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Structural evidence for Nap1-dependent H2A-H2B deposition and nucleosome assembly

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision (arbitration on reports from another journal)) 18 December 2014

1st Editorial Decision

Thank you again for submitting your manuscript for consideration at The EMBO Journal and my sincere apologies for the delay in communicating our decision to you.The editor who initially handled your submission has recently left The EMBO Journal and I have therefore taken over the decision process.

We had sent your revised manuscript file as well as the response to the referee concerns to two arbitrating advisors. We have now - after some delay for which I apologise - heard back from both arbitrating referees and their comments are included below. I am afraid that neither of the two offer support for publication of the revised manuscript in The EMBO Journal. As you will see, they both remain concerned about the conclusiveness of the study and find that significant additional data would be required to support the presented model.

We have rediscussed your manuscript in the editorial team and in light of the negative opinions from both of our expert advisors I am afraid we have to come to the conclusion that we cannot offer to publish your study in The EMBO Journal.

Thank you again for letting us consider your work and also for your patience in this matter. I am sorry that we cannot be more positive here.

Advisor #1:
I found the authors rebuttal to the points of stoichiometry and in vivo tests to be insufficent.

Stoichiometry: The mass spec based argument is still not clear. In the point-by-point response, the authors show the mass-spec data with excess H2A-H2B dimers over Nap1 and say they see an extra pair of H2A bound to the Nap1 dimer. However the figure in the rebuttal is hard to interpret as the authors do not deconvolve the data in terms of masses (as was asked by the referees). It is not clear how the authors can discriminate between a pair of H2A molecules vs. an H2A-H2B dimer given that the masses of H2A and H2B are very similar.

In vivo data: The authors say that testing the role of the Nap1 oligomerization in vivo would require mutants in the Nap1-Nap1 oligomerization interface as well as the H2A docking domain. They argue that mutation of the docking domain would cause other effects making the experiment hard to interpret. However histone binding only contributes in part to oligomerization of Nap1. So it is not clear why they do not simply test the in vivo effects of mutating the Nap1-Nap1 oligomerization interface (without altering the H2A docking domain)

Advisor #2:

I read the manuscript before reading the previous reviewers comments and, although it does address a very important question in the chromatin field, I found it on the whole rather unconvincing. The paper revolves around a low-resolution structure (~6 Angstroms) of the Nap1-H2A-H2B complex, and with this rather low-resolution structure high quality biochemical validation is very important. However, instead of providing this, the authors present too much data from too disparate approaches in a bid to convince the reader that their structure represents bona fide interactions in the context of Nap1's function.
From the previous reviewers comments it is clear that this manuscript has also failed to provide them with reasonable evidence that the crystallographic model is correct. The authors appear to have argued their corner rather than addressing the Reviewers comments experimentally. Taking this into consideration, I do not think that publication of this manuscript as it is would be well received by people within the chromatin field, and it is more likely to contribute to the general noise surrounding Nap1's function rather than solve any long standing questions.
That being said, I do see promise in the manuscript if the Nap1-histone interface seen in the crystal structure is properly validated biochemically. In agreement with the previous reviewers, I think the negative stain EM and nucleosome mapping bring little to the manuscript. A lot of emphasis is placed on the results from the native mass spectrometry, which, in many cases, is the sole source of validation for the interactions seen in the crystal structure. These interactions could easily be validated using simpler approaches that are less prone to artifacts (see below for more detailed comments). As it is, my personal opinion is that this study is too preliminary and inconclusive for publication in EMBO Journal at this stage.

More specific comments in addition to those from reviewers:
There are two main interactions that the authors report, and attempt to verify. First is the interaction between Nap1 and H2A-H2B. The low resolution crystal structure does offer some clues as to the residues that are important here, however, the attempts made at validating this interface fall short of convincing. It is a little frustrating that tried and tested biochemical methods, such as size exclusion chromatography, that offer a very straight forward interpretation of complexes under physiologically relevant conditions, have been omitted in favor of techniques such as native mass spectrometry, that are prone to artifacts and difficult to interpret with a high degree of confidence. Indeed, the authors use size exclusion chromatography for purifying the Nap1-H2A-H2B complex for crystalisation under physiological conditions. So why not use the same straight forward approach for testing mutants of the Nap1-histone interface? This approach would also have the benefit of isolating the Nap1-histone interaction from the confounding Nap1-Nap1 oligomeric interactions. Additionally, the GST pulldown experiments in Figure 2D are poorly controlled, with fluctuating levels of chaperone and free GST across the samples, with no attempt at quantification. Another concern is that no mutants on the histones are tested, only mutations on Nap1. As the interaction interface is

small, one would imagine point mutations on the histones could be made that disrupt their binding to Nap1. It is not clear why this has not been done.
The second interaction extracted from the crystal structure is that between Nap1 dimers, which the authors suggest is responsible for the oligomeric nature of Nap1, as has been report extensively in the literature. A major concern here is that the model for Nap1 oligomerisation goes against two independent structural papers, using complementary techniques, that find an alternative region of Nap1 is used to mediate oligomeric interactions. The authors do not test these established mutants in their assays.

2nd Editorial Decision (upon manuscript resubmission following discussion with author) 01 April 2016

Your study has now been seen by three referees whose comments are shown below. In addition, we have performed an additional round of cross-referee commenting given the somewhat different recommendations in the reports.

As you will see, the referees express interest in the findings reported in your manuscript, but they also still raise a number of concerns with the presentation and conclusiveness of the data that you will have to address before they can support publication of the manuscript. Just to let you know, refs #1 and #2 are the same referees that were involved in the assessment of the previous version here while ref #3 is new.

It is clear that both refs #1 and #2 acknowledge the efforts that have gone into this new version of the manuscript but while referee #1 consequently recommends publication after minor revision, ref #2 finds that a number of points still remain unclear thus reducing the overall advance provided. However, you will also see that many of the points raised by ref #2 appear to be addressable by textual changes or more extensive explanations. This latter point is in agreement with ref #3 who mainly raises concerns related to the presentation and discussion of the data.

Given the referees' overall positive recommendations (both in the reports and in cross-ref commenting), I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on clarifying and discussing the remaining ambiguities pointed out by the referees. As for the need to investigate nuclear transport further, I received the below input from ref #1 during cross-ref commenting and I would encourage you to follow it in the revised manuscript:

'I do not think the authors need to show direct evidence for a role in nuclear transport as these experiments would be outside the scope of the work. However, they do need to be clear about what they have shown and what they speculate on'

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

Histone chaperones play critical roles in regulating the chromatin landscape. Nap1 is the major chaperone promoting the correct assembly of histones H2A and H2B into nucleosomes. While Nap1 has been studied extensively by biochemical and biophysical studies, a crystal structure of this chaperone in complex with the H2A-H2B heterodimer has remained elusive. Here the authors

present a low-resolution (6.7 Angstroms) crystal structure of a truncated but functional form of yeast Nap1 in complex with the H2A-H2B dimer. The structure suggests the presence of specific self-limiting oligomerization interfaces and histone binding regions and provides the opportunity to resolve some fundamental aspects of Nap1 mechanism.

The work here goes beyond existing work on Nap1 action for the following reasons:

1. The authors provide convincing data for the stoichiometry of H2A/H2B on NAP1. This is an important parameter for constraining mechanistic models. The results suggest that contrary to previous inferences, one H2A-H2B dimer is bound to a NAP1 dimer. The authors use native mass-spec approaches under a few different concentration regimes to derive this conclusion together with the crystal structure.

2. The authors show in vitro and in vivo functions for NAP1 oligomerization. To my knowledge this has not been demonstrated previously. They find that the NAP1 oligomerization interfaces identified from the crystal structure are important for rescuing defective nucleosome assembly in vitro and mutating these interfaces has large effects on nucleosome occupancy and quality in vivo.

3. The authors identify specific histone binding regions and the functional role of these interactions in their vitro and in vivo assays.

4. The genome wide in vivo data strongly suggests that the in vivo role of NAP1 is primarily to deposit H2A/H2B and not H3/H4.

Based on the results, the authors propose a thoughtful model for the role of NAP1 oligomerization in nucleo-cytoplasmic shuttling of NAP-bound histones. This model and the accompanying data will help shape future discussions on NAP1 function and mechanism. Therefore the work will be of broad interest to the chromatin community and is very suitable for publication in EMBO Journal.

Minor points:

1. I could not easily find in the methods how the mutants were made for the in vivo assays. Were they integrated at the endogenous NAP1 locus or were they expressed on a plasmid? Is the pNAP1 construct the WT version of NAP1 re-introduced into the NAP1 delete? This would be an essential control. If so, it seems that pNAP1 this does not give the same MNase pattern as BY4741. This does not affect the core conclusion that the IF1 mutant and HBR2 mutants have a large defect. However the authors need to explicitly mention the pNAP1 result if this is the correct background in which to compare the mutants.

2. Is it possible to quantify the rescue assay data in Fig. S5? These are significant results with respect to the mutants and it would be useful to be able to compare to WT quantitatively.

3. That Nap1 disfavors H2A-H2B interactions with DNA has been previously shown by work from the Luger group using solution based and gel based assays. It does not seem be referenced as such when the authors describe their results in Fig. 6A.

Referee #2:

In this manuscript Aguilar-Gurrieri et al present the first crystal structure of yeast Nap1 bound to an H2A-H2B dimer. The crystal structure is of a low resolution, and includes only globular truncations of both histones and chaperone, and therefore it should be regarded as more of a model than a structure per se. Nonetheless, it is the first molecular insight at a resolution of <7 Angstroms of a Nap1 protein in complex with its histone cargo. The current lack of structural information regarding how the Nap1 family of histone chaperones interact with their histone substrates is testament to how difficult it is to crystalize these complexes. As such this manuscript does present an advance in the

understanding of how the chromatin assembly machinery functions. However, due to the low resolution and poor validation of the presented Nap1-H2A-H2B model, the manuscript does not unequivocally answer how Nap1 functions at the atomic level in nucleosome assembly. As such, it provides a limited advance on what is already known about Nap1, and is more suited to publication in specialised biochemical/biophysical journal than for the broad audience of EMBO Journal.

The model from the low-resolution crystal structure reveals a stoichiometry of one H2A-H2B dimer per Nap1 dimer, and identifies regions of Nap1 that mediate previously characterised self-association states of the yNap1-H2A-H2B. The stoichiometry observed is in conflict with previous observations from the Luger laboratory, who have suggested that two H2A-H2B dimers are bound to a single Nap1 dimer (1-3), but is in agreement with findings from the Rippe laboratory, and others, that also suggest one H2A-H2B dimer is bound to one Nap1 dimer (4,5).

The second aspect of the low-resolution crystal structure is that it identifies regions that are important in oligomerization of Nap1. Nap1 has previously been shown to oligomerize in both the presence and absence of histones (2,4-8). In the absence of histones, Nap1 proteins have been reported to form stable tetramers mediated by a beta-hairpin region (6,8), whereas in the presence of either H2A-H2B or H3-H4, Nap1 forms very large oligomeric complexes that have, as yet, only been defined using low resolution structural techniques and biophysical analysis (4,5).

Due to the low-resolution of the presented complex, extensive biochemical validation is required to demonstrate that (1) the interaction interface between H2A-H2B and Nap1 is the same as seen in solution, (2) the stoichiometry of binding is the same that is seen in solution, and (3) the oligomerisation interfaces are the same as seen in solution. However, the validation of the crystal structure is generally poor, the reasons for which are explained below under 'major concerns', and contributes more confusion in Nap1's function than provides clarity.

The authors try to link the low-resolution structure of Nap1 with genome wide analysis of chromatin structure in a Nap1 deletion strain of yeast (Figure 1). The link between structure and function in this case is extremely tenuous, and has most likely been included to try and increase the impact of the manuscript. In order for the genome wide analysis to be included, the authors should perform the same experiments on a Nap1Δ strain complemented with the HBR and IF mutants identified from the crystal structure. However, this would represent a significant undertaking, and most likely require publication in its own right.

The model of Nap1 function presented in Figure 7 is highly speculative, draws mostly on selective findings from the literature, and is suited to a review/essay style article rather than an original research article.

In its current form experiments presented in the manuscript provide only weak support for the low-resolution crystal structure.

Major concerns:
1) Authors should state that the crystal structure is of a low resolution in the abstract and introduction, so as not to mislead readers who would generally think of a crystal structure as being under 3.5 Angstrom resolution.
2) The introduction finishes with the authors stating that their findings indicate "two separable functions of Nap1: histone transport/storage and nucleosome assembly." How they came to this conclusion from their structural and functional analyses is unclear as they do not investigate nuclear transport/storage at all.
3) As stated in the main text above, Figure 1 has only tenuous links to the rest of the manuscript and should be removed.
4) Page 6. The authors mention that D'Arcy et al 2013 determine a stoichiometry of two H2A-H2B dimers to one Nap1 dimer, however, other studies have also identified the same stoichiometry, but are not mentioned (1,2). There is some overlap in the regions identified by D'Arcy et al 2013 and in the present study (H2A-L2 - incorporating the same K75 mutation identified here, for example). This is not discussed.
5) Figure 3D. The backgrounds are at a high contrast for some of the panels. Lane 13 has significantly less Nap1 than the rest of the conditions. Considering that the H2A-H2B band is

already quite weak, and the contrast is high, it can't be concluded that the HBR1-2 mutant does not bind histone in this case.
6) Figure 3D. Single and double mutations are present, but I cannot see any triple mutations (D201R, D205R, E310R) for HBR1 in the figure as stated in the main text. The same goes for triple mutations of the HBR2 region. In addition, it is not apparent which lanes of 13-15 are alanine mutations and which are charge reversal mutations.
7) The Nap1-histone interface is only validated by GST pull downs from recombinant protein in bacterial whole cell extracts. One would have hoped for a more extensive biochemical validation for a paper to appear in EMBO Journal.
8) Figure 3E. The labels are poorly aligned with the lane numbers to the extent that it is difficult to see which lane corresponds to which mutant.
9) Figure 3F. It is not stated exactly which charge reversal mutations are used (arginine, lysine, aspartate, glutamate?).
10) Figure 3. Were the mutants tested for structural integrity? Whilst the surface point mutations are probably accommodated fine, it may not be the case for multiple mutations. Indeed, in the discussion the authors state about D'Arcy et al 2013 "the reported mutations are located in the buried segments in the helical underside of yNap1 and most likely indirectly interfere with H2A-H2B binding by affecting the structural integrity of yNap1.". Were the mutations presented in this manuscript checked for structural integrity?
11) Figure 4 seems to be somewhat of a repetition of Figure 2.
12) Figure 5 - Cryo-EM. The Nap1-H2A-H2B structure observed by cryoEM does appear to show similarity to crystallography asymmetric unit. However, it is presented rather poorly and it is difficult for the reader to judge.
13) Native mass spectrometry. Although being called "native", native mass spectrometry is still far from an ideal method in determining the components of complexes that are present in solution. Protein complexes need to be vaporized for analysis, and the extent to which this affects the integrity of the complex is difficult to assess. The native mass spectrometry is the only technique that is used to address the stoichiometry of the Nap1-H2A-H2B oligomer. This question could have been addressed using much simpler methods.
14) Page 9-10. The authors state: "Previous analysis by ensemble averaging techniques such as size exclusion chromatography, sucrose gradients, analytical ultracentrifugation and small angle x-ray scattering did not converge on a unique solution for Nap1 oligomerization and histone binding stoichiometry". Have the authors considered that there may not be a unique solution for Nap1 oligomerisation and histone binding stoichiometry, and that what their crystal structure represents is a single trapped intermediate from a more complex and dynamic system?
15) Figure 5D-I. It is not clear what ratio of H2A-H2B to yNap1 was used for the experiments shown. If only one H2A-H2B dimer to one yNAp1 dimer was added, then one wouldn't expect to see two H2A-H2B dimers bound per yNap1 dimer. I think this point hits on a greater theme that runs throughout the manuscript in that the authors cannot sufficiently rule out that when histone H2A-H2B dimers are in excess of the yNap1, then a single yNap1 dimer may accommodate two H2A-H2B dimers, as has been characterised previously (1,2). Indeed, in the H2A-H2B titration experiments (Figure S4A), the titration stops at a ratio of one H2a-H2B dimer per yNap1 dimer. This should be very easy to test.
16) In the Materials and Methods the authors state that "reconstituted H2A-H2B dimers were added at a twofold molar excess to Nap1. The mixture was incubated for 30 minutes on ice and then loaded on a pre-equilibrated Superose 6 10/300 GL (GE Healthcare) in buffer 2.". If the solution state of the Nap1-H2A-H2B complex is one yNap1 dimer to one H2A-H2B dimer, then a molar excess of freely eluting H2A-H2B dimers should have been identified during gel filtration chromatography. Would this not be further evidence for the stoichiometry of the crystalized complex?
17) The reference that Park and Luger 2008 shows yNAP-1 eluting in a broad peak, alone in or complex with H2A-H2B (page 8), is wrongly cited. There is no size exclusion chromatography presented in Park and Luger 2008. Park et al 2008 (6) present size exclusion data showing that the elution of yNAP1 is dependent on ionic strength and pH (Figure 3), and that oligomerisation is dependent on a specific hairpin structure. McBryant et al 2003 (9) show a relatively discrete elution peak for both free yNAP1 and yNAP1 in complex with H2A-H2B (Figure 1).
18) Figure 6. The complementation of Nap1 in the MNase experiments is lacking important controls. Namely the authors do not show that levels of the Nap1 mutants expressed from plasmids are at the same level as the wild-type expressed from the plasmid, or even if the mutants are expressed and correctly folded at all. Without this it is impossible to interpret data.

19) Nap1 has also been shown to be stably associated with the evolutionary conserved histone H2A.Z variant(10-12). Are the Nap1 binding regions identified in the crystal structure conserved in the H2A.Z variant? No mention of this is found in the manuscript.
20) There is too much speculative discussion about the role of Nap1 in histone transport for a manuscript that does not address this aspect of Nap1 function.
21) The model represented in Figure 7 is highly speculative and draws mostly on results published elsewhere and not presented in the current manuscript.
22) There is a section of ITC measurements in the materials and methods, but no mention of ITC in the rest of the manuscript.

Minor points
1) Page 4. "molecular determinants of it role histone chaperoning" should read "molecular determinants of it role in histone chaperoning".

References

1. Andrews, A.J., Chen, X., Zevin, A., Stargell, L.A. and Luger, K. (2010) The Histone Chaperone Nap1 Promotes Nucleosome Assembly by Eliminating Nonnucleosomal Histone DNA Interactions. Molecular Cell, 37, 834-842.
2. Andrews, A.J., Downing, G., Brown, K., Park, Y.J. and Luger, K. (2008) A thermodynamic model for Nap1-histone interactions. Journal of Biological Chemistry, 283, 32412-32418.
3. D'Arcy, S., Martin, K.W., Panchenko, T., Chen, X., Bergeron, S., Stargell, L.A., Black, B.E. and Luger, K. (2013) Chaperone Nap1 shields histone surfaces used in a nucleosome and can put H2A-H2B in an unconventional tetrameric form. Mol Cell, 51, 662-677.
4. Toth, K.F., Mazurkiewicz, J. and Rippe, K. (2005) Association states of nucleosome assembly protein 1 and its complexes with histones. Journal of Biological Chemistry, 280, 15690-15699.
5. Newman, E.R., Kneale, G.G., Ravelli, R.B., Karuppasamy, M., Karimi Nejadasl, F., Taylor, I.A. and McGeehan, J.E. (2012) Large multimeric assemblies of nucleosome assembly protein and histones revealed by small-angle X-ray scattering and electron microscopy. J Biol Chem, 287, 26657-26665.
6. Park, Y.J., McBryant, S.J. and Luger, K. (2008) A beta-hairpin Comprising the Nuclear Localization Sequence Sustains the Self-associated States of Nucleosome Assembly Protein 1. Journal of Molecular Biology, 375, 1076-1085.
7. McBryant, S.J., Park, Y.J., Abernathy, S.M., Laybourn, P.J., Nyborg, J.K. and Luger, K. (2003) Preferential binding of the histone (H3-H4)2 tetramer by NAP1 is mediated by the amino-terminal histone tails. J Biol Chem, 278, 44574-44583.
8. Bowman, A., Hammond, C.M., Stirling, A., Ward, R., Shang, W., El-Mkami, H., Robinson, D.A., Svergun, D.I., Norman, D.G. and Owen-Hughes, T. (2014) The histone chaperones Vps75 and Nap1 form ring-like, tetrameric structures in solution. Nucleic Acids Res, 42, 6038-6051.
9. McBryant, S.J., Park, Y.J., Abernathy, S.M., Laybourn, P.J., Nyborg, J.K. and Luger, K. (2003) Preferential Binding of the Histone (H3-H4)2 Tetramer by NAP1 Is Mediated by the Amino-terminal Histone Tails. Journal of Biological Chemistry, 278, 44574-44583.
10. Kobor, M.S., Venkatasubrahmanyam, S., Meneghini, M.D., Gin, J.W., Jennings, J.L., Link, A.J., Madhani, H.D. and Rine, J. (2004) A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol, 2, E131.
11. Luk, E., Vu, N.D., Patteson, K., Mizuguchi, G., Wu, W.H., Ranjan, A., Backus, J., Sen, S., Lewis, M., Bai, Y. et al. (2007) Chz1, a nuclear chaperone for histone H2AZ. Mol Cell, 25, 357-368.
12. Mizuguchi, G., Shen, X., Landry, J., Wu, W.H., Sen, S. and Wu, C. (2004) ATP-Driven Exchange of Histone H2AZ Variant Catalyzed by SWR1 Chromatin Remodeling Complex. Science, 303, 343-348.

Referee #3:

The manuscript by Aguilar-Gurrieri investigates the structural and biochemical properties of NAP1, a histone chaperones that favors nucleosome assembly by promoting deposition of H2A-H2B dimer(s) onto the H3-H4 tetramer as last step in the process. The study involves several complementary and experimentally demanding approaches that lead a mechanistic model for NAP1

function. Key elements are the stoichiometry of the complex (1 NAP1 dimer/1 H2A-H2B dimer), the involvement of the H2A DNA-binding regions in association to NAP1, and the propensity of the complex to oligomerise, a feature which is suggested to be essential for function.
This is certainly an interesting topic, of great relevance in chromatin structure and molecular biology. The reported data also help to clarify inconsistencies present in the literature. However, I found this manuscript rather difficult to review as the presentation often lacks clarity, with statements that sometime apparently contract each other and tend to be difficult for the reader. The manuscript is long and would greatly benefit from shortening and a more concise and focused presentation.

Main points

-First paragraph of the results. Here, there is an example for the above comment:
"The distance between each band corresponds to the distance between the most distal nucleosome ends within an MNase-cleaved array of an integral number of nucleosomes. ..... Consequently, the interband distance corresponds to a population average spacing between adjacent nucleosome midpoints."
The distance between each band is first stated to correspond to the distance between nucleosomal ends and, later, it is stated to correspond to the distance between nucleosome midpoints. I think the reader can be easily lost at this point.

-Two paragraphs later, "MNase digestions indicate that the nucleosomal arrays maintained wild-type spacing (or ~165 bp inter-band distance), indicating that nucleosome spacing in bulk chromatin was generally unaltered in the nap1Δ strain."
However, Figure 1A shows that WT spacing is 160 bp, 150 bp, 152 bp (170, 330, 480, 632 size) whereas the mutant spacing seems to be partly different as it is 160, 150, and 125 bp (145, 305, 455, 570 size). The explanatory sentence "We interpret the fixed size reduction as follows: MNase is expected to cleave more frequently where linkers are longer, and this is expected to occur in the nap1Δ strain where H2A-H2B is lost (i.e., linker DNA plus the nucleosomal DNA that is vacated by H2A-H2B). This produces a fixed size reduction per array since nucleosome spacing is unaltered" is hard to understand. At the very least, some rewriting is needed together with a more careful description and analysis of the data. In particular, it is hard to understand how smaller mononucleosome-sized MNase fragments (145 bp versus 170 bp; previous paragraph in the main text) cannot affect inter-band distances (if this is really the case and if interband distance is interpreted as "distance between nucleosome midpoints").

-Likewise, the subsequent paragraph of this section is difficult to follow. The sentence "In these single-read sequencing experiments, MNase fragment size was deduced by pairing adjacent peaks located on opposite strands and in the 3' direction." is unclear. By analogy, the concept of fragment having fuzzier locations is rather obscure and should explained together with an improved description of Figure 1C.

-Page 9, analysis of oligomers. This section and associated figures are difficult to follow.
"Protomers A and B (A' and B' in layer 2) are related by a ~102{degree sign} rotation and ~8 Å displacement along the central axis (Figure 4A). Two non- crystallographic dyads relate protomer C to A and B in each layer, but because of displacement along the central axis, protomers C and C' pack through alternate protein interfaces: protomer C is located below the plane of protomers A and B whereas protomer C' is located above A' and B'. "
How can it be that a 102{degree sign} rotation superimposes A to B with both A and B related by 180{degree sign} rotations to C? This violates symmetry rules. If A to C is 180{degree sign} and C to B is 180{degree sign}, A and B must have the same orientation.

-Similarly, I cannot see the pseudohelical arrangement in Figure 4C. Presentation must be improved. If it is helical, why there is only a hexamer in cryoEM and crystallization? Why does the helix not propagate further? Is the asymmetry of the oligomer preventing extension of the helical assembly?

-To prove the significance of this packing interface, one would expect a more quantitative analysis. For instance, is the extent of the inter-complex surfaces compatible with those expected for oligomers (e.g. PISA analysis)? Gel-filtration or SAXS data are probably expected by the reader.

-Figure 4C, materials ("Fitting of the crystal structure into the EM map was done with Chimera") and associated main text: one would like to see a more quantitative analysis of the fitting of the model to the cryoEM maps.

-The discussion about the role of olimerization in protein function and nuclear export. It is proposed that isolated Nap1 oligomerises, shielding the NLS sequence. NLS is, however, fully accessible in the Nap1-H2A-H2B complex as shown by the crystal structure. At the same time, residues involved in oligomerisation of the complex are shown to be essential for function. Is it proposed that Nap1 exists in different oligomeric forms depending on whether unbound or bound to H2A-H2B? If this is the case, one would expect some experimental proof.

-Finally, possibly the most critical point:
Page 11: "As oligomerization of the yNap12-H2A-H2B complex results in burial of a segment that is required for docking onto H3-H4 tetrasomes, we argue that it is inhibitory for H2A-H2B deposition and nucleosome formation" and Page 16 (end of the manuscript): "As the histone-bound oligomer is inhibitory for H2A-H2B deposition,...."
Page 12: "The yNap1c and yNap1_IF1 mutants are not affected in this nucleosome assembly assay indicating that the flexible N- and C-terminal regions and oligomerization of yNap1 are not essential for H2A-H2B deposition (Figure S5C-D)"
PGE 13: "Surprisingly, the IF1 mutant, which showed only modest impact on H2A-H2B binding in vitro, showed the greatest defect on nucleosome assembly."
These statements are partly contradictory. In the general, these data indicate that oligomerisation is not inhibitory on the biochemical process. Rather, the explanation exist that it is not matter of oligomerisation. It can well be that the IF1 surface residues affect protein-protein interactions essential, for instance, for import-export or functions in the chromatin. An effect that has little to do with oligomerisation, which might simply result from promiscuous tendency to associate by these residues.

1st Revision - authors' response 09 April 2016

We want to thank the reviewers for taking the time to review our manuscript and for providing valuable comments and suggestions. We understand that the dynamic nature of the Nap1-H2A-H2B oligomer and the low resolution of the data make this a particularly challenging system to investigate and to review. Below we address the reviewers' concerns in a detailed point-by-point response. Although biological/technical limitations do not allow us to address every aspect of the reviewers concerns, we feel that the majority of findings of our paper are adequately supported by our data and that we can address most of the concerns in a revised manuscript. Concerning the final model for yNap1 as a H2A–H2B transport and chromatin assembly factor, we have clarified in the discussion which aspects of the model are based on data and which aspects are more speculative. We hope that the model will help shape future discussions on NAP1 function and mechanism and that the revised version of our MS is acceptable.

Reviewers' comments:
Referee #1:
Histone chaperones play critical roles in regulating the chromatin landscape. Nap1 is the major chaperone promoting the correct assembly of histones H2A and H2B into nucleosomes. While Nap1 has been studied extensively by biochemical and biophysical studies, a crystal structure of this chaperone in complex with the H2A-H2B heterodimer has remained elusive. Here the authors present a low-resolution (6.7 Angstroms) crystal structure of a truncated but functional form of yeast Nap1 in complex with the H2A-H2B dimer. The structure suggests the presence of specific self-limiting oligomerization interfaces and histone binding regions and provides the opportunity to resolve some fundamental aspects of Nap1 mechanism.

The work here goes beyond existing work on Nap1 action for the following reasons:

1. The authors provide convincing data for the stoichiometry of H2A/H2B on NAP1. This is an important parameter for constraining mechanistic models. The results suggest that contrary to previous inferences, one H2A-H2B dimer is bound to a NAP1 dimer. The authors use native mass-spec approaches under a few different concentration regimes to derive this conclusion together with the crystal structure.

2. The authors show in vitro and in vivo functions for NAP1 oligomerization. To my knowledge this has not been demonstrated previously. They find that the NAP1 oligomerization interfaces identified from the crystal structure are important for rescuing defective nucleosome assembly in vitro and mutating these interfaces has large effects on nucleosome occupancy and quality in vivo.

3. The authors identify specific histone binding regions and the functional role of these interactions in their vitro and in vivo assays.

4. The genome wide in vivo data strongly suggests that the in vivo role of NAP1 is primarily to deposit H2A/H2B and not H3/H4.

Based on the results, the authors propose a thoughtful model for the role of NAP1 oligomerization in nucleo-cytoplasmic shuttling of NAP-bound histones. This model and the accompanying data will help shape future discussions on NAP1 function and mechanism. Therefore the work will be of broad interest to the chromatin community and is very suitable for publication in EMBO Journal.

Minor points:

1. I could not easily find in the methods how the mutants were made for the in vivo assays. Were they integrated at the endogenous NAP1 locus or were they expressed on a plasmid? Is the pNAP1 construct the WT version of NAP1 re-introduced into the NAP1 delete? This would be an essential control. If so, it seems that pNAP1 this does not give the same MNase pattern as BY4741. This does not affect the core conclusion that the IF1 mutant and HBR2 mutants have a large defect. However the authors need to explicitly mention the pNAP1 result if this is the correct background in which to compare the mutants.
We thank the reviewer for the positive assessment of our paper. The pNAP1 construct is a single-copy plasmid in which *nap1* is expressed from the endogenous promoter (Miyaji-Yamaguchi et al, 2003). To clarify this point, we have included the genotypes of the strains and plasmids used in Appendix Table S1. The vectors containing wt *nap1* or mutants thereof were introduced into the *nap1D* strain to assess the impact on nucleosome assembly. The expression level of each *nap1* derivative was examined by western blot (Figure EV4 J). We would like to point out that the expression levels of Nap1p in the BY4741 strain are close to but not identical to the *nap1D* strains containing the pNAP1 plasmid.s These differences could account for the modified MNase pattern. Nevertheless, the nucleosome assembly defect of the HBR2 and IF1 mutants is very prominent.

2. Is it possible to quantify the rescue assay data in Fig. S5? These are significant results with respect to the mutants and it would be useful to be able to compare to WT quantitatively.
We have included a quantification in Figure 4EV, panel H. Quantification of these data is not easy and therefore we state (p11; bottom panel): 'To qualitatively compare the 'rescue' activity of the different yNap1 variants, the fluorescence intensity of the nucleosome band at 1 µM concentration of yNap1 was quantified'.

3. That Nap1 disfavors H2A-H2B interactions with DNA has been previously shown by work from the Luger group using solution based and gel based assays. It does not seem be referenced as such when the authors describe their results in Fig. 6A.
Thank you for pointing this out. To accommodate the reviewers' request, we state on p.12; bottom paragraph:' In accordance with our structure and previously published data (Andrews et al, 2010),

yNap1 is suppressing non–specific DNA binding of H2A–H2B and thereby facilitates specific deposition of H2A–H2B and nucleosome assembly.'

Referee #2:

In this manuscript Aguilar-Gurrieri et al present the first crystal structure of yeast Nap1 bound to an H2A-H2B dimer. The crystal structure is of a low resolution, and includes only globular truncations of both histones and chaperone, and therefore it should be regarded as more of a model than a structure per se. Nonetheless, it is the first molecular insight at a resolution of <7 Angstroms of a Nap1 protein in complex with its histone cargo. The current lack of structural information regarding how the Nap1 family of histone chaperones interact with their histone substrates is testament to how difficult it is to crystalize these complexes. As such this manuscript does present an advance in the understanding of how the chromatin assembly machinery functions. However, due to the low resolution and poor validation of the presented Nap1-H2A-H2B model, the manuscript does not unequivocally answer how Nap1 functions at the atomic level in nucleosome assembly. As such, it provides a limited advance on what is already known about Nap1, and is more suited to publication in specialised biochemical/biophysical journal than for the broad audience of EMBO Journal.

The model from the low-resolution crystal structure reveals a stoichiometry of one H2A-H2B dimer per Nap1 dimer, and identifies regions of Nap1 that mediate previously characterised self-association states of the yNap1-H2A-H2B. The stoichiometry observed is in conflict with previous observations from the Luger laboratory, who have suggested that two H2A-H2B dimers are bound to a single Nap1 dimer (1-3), but is in agreement with findings from the Rippe laboratory, and others, that also suggest one H2A-H2B dimer is bound to one Nap1 dimer (4,5).

The second aspect of the low-resolution crystal structure is that it identifies regions that are important in oligomerization of Nap1. Nap1 has previously been shown to oligomerize in both the presence and absence of histones (2,4-8). In the absence of histones, Nap1 proteins have been reported to form stable tetramers mediated by a beta-hairpin region (6,8), whereas in the presence of either H2A-H2B or H3-H4, Nap1 forms very large oligomeric complexes that have, as yet, only been defined using low resolution structural techniques and biophysical analysis (4,5).

Due to the low-resolution of the presented complex, extensive biochemical validation is required to demonstrate that (1) the interaction interface between H2A-H2B and Nap1 is the same as seen in solution, (2) the stoichiometry of binding is the same that is seen in solution, and (3) the oligomerisation interfaces are the same as seen in solution. However, the validation of the crystal structure is generally poor, the reasons for which are explained below under 'major concerns', and contributes more confusion in Nap1's function than provides clarity.
We have addressed all three issues in our MS as discussed below.

The authors try to link the low-resolution structure of Nap1 with genome wide analysis of chromatin structure in a Nap1 deletion strain of yeast (Figure 1). The link between structure and function in this case is extremely tenuous, and has most likely been included to try and increase the impact of the manuscript. In order for the genome wide analysis to be included, the authors should perform the same experiments on a Nap1Δ strain complemented with the HBR and IF mutants identified from the crystal structure. However, this would represent a significant undertaking, and most likely require publication in its own right.
The genome wide analysis of the impact of yNap1 on nucleosome assembly has been undertaken to understand the contribution of Nap1 to nucleosome assembly in vivo (see comment below). The experiment suggested has been done and is shown in Figure 6 C,D.

The model of Nap1 function presented in Figure 7 is highly speculative, draws mostly on selective findings from the literature, and is suited to a review/essay style article rather than an original research article.
We would like to put our findings in context of the prior literature. As this is in the discussion section, we think it is appropriate.

In its current form experiments presented in the manuscript provide only weak support for the low-

resolution crystal structure.

Major concerns:
1) Authors should state that the crystal structure is of a low resolution in the abstract and introduction, so as not to mislead readers who would generally think of a crystal structure as being under 3.5 Angstrom resolution.
We now state in the introduction (page 4): The structure of yeast Nap1 in complex with H2A–H2B, determined at 6.7Å resolution, demonstrates that Nap1 uses an acidic binding surface to engage histone H2A of a single H2A–H2B heterodimer.

2) The introduction finishes with the authors stating that their findings indicate "two separable functions of Nap1: histone transport/storage and nucleosome assembly." How they came to this conclusion from their structural and functional analyses is unclear as they do not investigate nuclear transport/storage at all.
Our structural result shows that Nap1–H2A–H2B oligomerization results in burial of surfaces required for deposition of H2A–H2B. Thus, as Nap1 has already been implicated in nuclear transport of H2A-H2B (REF) and as mutation of the oligomerization interface results in strong nucleosome assembly defects (Figure 6), we suggest that oligomerization provides an additional function, presumably histone transport or storage. We agree with the reviewer that further experiments along these lines are required to investigate this issue. While beyond the scope of our MS, we feel that we provide the field with the tools to resolve this question in the future.

3) As stated in the main text above, Figure 1 has only tenuous links to the rest of the manuscript and should be removed.
The role of Nap1 in nucleosome assembly has been clearly established by in vitro assays. Such assays are suggestive but do not prove such a function in vivo. It is therefore important to confirm the findings of biochemical analyses with in vivo studies. However, the interpretation of in vivo studies becomes complicated when the histone chaperones are involved in multiple pathways, or when they are functionally redundant with other chaperones. Data available on Nap1 function in vivo indicates that Nap1 deletion results in excessive H2A-H2B binding to DNA (Andrews et al, 2010). The implications for nucleosome assembly have remained unclear. Data presented in Figure 1 shows that lack of Nap1 directly impacts nucleosome assembly in vivo. We think this is a critical piece of data that also allows us to analyze the impact of Nap1 mutants (Figure 6) on nucleosome assembly in vivo. Therefore we have not removed these data.

4) Page 6. The authors mention that D'Arcy et al 2013 determine a stoichiometry of two H2A-H2B dimers to one Nap1 dimer, however, other studies have also identified the same stoichiometry, but are not mentioned (1,2). There is some overlap in the regions identified by D'Arcy et al 2013 and in the present study (H2A-L2 - incorporating the same K75 mutation identified here, for example). This is not discussed.
We thank the reviewer for pointing this out. While it is not apparent to us why the reviewer refers to (Andrews et al, 2010), we have included a reference to (Andrews et al, 2008) on p.15 (bottom paragraph). Clearly the exact binding stoichiometry of the Nap1–H2A–H2B complex has remained controversial until now. While there is some overlap in the regions identified by D'Arcy et al. 2013, others do not match with the available structure. As mentioned in the discussion section: 'Such low–resolution techniques can produce confounding results, especially with self–assembling systems where it is difficult to determine from which interface (Nap1–Nap1, Nap1–histone or histone–histone) protection arises'. Eg. none of the two sites of yNap1 that show H/DX protection upon addition of H2A-H2B correspond to H2A-H2B binding sites. We think it would be wrong to 'cherry-pick' parts of the H/DX data and prefer not to discuss these data further.

5) Figure 3D. The backgrounds are at a high contrast for some of the panels. Lane 13 has significantly less Nap1 than the rest of the conditions. Considering that the H2A-H2B band is already quite weak, and the contrast is high, it can't be concluded that the HBR1-2 mutant does not bind histone in this case.
The only image processing done was to crop the gels in order to focus on the relevant sections. The contrast of the individual panels was not readjusted. Maybe inspection of the marker lane, which has

similar contrast to the top panel, can help to convince this reviewer? Lane 13 has indeed less Nap1 (inputs are similar) indicating some loss of the glutathione–affinity resin upon the repeated wash cycles. However, we maintain that residual H2A-H2B binding would have been detected: Lanes 2-4 have similar levels of yNap1 and reveal a H2A-H2B band. Other experiments back up the claim that the HBR1-2 mutant does not bind histones: Panel 3F (lane 2) also shows abolishment of H2A-H2B binding with this mutant upon co-overexpression with H2A-H2B in Ecoli. In addition, experiments shown in Figure 3D, Lane 15 (containing the identical HBR1+2 mutations in addition to IF1) as well as lane 14 (Alanine mutation of the HBR1+2 interfacial residues) backup this result.

6) Figure 3D. Single and double mutations are present, but I cannot see any triple mutations (D201R, D205R, E310R) for HBR1 in the figure as stated in the main text. The same goes for triple mutations of the HBR2 region. In addition, it is not apparent which lanes of 13-15 are alanine mutations and which are charge reversal mutations.
The HBR1 triple mutant is shown in lane 6 and the HBR2 triple mutation in lane 12. The confusion arises as the single and double mutants are labeled explicitly while the triple mutants are labeled with their abbreviated 'HBR' label (explicit labels become too long). By default all mutations are charge reversals except the HBR1+2A mutant (lane 14) containing 6 Ala mutations. We have clarified this in the figure legend. Appendix Table 2 also shows the identity of these mutants.

7) The Nap1-histone interface is only validated by GST pull downs from recombinant protein in bacterial whole cell extracts. One would have hoped for a more extensive biochemical validation for a paper to appear in EMBO Journal.
We have used two assays to validate the various interfaces (some of us would say that the structure validates the less rigorous conclusions from the biochemistry…): 1. A GST pulldown using purified Nap1 variants and H2A–H2B in vitro (Figure 3D). 2. Co–purification after recombinant overexpression from Ecoli. We have also attempted to detect Nap1-H2A-H2B complexes by co-immunoprecipitation in Yeast cells. While we could detect Nap1, we were unable to co-immunoprecipitate H2A-H2B. Our interpretation is that the free concentration of histones H2A-H2B in the cell (non-incorporated into nucleosomes) is too low to be detectable in this Nap1 co-IP assay. This fact that is well known in the field and is likely due to the fact that the majority of cellular histones are part of chromatin and that the free pool of H2A-H2B (that would be substrates for Nap1) is very low (Loyola et al, 2006). We therefore have used the co-overexpression system in Ecoli for further 'validation'.

8) Figure 3E. The labels are poorly aligned with the lane numbers to the extent that it is difficult to see which lane corresponds to which mutant.
We have adjusted the alignment of the labels.

9) Figure 3F. It is not stated exactly which charge reversal mutations are used (arginine, lysine, aspartate, glutamate?).
The details of the mutants are shown in Appendix Table 2: The HBR1+2 mutant contains 6 charge reversal mutations: D201R, D205R, E310R (HBR1) and E332R, D333R, E336R (HBR2). H2A-H2B contains the NBR1+2 mutations. The most definitive 'validation' is shown in Figure 3F, lane 3: The charged interfacial residues are 'swapped' and the Nap1 mutant carrying the mutations D201R, D205R, E310R, E332, D333, E336 (HBR1+2), which can not bind to wild-type H2A-H2B (lane 2), now binds to the H2A-H2B variant containing the R30E, R33E, R36E, K37E, K75E, R78E, R82E (NBR1+2) mutations. To clarify this, we have added the following to the discussion section (p.14, top paragraph): A rigorous test for the specific yNap1–H2A–H2B interaction comes from a charge swap experiment: Replacement of the basic HBR1+2 interface in yNap1 with cationic residues abolishes binding to H2A–H2B. Also replacement of the basic NBR1+2 residues of H2A with acidic residues abolishes binding to yNap1. Binding is restored when such mutants are tested against each other (Figure 3F).

10) Figure 3. Were the mutants tested for structural integrity? Whilst the surface point mutations are probably accommodated fine, it may not be the case for multiple mutations. Indeed, in the discussion the authors state about D'Arcy et al 2013 "the reported mutations are located in the buried segments in the helical underside of yNap1 and most likely indirectly interfere with H2A-H2B binding by affecting the structural integrity of yNap1.". Were the mutations presented in this manuscript checked for structural integrity?
All Nap1 mutants analyzed here are in surface exposed residues and were purified using GST

affinity purification and size exclusion chromatography. All mutants maintain structural integrity as judged from their size exclusion chromatography profiles. This biochemical result was further validated by computational verification of protein stability upon mutation by using DUET (Pires et al, 2014).

11) Figure 4 seems to be somewhat of a repetition of Figure 2.
Figure 4 shows the details of the oligomerization interfaces and a schematic cartoon of the oligomer. We have been asked to supply such a figure to help the reader to understand the (non-trivial) oligomerization mode.

12) Figure 5 - Cryo-EM. The Nap1-H2A-H2B structure observed by cryoEM does appear to show similarity to crystallography asymmetric unit. However, it is presented rather poorly and it is difficult for the reader to judge.
As indicated, the data represent a negative–stain single particle EM reconstruction (not CryoEM) of the yNap1–H2A–H2B particle. The purpose here is to show that the particle obtained in EM is identical to the particle seen in the crystallographic asymmetric unit. We have increased the size of these panels to help the reader better judge these data.

13) Native mass spectrometry. Although being called "native", native mass spectrometry is still far from an ideal method in determining the components of complexes that are present in solution. Protein complexes need to be vaporized for analysis, and the extent to which this affects the integrity of the complex is difficult to assess. The native mass spectrometry is the only technique that is used to address the stoichiometry of the Nap1-H2A-H2B oligomer. This question could have been addressed using much simpler methods.
None of the 'simpler' ensemble averaging techniques that were used previously resulted in an unambiguous answer (Andrews et al, 2008; Chang et al, 1997; D'Arcy et al, 2013; Fujii-Nakata et al, 1992; Ishimi et al, 1984; Ishimi et al, 1983; McBryant & Peersen, 2004; Mosammaparast et al, 2002; Newman et al, 2012; Noda et al, 2011; Park et al, 2008; Toth et al, 2005). In part, the problem stems from the fact that these 'simpler' methods are frequently too imprecise especially with such dynamic, self-assembling systems. Native mass spectrometry affords unparalleled mass accuracy and enables unambiguous determination of protein stoichiometry. However, we do not only use native MS to address the stoichiometry: The xray structure provides the most definitive confirmation of the observed stoichiometry and is nicely supported by (numerous) native MS and mutagenesis experiments.

14) Page 9-10. The authors state: "Previous analysis by ensemble averaging techniques such as size exclusion chromatography, sucrose gradients, analytical ultracentrifugation and small angle x-ray scattering did not converge on a unique solution for Nap1 oligomerization and histone binding stoichiometry". Have the authors considered that there may not be a unique solution for Nap1 oligomerisation and histone binding stoichiometry, and that what their crystal structure represents is a single trapped intermediate from a more complex and dynamic system?

We agree with the reviewer that Nap1 oligomerization is quite dynamic and that is probably why crystallization has presented such a challenge. These properties of Nap1 are remarkably similar to that of ATP-independent molecular chaperones such as small heat shock proteins (sHSP), that typically form dynamic assemblies with cargo proteins and populate ensembles of inter–converting oligomeric states at equilibrium. Not unlike sHSPs, we predict that yNap1 does not form a discreet oligomer *in vivo* but rather populates a continuum of oligomeric states depending on the concentration of free H2A–H2B and Nap1. We agree with the scenario that the crystal structure represents a single trapped intermediate. However the same particle is also seen in EM and thus represents a significant fraction of the oligomer in solution. The xray structure together with the EM imaging indicate that the oligomer becomes self-limiting which arises due to the interlayer contacts upon oligomerization of the yNap1-H2A-H2B complex. That is, our data supports the notion that, while the assembly is quite dynamic, there is a preferred oligomerization mode/state.

15) Figure 5D-I. It is not clear what ratio of H2A-H2B to yNap1 was used for the experiments shown. If only one H2A-H2B dimer to one yNAp1 dimer was added, then one wouldn't expect to see two H2A-H2B dimers bound per yNap1 dimer. I think this point hits on a greater theme that runs throughout the manuscript in that the authors cannot sufficiently rule out that when histone H2A-H2B dimers are in excess of the yNap1, then a single yNap1 dimer may accommodate two H2A-H2B

dimers, as has been characterised previously (1,2). Indeed, in the H2A-H2B titration experiments (Figure S4A), the titration stops at a ratio of one H2a-H2B dimer per yNap1 dimer. This should be very easy to test.
Indeed as shown in Table EV2, the samples were typically mixed at an equimolar ratio. If the preferred binding mode would have been two histone H2A-H2B dimers/yNap1 dimer, one would have expected to see a distribution of masses with the centroid containing two H2A-H2B dimers/Nap1 dimer at equilibrium. However only one H2A-H2B/yNap1 dimer is seen. To allay the concerns of the reviewer, we have added excess of H2A-H2B to Nap1 and verified these preparations by native MS. Most samples did not spray well, presumably because of the lack of available H2A-H2B binding sites on Nap1. With one sample, Nap1FL, we observed unconventional complexes with an additional histone pair bound to each subcomplex when histones were in excess (see figure). We found that an extra copy of weakly bound H2A-H2B can be obtained when histones are in excess. However, the stoichiometry of these complexes is not two H2A-H2B dimers per Nap1 dimer as would be expected according to D'Arcy et al. 2013. We observed that this extra H2A-H2B copy is easily dissociated upon sample dilution and therefore our interpretation is that this extra copy arises through non-specific binding. Overall, the crystal structure in combination with the mutagenesis (indicating the validity of the various interfaces) and mass spectrometry provides sufficient validation of the nature of the Nap1-H2A-H2B structure, its oligomerization mode and stoichiometry.

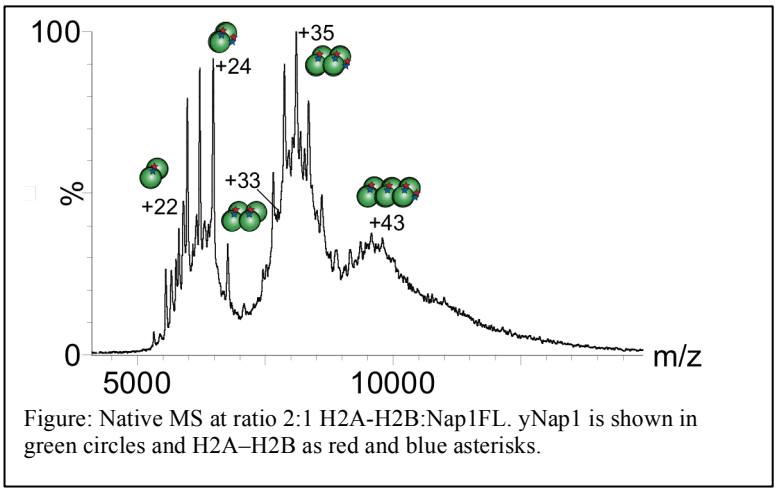

Figure: Native MS at ratio 2:1 H2A-H2B:Nap1FL. yNap1 is shown in green circles and H2A–H2B as red and blue asterisks.

16) In the Materials and Methods the authors state that "reconstituted H2A-H2B dimers were added at a twofold molar excess to Nap1. The mixture was incubated for 30 minutes on ice and then loaded on a pre-equilibrated Superose 6 10/300 GL (GE Healthcare) in buffer 2.". If the solution state of the Nap1-H2A-H2B complex is one yNap1 dimer to one H2A-H2B dimer, then a molar excess of freely eluting H2A-H2B dimers should have been identified during gel filtration chromatography. Would this not be further evidence for the stoichiometry of the crystalized complex
As shown previously by others (eg.) yNAP-1 alone or in complex with H2A-H2B elutes as a broad peak during gel filtration chromatography (Park et al, 2008). It is difficult to determine the stoichiometry from such data. It is not clear to us how one could have rigorously deduced the stoichiometry of the Nap1-H2A-H2B complex from the freely eluting H2A-H2B dimers.

17) The reference that Park and Luger 2008 shows yNAP-1 eluting in a broad peak, alone in or complex with H2A-H2B (page 8), is wrongly cited. There is no size exclusion chromatography presented in Park and Luger 2008. Park et al 2008 (6) present size exclusion data showing that the elution of yNAP1 is dependent on ionic strength and pH (Figure 3), and that oligomerisation is dependent on a specific hairpin structure. McBryant et al 2003 (9) show a relatively discrete elution peak for both free yNAP1 and yNAP1 in complex with H2A-H2B (Figure 1).
We thank the reviewer for pointing this out: We have included the proper citation (Park et al, 2008).

18) Figure 6. The complementation of Nap1 in the MNase experiments is lacking important controls. Namely the authors do not show that levels of the Nap1 mutants expressed from plasmids are at the same level as the wild-type expressed from the plasmid, or even if the mutants are expressed and correctly folded at all. Without this it is impossible to interpret data.
The expression controls are shown in Figure S5J. We have purified all the mutants that were used in this experiment: HBR1, HBR2, HBR1+2 and IF1 mutants. Structural integrity is maintained as

judged by size exclusion chromatography and further validated by computational verification of protein stability by using DUET (Pires et al, 2014).

19) Nap1 has also been shown to be stably associated with the evolutionary conserved histone H2A.Z variant(10-12). Are the Nap1 binding regions identified in the crystal structure conserved in the H2A.Z variant? No mention of this is found in the manuscript.

The binding interfaces are conserved. We have added the following sentence to refer to these data (p12, bottom): 'The DNA binding surfaces are conserved in other H2A variants such as H2A.Z, and therefore our structure also explains why Nap1 can assist remodeling complexes such as SWR1 in incorporating H2A.Z into chromatin (Kobor et al, 2004; Luk et al, 2007; Mizuguchi et al, 2004; Park et al, 2005).'

20) There is too much speculative discussion about the role of Nap1 in histone transport for a manuscript that does not address this aspect of Nap1 function.

21) The model represented in Figure 7 is highly speculative and draws mostly on results published elsewhere and not presented in the current manuscript.
We address comments 20+21 together: While we do not investigate the role of Nap1 in histone transport directly, our structural result shows that Nap1–H2A–H2B oligomerization results in burial of surfaces required for deposition of H2A–H2B. The observation that mutagenesis of the oligomerization interface results in strong nucleosome assembly defects indicates a dominant negative effect and suggests that oligomerization is an important aspect of yNap1 function (Figure 6). We therefore suggest that oligomerization provides an additional function, presumably histone transport or storage. The model presented in Figure 7 draws on these observation and also on research published elsewhere. Does the reviewer suggest that we should not put your findings into the context of data published previously? To accommodate the reviewer, we have defined more clearly which aspects of the model are based on available data and which aspects are more speculative. We hope the model and the accompanying data will help shape future discussions on NAP1 function and mechanism. While we agree that the model is speculative, some speculation surely should be permitted in the discussion section.

22) There is a section of ITC measurements in the materials and methods, but no mention of ITC in the rest of the manuscript.
It has been expunged.

Minor points
1) Page 4. "molecular determinants of it role histone chaperoning" should read "molecular determinants of it role in histone chaperoning".
Fixed

References

1. Andrews, A.J., Chen, X., Zevin, A., Stargell, L.A. and Luger, K. (2010) The Histone Chaperone Nap1 Promotes Nucleosome Assembly by Eliminating Nonnucleosomal Histone DNA Interactions. Molecular Cell, 37, 834-842.
2. Andrews, A.J., Downing, G., Brown, K., Park, Y.J. and Luger, K. (2008) A thermodynamic model for Nap1-histone interactions. Journal of Biological Chemistry, 283, 32412-32418.
3. D'Arcy, S., Martin, K.W., Panchenko, T., Chen, X., Bergeron, S., Stargell, L.A., Black, B.E. and Luger, K. (2013) Chaperone Nap1 shields histone surfaces used in a nucleosome and can put H2A-H2B in an unconventional tetrameric form. Mol Cell, 51, 662-677.
4. Toth, K.F., Mazurkiewicz, J. and Rippe, K. (2005) Association states of nucleosome assembly protein 1 and its complexes with histones. Journal of Biological Chemistry, 280, 15690-15699.
5. Newman, E.R., Kneale, G.G., Ravelli, R.B., Karuppasamy, M., Karimi Nejadasl, F., Taylor, I.A. and McGeehan, J.E. (2012) Large multimeric assemblies of nucleosome assembly protein and histones revealed by small-angle X-ray scattering and electron microscopy. J Biol Chem, 287, 26657-26665.
6. Park, Y.J., McBryant, S.J. and Luger, K. (2008) A beta-hairpin Comprising the Nuclear Localization Sequence Sustains the Self-associated States of Nucleosome Assembly Protein 1. Journal of Molecular Biology, 375, 1076-1085.

7. McBryant, S.J., Park, Y.J., Abernathy, S.M., Laybourn, P.J., Nyborg, J.K. and Luger, K. (2003) Preferential binding of the histone (H3-H4)2 tetramer by NAP1 is mediated by the amino-terminal histone tails. J Biol Chem, 278, 44574-44583.
8. Bowman, A., Hammond, C.M., Stirling, A., Ward, R., Shang, W., El-Mkami, H., Robinson, D.A., Svergun, D.I., Norman, D.G. and Owen-Hughes, T. (2014) The histone chaperones Vps75 and Nap1 form ring-like, tetrameric structures in solution. Nucleic Acids Res, 42, 6038-6051.
9. McBryant, S.J., Park, Y.J., Abernathy, S.M., Laybourn, P.J., Nyborg, J.K. and Luger, K. (2003) Preferential Binding of the Histone (H3-H4)2 Tetramer by NAP1 Is Mediated by the Amino-terminal Histone Tails. Journal of Biological Chemistry, 278, 44574-44583.
10. Kobor, M.S., Venkatasubrahmanyam, S., Meneghini, M.D., Gin, J.W., Jennings, J.L., Link, A.J., Madhani, H.D. and Rine, J. (2004) A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol, 2, E131.
11. Luk, E., Vu, N.D., Patteson, K., Mizuguchi, G., Wu, W.H., Ranjan, A., Backus, J., Sen, S., Lewis, M., Bai, Y. et al. (2007) Chz1, a nuclear chaperone for histone H2AZ. Mol Cell, 25, 357-368.
12. Mizuguchi, G., Shen, X., Landry, J., Wu, W.H., Sen, S. and Wu, C. (2004) ATP-Driven Exchange of Histone H2AZ Variant Catalyzed by SWR1 Chromatin Remodeling Complex. Science, 303, 343-348.

Referee #3:

The manuscript by Aguilar-Gurrieri investigates the structural and biochemical properties of NAP1, a histone chaperones that favors nucleosome assembly by promoting deposition of H2A-H2B dimer(s) onto the H3-H4 tetramer as last step in the process. The study involves several complementary and experimentally demanding approaches that lead a mechanistic model for NAP1 function. Key elements are the stoichiometry of the complex (1 NAP1 dimer/1 H2A-H2B dimer), the involvement of the H2A DNA-binding regions in association to NAP1, and the propensity of the complex to oligomerise, a feature which is suggested to be essential for function.
This is certainly an interesting topic, of great relevance in chromatin structure and molecular biology. The reported data also help to clarify inconsistencies present in the literature. However, I found this manuscript rather difficult to review as the presentation often lacks clarity, with statements that sometime apparently contract each other and tend to be difficult for the reader. The manuscript is long and would greatly benefit from shortening and a more concise and focused presentation.

Main points

-First paragraph of the results. Here, there is an example for the above comment:
"The distance between each band corresponds to the distance between the most distal nucleosome ends within an MNase-cleaved array of an integral number of nucleosomes. ..... Consequently, the interband distance corresponds to a population average spacing between adjacent nucleosome midpoints."
The distance between each band is first stated to correspond to the distance between nucleosomal ends and, later, it is stated to correspond to the distance between nucleosome midpoints. I think the reader can be easily lost at this point.
They mean the same thing, but we see how this can be confusing, and so we have now deleted the word “midpoints” (p. 4; first paragraph result section)

-Two paragraphs later, "MNase digestions indicate that the nucleosomal arrays maintained wild-type spacing (or ~165 bp inter-band distance), indicating that nucleosome spacing in bulk chromatin was generally unaltered in the nap1Δ strain."
However, Figure 1A shows that WT spacing is 160 bp, 150 bp, 152 bp (170, 330, 480, 632 size) whereas the mutant spacing seems to be partly different as it is 160, 150, and 125 bp (145, 305, 455, 570 size). The explanatory sentence "We interpret the fixed size reduction as follows: MNase is expected to cleave more frequently where linkers are longer, and this is expected to occur in the nap1Δ strain where H2A-H2B is lost (i.e., linker DNA plus the nucleosomal DNA that is vacated by H2A-H2B). This produces a fixed size reduction per array since nucleosome spacing is unaltered" is hard to understand. At the very least, some rewriting is needed together with a more careful description and analysis of the data. In particular, it is hard to understand how smaller

mononucleosome-sized MNase fragments (145 bp versus 170 bp; previous paragraph in the main text) cannot affect inter-band distances (if this is really the case and if interband distance is interpreted as "distancebetween nucleosome midpoints").
We now revised the text to explain this more clearly. In short, we believe that the spacing is derived from the full nucleosomes, with the relatively rare dissociation of H2A-H2B providing location for MNase cleavage. (p. 4-p.5)

-Likewise, the subsequent paragraph of this section is difficult to follow. The sentence "In these single-read sequencing experiments, MNase fragment size was deduced by pairing adjacent peaks located on opposite strands and in the 3' direction." is unclear. By analogy, the concept of fragment having fuzzier locations is rather obscure and should explained together with an improved description of Figure 1C.
"MNase fragments" were intended to describe full nucleosomes and subnucleosomes, using a more technically-precise term. However, we see how this can be unclear, and thus have changed the wording to reflect nucleosomes/subnucleosomes and putative hexasomes (p. 5; middle paragraph).

-Page 9, analysis of oligomers. This section and associated figures are difficult to follow. "Protomers A and B (A' and B' in layer 2) are related by a ~102° rotation and ~8 Å displacement along the central axis (Figure 4A). Two non- crystallographic dyads relate protomer C to A and B in each layer, but because of displacement along the central axis, protomers C and C' pack through alternate protein interfaces: protomer C is located below the plane of protomers A and B whereas protomer C' is located above A' and B'. "
How can it be that a 102° rotation superimposes A to B with both A and B related by 180° rotations to C? This violates symmetry rules. If A to C is 180° and C to B is 180°, A and B must have the same orientation.
It does not violate symmetry rule, as each Nap1-H2A-H2B protomer is asymmetric, carrying the histone H2A-H2B heterodimer below or above the plane of the Nap1 dimer. This is illustrated in the cartoon in Figure 4A (the blue squares (H2A-H2B) are above or below the yellow/green squares (yNap1 dimers)). Eg. protomer A and B in each layer has the H2A-H2B dimer on the same face of the Nap1-H2A-H2B protomer, related by a ~102° rotation. Promoter C is oriented such that the H2A-H2B dimer is located on the opposite face of the Nap1-H2A-H2B protomer such that it is related by a 180° rotation to either A or B. The symmetry of the particle is non-trivial and therefore we have attempted to help the reader to understand the oligomerization mode by drawing the cartoon shown in Figure 4A. However, it has not been easy to reduce the 3D model into a 2D cartoon and we apologize that we have not found a more straightforward/elegant way how to illustrate the oligomerization mode.

-Similarly, I cannot see the pseudohelical arrangement in Figure 4C. Presentation must be improved. If it is helical, why there is only a hexamer in cryoEM and crystallization? Why does the helix not propagate further? Is the asymmetry of the oligomer preventing extension of the helical assembly?
There is no helical symmetry that relates these protomers. The oligomer is indeed asymmetric causing the oligomerization to be self-limiting and thus preventing filament formation: Self-limitation arises as the two layers of the oligomer are held together by TWO IF2 (between the histones H2A) interlayer contacts. These contacts occur between the A -A' and B-B' protomers. The contact to the next crystallographic unit cell is mediated by a SINGLE IF2 contact mediated by C or C'. That is, two IF2 contacts pointing inwards (stabilizing packing of layers 1+2) whereas only one pointing outwards. This feature explains the presence of particles rather than filaments in EM. We have tried to illustrate this in Figure 4F where the IF2 contacts are illustrated with arrows. The IF2 contacts are through histones, accounting for increased oligomerization when histones bind.

-To prove the significance of this packing interface, one would expect a more quantitative analysis. For instance, is the extent of the inter-complex surfaces compatible with those expected for oligomers (e.g. PISA analysis)? Gel-filtration or SAXS data are probably expected by the reader.
We have extensively analysed the complex by PISA to understand the inter-protomer interfaces that drive assembly and have designed our mutational analysis around these results. The size of the buried area surface of the HBR1 (Nap1-H2A) interfaces (~455Å^2 each) is in a similar range to that seen in the IF2 oligomerisation interfaces (H2A–H2A; ~460 Å^2). This is followed by the Nap1-Nap1 IF1 oligomerisation interface (~446 Å^2) and the HBR2 interface (Nap1-H2A; ~155-266 Å^2). Thus a series of small binding interfaces drive Nap1-H2A-H2B binding and oligomerisation. While

these interfaces in isolation are relatively small, the combined surface area buried is large (presumably accounting for the high H2A-H2B binding avidity). None of the ensemble averaging techniques used previously (including the ones suggested by the reviewer (SAXS/Gel filtration), are suitable for the analysis of such dynamic oligomers and have already been used in the past (Andrews et al, 2008; Chang et al, 1997; D'Arcy et al, 2013; Fujii-Nakata et al, 1992; Ishimi et al, 1984; Ishimi et al, 1983; McBryant & Peersen, 2004; Mosammaparast et al, 2002; Newman et al, 2012; Noda et al, 2011; Park et al, 2008; Toth et al, 2005). None of them has resulted in an unambiguous result.

-Figure 4C, materials ("Fitting of the crystal structure into the EM map was done with Chimera") and associated main text: one would like to see a more quantitative analysis of the fitting of the model to the cryoEM maps.
The data shown represent a negative–stain single particle EM reconstruction (not CryoEM) of the yNap1–H2A–H2B particle. Compared to CryoEM, the resolution of the reconstruction is low (~35Å). Therefore we used the Chimera function 'Fit in map' to model the coordinates into the negative stain EM map. Considering the low resolution of the negative stain reconstruction, we do not attempt a more quantitative analysis. Visual inspection already illustrates that the fit is reasonable. We have increased the size of panel Figure 5C to better show these data.

-The discussion about the role of oligomerization in protein function and nuclear export. It is proposed that isolated Nap1 oligomerises, shielding the NLS sequence. NLS is, however, fully accessible in the Nap1-H2A-H2B complex as shown by the crystal structure. At the same time, residues involved in oligomerisation of the complex are shown to be essential for function. Is it proposed that Nap1 exists in different oligomeric forms depending on whether unbound or bound to H2A-H2B? If this is the case, one would expect some experimental proof.

Experimental proof for the different modes of oligomerization is available: Park et al have shown that isolated Nap1 oligomerises (as shown by a crystal structure and mutagenesis) through the region involving the proposed NLS (Park et al, 2008). We show here that a different oligomer arises upon histone H2A-H2B binding. Thus, whereas in the histone-bound form the proposed NLS is accessible in the unbound form it is not. We feel that it is important to clarify which aspects of he proposed model are based on data and which aspects are more speculative. Eg. while there is experimental evidence for function of the NES (Miyaji-Yamaguchi et al, 2003; Mosammaparast et al, 2002), the NLS has been deduced from sequence analysis and never been experimentally validated. To clarify, we state on p. 15:' Future studies need to experimentally demonstrate the role of the NLS and how defective oligomerization or histone loading interferes with the yNap1 functional cycle.' We believe that the mutants derived from our work will allow investigation of NAP1 function and the role of oligomerization in nuclear transport in the future.

-Finally, possibly the most critical point:
Page 11: "As oligomerization of the yNap12-H2A-H2B complex results in burial of a segment that is required for docking onto H3-H4 tetrasomes, we argue that it is inhibitory for H2A-H2B deposition and nucleosome formation" and Page 16 (end of the manuscript): "As the histone-bound oligomer is inhibitory for H2A-H2B deposition,...."
Page 12: "The yNap1c and yNap1_IF1 mutants are not affected in this nucleosome assembly assay indicating that the flexible N- and C-terminal regions and oligomerization of yNap1 are not essential for H2A-H2B deposition (Figure S5C-D)"
PGE 13: "Surprisingly, the IF1 mutant, which showed only modest impact on H2A-H2B binding in vitro, showed the greatest defect on nucleosome assembly."

These statements are partly contradictory. In the general, these data indicate that oligomerisation is not inhibitory on the biochemical process. Rather, the explanation exist that it is not matter of oligomerisation. It can well be that the IF1 surface residues affect protein-protein interactions essential, for instance, for import-export or functions in the chromatin. An effect that has little to do with oligomerisation, which might simply result from promiscuous tendency to associate by these residues.

Our analysis shows that yNap1 oligomerization is quite dynamic especially in the presence of H2A-H2B. These properties of yNap1 are similar to that of ATP-independent molecular chaperones such as small heat shock proteins (sHSP), that typically form dynamic assemblies with cargo proteins and

populate ensembles of inter–converting oligomeric states at equilibrium. We attribute the lack of a strong (gain-of-function) phenotype of the IF1 mutant in the in vitro assays to the fact that a fraction of yNap1 is not oligomerized to begin with and will be competent to deposit H2A-H2B (eg. see native MS results in Table EV2). However the strong phenotype with the IF1 mutant in cells clearly indicates that this oligomerization interface provides an important function during the yNap1 functional cycle. Considering that oligomerization results in burial of interfaces required for H2A-H2B deposition, we suggest that oligomerization provides an additional function to Nap1 (in addition to the nucleosome assembly activity). This additional function is probably histone transport or storage, aspects that we can not easily address with our assays.

We agree however with the reviewer in that these observations at first appear contradictory. We therefore state on p15; bottom paragraph: 'This observation at first seems counterintuitive: As the histone–bound oligomer is inhibitory for H2A–H2B deposition, mutagenesis of the IF1 interface might have been expected to result in a gain–of–function phenotype. One plausible interpretation is that the yNap1_IF1 mutant acts as a dominant negative by sequestering H2A–H2B from other chaperones or by interfering with nucleocytoplasmic transport thus causing the severe assembly defect observed here.'

References

Andrews AJ, Chen X, Zevin A, Stargell LA, Luger K (2010) The histone chaperone Nap1 promotes nucleosome assembly by eliminating nonnucleosomal histone DNA interactions. Mol Cell **37:** 834-842

Andrews AJ, Downing G, Brown K, Park YJ, Luger K (2008) A thermodynamic model for Nap1-histone interactions. J Biol Chem **283:** 32412-32418

Chang L, Loranger SS, Mizzen C, Ernst SG, Allis CD, Annunziato AT (1997) Histones in transit: cytosolic histone complexes and diacetylation of H4 during nucleosome assembly in human cells. Biochemistry (Mosc) **36:** 469-480

D'Arcy S, Martin KW, Panchenko T, Chen X, Bergeron S, Stargell LA, Black BE, Luger K (2013) Chaperone Nap1 shields histone surfaces used in a nucleosome and can put H2A-H2B in an unconventional tetrameric form. Mol Cell **51:** 662-677

Fujii-Nakata T, Ishimi Y, Okuda A, Kikuchi A (1992) Functional analysis of nucleosome assembly protein, NAP-1. The negatively charged COOH-terminal region is not necessary for the intrinsic assembly activity. J Biol Chem **267:** 20980-20986

Ishimi Y, Hirosumi J, Sato W, Sugasawa K, Yokota S, Hanaoka F, Yamada M (1984) Purification and initial characterization of a protein which facilitates assembly of nucleosome-like structure from mammalian cells. Eur J Biochem **142:** 431-439

Ishimi Y, Yasuda H, Hirosumi J, Hanaoka F, Yamada M (1983) A protein which facilitates assembly of nucleosome-like structures in vitro in mammalian cells. J Biochem **94:** 735-744

Kobor MS, Venkatasubrahmanyam S, Meneghini MD, Gin JW, Jennings JL, Link AJ, Madhani HD, Rine J (2004) A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS biology **2:** E131

Loyola A, Bonaldi T, Roche D, Imhof A, Almouzni G (2006) PTMs on H3 variants before chromatin assembly potentiate their final epigenetic state. Mol Cell **24:** 309-316

Luk E, Vu ND, Patteson K, Mizuguchi G, Wu WH, Ranjan A, Backus J, Sen S, Lewis M, Bai Y, Wu C (2007) Chz1, a nuclear chaperone for histone H2AZ. Mol Cell **25:** 357-368

McBryant SJ, Peersen OB (2004) Self-association of the yeast nucleosome assembly protein 1. Biochemistry (Mosc) **43:** 10592-10599

Miyaji-Yamaguchi M, Kato K, Nakano R, Akashi T, Kikuchi A, Nagata K (2003) Involvement of nucleocytoplasmic shuttling of yeast Nap1 in mitotic progression. Mol Cell Biol **23:** 6672-6684

Mizuguchi G, Shen X, Landry J, Wu WH, Sen S, Wu C (2004) ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science **303:** 343-348

Mosammaparast N, Ewart CS, Pemberton LF (2002) A role for nucleosome assembly protein 1 in the nuclear transport of histones H2A and H2B. EMBO J **21:** 6527-6538

Newman ER, Kneale GG, Ravelli RB, Karuppasamy M, Karimi Nejadasl F, Taylor IA, McGeehan JE (2012) Large multimeric assemblies of nucleosome assembly protein and histones revealed by small-angle X-ray scattering and electron microscopy. J Biol Chem **287:** 26657-26665

Noda M, Uchiyama S, McKay AR, Morimoto A, Misawa S, Yoshida A, Shimahara H, Takinowaki H, Nakamura S, Kobayashi Y, Matsunaga S, Ohkubo T, Robinson CV, Fukui K (2011) Assembly states of the nucleosome assembly protein 1 (NAP-1) revealed by sedimentation velocity and non-denaturing MS. Biochem J **436:** 101-112

Park YJ, Chodaparambil JV, Bao Y, McBryant SJ, Luger K (2005) Nucleosome assembly protein 1 exchanges histone H2A-H2B dimers and assists nucleosome sliding. J Biol Chem **280:** 1817-1825

Park YJ, McBryant SJ, Luger K (2008) A beta-hairpin comprising the nuclear localization sequence sustains the self-associated states of nucleosome assembly protein 1. J Mol Biol **375:** 1076-1085

Pires DE, Ascher DB, Blundell TL (2014) DUET: a server for predicting effects of mutations on protein stability using an integrated computational approach. Nucleic Acids Res **42:** W314-319

Toth KF, Mazurkiewicz J, Rippe K (2005) Association states of nucleosome assembly protein 1 and its complexes with histones. J Biol Chem **280:** 15690-15699

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