Supporting Information

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SI Materials and Methods

Chromatin Preparation. HeLa nuclei preparation, followed by mild nuclease digestion and standard chromatin extractions were performed essentially as stated (1, 2) with the following adaptations. For nuclei derived from $5 \times 150\text{-mm}^3$ flasks of confluent cells, 0.5 units/mL [equivalent to 42.5 units of Worthington micrococcal nuclease (MN)] MN (Sigma) was added for 0.5, 1, 2, 4, 8 min at 37 °C, and 30 min at room temperature (RT). MN digests were stopped with 5 mM EGTA to preserve magnesium for chromatin fiber folding. Buffers used for extraction were low salt (LS) (0.5× PBS or 50 mM NaCl, 10 mM Tris, pH 8.0, 0.2 mM EDTA, 0.5 mM PMSF) and moderate salt (MS) (350 mM NaCl supplemented 1× PBS, 0.2 mM EDTA, 0.5 mM PMSF).

Immunopurification. Twenty-five microliters anti-CENP-A (centromere-protein-A) antibodies were attached to 50 uL magnetic beads (Invitrogen) so that chromatin complexes could be gently brought down using magnets. Native CENP-A chromatin immunopurifications (NChIP) were performed for 8–12 h as described (1, 3). Elution was performed either by using 0.1 mg/mL of the peptide to which the anti-CENP-A antibody was raised, or in 0.2 M Glycine in 1× PBS, pH 6.5, or in 2 mM biotin [for tagged-CENP-A, EM, and atomic force microscopy (AFM)]. Chromatin was dialyzed (Millipore 0.025-uM discs) for 1 h against 1× PBS prior to imaging by AFM.

Strep-Tag CENP-A Expression and Purification. Two copies of the eight amino acid biotin-mimic peptide (from the *Strep*-tag™ II system (IBA BioTAGnology) fused to the N terminus of CENP-A was expressed in human HEp-2 cells. Chromatin was extracted from lightly digested nuclei using the low-salt nuclear lysis protocol as above. The nuclear extract was added to a high affinity Strep-Tactin column (IBA BioTAGnology) and washed twice with low-salt buffer. CENP-A chromatin was eluted using 2 mM biotin in 1× PBS and used directly for AFM and EM.

Protein Gels, Western Blots, PCR, and Antibodies. Two percent input and 50% immunopurification (IP) proteins were separated using 4–12% SDS-PAGE gels in the NuPAGE system (Invitrogen), stained with Krypton-X (Invitrogen), and visualized using the Typhoon 9140. Bands were sliced out for mass spectrometry confirmation. Proteins were transferred to Nitrocellulose membranes (Optitran) and Western blots per the manufacturer's recommendation. For codetection of CENP-C, CENP-B, H3, and H2B, the blot was sliced along the marker bands at 20 and 80 kDa and used in parallel, or stripped and reused, or parallel gels were used. PCR was performed from eluted, deproteinized, and purified nucleosomal DNA from 2% input and 50% IP, using primers spanning the 171 bp D17Z1 repeat (4).

Antibodies used were CENP-A [EM, Upstate 07-240; NChIP, Santacruz sc11277 (2009), Abcam ab13939; Western blot (WB), Millipore 07-574], H2B (EM and WB, Abcam ab47190 and Santacruz sc10808), H3 (WB, Abcam ab1791 and ab8654; EM, Sigma H9289), CENP-C (Santa Cruz sc11285), and CENP-B (Abcam ab25734) at dilutions recommended by the manufacturer. Five nanometer gold beads used for immuno-EM were goat anti-rabbit IgG (Abcam ab27235) and goat anti-human IgG (British Biocell EM GAHL5).

AFM Methods. Samples were diluted 1:100 in 1× PBS and deposited onto freshly cleaved mica that had previously been treated with aminopropyl-silatrane (APS) according to the protocol described (5). Samples were incubated on the mica for 10 min, excess salts rinsed with 400 μL ultrapure, deionized water, and gently dried under an argon stream. Imaging was performed with a commercial AFM (MultiMode/Picoforce AFM with Nanoscope V controller and the 10 μm E-scanner, Veeco Instruments, Inc.) using silicon probes (TESP-SS by Veeco or OMCL by Olympus). The nominal radius of curvature at the tip of the probes was 2–5 nm for TESP-SS and 7–10 nm for OMCL, and the nominal stiffness of the cantilevers was 42 N/m (resonance frequency of 280 kHz for TESP-SS and 340 kHz for OMCL). Imaging was performed in the noncontact/tapping mode, with a small amount of desiccant placed within the AFM chamber to reduce humidity

Height and Volume Measurements. Images were preprocessed using the instrument image analysis software (Nanoscope v7.30) and grayscale images were exported to National Institutes of Health image analysis software (ImageJ, v1.38). Images of nucleosome populations were first thresholded at the substrate level. The volumes of all the particles in each image were measured automatically using the particle analysis utility function. This utility adds the grayscale pixel values for each particle. The height of each particle was also measured in the same particle analysis procedure.

Height and Volume Filters. Three particle filters were used to minimize contributions from nonnucleosomal molecules in order to carry out unbiased automated measurements. (*i*) A particle filter was set to exclude excessively elongated (i.e., denatured) particles whose circularity was less than 0.5 ($4 \cdot \pi \cdot$ base area/perimeter^2). (*ii*) A second filter excluded particles whose diameter was less than 10 nm. Nucleosomal width will be dilated by the AFM tip used (sharp tips have radius of curvature of 2 nm), resulting in diameters >10 nm for true nucleosomes. (*iii*) A third filter excluded particles whose heights were >10 nm, because the crystallographic height of single nucleosomes is 5 nm. DNA provides an internal minimal height baseline of 0.5 nm and particles below this height were excluded. Particle analysis data were processed and plotted in Origin (v.8.0 by OriginLab Corp.). Note that heights measured by AFM are typically smaller compared to crystallographic values due to sample compression and adsorbed salts.

A regular nucleosome with its octameric histone core and 147 bp of DNA has a mass of approximately 220–240 kDa (110 kDa of protein and 110 kDa DNA). Therefore, the predicted volume of an octameric nucleosome in air is 400 nm^3, based on an assumed hydration ratio (6)

$$V_n = M_h \frac{V_p + d_p V_w}{N_a} + M_{\mathrm{DNA}} \frac{V_{\mathrm{DNA}} + d_{\mathrm{DNA}} V_w}{N_a}$$

where M_h is the total protein mass (~110 kDa), M_{DNA} is the mass of the wrapped DNA (147 bp or ~91 kDa), V_p is the partial specific volume of the protein (~0.74 cm^3/g), V_{DNA} is the partial specific volume of DNA (0.55 cm^3/g), V_w is the partial specific volume of water (1 cm^3/g), d_p is the hydration ratio of the protein in grams of water per gram of anhydrous protein (~0.3–0.4), and d_{DNA} is the hydration ratio of DNA (~0.6).

Stable and Transient CENP-A/GFP Cell Lines. Stable cell lines were created by selection using neomycin for 2 w posttransfection and maintained by selection of stable clones for 8–10 w. Transient transfection was performed using Lipofectamine and cells

harvested 4 d posttransfection. In both cases, the CENP-A/GFP construct was expressed from the CMV promoter (7).

Electron Microscopy of Tagged CENP-A Chromatin. The chromatin preparations were imaged as described (2, 8, 9). Chromatin without antibody labeling displayed mainly three types of structures: We identified single nucleosomes stained with uranyl acetate having a diameter of 10 nm (Fig. 3*A*). A quantitative analysis yielded diameters of 10 ± 1 nm. Single nucleosomes represented ~85% of observed structures. In addition, we observed ~13% multinucleosomal structures having diameters of 20 ± 2 nm. Several multinucleosomes have well-defined substructures which each resemble single nucleosomes. Following this interpretation, each of these multinucleosomal structures contained 5 ± 1 nucleosomes. In 2% of cases, larger undefined structures were observed, which might represent larger chromatin arrays bound to kinetochore proteins or aggregates, and were excluded from analysis.

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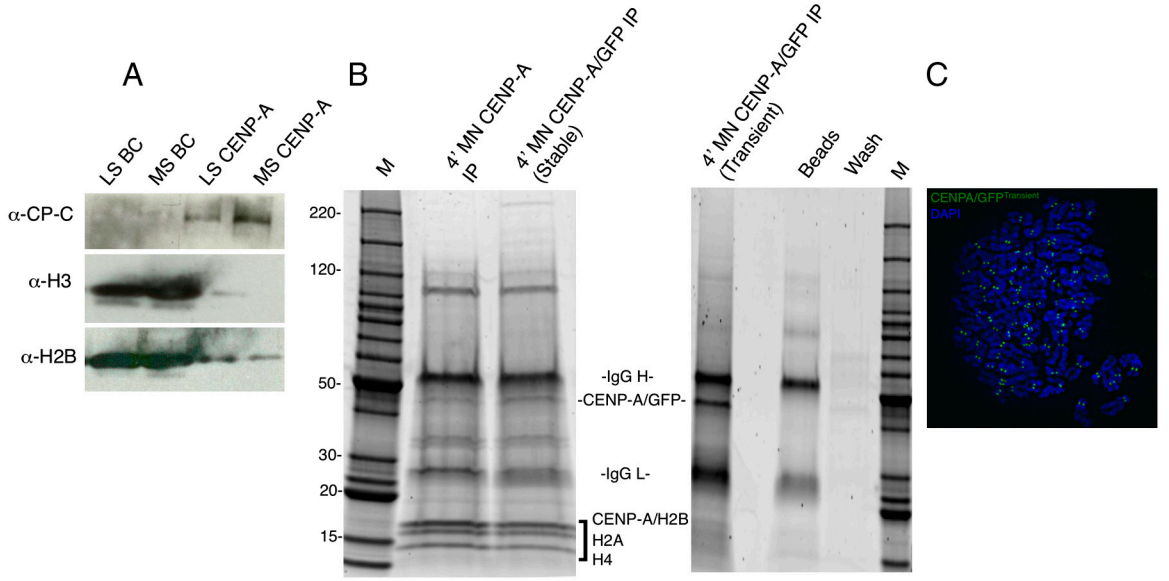

Fig. S1. Native and tagged CENP-A nucleosomes are heterotypic in stable cell lines. (*A*) Western blot analysis shows that MS and LS CENP-A IP from moderate length chromatin (2.5′ MN) is enriched in CENP-C and H2B, whereas H3 is depleted. Krypton-X stained protein gels show that (*B*) LS CENP-A IP and LS CENP-A/GFP IP obtained from short chromatin input (4′ MN) derived from a HeLa cell line stably expressing CENP-A/GFP, yields equimolar amounts of H2A, H2B, and H4, whereas kinetochore proteins are depleted. (*C*) Transiently overexpressed CENP-A/GFP is not associated with H2A, H2B, or H4 but (*D*) is still able to localize to mitotic human centromeres.

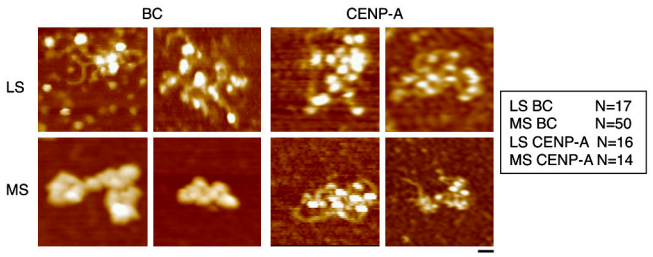

Fig. S2. CENP-A chromatin arrays have distinctive folding motifs. Representative AFM images demonstrating that LS bulk chromatin (BC) has open beads-on-a-string structure ($N = 17$), whereas LS CENP-A tends to be more compacted ($N = 16$). MS BC forms highly compacted chromatin ($N > 50$), whereas MS CENP-A resists ionic condensation ($N = 14$). BC arrays counted range from 5 to 10 nucleosomes; CENP-A arrays counted range from 5 to 15 nucleosomes. (Scale bar: 20 nm.)

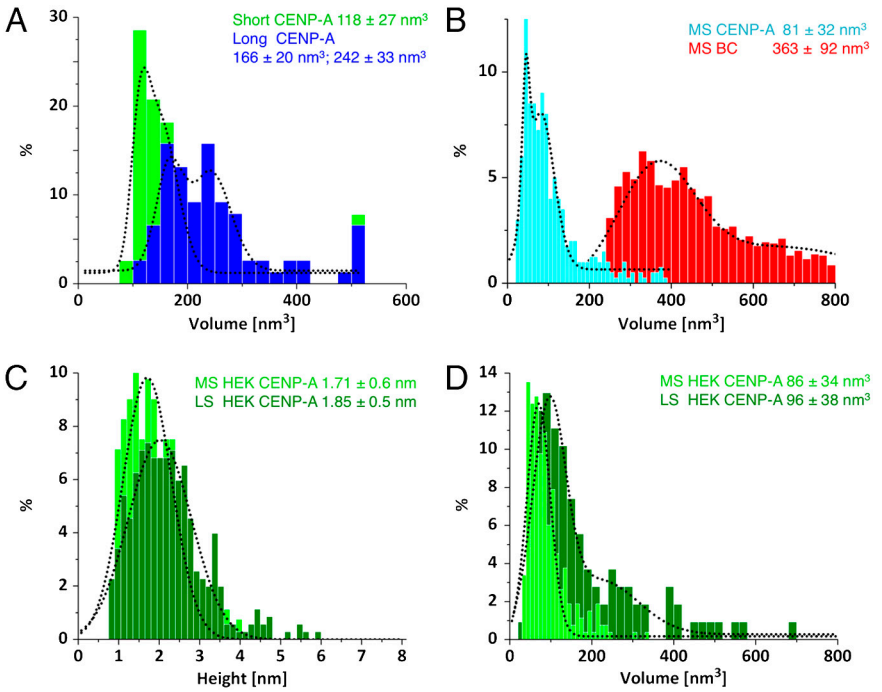

Fig. S3. CENP-A nucleosomes from HeLa and HEK cells have tetrameric dimensions. (*A*) Long chromatin LS CENP-A yields volumes consistent with mixtures of tetramers and bulk nucleosomes, whereas short LS CENP-A yields exclusively tetrameric volumes. (*B*) Volume analysis from MS conditions show that HeLa CENP-A IP has volumes smaller than those of bulk chromatin (light blue vs. red). (*C*) HEK CENP-A IP from LS (dark green) and MS (light green) has tetramer heights consistent with HeLa CENP-A IP. (*D*) HEK CENP-A IP from LS and MS has tetramer volumes consistent with HeLa CENP-A nucleosomes.

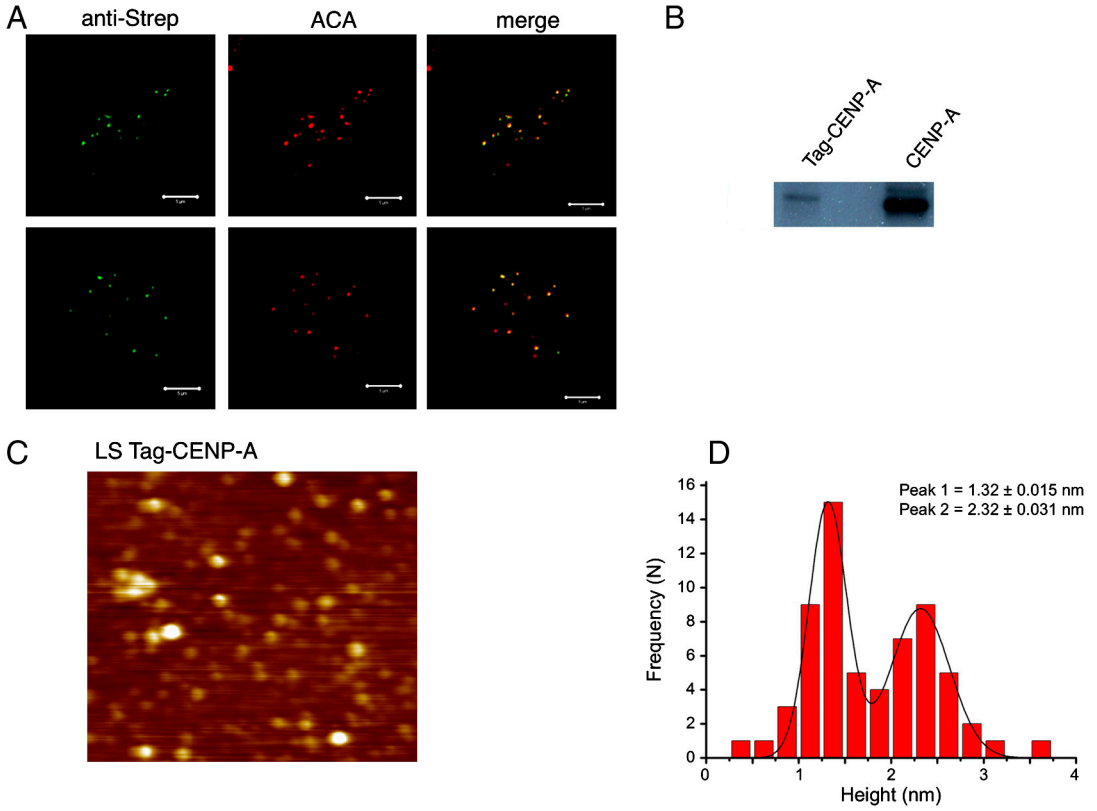

Fig. S4. Tagged CENP-A nucleosomes are enriched in tetrameric heights. (*A*) Strep^{peptide}-tagged CENP-A localizes to interphase centromere spots detected by anti-CREST antiserum (ACA) antiserum and by antistrep antibody. (Scale bar: 5 μM.) (*B*) Western blot showing levels of tagged CENP-A are lower than those of native CENP-A. (*C*) AFM image of tagged-CENP-A chromatin yields nucleosomal beads. (*D*) AFM heights of tagged CENP-A nucleosomes yields tetrameric heights.

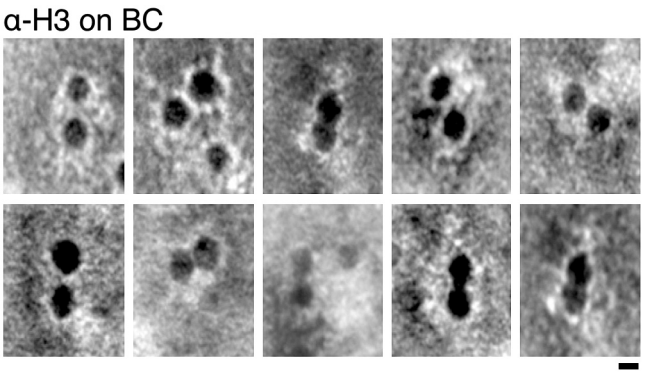

Fig. S5. Immuno-EM of bulk chromatin detects single and dual epitopes of H3. Anti-H3 staining of bulk chromatin using an N-terminal epitope yields examples of dual-labeled nucleosomes. (Scale bar: 5 nm.)

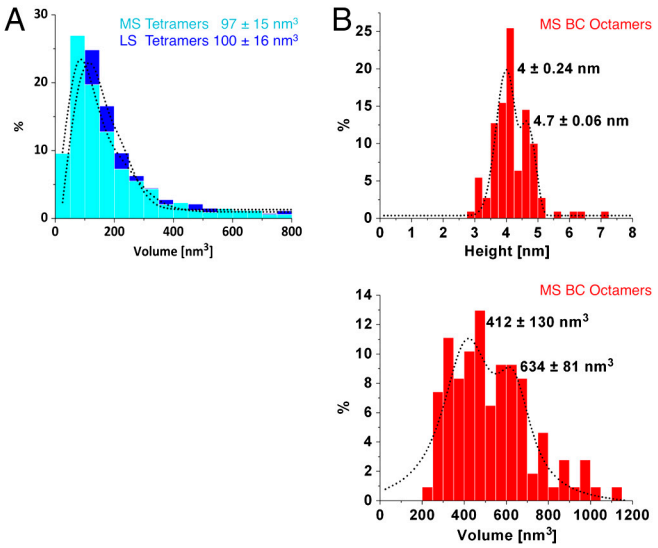

Fig. S6. Bulk nucleosomes in whole nuclear spreads have octameric dimensions. Heights and volumes of taller nucleosomes in whole nuclear extracts are consistent with octameric and dinucleosomal species.

Table S1. CENP-A nucleosomes have tetrameric dimensions under a range of conditions

Nucleosome	Chromatin length	N	Height, nm	Range, nm	Volume, nm^3	Organization
LS BC	Medium	2211	**2.81 ± 0.33**	**2.5-4.5**	**260 ± 61**	Octamer
			3.69 ± 0.77			
MS BC	Medium	2720	**3.56 ± 0.47**	**3.1-6.1**	**363 ± 92**	Octamer
			4.80 ± 1.26			
LS CENP-A	Medium	2631	1.64 ± 0.34	1.3-2.0	115 ± 65	Tetramer
			2.28 ± 0.67	1.7-**3.0**		
MS CENP-A	Medium	587	1.25 ± 0.35	0.9-1.6	81 ± 32	Tetramer
LS HEKCENP-A	Medium	266	1.85 ± 0.49	1.4-2.3	96 ± 38	Tetramer
MS HEKCENP-A	Medium	107	1.71 ± 0.60	1.2-2.3	86 ± 34	Tetramer
Tag-CENP-A	Long	63	1.32 ± 0.02	1.3-1.3	—	Tetramer
			2.32 ± 0.03	2.3-2.3		
LS CENP-A	Long	76	2.03 ± 0.25	1.8-2.3	166 ± 20	Tetramer
			2.90 ± 0.24	**2.7-3.2**	**242 ± 33**	Octamer
LS CENP-A	Short	154	1.40 ± 0.18	1.2-1.6	118 ± 10	Tetramer
LS CENP-A	Very short	None seen	<0.5	0.1-0.5	<50	—
LS Tetramers in bulk	Very long	345	1.95 ± 0.36	1.6-2.3	100 ± 16	Tetramer
MS Octamers in bulk	Very long	112	**4.0 ± 0.24**	**3.8-4.2**	**412 ± 130**	Octamer
			4.7 ± 0.06	**4.6-4.8**	**634 ± 81**	Octamerx2
MS Tetramers in bulk	Very long	6980	1.25 ± 0.31	0.9-1.6	97 ± 15	Tetramer

Bolded numbers denote values within the predicted range for canonical octameric nucleosomes. Range = mean height ± 1 SD. LS, 50mM NaCl; MS, 350mM NaCl; N, number of nucleosomes measured..