CENP-A octamers do not confer a reduction in nucleosome height by AFM

To the Editor:

Centromeric chromatin comprises nucleosomes with the universal cenH3 (CENP-A) histone variant in place of histone H3. Although there is general agreement as to the function of cenH3 nucleosomes in organizing the kinetochore, the structure of the particle itself has been controversial. One of the methods applied to addressing this issue has been atomic force microscopy (AFM), which measures heights of single nucleosomes on a cleaved mica surface and so can distinguish whether a particle consists of four or eight histones wrapped by DNA. AFM of various reconstituted tetrameric nucleosomes, including cenH3 'hemisomes' (cenH3–H4–H2A–H2B), showed them to be ~40% shorter than are octameric nucleosomes ('octasomes')[1,2], and similar tetrameric dimensions were reported for cenH3 nucleosomes purified from native chromatin[3–5] (**Supplementary Note** and **Supplementary Fig. 1a**).

In a recent report, Miell *et al.*[6] measured cenH3 octasomes to be 22–32% shorter than were H3 octasomes. They surmised that previous comparisons of cenH3 particle heights to those of bulk nucleosomes would have been unable to distinguish hemisomes from octasomes. To resolve this issue, we have reconstituted both octasomes and hemisomes, using *Saccharomyces cerevisiae* histones. Budding-yeast centromeres are genetically defined by an ~120-bp sequence with a tripartite structure comprising centromere DNA element (CDE) I, II and III[7]. CDEI is bound by the Cbf1 transcription factor and CDEIII is bound by the centromere-specific CBF3 complex. A Cse4 (budding-yeast cenH3) nucleosome that contains H2A precisely localizes to the AT-rich ~80-bp CDEII, which contains only enough DNA for a single wrap around the histone core[8]. Whereas octasomes are left handed, Cse4 nucleosomes at functional centromeres are right handed, results consistent with a hemisome structure[9].

To obtain homogeneous particles for accurate AFM analysis, we reconstituted nucleosomes, using the same conventional salt dialysis procedure for both hemisomes and octasomes, with DNAs of 62–78 bp producing hemisomes and DNAs of 145–147 bp producing octasomes. Both short and long DNAs were derivatives of human centromeric α-satellite (α62 and Widom 601) DNA or of budding-yeast centromeres (Cen4 CDEII and Cen3). The resultant nucleosomes were dialyzed into 4 M urea to minimize aggregation prior to fixation and gel purification. Gel-based fluorescence resonance energy transfer (gelFRET) between Alexa488 and Cy3 fluorophores at opposite ends of the short duplexes confirmed hemisome wrapping[2] (**Fig. 1a**). We further confirmed the intactness of gel-purified hemisomes and octasomes by PAGE (**Fig. 1b**). AFM imaging of Cse4 and H3 particles reconstituted and gel-purified in parallel (**Fig. 1c**) showed that the hemisomes were 35–44% shorter than were their corresponding octasomes, but Cse4 octasomes (median = 2.16, $n = 72$) and H3 octasomes (median = 1.95, $n = 116$) displayed similar size distributions (**Fig. 1d**). We also detected no difference between the heights of canonical H3 nucleosomes and nucleosomes with histone H2A.Z in place of H2A (median = 2.17, $n = 103$) (**Fig. 1d**), and this further confirms that our AFM measurement protocol is insensitive to histone-variant composition.

We also tested the possibility that histone tail length is a factor in AFM height measurements. When the Cse4 tails (129 amino acids) were deleted, octasomes showed at most a slight reduction in particle height (**Fig. 1e**). To partially

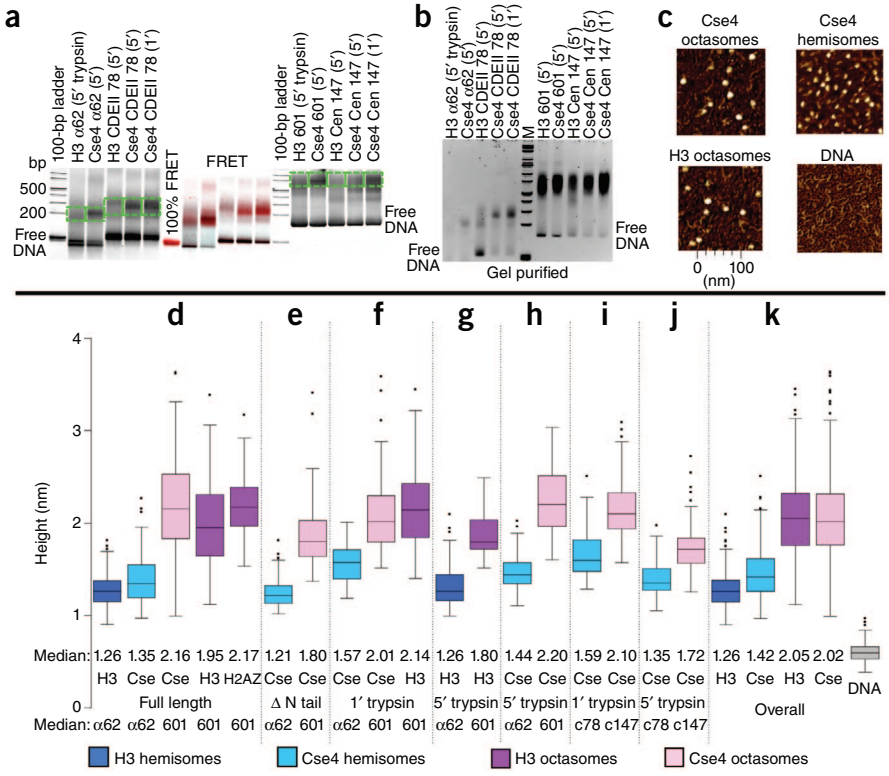

Figure 1 Octameric and tetrameric nucleosomes differ in height, but variants do not. (**a**) Gel purification of reconstituted hemisomes and octasomes with partially trypsinized cores and centromeric and noncentromeric DNAs. (**b**) Gel-purified samples re-electrophoresed on a 7% native gel. M, marker. (**c**) Images of trypsinized reconstituted yeast hemisomes and octasomes and control 145-bp DNA (contour length 44.7 ± 3.9 nm). Bottom left, trypsinized H3 hemisomes reconstituted with 78-bp Cen4 CDEII DNA showing only the DNA, indicative of inherent instability of these particles. (**d**–**k**) Box-and-whisker plots of AFM heights for reconstituted particles. Groupings represent particles prepared in parallel in single representative experiments. Central lines, medians; box outer edges, first and third interquartile ranges; whiskers, range; outliers, single dots. (**d**) Heights of full-length hemisomes and octasomes with indicated histone variants and DNA (left to right: H3 + α62, Cse4 + α62, Cse4 + 601, H3 + 601 and H2A.Z + 601) in parallel assembly reactions. (**e**) Same as **d** except with Cse4Δ129 in place of Cse4 (left to right: Cse4 + α62 and Cse4 + 601). (**f**) Same as **d** except that octamers were trypsinized for 1 min before assembly (left to right: Cse4 + α62, Cse4 + 601 and H3 + 601). (**g**) Same as **d** except that octamers were trypsinized for 5 min (left to right: H3 + α62 and H3 + 601). (**h**) Same as **g** except with Cse4 instead of H3 (left to right: Cse4 + α62 and Cse4 + 601). (**i**) Same as **h** except with CDEII DNAs for 1-min trypsin digestion (left to right: Cse4 + Cen78 and Cse4 + Cen147). (**j**) Same as **i** except for 5-min trypsin digestion (left to right: Cse4 + Cen78 and Cse4 + Cen147). (**k**) Overall height distributions for H3 and Cse4 hemisomes and octasomes. Raw data are presented in **Supplementary Data 1**.

truncate all histone tails, we trypsinized H3 and Cse4 cores for 1 or 5 min in 2 M NaCl before reconstitution with various DNAs[2], dialysis against 4 M urea, fixation and gel purification. We again observed that hemisomes were shorter than octasomes were but detected no difference between Cse4 and H3 octasomes (**Fig. 1f–h**). We obtained similar results for Cse4 hemisomes and octasomes prepared on CDEII DNAs (**Fig. 1i,j**). Overall, H3 hemisomes (median = 1.26, n = 125) were 39% shorter than were H3 octasomes (median = 2.05, n = 319), and Cse4 hemisomes (median = 1.42, n = 441) were 30% shorter than were Cse4 octasomes (median = 2.02, n = 362) (**Fig. 1k** and **Supplementary Fig. 1b**). However, there was no significant overall height difference between Cse4 and H3 octasomes ($P > 0.9$; Kolmogorov–Smirnov test).

Our inability to detect any difference in height between Cse4 octasomes and H3 octasomes contrasts with measurements suggesting that octasomes reconstituted with human CENP-A and fission-yeast CENP-A^{Cnp1} are shorter than are their H3 counterparts[6]. Miell *et al.*[6] reported a median height of 0.96 nm for *Schizosaccharomyces pombe* octasomes, 41% less than that for those of humans (1.64 nm), and for H3 octasomes the difference was 32% (1.43 versus 2.09 nm). These large differences between particles that should have identical properties and dimensions are equal to or greater than the 22–32% differences between H3 and CENP-A octasomes that led these authors to conclude that they differ in height. What is the basis for these height differences? Possible sources of technical variation include differential compression, differential loss of H2A–H2B dimers, differential adhesion between the AFM tip and the surface and variation in surface properties[10,11]. It is also possible that CENP-A octasomes are more susceptible to loss of H2A–H2B dimers during dilution for AFM, because the crystal structure of the CENP-A octasome shows that it is partially unwrapped relative to H3 octasomes[12]. In agreement with this interpretation, we found that even a 30,000-fold to 60,000-fold molar excess of glutaraldehyde for cross-linking was insufficient to prevent some DNA release from both octasomes and hemisomes (**Fig. 1b**), a result that we attribute to sporadic unraveling of nucleosomes. It is also possible that preparation for AFM could cause particles to unravel, even after strong cross-linking. For example, we were able to recover H3 hemisomes reconstituted with Cen4 CDEII DNA from gels, albeit at low concentrations, but we observed only the DNA by AFM, a result suggesting that attachment to a solid surface puts stresses on particles that are not encountered when the particles are in solution. Taken together, these observations indicate that loss of dimers could plausibly account for the height differences that Miell *et al.*[6] attributed to differential compressibility of particles with virtually identical dimensions.

In summary, we find that hemisomes are consistently much shorter than octasomes are, regardless of differences in histone-variant composition, tail length and DNA sequence. This confirms for reconstituted yeast Cse4 particles what has been previously shown for reconstituted $(H3–H4)_2$ tetrasomes, archaeal tetrameric nucleosomes and *Drosophila* and human cenH3 particles isolated by ChIP[1,3–5]. Given that the overall dimensions of H3 and CENP-A octasomes are virtually identical on the basis of X-ray crystallography[12], our evidence that they are also indistinguishable on the basis of AFM confirms the appropriateness of this single-molecule method for comparing nucleosome heights.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nsmb.2743).

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Miell *et al.* reply:

Previous studies from the Dalal and Henikoff groups compared atomic force microscopy (AFM) measurements of heights of nucleosomes containing histone H3 and CENP-A from chromatin arrays that had been extracted from human or *Drosophila* cells and enriched by immunoprecipitation[1–4]. In each study, CENP-A nucleosomes were observed to be lower in height than were H3 nucleosomes, and the authors concluded that CENP-A nucleosomes are tetrameric hemisomes with one copy of each histone—half the components of regular octameric H3 nucleosomes. To date, the suggestion that CENP-A nucleosomes are hemisomal *in vivo* remains heavily reliant on AFM data and has proven controversial because it conflicts with several *in vitro* and *in vivo* studies demonstrating that CENP-A nucleosomes contain CENP-A dimers and are octameric, as are all other known histone-variant nucleosomes[5].

We recently presented control analyses that we felt had been omitted from previous studies[6]. Specifically, we hypothesized that the apparently conflicting data for CENP-A–nucleosome height and stoichiometry could be reconciled if octameric CENP-A nucleosomes have a more compacted structure, in keeping with previous deuterium exchange analyses[7], and thus present a lower height than do octameric H3 nucleosomes in AFM measurements. We therefore prepared CENP-A and H3 nucleosomal arrays *in vitro* and validated that they were octameric, containing two copies of each histone. Our AFM analysis showed that octameric CENP-A nucleosomes were 21–33% lower in height than H3 nucleosomes. This difference was similar to that observed between CENP-A and H3 nucleosomes *in vivo*, a result previously interpreted to indicate a hemisomal composition for CENP-A nucleosomes[1–3]. Thus, our data suggested that it may not be appropriate to use the relative AFM height measurements of H3 and CENP-A nucleosomes as the sole assay by which to infer the stoichiometry of CENP-A nucleosomes. Crucially, our data were consistent with numerous persuasive *in vitro* and *in vivo* biochemical analyses demonstrating that CENP-A nucleosomes are actually octameric (described below) and provided an alternative explanation for previous AFM analyses that did not require CENP-A nucleosomes to be hemisomes.

Walkiewicz *et al.*[8] and Codomo *et al.*[9] have now also completed these same controls. In contrast to our analyses, they conclude that *in vitro*–assembled CENP-A and H3 nucleosomes do not differ in height. Codomo *et al.*[9] were further able to assemble hemisome-like CENP-A^{Cse4} and H3 particles *in vitro*, which they found to be substantially smaller than were canonical nucleosomes in both cases (also described in ref. 4).

We suspect that the discrepancy between the relative heights of CENP-A and H3 recombinant