (TFS). Details of data collection for the tri-nucleosomes are denoted in Table 2.

Image Processing

All frames of the tri-nucleosomes were aligned using MOTIONCOR2 (Zheng et al., 2016) with dose weighting. For the defocused images, the values for defocus and objective lens astigmatism were estimated by fitting the phase contrast transfer function (CTF) with CTFFIND4 (Mindel et al., 2003) from digital micrographs without dose weighting. RELION 2.1 (Scheres, 2016) was used for all subsequent image processing operations. The particles of the tri-nucleosomes were picked semi-automatically with a box-size of 280 x 280 pixels, followed by a few rounds of 2D classification, which discarded junk particles. For the H3-CA-H3 with the 22 base-pair linker DNA, the H3-H3-H3 with the 22 base-pair linker DNA, the H3-CA-H3 with the 30 base-pair linker DNA, and the H3-H3-H3 with the 30 base-pair linker DNA, the selected particles were used for 3D classification. A cylinder generated with SPIDER (Frank et al., 1996) was used as the initial alignment model. After a few rounds of 3D classification, the best

classes were selected for 3D refinement. C2 symmetry was applied to the 3D reconstructions for the H3-CA-H3 with the 22 base-pair linker DNA, the H3-H3-H3 with the 22 base-pair linker DNA, and the H3-H3-H3 with the 30 base-pair linker DNA. The 3D reconstruction of the H3-CA-H3 with the 30 base-pair linker DNA was unsuccessful when C2 symmetry was applied, probably due to the flexibility of the structure. Therefore, C2 symmetry was not applied to the 3D reconstruction for the H3-CA-H3 with the 30 base-pair linker DNA. Final 3D map resolutions were estimated from independent datasets at an FSC=0.143 (Scheres, 2016). The local resolution of the tri-nucleosomes was calculated with RELION 2.1 (Scheres, 2016). The maps of the tri-nucleosomes were normalized with MAPMAN (Kleywegt et al., 2004) and visualized with UCSF Chimera (Pettersen et al., 2004). Detailed processing statistics for the tri-nucleosomes are listed in Table 2.

Model Building

For the H3-CA-H3 with the 22 base-pair linker DNA, two copies of the nucleosome (PDB: 3LZ0) and a single copy of the CENP-A nucleosome (PDB: 3AN2) were manually fitted into the 20.8-Å-resolution cryo-EM map by UCSF Chimera (Pettersen et al., 2004), and positionally refined with rigid-body docking with colors, using the SITUS program package (Chacón and Wriggers, 2002). The linker DNA regions missing in the partial model were modeled as double-stranded DNA, and the gaps were manually modeled by COOT (Emsley and Cowtan, 2004) with real-space refinement and regularization. For the H3-H3-H3 with the 22 base-pair linker DNA, three copies of the nucleosome (PDB: 3LZ0) were manually fitted into the 12.4-Å-resolution cryoEM map by UCSF Chimera (Pettersen et al., 2004), and were positionally refined by rigid-body docking with colors, using the SITUS program package (Chacón and Wriggers, 2002). The linker DNA regions missing in the partial model were built as described above. The amino acid sequence and coordinates of the crystal structure of the *Xenopus laevis* nucleosome (PDB: 3LZ0) were replaced with those of human histones.

QUANTIFICATION AND STATISTICAL ANALYSIS

The cryo-EM single particle analysis was performed within published software as

described in Image Processing Method Details section.

DATA AND SOFTWARE AVAILABILITY

The cryo-EM reconstructions of the tri-nucleosomes have been deposited in the Electron Microscopy Data Bank, under the accession codes EMD-0768 (Figure 4B), EMD-0769 (Figure 5A), EMD-0770 (Figure 4A), EMD-0771 (Figure S6), and EMD-0772 (Figure 5B). The cryo-EM models of the tri-nucleosomes have been deposited in the Protein Data Bank, under the accession codes 6L4A (Figure 4A) and 6L49 (Figure 4B). Original gel scans of purified tri-nucleosomes have been deposited in the Mendeley Data repository (http://dx.doi.org/10.17632/72rdvsvs2r.1).

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