Cryo–electron microscopy structure of the H3-H4 octasome: A nucleosome-like particle without histones H2A and H2B

Kayo Nozawa, Yoshimasa Takizawa, Leonidas Pierrakeas, Chizuru Sogawa-Fujiwara, Kazumi Saikusa, Satoko Akashi, Ed Luk, and Hitoshi Kurumizaka

The canonical nucleosome, which represents the major packaging unit of eukaryotic chromatin, has an octameric core composed of two histone H2A-H2B and H3-H4 dimers with ~147 base pairs (bp) of DNA wrapped around it. Non-nucleosomal particles with alternative histone stoichiometries and DNA wrapping configurations have been found, and they could profoundly influence genome architecture and function. Using cryo–electron microscopy, we solved the structure of the H3-H4 octasome, a nucleosome-like particle with a di-tetrameric core consisting exclusively of the H3 and H4 histones. The core is wrapped by ~120 bp of DNA in 1.5 negative superhelical turns, forming two stacked disks that are connected by a H4-H4’ four-helix bundle. Three conformations corresponding to alternative interdisk angles were observed, indicating the flexibility of the H3-H4 octasome structure. In vivo crosslinking experiments revealed that the core of the H3-H4 octasome is composed of two (H3-H4)2 tetramers, separated by ~1.7 left-handed superhelical turns. The nucleosome limits the accessibility of the underlying DNA sequence and generally inhibits the binding of sequence-specific factors. Nucleosomes are structurally heterogeneous. The core histones can be replaced by histone variants or altered with covalent modifications, generating a repertoire of structurally distinct nucleosomes along the chromatin with diverse biophysical and biochemical properties that contribute to the regulation of chromatin-structure and nuclear activities (2–4).

While the nucleosome represents the major histone-DNA assembly in cellular chromatin, histone-DNA complexes with alternative stoichiometries, such as subnucleosomes, have also been observed (5, 6). For example, the hexasome, which has one less H2A-H2B dimer than the canonical nucleosome, can form when the transcription machinery traverses a nucleosome during elongation (7, 8). Indeed, hexasomes are detected throughout the genome (9, 10). The tetrasome, which contains an (H3-H4)2 tetramer core without H2A-H2B, is an intermediate structure involved in nucleosome formation (11). During this step, two H3-H4 dimers associate with a DNA fragment to form a tetrasome, followed by the deposition of two H2A-H2B dimers in a process mediated by histone chaperones in vivo (12, 13).

The H3-H4 tetrasome, which is associated with ~70 bp of DNA, can be reconstituted by combining (H3-H4)2 tetramers and DNA at an equimolar ratio (2, 14). At higher protein-to-DNA ratios, H3-H4 can form a nucleosome-size particle consisting of an octameric H3-H4 core wrapped by ~130 bp of DNA (15, 16). This nucleosome-like particle, called the H3-H4 octasome hereafter, exhibits a bead-like structure with a diameter comparable to that of the nucleosome, as evidenced by early electron microscopy (EM) studies and a recent atomic force microscopy analysis (2, 17, 18). The (H3-H4)2 tetramer alone is sufficient to be positioned around the center of the Lytechinus variegatus 5S rRNA gene (rDNA) sequence, a naturally occurring nucleosome positioning sequence (14). Interestingly, when reconstituted with a 2:1 tetramer-to-DNA concentration, the tetramers redistribute equally to the two halves of the 5S rDNA sequence to form a “di-tetrasome” particle, consistent with the H3-H4 octasome configuration (19).

In this study, we determined cryo-EM structures of the H3-H4 octasome. The data revealed that the core of the H3-H4 octasome is composed of two (H3-H4)2 tetramers, wrapped by ~120 bp of DNA in 1.5 left-handed superhelical turns. Along the dyad axis where the two tetramers meet, two H4 molecules form a four-helix bundle (FHB). To assess the biological relevance of the H3-H4 octasome structure, we interrogated cryo-electron microscopy | nucleosome | chromatin structure

Significance

Genetic information is stored in chromatin, with nucleosomes as the basic unit. A typical nucleosome comprises an octameric core, consisting of two copies of the histone H2A-H2B dimers and H3-H4 dimers, wrapped by one and a half turns of DNA. In the present study, we determined the structure of an unconventional nucleoprotein particle called the H3-H4 octasome, which has a core composed of four dimers of human H3-H4 without H2A-H2B, with DNA wrapped around this core in a nucleosome-like configuration. Histone–histone interactions observed in the human H3-H4 octasome structure were found in yeast. The incorporation of H3-H4 octasomes into the eukaryotic genome will likely alter chromatin structure and dynamics, representing a paradigm shift in our understanding of epigenome regulation.

Author contributions: K.N. and C.S.-F. prepared the nucleosomes and carried out biochemical analyses. K.N. and Y.T. performed cryo-EM analyses. K.S. and S.A. performed ESI-MS experiments. L.P. and E.L. prepared yeast strains and performed the in vivo crosslinking experiments. H.K. and E.L. designed and supervised the research. K.N., E.L. and H.K. interpreted the data and wrote the manuscript. All of the authors discussed the results and commented on the manuscript. The authors declare no competing interest.

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To whom correspondence may be addressed. Email: kurumizaka@ibp.u-tokyo.ac.jp or ed.luk@stonybrook.edu.

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yeast chromatin with in vivo crosslinking assays and detected histone–histone interactions observed in the human H3-H4 octasome structure. The implications of the H3-H4 octasome on chromatin architecture and genome function are discussed.

Results

Reconstitution of the H3-H4 Octasome. Nucleoprotein complexes with human histones H3 and H4 and a 145-bp DNA fragment containing the Widom 601 positioning sequence were reconstituted by the salt dialysis method (20). Two distinct protein complexes were detected by native polyacrylamide gel electrophoresis (PAGE) (Fig. 1A). Electrospray ionization mass spectrometry (ESI-MS) revealed that the molecular weights of the nucleoproteins corresponding to the upper and lower bands were 149,789 and 201,681, respectively. These values are consistent with but greater than the theoretical molecular weights of the H3-H4 tetrasome (144,305) and the H3-H4 octasome (198,973) formed on the 145-bp Widom DNA (Fig. 1B and C and SI Appendix, Fig. S1). The discrepancies are likely due to the replacement of protons in the phosphate groups of the DNA by monovalent cations, such as K⁺, Na⁺, and NH₄⁺, which were present in the sample buffer and concentrated during the ionization step (21).

To assess the extent of histone-DNA contacts within the H3-H4 octasome, nuclease sensitivity assays were performed with H3-H4 octasomes, along with canonical nucleosomes and H3-H4 tetrasomes as controls (Fig. 1D). Micrococcal nuclease (MNase) is an endo/exo nuclease that preferentially digests DNA detached from the histone surface but not DNA stably wrapped around histones, as in the nucleosome (22). Time courses of MNase digestion revealed that canonical nucleosomes protect a dominant DNA species at ~145 bp, corresponding to the stably wrapped nucleosomal DNA, and an ~120-bp species at later time points, consistent with partial unwrapping of the DNA ends (23) (Fig. 1D, lanes 2 to 6). By contrast, the H3-H4 tetrasome was highly susceptible to MNase attack, indicating substantial exposure of its DNA to the solvent (Fig. 1D, lanes 7 to 11). The DNA was more strongly protected in the H3-H4 octasome compared to the H3-H4 tetrasome, although the full-length 145-bp DNA fragment was progressively trimmed to ~130 bp and further to ~120 bp and ~70 bp at later time points (Fig. 1D, lanes 12 to 16). The DNA protection pattern of the H3-H4 octasome suggested that the octasomal histone core is stably wrapped by a DNA fragment, but in a manner that differs from that of the canonical nucleosome.

Overall Structure of the H3-H4 Octasome. The structure of the H3-H4 octasome was determined by cryo-EM (Fig. 2A). The...
purified H3-H4 octasome sample was analyzed with a 300-kV electron microscope. Approximately 1.43 million particles related to the H3-H4 octasome were identified from 5,517 electron micrographs (SI Appendix, Figs. S2 and S3). Single-particle analysis identified three well-resolved structures, indicating that the H3-H4 octasome exists in alternative conformations. The three structures, namely the open, closed, and intermediate forms, were similar but differed by the clamshell opening angle of the two stacked disks formed by the symmetrical halves of the H3-H4 octasome (Fig. 2B and SI Appendix, Figs. S2 and S3). The closed form, with a resolution of 3.6 Å, was best resolved (Fig. 2A). The structure showed that the H3-H4 octasome has a core composed of two (H3-H4)2 tetramers, forming a left-handed ramp allowing ~120 bp of DNA to wrap around the core 1.5 times (Fig. 2A). Each (H3-H4)2 tetramer engaged an ~60-bp DNA segment on either side of the dyad to form a disk. A novel H4-H4’FHB, assembled by the helix–loop–helix regions of the two inward-facing H4 histones, connected the two disks (Fig. 2A). In the nucleosome, the H3-H3’ FHB of the (H3-H4)2 tetramer coincided with the nucleosomal dyad; however, in the H3-H4 octasome, there were two H3-H3’ FHBs, and they were positioned at the superhelical locations (SHLs) +3 and −3 (Fig. 2D) on either side of the dyad (SHL 0).

The open and intermediate forms of the H3-H4 octasome structure were determined at 3.9 Å and 4.3 Å resolution, respectively (SI Appendix, Figs. S2 and S3). The histone core arrangement and DNA wrapping configuration were similar to those of the closed form, but differed in the distances of the opening measured between the two DNA gyres at the farthest points from the dyad, which were 7.7 Å and 1.7 Å wider in the open and intermediate forms, respectively (Fig. 2C). By contrast, the opening between the two disks of the nucleosome was much smaller. When the lower disk of the nucleosome was superimposed on the H3-H4 octasome, the upper disk of the H3-H4 octasome showed an ~20 Å outward displacement relative to the nucleosome (Fig. 3A, Right).

Further comparison of the H3-H4 octasome with the canonical nucleosome revealed additional H3-H4 octasome-specific features. First, the H3-H4 octasome lacked the acidic patch provided by H2A and H2B, which functions as a docking site for a variety of nucleosome binding proteins (24) (Fig. 3B). Second, the αN regions of the two inward-facing H3 histones were predicted to occupy the interdisk space of the H3-H4 octasome; however, the expected alpha helical structures were not visible, suggesting that

Fig. 2. Cryo-EM structures of the H3-H4 octasome. (A) The cryo-EM structure of the H3-H4 octasome (closed form). The electron densities of the N-terminal regions (amino acid residues 1 to 58) of H3.1 and the DNA termini were not observed (indicated by dashed lines). (B) Cryo-EM densities of the H3-H4 octasome superimposed by aligning the bottom disk. The dots indicate the positions of G39 in the DNA strand of the H3-H4 octasome (pink: closed, orange: intermediate, blue: open). The double arrow indicates the difference of the measured distances between the DNA gyres in these conformations. (C) Ribbon models of the H3-H4 octasome (Left) and the canonical nucleosome (Right). In the H3-H4 octasome, the two H3-H3' FHBs are positioned three helical turns away from the H3-H4 octasomal dyad at the SHLs +3 and −3. Orange lines in the schematic representations of the histone core assemblies indicate the H4-H4’ interface and the H4-H2B interface of the H3-H4 octasome and the nucleosome, respectively. The white line between H4 and H2A in the nucleosome schematic emphasizes that the two proteins are not connected.
the H3 αN regions in the interdisk space are dynamic (Fig. 3A). The remaining two H3 αN regions exposed on the outer surfaces of the H3-H4 octasome were also unstructured. Finally, the FHB at the octosomal H4-H4' interface resembled the one at the nucleosomal H4-H2B interface (SI Appendix, Fig. S4 A–C). For example, in the nucleosome, the sidechains of H4 Tyr72, Glu74, His75, and Arg92 interacted with those of H2B Glu76, Arg99, Glu93, and Leu100, respectively. In the H3-H4 octasome, the same H4 sidechains were oriented in a similar manner, but they interacted with the sidechains of Arg92, Tyr88, Asp85, and Asp68 on the opposite H4' instead (Fig. 3C).

**Detection of H3-H4 Octasome-Specific Interactions In Vivo.** To assess the biological relevance of the H3-H4 octasome, in vivo crosslinking experiments were performed in *Saccharomyces cerevisiae* to determine whether the histone-histone contacts predicted by the H3-H4 octasome structure occur inside cells. Arg49 of yeast H3 was substituted with cysteine so that the inward-facing H3 R49C sites would be close enough to allow disulfide crosslinking at the interdisk interface of the H3-H4 octasome (Fig. 4A, Bottom; Cβ-Cβ' of 12.8 Å). However, these same sites should be too far apart to crosslink in the nucleosome (Fig. 4B; Cβ-Cβ' of 54.3 Å) (25).

The yeast histone H3 gene *HHT2* with or without R49C was expressed as the sole source of H3, under endogenous promoter control on a low-copy plasmid (26). *hht2(R49C)* cells were viable, albeit slow growing, indicating that the *hht2(R49C)* allele is at least partially functional (SI Appendix, Fig. S5 A and B). After...
HHT2 and hht2(R49C) cells were treated with 4,4'-dipyridyl disulfide (4-DPS), a cell-permeable oxidant, total proteins were analyzed by anti-H4 immunoblotting (25) (Fig. 4 C). An ~30-kDa band was observed in the hht2(R49C) strain, but not HHT2 (Fig. 4 C, lanes 2, 4). This 30-kDa band was absent when the extracts were pretreated with beta-mercaptoethanol (βME), confirming that the H3 adduct had a cysteine linkage (Fig. 4 C, lane 6). In vivo crosslinking was also observed for hht2(V46C) and hhf2(A47C) (within the unstructured H3 αN region at the inter-disk interface) but not hhf2(S102C) (within the α2 helix of H3), consistent with the H3-H4 octasome structure (SI Appendix, Fig. S5 C–E).

To verify that the 30-kDa band was due to a crosslinked adduct between two H3 molecules (15 kDa each) but not H3 with another cysteine-containing protein, we cotransformed a V5-tagged hht1(R49C) with an untagged hht2(R49C) into yeast to generate a “hybrid” strain heterozygous for the tag. Anti-H3 and anti-V5 immunoblotting analyses collectively showed that the hybrid strain has three H3 crosslinking bands, confirming that the crosslinked species in hht2(R49C) are between two H3 molecules (SI Appendix, Fig. S5F).

As a second test for the in vivo presence of the H3-H4 octasome, the unique H4-H4' FHB interaction was targeted using a yeast strain in which the histone H4 gene HHF2 contains a cysteine substitution at Arg92 (SI Appendix, Fig. S5G). In the H3-H4 octasome, the two H4 R92C sites on the FHB were 9.4 Å apart and facing each other (Fig. 4 A). By contrast, in the nucleosome, the same sites were 16.8 Å apart (Cβ-Cβ') and facing away from each other (Fig. 4 B). The bifunctional sulphydryl crosslinker bis-maleimidoethane (BMOE), instead of 4-DPS, was used to crosslink the R92C sites because the FHB structure lacks the structural flexibility required for disulfide crosslinking (27, 28). To validate the specificity of BMOE, we first treated hht2(V46C) cells with BMOE or 4-DPS. An ~30-kDa H3-H3' adduct was observed in both cases, suggesting that BMOE successfully detected the interdisk H3-H3' interaction (SI Appendix, Fig. S5H). However, unlike the 4-DPS–induced cysteine, the linkage induced by BMOE was not cleaved by βME. Importantly, when hhf2(R92C) cells were treated with BMOE, an adduct consistent with H4-H4' crosslinking was observed (Fig. 4 D, lanes 3 and 4), but no crosslinking was detected in wild-type cells (Fig. 4 D, lanes 1 and 2). This result suggests that the H4-H4' FHB interaction occurred in vivo.

To confirm that the crosslinking adducts observed in hht2(R49C) and hhf2(R92C) cells were due to interactions specific to H3-H4 octasomes but not nucleosomes, we reconstituted nucleoprotein assemblies containing human histone H3.1 bearing R49C or H4 bearing R92C and subjected them to...
Discussion

Nucleosomes act as a physical barrier for DNA-binding proteins, which regulate genomic DNA functions such as transcription, replication, recombination, and repair (29). In these processes, nucleosomes must be disassembled and reassembled. Misregulation of these cycles is linked to various diseases, including cancer (30). Subnucleosomes and nucleosome-like particles are dynamic structures that emerge and/or function during these cycles (31, 32). In the present study, we determined the cryo-EM structures of a nucleosome-like particle, the H3-H4 octasome, which has unique features not found in the canonical nucleosome. In these structures, an ~120-bp DNA segment and four copies of H3-H4 dimers formed a stable core particle without H2A and H2B.

The H3-H4 octasome surface lacks the common footpath known as the acidic patch, which functions as an anchoring site for nucleosome-binding proteins, including histone modifiers and nucleosome remodelers (24). Thus, the presence of H3-H4 octasomes could interfere with the propagation of epigenetic marks and the spacing of nucleosomal arrays. Notably, the acidic patch serves as the docking site for the H4 N-terminal tail of a neighboring nucleosome, an interaction critical for chromatin compaction (1, 33). The absence of the acidic patch in the H3-H4 octasome could therefore interrupt chromatin fiber formation. In addition, the unstructured N termini on the two outer H3 molecules are expected to occupy the spaces above and below the H3-H4 octasome disks, interfering with stacking interactions between nucleosomes. Thus, chromatin fibers punctuated with H3-H4 octasomes could contribute to alternative higher-order conformations that may influence a wide variety of genomic functions.

The H3-H4 octasome exhibits larger interdisk spacing between DNA superhelical gyres compared to the nucleosome. This is probably due to the lack of the H2A-mediated L1-L1’ interaction and the steric repulsion of the extended unstructured N termini on the two inward-facing H3 molecules. The interdisk spacing appears to be dynamic, as evidenced by the alternative conformations of the H3-H4 octasome structure. The wider interdisk space and the dynamic nature of the clamshell structure suggest that the H3-H4 octasome could provide greater access to DNA-binding factors, such as pioneer factors, which prefer to interact with the DNA along the gyre (34).

We note that the observed in vivo crosslinking of cysteine-modified histones is not proof for the existence of discrete H3-H4 octasome particles within the cell. An alternative explanation is that the observed crosslinking may represent interactions of subnucleosomal particles, such as hexasomes, which are distributed broadly across the genome and are robust substrates for chromatin remodelers (9, 10). It is conceivable that when two hexasomes slide into each other, the interactions of the two sides with an exposed H4 could generate an interface that is structurally similar to the interdisk interface of the H3-H4 octasome. Although this configuration would have two H2A-H2B dimers on the terminal sides of the H3-H4 octasome, our data do not rule out such a possibility.

In the H3-H4 octasome, the formation of the H4-H4’ FHB involves the conserved H4 residues Asp68, Tyr72, Glu74, His75, Asp85, Tyr68, and Arg92 in the a2-L2-a3 region (SI Appendix, Fig. S6B). The fact that H4 can pair with another H4 (as opposed to H2B in the nucleosome) in vitro was previously noted by the Richmond laboratory, but not structurally resolved until now (19).

The two outward-facing H4 molecules in the H3-H4 octasome each have an unpaired α2-L2-α3, raising the possibility...
that additional (H3-H4)2 tetramers can stack onto the H3-H4 octasome and polymerize along the DNA. In fact, a previous report suggested the stacking of up to four (H3-H4)2 tetramers based on a minor but distinct peak observed in an MNase sensitivity assay of nucleohistone assemblies reconstituted with H3 and H4 (15).

The eukaryotic (H3-H4)-exclusive fiber is perhaps not unlike the archaeal chromatin fiber, in which DNA wraps around a polymer of archaeal histone homodimers to form a quasi-continuous superhelical structure (35–38). However, the eukaryotic (H3-H4)-exclusive fiber would likely exhibit a strong bend. Comparisons of the H3-H4 octasome with the archaeal nucleosome-like particle, known as the archaeasome, indicated a much wider DNA gyre separation (12.7 Å). This is because the interdisk space of the H3-H4 octasome is occupied by the two extended, unstructured H3 N termini, whereas in the archaeasome, the disks are held together by stacking interactions, contributed in part by the interdisk L1-L1’ contact between the dimers at positions N and N+3 (36). Interestingly, a recent study that combined cryo-EM and molecular simulation analyses indicated that archaeal chromatin is not a straight rod, but perhaps more slinky-like (35). In fact, the archaeasome disks can open, like a clam, with a 90° angle (36). Therefore, eukaryotic chromatin may share more similarity with archaeal chromatin than previously thought.

In summary, the structural insights of the H3-H4 octasome provided in this study underscore how eukaryotes may utilize alternative histone arrangements to modulate chromatin structure and dynamics. The next major challenge is to understand how H3-H4 octasomes interact with nuclear factors to modulate genomic functions.

**Materials and Methods**

_Purification of DNA Fragments._ The 145-bp DNA fragment derived from the Widom 601 sequence (ATCAGAATTCCGCGCAACGTGATTCGCAACGACCTGCAAAACGTACGCGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTA) was prepared as previously described (40). Tandem repeats of the DNA fragment were inserted into the Candida albicans 2μ DNA at the EcoRI site. The resulting 1.9-kb DNA construct was cleaved by digestion with EcoRI and dephosphorylated with calf intestine phosphatase. The DNA fragment was applied to a glow-discharged Quantifoil R1.2/1.3 200-mesh Cu grid and blotted for 8 s under 100% humidity at 12°C in liquid ethane using a MicroGraft TF-8 coater (Beckman Coulter). The fractions containing the H3-H4 octasome were subjected to buffer exchange chromatography using Micro Bio-Spin Columns (Bio-Rad) equilibrated with 20 mM Tris-HCl (pH 7.5) with 1 mM DTT.

_Prepurification and Extension._ The DNA fragment was further purified by gel filtration 2000 Superdex 200 20-ml column (Cytiva). H3-H4 octasomes and tetrasomes were reconstituted by mixing the archaeal chromatin and the archaeasome disks. The tips were coated using a microforge MF-900 instrument (Narishige). The tips were coated with gold at a 10% transmission electron microscope (Thermo Fisher Scientific), operated at 300 kV with a pixel size of 1.05 Å and a defocus range from −1.25 to −2.5 μm. Images of the H3-H4 octasome were recorded at 13.2 e/Å2 with a total dose of ~63 electron/Å2.

**Cryo-EM Sample Preparation and Data Collection.** To stabilize the purified H3-H4 octasome, the gradient fixation method (Graph) was performed during sucrose gradient centrifugation (46). A linear gradient was prepared using a Gravity Master instrument (SKB), with low buffer containing 20 mM N,N,N,N'-tetraethylenepiperazine-N’-ethylene glycol 6,000. The DNA fragment was mixed with 0.01 unit/μL of MNase (Takara), and the vector DNA region was removed by precipitation with polyethylene glycol 6,000. The DNA fragment was further purified by TSKgel DEAE-5 PM (TOSOH) gel chromatography. The DNA fragment for trunclesosome reconstitution contains three Widom 601 sequences separated by two 22-bp linker regions and was prepared as previously described (40).

_Prepurification of Human Histones, Histone Complexes, and Nucleosomes._ The human histones H2A, H2B, H3.1, H3.1C96S, C110A, H3.1C96S, C110A, R49C, H4, and H4(R92C) were expressed and purified as previously described (40). The histone octamer and the H3-H4 tetramer were refolded by dialysis and purified by gel filtration chromatography on a HiLoad 16/60 Superdex 200 20-ml column (Cytiva). H3-H4 octasomes and tetrasomes were reconstituted by mixing H3-H4 tetramers with the Widom 601 DNA (145 bp) (20), followed by refolding using the salt dialysis method (7). More specifically, the purified DNA was mixed with H3-H4 tetramers at a DNA-to-histone molar ratio of 1:2.2 (for H3-H4 octasomes) or 1:1 (for tetrasomes) in 2 M KCl buffer. The nucleoprotein mixtures were then dialyzed against buffer containing 10 mM Tris-Cl (pH 7.5), 2 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). The KC1 concentration was gradually reduced to 0.25 M by exchanging the buffer with a peristaltic pump. The resulting H3-H4 octasomes (but not tetrasomes) were incubated at 55°C for 2 h and then purified by 6% nondenaturing PAGE using a Prep Cell apparatus (Bio-Rad). The 485-bp trunclesosome with the 22-bp linker was prepared as previously described (40).

**Cryo-EM Sample Preparation and Data Collection.** The final conditions of the H3-H4 octasome were chosen based on the resolution. The structures were subjected to 3D reconstruction, followed by Bayesian polishing and two rounds of CTF refinement. C2 symmetry was applied to the 3D reconstruction of the H3-H4 octasome at 3.6 Å resolution. The final models of the H3-H4 octasome were visualized with Chimera software (53). The processing statistics for the H3-H4 octasome structures are presented in SI Appendix, Table S1.

**Cryo-EM Image Processing.** In total, 5,517 movies of the H3-H4 octasome were aligned by MotionCor2 (48) with dose weighting. The contrast transfer function (CTF) parameters for each micrograph were estimated by CTFFIND4 (49). RELION 3.0 was used to process the images of the H3-H4 octasome sample as follows (50). From 3,922 micrographs, 1,427,558 particles were picked automatically using the two-dimensional (2D) template-based picker function and subjected to reference-free 2D classification to remove bad particles. Subsequently, 990,623 selected particles were used for three-dimensional (3D) classification with global soft mask applied. The three best classes from the 3D classification were chosen based on the resolution. The structures were subjected to 3D refinement, followed by Bayesian polishing and two rounds of CTF refinement. C2 symmetry was applied to the 3D reconstruction of the H3-H4 octasome. The final conformations of the H3-H4 octasome were 3.6 Å, 3.9 Å, and 4.3 Å, respectively, and were determined based on the gold standard Fourier Shell Correlation with the 0.143 criterion (51). The final maps of the H3-H4 octasome were visualized with MAPMAN (52) and visualized with University of California, San Francisco (UCSF) Chimera software (53). The processing statistics for the H3-H4 octasome structures are presented in SI Appendix, Table S1.

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**Cryo-EM Image Processing.** In total, 5,517 movies of the H3-H4 octasome were aligned by MotionCor2 (48) with dose weighting. The contrast transfer function (CTF) parameters for each micrograph were estimated by CTFFIND4 (49). RELION 3.0 was used to process the images of the H3-H4 octasome sample as follows (50). From 3,922 micrographs, 1,427,558 particles were picked automatically using the two-dimensional (2D) template-based picker function and subjected to reference-free 2D classification to remove bad particles. Subsequently, 990,623 selected particles were used for three-dimensional (3D) classification with global soft mask applied. The three best classes from the 3D classification were chosen based on the resolution. The structures were subjected to 3D refinement, followed by Bayesian polishing and two rounds of CTF refinement. C2 symmetry was applied to the 3D reconstruction of the H3-H4 octasome. The final conformations of the H3-H4 octasome were 3.6 Å, 3.9 Å, and 4.3 Å, respectively, and were determined based on the gold standard Fourier Shell Correlation with the 0.143 criterion (51). The final maps of the H3-H4 octasome were visualized with MAPMAN (52) and visualized with University of California, San Francisco (UCSF) Chimera software (53). The processing statistics for the H3-H4 octasome structures are presented in SI Appendix, Table S1.
Model Building. The structural models of the closed, open, and intermediate forms of the H3-H4 octamer were built from the H3-H4 tetramer and its proximal DNA fragment in the crystal structure of the nucleosome containing Xenopus laevis histones and 145-bp Widom 601 DNA (Protein Data Bank [PDB]: 3L20), which represented roughly half of the symmetric structure of the H3-H4 octamer (54). The amino acid residues of the histones were adjusted to those of human histones. The model coordinates were refined automatically with phenix-real_space_refine and manually using Coot (55, 56). The DNA sequence of the H3-H4 octamer was estimated based on M Nassir and restriction enzyme analyses of H3-H4 octamer samples. All structure figures were prepared using UCSF Chimera and PyMOL (Schrodinger; http://www.pymol.org).

Yeast Plasmids. Yeast histone expression plasmids were derived from the HHT1-HHF1 URA3 CEN ARS plasmid pMS329 (gift from Mitchell Smith, University of Virginia) and the HHT2-HHF2 TRP1 CEN ARS plasmid pWZ414-F12 (gift from Rolf Strenglanz, Stony Brook University) (26, 57). The plasmid for hht1(R49C) (pEL649) was constructed by digesting pMS329 with Clal and AgeI to remove a segment of the H3 gene, followed by recombination with a synthetic DNA fragment (Twist Bioscience) containing the R49C mutation, using the Gibson assembly protocol (New England Biolabs). The plasmids for hht2(R49C) (pEL629), hht2(V46C) (pEL651), hht2(A47C) (pEL652), and hht2(S102C) were similarly constructed by digesting pWZ414-F12 with Bphi and BamHI to remove the H3 fragment followed by recombination with the mutant fragment. The 2xVS-HHT1 (pEL656) and 2xVS-HHT2(R49C) (pEL650) plasmids were generated by digesting pMS329 and pEL649, respectively, with AgeI and Pmel followed by recombination with a synthetic fragment containing the N-terminal 2xVS tag. The hht2(R292C) plasmid (pEL626) was generated by digesting pWZ414-F12 with BamHI and NcoI and replacing the H4 fragment with a synthetic DNA fragment containing the R92C mutation. The integrity of all plasmids was confirmed by Sanger sequencing (Genewiz).

Yeast Strains. The histone knockout strain YYY67 (gift from R. Sternglanz) was supplemented with pMS329 (26, 58). The HHT2 TRP1 CEN ARS plasmid and the mutant variants were introduced into YYY67 by standard yeast transformation. Transformants were selected on synthetic complete media lacking uracil and subjected to uridine selection to recover intact plasmids. Survivors were screened for growth on synthetic complete media lacking uracil and 5-FOA. When the hht2(A47C) strain was tested for sensitivity to 5-FOA, it was found to be more sensitive to 5-FOA than the wild-type strain. For the experiment in Fig. 4C and SI Appendix, Figs. S5 and S6, the hybrid mutant strain in S1 Appendix, Fig. S5F, hht1(R49C) and HHT1, with or without an N-terminal 2xVS tag, were introduced into YEL699 and YLT05 on uracil and 5-FOA media at 30 °C for 30 min, where indicated. For reducing SDS-PAGE, aliquots of the reactions were mixed at a 1:1 ratio with sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 200 mM βME. The samples (280 ng each) were heated at 95 °C for 5 min and then analyzed by SDS-PAGE (16% polyacrylamide) with Coomassie Brilliant Blue (CBB) staining. For nonreducing SDS-PAGE, aliquots of samples were mixed with a similar sample buffer without βME and analyzed by SDS-PAGE without prior heating. For BOE crosslinking, the nucleoproteins were incubated with 80 μM BOE for 30 min at 25 °C. Trinitrocellulose was incubated at 0.27 μM, which is equivalent to the molar concentration of the nucleosome (0.8 μM) used in the in vitro crosslinking reaction for the mononucleosome. Site-specific crosslinking with BOE was analyzed by SDS-PAGE under reducing conditions. The integrity of the nucleoproteins before and after 4-DPS and BOE treatment was verified by native PAGE analysis (with 6% polyacrylamide in 0.5x TBE).

Data, Materials, and Software Availability. The cryo-EM reconstructions and atomic models of the H3-H4 octamer have been deposited to the Electron Microscopy Data Bank and the Protein Data Bank under the following accession codes: EMDB-33010 and 7X57 for the closed form (59, 60), EMDB-33011 and 7X58 for the open form (61, 62), and EMDB-33991 and 7Y0Z for the intermediate form (63, 64). All data presented in this study are included in the main article or the SI Appendix.

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