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Reply to: “CENP-A octamers do not confer a reduction in nucleosome height by AFM”

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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 nucleosomes⁵.

We recently presented control analyses that we felt had been omitted from previous studies⁶. 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 analyses⁷, 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.

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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 nucleosomes in our data and those now provided by Walkiewicz et al.⁸ and Codomo et al.⁹ are symptomatic of the variability inherent to AFM nucleosome height measurement and thus reinforce our previous conclusion that care must be taken when making inferences about nucleosome stoichiometry from AFM measurements. To illustrate this point, we have collated data from Walkiewicz et al.⁸ and Codomo et al.⁹ and from several other publications for the median and estimated interquartile range (IQR) of nucleosome heights measured by AFM (Figure 1)^{1-4,6,8-12}. When viewed together, the large variations in height measurements for both CENP-A and H3 nucleosomes become strikingly apparent.

In the two H3 recombinant nucleosome samples used by Walkiewicz et al.⁸, most of the heights of human H3 nucleosomes are between 2.1 nm and 2.5 nm, a range of approximately 17% of the average H3-nucleosome height. Similarly, the majority of their CENP-A-nucleosome heights are between 2.15 nm and 2.75 nm, equivalent to about 25% of the average CENP-A-nucleosome height. We note a similar level of variability in our data, with most of the recombinant nucleosome heights recorded within a range that is 21-26% of the median height for H3 and CENP-A nucleosomes, respectively. Codomo et al.⁹ observed even greater variation for recombinant *Saccharomyces cerevisiae* nucleosome heights, with most of the data spread over 33% and 32% of the median H3 and CENP-A^{Cse4} nucleosome heights, respectively. Thus, even with these highly purified in vitro-assembled samples, there is a broad distribution of heights for both CENP-A and H3 nucleosomes, with estimated IQRs that are equivalent to approximately 20-30% of the median nucleosome height.

For chromatin extracted from cells, this variability in nucleosome height measurements obtained with AFM is noticeably larger. The average height of H3 nucleosomes extracted from human cells spans from 2.4 to 3.25 nm and for CENP-A nucleosomes spans from 1.64 to 2.7 nm. These measurements become even more varied with consideration of seminal AFM data for the heights of H3 nucleosomes, which record estimated median heights of 3.77 nm and even up to 4.5 nm^{10,13}. Thus AFM measurements from a number of different laboratories have recorded median heights between 2.4 and 4.5 nm for H3 nucleosomes, a difference of 47%, and yet these data were not interpreted as indicating that H3 nucleosomes have an assortment of different component stoichiometries, as proposed for CENP-A nucleosomes.

In this context, our data are similar to those observed by Walkiewicz et al.⁸ and Codomo et al.⁹. Our measurements for the median height of H3 nucleosomes differ from those of Walkiewicz et al.⁸ by 8–13% and from those of Codomo et al.⁹ by 7%. For CENP-A the differences are greater; our data differ from those of Walkiewicz et al.⁸ by 31% and 42% (for unfixed and fixed samples, respectively) and from Codomo et al.⁹ by 24% (comparing CENP-A with full-length CENP-A^{Cse4}). However, we note that when Codomo et al.⁹ delete

the N-terminal tail from CENP-A^{Cse4} to better mimic human CENP-A nucleosomes, our measurements differ by only 0.16 nm (9%). Inconsistencies between data collected by different groups are not unusual for AFM measurements of nucleosome height. This is perhaps most clearly demonstrated by comparison of data from Walkiewicz et al.⁸ and Codomo et al.⁹ for the height of recombinant CENP-A^{Cse4} nucleosomes (no data are presented by Walkiewicz et al.⁸ to allow comparison of *S. cerevisiae* H3 nucleosomes). Codomo et al.⁹ measured a median height of 2.16 nm, whereas Walkiewicz et al.⁸ recorded a median height of 2.80 nm for the same nucleosome type, which corresponds to a 30% height difference. Moreover, Henikoff and colleagues⁴ recently obtained an estimated median height of 2.67 nm for recombinant *S. cerevisiae* H3 nucleosomes, results representing a 37% difference from the median height of 1.95 nm reported by the same group (Codomo et al.). We note that in the same report⁴, the authors measure a median height for CENP-A^{Cse4} hemisomes that is actually close to that of the octameric particles measured by Codomo et al. (2-8% of the median H3 and CENP-A^{Cse4} heights). These comparisons highlight the great variability of AFM nucleosome height measurements, even for data collected by the same research group for the same recombinant nucleosome type. Thus, we conclude that the subunit stoichiometry of nucleosomes cannot be inferred from AFM height measurements alone.

The most common reasons given for inconsistency in AFM data are differences in the imaging conditions, such as the force or frequency at which the AFM tip presses down onto the surface, the local humidity (to which AFM measurements are particularly sensitive) or the adsorption of salts by the surface^{4,14,15}. Such environmental differences are likely to be contributing factors here, too, and may even be accentuating subtle differences between CENP-A and H3 nucleosomes. The data from Walkiewicz et al.⁸ show a greater difference from our data in terms of heights of both CENP-A and H3 nucleosomes than do the data of Codomo et al.⁹, thus suggesting that the reason why CENP-A nucleosomes report lower heights in our hands might partly be due to differences in sample preparation. Our samples were typically deposited onto the coated-mica surfaces in 10 mM NaCl and 1 mM EDTA, whereas the samples of Walkiewicz et al.⁸ were deposited in PBS (150 mM NaCl) supplemented with 2 mM MgCl₂. Codomo et al.⁹ deposited their samples in just 1 mM EDTA and observed a 7% and 18% reduction in the respective heights of H3 and CENP-A nucleosomes as compared with measurements in Walkiewicz et al.⁸ It is known that, depending on the salt concentration, nucleosomes can aggregate or subunits can dissociate¹⁶⁻¹⁸. Moreover, *Drosophila* CENP-A nucleosomes extracted from cells are more sensitive to higher salt concentrations than are H3 nucleosomes¹⁹. It would therefore be worth testing whether CENP-A-nucleosome height, as measured by AFM, is more sensitive to changes in salt concentration than is H3 nucleosome height.

In light of the shortcomings of AFM measurements, appropriate controls become critical. Our analyses recorded the height of a large number of nucleosomes, showed the same result with both fixed and unfixed nucleosomes and notably also demonstrated that a mixed population of CENP-A and H3 nucleosomes closely matched the height distributions of an *in silico* mix of separately recorded heights of CENP-A and H3 nucleosomes. We were also careful to record the height of DNA in each image as an internal control between images. These controls exclude differences in the AFM setup and fluctuations in environmental

conditions as explanations for the difference that we observed between heights of CENP-A and H3 nucleosomes. Furthermore, our analyses showed that octameric nucleosomes containing chimeric histone H3 with the CENP-A-targeting domain (CATD) region from CENP-A (H3-CATD) also exhibited a reduced height and therefore demonstrated that the CATD is sufficient to account for the relative reduction in height observed between CENP-A and H3 nucleosomes. Thus, our data are consistent with CENP-A nucleosomes that exhibit distinct, more compact structural properties as reported for chimeric (H3-CATD-H4)₂ tetramers relative to canonical (H3-H4)₂ tetramers⁷.

The discrepancy in reported height difference of CENP-A and H3 nucleosomes between our data and the data now provided by Walkiewicz et al.⁸ and Codomo et al.⁹, along with the wide variation observed for nucleosome height measurements in general, highlights the need for other analyses that are distinct from AFM when determining whether CENP-A nucleosomes released from cellular chromatin are hemisomes or octasomes. Indeed, several studies that involved completely different approaches have now tested the hemisome model.

First, in agreement with the octameric CENP-A nucleosome crystal structure, CENP-A nucleosomes within a neocentromere also protect slightly less DNA than do bulk (H3) nucleosomes^{20,21}. Upon exposure to micrococcal nuclease, size classes of ~150-160, ~120-139 and ~100-119 bp are progressively released from the same particles. Given that hemisomes created artificially wrap substantially less DNA (61 bp)⁴, the protection of >100 bp implies that CENP-A particles at centromeres are actually octameric.

Second, the number of CENP-A-GFP signals in single particles released from human chromatin, as counted by total internal reflection fluorescence microscopy²², indicates that the vast majority of particles assayed contain two CENP-A-GFP subunits, regardless of cell-cycle stage.

Third, cysteine residues within the C-terminal region of both CENP-A and H3 can be cross-linked in octameric nucleosomes, and in *Drosophila* S2 cells it has been shown that the majority of nucleosomal CENP-A can be cysteine-cysteine cross-linked as a dimer¹⁹. This indicates that most CENP-A resides in chromatin with its C terminus in proximity to another CENP-A molecule, and if they are not within octameric particles they must at least be in (CENP-A-H4)₂ tetramers.

Finally, bimolecular fluorescence complementation, fluorescence resonance energy transfer and photobleaching-assisted counting assays demonstrate the presence of at least two CENP-A^{Cse4} molecules in physical proximity at *S. cerevisiae* centromeres²³. From these four studies, the most parsimonious explanation is that, as with H3, the majority of CENP-A is incorporated into octameric nucleosomes.

The Dalal and Henikoff groups have repeatedly used comparisons of CENP-A and H3 nucleosome heights measured with AFM to imply the stoichiometry of CENP-A nucleosomes. We have highlighted here that the measurement of nucleosome height with AFM is prone to variability and inconsistency and therefore cannot be used in isolation to infer nucleosome stoichiometry. Future studies involving AFM nucleosome height measurements must be performed alongside more robust analyses, such as those mentioned

above, in order to allow concrete conclusions to be drawn about CENP-A nucleosome composition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Variability in the distribution of nucleosome heights measured using AFM
 Points represent the median height of H3 (red diamonds) nucleosomes and CENP-A (blue squares) nucleosomes measured by AFM from several publications. Whiskers represent estimates of the interquartile range, taken from the nucleosome height distributions shown in each publication. In cases in which the median was not explicitly stated, we estimated it from the available data, as detailed in Supplementary Table 1. Unless otherwise stated, the sample type is that of canonical octameric nucleosomes.