BRIEF COMMUNICATIONS

CENP-A confers a reduction in height on octameric nucleosomes

Matthew D D Miell[1], Colin J Fuller[2], Annika Guse[2,5], Helena M Barysz[1], Andrew Downes[3], Tom Owen-Hughes[4], Juri Rappsilber[1], Aaron F Straight[2] & Robin C Allshire[1]

Nucleosomes with histone H3 replaced by CENP-A direct kinetochore assembly. CENP-A nucleosomes from human and *Drosophila* have been reported to have reduced heights as compared to canonical octameric H3 nucleosomes, thus suggesting a unique tetrameric hemisomal composition. We demonstrate that octameric CENP-A nucleosomes assembled *in vitro* exhibit reduced heights, indicating that they are physically distinct from H3 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 histones H2A, H2B, H3 and H4 (ref. 1). A distinguishing feature of all centromeres is the presence of specialized nucleosomes in which the histone H3 variant CENP-A replaces canonical histone H3 (ref. 2). Atomic force microscopy (AFM) has shown that *ex vivo* CENP-A nucleosomal arrays, in chromatin extracted from fly and human cells, are reduced in height relative to histone H3 nucleosomal arrays. 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 remains the principal assay supporting a hemisomal organization of CENP-A nucleosomes in chromatin. However, it remains possible that these 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 histone H3 nucleosomes in arrays assembled *in vitro* from recombinant histones. *In vitro*–assembled CENP-A and histone H3 nucleosomes were measured in arrays to closely emulate *ex vivo* measurement conditions[3–5].

We prepared CENP-A and histone H3 nucleosomes by using untagged recombinant histones from two evolutionarily distant organisms, humans and fission yeast (*Schizosaccharomyces pombe*)[6]. Nucleosomes were assembled onto arrays of DNA containing 19×197 bp repeats of the well-characterized 601 sequence[7]. Particles assembled by this procedure are consistently octameric[1,8,9].

To confirm that the assembled CENP-A and histone H3 nucleosomes were octameric, we performed cross-linking and gel mobility assays. To estimate the molecular weights, we exposed assembled nucleosome arrays to the BS(PEG)$_5$ cross-linker at nanomolar nucleosome concentrations and analyzed them by SDS-PAGE (**Fig. 1a** and **Supplementary Fig. 1a**). Both CENP-A and histone 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 demonstrated that both CENP-A and histone 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 did not result from the progressive cross-linking of tetramers. We also assessed the relative size of recombinant CENP-A and histone H3 particles by comparing their mobility. Assembled CENP-A and histone H3 nucleosome arrays were digested to monomers with AvaI, which cuts between each 601 repeat. Analyses by native PAGE showed that CENP-A and histone H3 nucleosomes have identical motilities (**Fig. 1b** and **Supplementary Fig. 2**). Thus, as previously observed, these *in vitro*–assembled nucleosomes consisted of octamers of histones[1,8,9]. Moreover, MS analyses of these same nucleosome bands, extracted from the native PAGE gel, revealed that each nucleosome type contained the full complement of expected histones (**Supplementary Fig. 3**). To assay the length of DNA wrapped around these histone H3 and CENP-A nucleosomes, we digested arrays with micrococcal nuclease (MNase) and determined
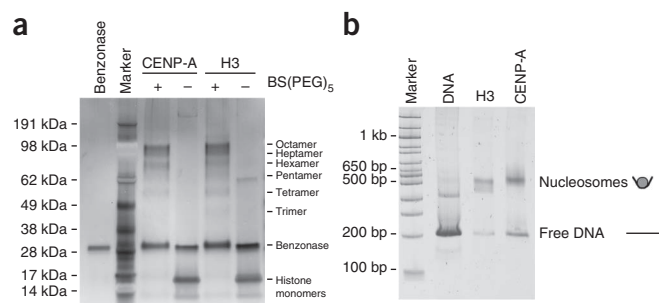

Figure 1 *In vitro*–assembled histone H3 and CENP-A nucleosomes behave as octamers. (**a**) Silver stained SDS-PAGE gel of recombinant human nucleosomes following BS(PEG)$_5$ fixation and digestion with Benzonase. (**b**) SYBR Green–stained native PAGE of *in vitro*–assembled human histone H3 and CENP-A nucleosome arrays digested to mononucleosomes with AvaI. H3, histone H3; kb, kilobases.

Figure 2 Octameric CENP-A nucleosomes are lower in height. (**a**) Box plots of AFM peak heights for individual human and *S. pombe* histone H3 and CENP-A^{Cnp1} nucleosomes, fixed nucleosomes and mixed nucleosomes. Naked DNA controls for each image (white box plots) are shown below. Inset, AFM image example; scale bars, 50 nm. (**b**) Distribution of nucleosome diameters for 95 histone H3 and 98 CENP-A human particles. In box plots: central lines with values, medians; box outer edges, first and third interquartile ranges; whiskers, range; outliers, single dots; *n*, particle number counted per sample.
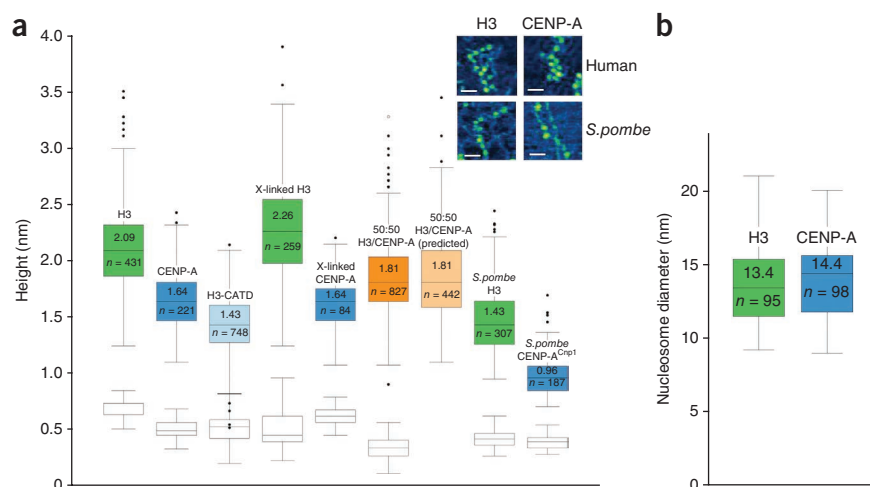
the length of protected DNA by gel electrophoresis or microfluidic measurements. Although both particle types protected discrete lengths of DNA with some variability, CENP-A nucleosomes protected approximately 20 bp less than histone H3 nucleosomes (**Supplementary Fig. 4**), which is consistent with other *in vitro* and *in vivo* analyses of CENP-A nucleosomes[10,11].

We used these same *in vitro*–assembled nucleosome arrays to determine the heights of octameric CENP-A and histone H3 particles by using AFM. At least 180 individual particles of each type that were clearly part of arrays were measured. We found that CENP-A nucleosomes assembled *in vitro* from both human and *S. pombe* recombinant histones were consistently lower in height than histone H3 nucleosomes (**Fig. 2a**). For human nucleosomes, CENP-A nucleosomes had a median height of 1.64 nm (s.e.m. ± 0.02 nm), compared with 2.09 nm (± 0.02 nm) for histone 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 histone H3 nucleosomes. These nucleosomal heights are less than those observed in crystal structures; however, they are typical of AFM images collected in air, owing to a combination of sample compression and dehydration[3,12]. As the data were distributed nonparametrically (Shapiro-Wilk test $W = 0.969$, $P = 6.383 \times 10^{-8}$), we used two-sample Kolmogorov-Smirnov (KS) tests to compare CENP-A and histone H3 nucleosome height distributions. The recorded CENP-A particle heights were significantly lower than those of histone H3 particles; $D = 0.5662$, $P < 2.2 \times 10^{-16}$ and $D = 0.7723$, $P < 2.2 \times 10^{-16}$ for human and *S. pombe* CENP-A nucleosomes, respectively.

One explanation for this height difference is that CENP-A nucleosomes might disassemble during preparation for AFM imaging, whereas histone H3 nucleosomes remain intact. However, we also measured histone H3 and CENP-A nucleosomes that were cross-linked with BS(PEG)$_5$ before preparation for AFM (**Fig. 2a**). The level of cross-linking was equivalent to that for which mainly octameric complexes for histone H3 and CENP-A nucleosomes were detected on denaturing gels (**Fig. 1a**). We found that height measurements of nucleosomes in these samples were very similar to those of un–cross-linked material (**Fig. 2a**). Thus, sample preparation does not account for the height difference observed between histone H3 and CENP-A nucleosomes. We also discounted the possibility that height differences between histone H3 and CENP-A nucleosomes are due to variable AFM imaging conditions. When we mixed histone H3 and CENP-A at an equal ratio before imaging, the resulting distribution of observed nucleosome heights lay exactly as predicted for an equal mix of data randomly collected from the individual histone H3 and CENP-A nucleosome samples (**Fig. 2a**). An additional explanation for the height difference is that CENP-A nucleosomes

might deform more readily than histone H3 nucleosomes under the AFM tip. However, we found the diameters of both human CENP-A (14.4, s.d. ± 2.5 nm, $n = 98$) and histone H3 (13.4 ± 2.7 nm, $n = 95$) recombinant particles to be similar (**Fig. 2b**). To further investigate the observed height difference between CENP-A and histone H3 particles, we assembled nucleosome arrays *in vitro* that contained a chimeric human histone H3 (H3^{CATD}), including the CENP-A–targeting domain (CATD) region from CENP-A. A functional CATD region in H3 consists of 22 amino acid substitutions from CENP-A that span the loop 1 and α2 helix and are sufficient to target H3CATD to centromeres[13]. AFM measurements of these *in vitro*–assembled human H3^{CATD} nucleosomes had a median height of 1.43 nm (s.e.m. ± 0.01 nm), significantly less than the median height of 2.09 nm (± 0.02 nm) recorded for histone H3 (**Fig. 2a**); KS test $D = 0.7676$, $P < 2.2 \times 10^{-16}$. Thus the CATD region is sufficient to account for the reduced height of human CENP-A nucleosomes. Notably, the CATD region is known to impart a rigid and compact nature to CENP-A–histone H4 tetramers in solution[13]. Although these features were not apparent in the CENP-A–nucleosome crystal structure[9], our data support the conclusion that the CATD region also confers distinct biophysical properties to octameric CENP-A nucleosomes that result in their having a reduced height measurement by AFM.

The *in vitro*–assembled CENP-A nucleosomes used here migrated through native PAGE gels similarly to octameric histone H3 nucleosomes and cross-linked as octameric complexes (**Fig. 1** and **Supplementary Figs. 1** and **2**). However, when CENP-A and histone H3 nucleosome heights were compared by using AFM, CENP-A particles registered a markedly lower height. This difference was apparent whether using independently produced human or *S. pombe* nucleosomal arrays. Previously, the observed difference in height between CENP-A and histone H3 nucleosomes in *ex vivo* arrays 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 histone H3 counterparts when assembled as nucleosomal arrays *in vitro*. Moreover, the CATD region that confers specific biological properties to CENP-A is sufficient to account for this difference.

The shorter length of DNA protected by human CENP-A nucleosomes *in vivo* is similar to that of *in vitro*–assembled CENP-A nucleosomes (**Supplementary Fig. 4**), which indicates that both sources of CENP-A nucleosomes have similar properties[14]. This reduced

protection most probably results from the slacker association of DNA at the entry and exit points of CENP-A nucleosomes, owing to the less extensive αN helix[9]. Both the length of wrapped DNA and the integrity of the αN helix of histone H3 have previously been observed to alter nucleosome height[12,15]. Our analyses show that the CATD domain also influences nucleosome height (**Fig. 2a**). It remains to be determined whether reduced DNA wrapping, or some other structural property conferred by the CATD region (for example, increased rigidity[16]), results in decreased particle height.

The heights reported here for octameric *in vitro*–assembled human nucleosome arrays are consistent with previously observed heights for CENP-A and histone H3 nucleosomes on arrays extracted from human cells[3,5]. Furthermore, most CENP-A residing in mononucleosomes extracted from *Drosophila* cells can be cross-linked as dimers, which is consistent with it forming mainly octameric particles[17]. Thus our analyses suggest that CENP-A nucleosomes extracted from human and *S. pombe* cells are also likely to be octameric and that they are unlikely to be hemisomes as has been proposed[3–5].

METHODS

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

Note: Supplementary information is available in the online version of the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Nucleosome fixation with BS(PEG)$_5$. Nucleosomes were dialyzed into a fixation buffer of 20 mM HEPES, pH 7, and 2 mM EDTA. The primary amine cross-linker BS(PEG)$_5$ (Thermo Scientific) was added at the required molar excess (1,000–5,000× 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 min then boiled in SDS-PAGE loading buffer (Life Technologies) and run on a 4–12% NuPAGE SDS-PAGE gel in MES buffer (Life Technologies) alongside an unfixed control sample, and the gel was 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 and 0.5 mM MgCl$_2$. Digests were left to proceed overnight at room temperature, then run on 5% nondenaturing acrylamide gels (29:1 acrylamide/bisacrylamide) in 0.5× TBE (45 mM Tris base, 45 mM boric acid and 1 mM EDTA), with 0.5× TBE as the running buffer and standard DNA-gel glycerol loading buffer.

Micrococcal nuclease digests of nucleosome arrays. Nucleosome arrays were digested on ice for 1 min with micrococcal nuclease (Worthington Biochemical) in a buffer containing 10 mM Tris, pH 8, 50 mM NaCl and 1 mM CaCl$_2$. Reactions were quenched with the addition of 0.5 volumes of 600 mM NaCl, 0.3% SDS, 30 mM EGTA and 10 mM Tris, pH 8. Proteins were digested by incubation with proteinase K (Fermentas) at 37 °C for 15 min, and the DNA was extracted with phenol-chloroform. DNA extracted from digested and undigested control samples of both histone H3 and CENP-A nucleosome arrays was separated on 5% nondenaturing acrylamide gels (29:1 acrylamide/bisacrylamide) in 0.5× TBE (45 mM Tris base, 45 mM boric acid and 1 mM EDTA), with 0.5× TBE as the running buffer and standard DNA-gel glycerol loading buffer. DNA was visualized by staining with SYBR Gold and scanned on a versadoc gel imager (Bio-Rad).

Analysis of nucleosome components by mass spectrometry. Gel bands were excised, cut into small pieces and digested as described previously[18]. The resulting peptides were desalted by using StageTips and analyzed by using a nanoLC (Dionex UltiMate 3000 system) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific)[19]. Full MS scans were acquired in the Orbitrap mass analyzer over the range m/z 300–1,750, and the ten most intense peaks were fragmented in the HCD collision cell. The MS data were analyzed by using Mascot version 2.2.0 (Matrix Science). MS/MS data were searched against the UniProtKB human database, containing 807,454 protein sequences (released February 2013).

Preparation of samples and surfaces for atomic force microscopy. Freshly cleaved V1 grade mica (SPI supplies) was functionalized with 3-aminopropyltriethoxysilane (APTES, Sigma) as described in a previous study[20]. Samples were pipetted onto the functionalized surface at a titration of nucleosome concentrations centered on 0.1 nM. Deposited samples were left for 5 min 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.

Atomic force microscopy imaging. AFM imaging was performed in air at minimal force in intermittent contact mode by 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 were essentially identical from either machine. Images were collected over an area of between 1 and 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.

Atomic force microscopy image processing. AFM images were first leveled by using the NanoScope Analysis software (Veeco) then exported for further analysis with ImageJ (http://imagej.nih.gov/ij/). 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 for 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 were recorded from the original background-subtracted image. The height of DNA was recorded from at least ten 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.m. ± 0.01 nm, n = 323), which is typical of dehydrated DNA under pressure from the AFM probe and absorbed on a surface[4].

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