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Abstract

Nucleosomes in which histone H_3 is replaced by CENP-A direct kinetochore assembly. CENP-A nucleosomes extracted from cells have been reported to have a reduced height relative to canonical octameric H_3 nucleosomes, suggesting a unique tetrameric, hemisomal composition. We demonstrate that even octameric CENP-A nucleosomes assembled *in vitro* exhibit a reduced height, indicating that they are physically distinct from H_3 nucleosomes, and negating the need to invoke the presence of hemisomes.

Conventional nucleosomes wrap 147 base pairs (bp) of DNA 1.65 times around an octameric protein core containing two copies of the histone: H2A, H2B, H3 and H4[1]. A distinguishing feature of all centromeres is the presence of specialized nucleosomes in which the histone H3 variant, CENP-A (or cenH3), replaces canonical histone H3[2]. Atomic Force Microscopy (AFM) has shown that CENP-A nucleosomes are reduced in height relative to H3 nucleosomes in chromatin extracted from fly and human cells. This finding underpins the proposal that CENP-A nucleosomes are atypical tetrameric particles containing only a single subunit of H2A, H2B, CENP-A and H4 (hemisomes)[3,4]. Height measurement has remained the principal assay used to indicate that *ex vivo* CENP-A nucleosomes are hemisomal. However, it remains possible that CENP-A nucleosomes are actually octameric, but that fundamental physical differences between CENP-A and H3 particles makes them appear shorter in height by AFM. To test this we have examined the height of octameric CENP-A and H3 nucleosomes in arrays assembled *in vitro* from recombinant histones.

CENP-A and H3 nucleosomes were prepared using untagged recombinant histones from two evolutionarily distant organisms, human and fission yeast (*Schizosaccharomyces pombe*)[5]. Nucleosomes were assembled onto arrays of DNA containing 19 x 197 bp repeats of the well-characterized 601 sequence[6]. Particles assembled by this procedure are consistently octameric[1,7,8].

To confirm that the assembled CENP-A and H3 nucleosomes were octameric, we performed cross-linking and gel mobility assays. To approximate their molecular weight assembled nucleosome arrays were exposed to the $BS(PEG)_5$ cross-linker at nanomolar nucleosome concentrations and analyzed by SDS-PAGE (Fig. 1a, and Supplementary Fig. 1a). Both CENP-A and H3 chromatin yielded the expected molecular weights for octameric nucleosomes and intermediate complexes were observed that are consistent with the progressive fixation of individual histones from monomers up to octameric complexes. This demonstrates that both CENP-A and H3 assemble into similar octameric complexes *in vitro*. Moreover, increased concentrations of cross-linker did not lead to the formation of complexes with a higher molecular weight than octamers (Supplementary Fig. 1b). The absence of larger complexes indicates that the observed octamers do not result from the progressive cross-linking of tetramers. The relative size of recombinant CENP-A and H3 particles was also assessed by comparing their mobility. Assembled CENP-A and H3 nucleosome arrays were digested to monomers with AvaI, which cuts between each 601 repeat. Analyses by native PAGE shows that CENP-A and H3 nucleosomes

have identical mobilities (Fig. 1b). Thus, as previously observed, these *in vitro* assembled nucleosomes consist of an octamer of histone[1,7,8].

We used these same *in vitro* assembled samples to determine the height of octameric CENP-A and H3 particles using AFM. At least 180 individual particles of each type, that were clearly part of arrays, were measured. CENP-A nucleosomes assembled *in vitro* from both human and *S. pombe* recombinant histones were consistently found to have a lower height than H3 nucleosomes (Fig. 2a). For human nucleosomes, hsCENP-A nucleosomes had a median height of 1.64 nm (S.E. ±0.02 nm), compared with 2.09 nm (±0.02 nm) for H3. *S. pombe* CENP-A^{Cnp1} nucleosomes had a median height of 0.96 nm (±0.01 nm), compared with 1.43 nm (±0.02 nm) for H3 nucleosomes. These nucleosomal heights are less than those observed in crystal structures, however they are typical of AFM images collected in air due to a combination of sample compression and dehydration[3,9]. As the data is distributed non-parametrically (Shapiro-Wilk test $W = 0.969$, $p = 6.383\times10^{-8}$), two-sample Kolmogorov-Smirnov (KS) tests were used to compare CENP-A and H3 nucleosome height distributions. The recorded CENP-A particle heights were significantly lower than those of H3 particles; $D = 0.5662$, $p < 2.2\times10^{-16}$ and $D = 0.7723$, $p < 2.2\times10^{-16}$ for human and *S. pombe* CENP-A nucleosomes, respectively.

One explanation for this height difference is that CENP-A nucleosomes might deform more readily than H3 nucleosomes under the AFM tip. However, the diameter of both human CENP-A (14.4 nm S.D. ±2.5 nm, n = 98) and H3 (13.4 ±2.7 nm, n = 95) recombinant particles was found to be similar (Fig. 2b). To further investigate the observed height difference between CENP-A and H3 particles a chimeric human H3 (H3^{CATD}), containing the CENP-A targeting domain (CATD) region from CENP-A, was used to assemble nucleosome arrays *in vitro*. The CATD region consists of 22 amino acid substitutions from CENP-A that span the loop 1 and α2 helix and are sufficient to target chimeric H3^{CATD} to centromeres[10]. AFM measurement of these *in vitro* assembled human H3^{CATD} nucleosomes had a median height of 1.43 nm (S.E. ±0.01 nm), significantly less than the median height of 2.09 nm (±0.02 nm) recorded for H3 (Fig. 2c); KS test $D = 0.7676$, $p < 2.2\times10^{-16}$. Thus the CATD region is sufficient to account for the reduced height of human CENP-A nucleosomes. Interestingly, the CATD region is known to impart a rigid and compact nature to hsCENP-A:H4 tetramers in solution[10]. Whilst these features were not apparent in the CENP-A nucleosome crystal structure[8], our data support the conclusion that the CATD region also confers distinct biophysical properties to octameric CENP-A nucleosomes that results in them having a reduced height measurement by AFM.

The *in vitro* assembled CENP-A nucleosomes utilized here migrate through native PAGE gels similarly to octameric H_3 nucleosomes and cross-link as octameric complexes (Fig. 1). However, when CENP-A and H_3 nucleosome heights were compared using AFM, CENP-A particles register a significantly lower height. This difference was apparent whether using independently produced human or *S. pombe* nucleosomes. Previously, the observed difference in height between CENP-A and H_3 nucleosomes was considered to support the conclusion that CENP-A particles are hemisomal complexes. In contrast, our analyses demonstrate that AFM actually detects an intrinsic difference in the biophysical properties of octameric CENP-A nucleosomes that causes them to appear lower in height than their H_3 counterparts. Moreover, the CATD region that confers specific biological properties to CENP-A is sufficient to account for this difference.

The height measurements for *in vitro* assembled human nucleosomes obtained here are consistent with previously observed heights for CENP-A and H_3 nucleosomes extracted from human cells[3,11]. Thus, our analyses of these *in vitro* assembled octameric nucleosomes suggests that CENP-A nucleosomes extracted from cells are also likely to be octameric, and that they are unlikely to be hemisomes as has been proposed[3,4,11].

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Figure 1 *In vitro* assembled H3 and CENP-A nucleosomes behave as octamers

(a) Silver stained SDS-PAGE gel of recombinant human nucleosomes following $BS(PEG)_5$ fixation and digestion with Benzonase. (b) Sybr Green stained native PAGE of *in vitro* assembled human H3, CENP-A and chimeric $H3^{CATD}$ nucleosome arrays, digested to mono-nucleosomes with AvaI.

Figure 2 Octameric CENP-A nucleosomes are lower in height

Box plots of AFM peak heights for individual (a) human (hs) and *S. pombe* (sp) H3 and CENP-A (Cnp1) nucleosomes. (c) Chimeric $hsH3^{CATD}$ nucleosomes. Naked DNA control for each image (white box plots). Inset (a): AFM image example, bar = 50 nm.

(b) Distribution of nucleosome diameters for 95 H3 and 98 CENP-A human particles.

Box Plots: central lines with values = medians; box outer edges = first/third interquartile ranges; whiskers = range; outliers = single dots. n = particle number counted per sample.

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Methods

Nucleosome fixation with $BS(PEG)_5$

Nucleosomes were dialyzed to a fixation buffer of 20 mM Hepes pH 7, 2 mM EDTA. Then the primary amine cross-linker $BS(PEG)_5$ (Thermo Scientific) was added at the required molar excess (1000 – 5000 x for full fixation of the histone octamer). Samples were fixed for 2 h at 37 C with gentle shaking before addition of Tris pH 7 to a final concentration of 200 mM to quench the fixative. To check the extent of fixation, 15 pmol of nucleosomes were digested with 0.5 µl of Benzonase (Novagen) at RT for 10 minutes then boiled in SDS-PAGE loading buffer (Life Technologies), run on a 4-12% NuPAGE SDS-PAGE gel in MES buffer (Life Technologies) alongside an unfixed control sample and the gel stained with a silver staining kit (Life Technologies).

Ava1 digests of nucleosome arrays

Nucleosome arrays were digested with Ava1 (NEB) in buffer containing 50 mM KOAc, 20 mM Tris-OAc pH 7.9, 1 mM DTT, 0.1 mg mL^{-1} BSA, 0.5 mM $MgCl_2$. Digests were left to proceed overnight at room temperature then run on 5% non-denaturing acrylamide gels (29:1 acrylamide:bis-acrylamide) in 0.5 x TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA), with 0.5 x TBE as the running buffer and standard DNA-gel glycerol loading buffer.

Preparation of samples and surfaces for Atomic force microscopy (AFM)

Freshly cleaved V1 grade Mica (SPI supplies) was functionalized with 3-aminopropyltriethoxysilane (APTES - Sigma) as described in Lyubchenko *et al* (2009)[12]. Samples were pipetted onto the functionalized surface at a titration of nucleosome concentrations centered on 0.1 nM. Deposited samples were left for 5 minutes to adhere at RT then rinsed twice with molecular biology grade water (Sigma). A stream of argon was used to gently dry the surfaces and they were imaged immediately. At least two biological replicates were imaged for each sample.

AFM imaging

AFM imaging was performed in air at minimal force in intermittent contact mode using either a Veeco Explorer or a Veeco Nanoman VS with a Dimension 3100 controller (Bruker). In our hands both machines gave images of comparable quality and the nucleosome height data collected was

essentially identical from either machine. Images were collected over an area of between 1 – 5 µm at a typical scan rate of ~ 1.2 Hz. The DLC-10 probes used (Bruker) had a nominal resonance of 160 kHz, stiffness of 5 N m^{-1}, and a nominal tip radius of 1 nm.

AFM image processing

AFM images were first leveled using the NanoScope Analysis software (Veeco) then exported for further analysis using ImageJ (NIH). The background height was subtracted from the image and a mask layer used to remove particles above 5 nm in height. All nucleosome-like particles that could be clearly distinguished as round "bead-on-a-string" particles were selected manually. Manual selection of nucleosomes was preferred as in our hands this was found to include fewer non-nucleosomal particles in the analysis than an automated, filter-based approach. Particles were classed as non-nucleosomal or excluded from analysis if they were located within regions compacted such that individual nucleosomes could not be easily distinguished, if they were potentially deposited on top of DNA or other particles or if the particle diameter was above 25 nm. The maximum height and diameter of selected particles was recorded from the original, background-subtracted image. The height of DNA was recorded from at least 10 points within each image to be used as an internal control of DNA height. The median height of DNA across all images was 0.49 nm (S.E. ±0.01 nm, n = 323), which is typical of dehydrated DNA under pressure from the AFM probe and absorbed on a surface[4].

Figure-1 Allshire

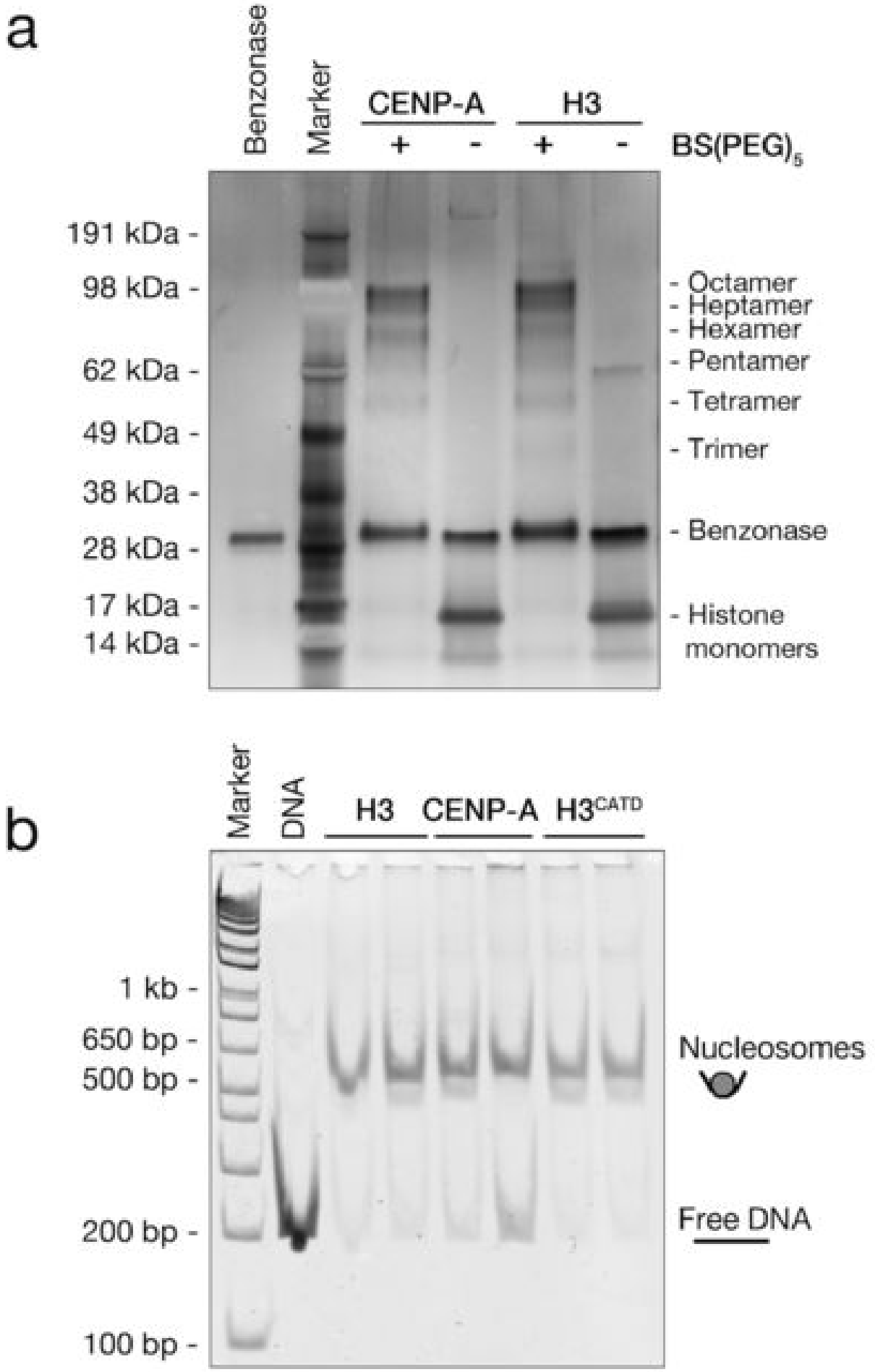

Figure-2 Allshire