THE EMBO JOURNAL

# Frozen-hydrated chromatin from metaphase chromosomes has an interdigitated multilayer structure

Andrea Chicano, Eva Crosas, Joaquín Otón, Roberto Melero, Benjamin D Engel and Joan-Ramon Daban.

**Review timeline:**

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|---|---|
| Submission date: | 4th May 2018 |
| Editorial Decision: | 9th May 2018 |
| Authors' correspondance | 9th May 2018 |
| Revision received: | 11th May 2018 |
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Editor: Anne Nielsen

## 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 9th May 2018

Thank you for submitting your manuscript to The EMBO journal. I have now read your study carefully and discussed the work with other members of the editorial team. I am afraid the outcome of these discussions is that we have decided not to pursue publication of this manuscript.

We appreciate that you extend your previous work on the formation of planar structures in metaphase chromosomes to samples that are frozen in vitreous ice rather than immobilized on a carbon grid. You are thus able to use cryo-ET and SAXS to visualize planar stacks and the possible interdigitation of nucleosomes. However, while your study thereby adds to the ongoing discussion of higher-orger chromosome organisation we are concerned that the notion of a planar organisation had already been put forward in previous papers from your lab. We recognise that the current study extends this principle to cryoET of frozen-hydrated chromosomes but while your findings are thus certain to raise interest within the field, your manuscript does not in our view provide the level of compelling conceptual and functional advance that we have to require for papers published in The EMBO Journal. I am therefore sorry to say that we have decided not to send it out for peer-review.

The EMBO Journal is only able to publish a small percentage of the many manuscripts submitted, and we can only subject those manuscripts to external review that have a high probability of timely publication. Thank you for giving us the opportunity to consider this manuscript. I regret that we have to disappoint you on this occasion.

 

Authors' correspondance 9th May 2018

A significant motivation to submit our work to The EMBO journal was the fact that previously Nishino et al (ref 15 in our manuscript) published a paper in this journal, in which using exclusively Synchrotron SAXS they concluded that chromatin in metaphase chromosomes consists of irregularly folded nucleosome fibers. Nishino el al. interpreted the dominant scattering peak at 6 nm as face-to-face nucleosome interactions in irregularly folded nucleosome fibers. Using the same technique we have observed also a dominant 6-nm scattering peak in different structuring conditions, but according to our cryo-ET results, necessarily our interpretation has to be different; the face-to-face nucleosome interactions take place between nucleosomes ordered in interdigitating stacked layers of chromatin. The model of Nishino et al. is completely different from the multilayer organization demonstrated in our work using cryo-ET, which is the best technique available at present to study large and complex structures. In our work, cryo-ET has been successfully applied using the state-of-the-art technology developed in the best platforms in Europe.

I am sending you this information because I think that many scientists are interested now in the difficult problem of the 3D organization of chromatin within chromosomes, and probably many of them consider that the interpretation of Nishino et al. is definitive because it was published in the respected EMBO journal. I would appreciate very much if on the basis of this information you can reconsider your editorial decision. We would like to have the opportunity to publish our advanced research with respect to the work of Nishino et al. in The EMBO journal.

Thank you very much for your consideration.

2nd Editorial Decision 5th July 2018

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, the referees express interest in the findings reported in your manuscript but also raise a number of concerns that you will have to address before they can support publication here. In particular, while referee #1 asks for relatively minor clarifications, referees #2 and #3 are more critical and find that both resolution and functional/physiological relevance will have to be significantly improved. Given the discrepancy in these recommendations, I've consulted with an additional arbitrating advisor. This person agreed with ref #1 and #2 on the importance of the proposed model but also emphasised ref #2's point that an improved resolution is needed to convincingly support this model.

With the regard to the functional relevance of the observations here, I appreciate that several comparable studies in the field have also been based on in vitro analysis. I will therefore be willing to partly overrule on the need for in vivo validation but I will ask you to clearly state the limitations of the current approach and discuss your model more extensively in the context of the existing literature.

Given the recommendations from the referees and the arbitrating advisor, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers.

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REFEREE REPORTS

Referee #1:

The authors made use of cryoEM and SAXS methods and report that metaphase chromosomes form planar structures consisting of stacked nucleosomes. The quality of the data is very good and supports a planar model of chromosome organization. Application of SAXS and CryoEM to isolated

 

chromosomes is a good step toward understanding chromatin structural organization. I recommend this paper for publication after minor revision.

• Do the authors see the sheets oriented almost horizontally? Would they even be visible in cryo-EM? One would imagine that a more flat orientation would be preferred in a thin layer of ice on a cryo grid. If there are horizontal sheets - please, add a few sentences describing those. Even if not, it may be worth mentioning in the text.
• If the authors looked at the coordinates and orientations of the nucleosomes in the tomograms containing compact plates, did they see that the nucleosomes are positioned similarly to the proposed model (Figure 6)? It would be good if the authors added a panel where they show such nucleosome "lattice-maps".
• Fig 3C: If there were any other positions on the grid where the authors have found large structures formed by stacked layers, it would be nice to see several examples in Figure 3.
• The speculation "Therefore, the left (L) and right (R) regions of the multilayer structures shown in Fig 3C may correspond to a group of stacked layers of two sister chromatids that broke apart during the preparation and deposition procedures. " should be omitted. It seems more likely that the two "wings" of that structure are simply parts of the same chromatid disturbed by the preparation.
• Did the authors try focused refinement of the structures shown in the Figure 2F? It should be possible to get a better map of the nucleosome - comparable to the EM map shown in panel G of the Figure 2.
• In the methods section, please, add information about the masks used for subtomogram alignment and mention if the EM densities shown in the Figures EV2, EV3, Figure 2F,G were masked and what was the shape of the mask.
• Please, label the main peaks not only in the Figure 5B, but also in the panels A and C.

Referee #2:

In this manuscript authors look at metaphase chromosomes isolated from cells by cryo-ET. They observe that frozen hydrated chromatin from metaphase chromosomes is planar and forms multilayered plates. The study is important and timely and authors provide an interesting model about chromatin organization in metaphase chromosomes.

For a publication in EMBO I have two major concerns.

1) The model authors propose is very interesting, but I am not sure if it recapitulates in vivo conditions. It is possible that observed nucleosome organization might have been influenced by in vitro condition used to isolate chromatin. The authors should provide some kind of in vivo data that might support nucleosome organization they observe.

2) The resolution of the data is not great and single nucleosomes cannot be recognized in two interacting plates, weakening the significance of the manuscript. The authors should try to improve the resolution to see the nucleosomes in interacting plates. Current data for interdigitated model are weak and this would make the finding much more interesting.

If the authors could address one of the two concerns, I would support the publication in the EMBO journal.

Referee #3:

In this paper, the Chicano et al investigate the structure of denatured metaphase chromosomes using cryo electron tomography. They shear sheets of material from condensed isolated mitotic chromosomes. The cryo-ET images from this material shows thin, deformed sheets. Some sheets (relaxed plates) which exhibit nucleosome-like particles at the edges. Similarly, subtomogramm averaging of volume units within compact sheets show nucleosome-like shapes. The resulting data is interpreted that the sheets (or plates) are composed of tightly packed nucleosomes, that are tilted relative to the sheet plane. Further observations show multilayered plates, with layer separation of ~10 nm and a lateral expansion of roughly the dimension of a metaphase chromosome. Contacts between two layers appear thinner than expected from a direct layer stack. Interdigitation between

 

nucleosomes is proposed as a mechanism for these contact points. To further analyze the molecular chromatin properties, the authors apply synchrotron SAXS to whole metaphase chromosomes. The observed scattering plots reveal peaks corresponding to individual nucleosomes and their expected face-to-face stacking distance (6 nm) and no indication of the presence of 30-nm chromatin fibers. In conclusion, this combined cryoET and SAXS analysis thus demonstrates that plates obtained from isolated mitotic chromosomes correspond to extended sheets of stacked nucleosomes.
This study is an extension of previous extensive work from these authors on the nature of chromatin plates. The potential significance of the study can thus be assessed based on the added value of this study and the biological relevance of the discoveries. With regards to the advancements, the authors investigate the previously observed phenomena with cryo-ET (as opposed to TEM & AFM). As a main advancement they obtain sub-tomogram reconstructions that exhibit nucleosome-like particles from the plates. I am not sure if that qualifies as a significant advancement over previous data (see also comment below).
Secondly, and more importantly, with regards to the biological relevance: It is not at all clear that the chromatin structure corresponding to the observed plates represents a physiological state of chromatin. Based on the reported methods, the extracted chromosomes are extensively manipulated, sheared, dialyzed into various buffers, conditions which might induced non-native clustering of nucleosomes over time. At this point it would increase the significance of the study if the obtained models would be compared to Hi-C data from mitotic chromosomes, or corroborated with alternative less invasive methods.
With regards to the performed experiments: I was wondering how the sub-tomograms of the particles making up the plates were constructed. The authors write that 1243 particles were manually picked. From the figure 2a, structure P2, no particles can be discerned. It is thus unclear to me where these particle reconstructions come from and how they were obtained.
With these reservations, I believe that the study is too preliminary for acceptance in EMBO J.

2nd Revision - authors' response — 11th October 2018

**Frozen-hydrated chromatin from metaphase chromosomes has an interdigitated multilayer structure**
Andrea Chicano, Eva Crosas, Joaquín Otón, Roberto Melero, Benjamin D Engel, and Joan-Ramon Daban

**Response to the reviewers' comments**
**Reviewer #1:**
*The authors made use of cryoEM and SAXS methods and report that metaphase chromosomes form planar structures consisting of stacked nucleosomes. The quality of the data is very good and supports a planar model of chromosome organization. Application of SAXS and CryoEM to isolated chromosomes is a good step toward understanding chromatin structural organization.*

*I recommend this paper for publication after minor revision.*

**We thank this reviewer for the positive comments.**

• *Do the authors see the sheets oriented almost horizontally? Would they even be visible in cryo-EM? One would imagine that a more flat orientation would be preferred in a thin layer of ice on a cryo grid. If there are horizontal sheets - please, add a few sentences describing those. Even if not, it may be worth mentioning in the text.*

**The typical slices through the x-y plane (perpendicular to the electron beam) of the cryo-tomograms presented in Figs 1-4 show well-defined lines corresponding to plates oriented more or less perpendicular to this plane. The ice in our preparations is relatively thick (0.2-0.4 µm; page 3), and we do see plates adopting a variety of orientations. However, due to the tomographic missing-wedge (Lucic et al. 2005), plates approximately parallel to the x-y plane are not well-resolved, but those that are roughly perpendicular to the x-y plane can be easily analyzed following the successive slices of the tomograms. We have included this information in the revised version (Results, page 3).**

 

*• If the authors looked at the coordinates and orientations of the nucleosomes in the tomograms containing compact plates, did they see that the nucleosomes are positioned similarly to the proposed model (Figure 6)? It would be good if the authors added a panel where they show such nucleosome "lattice-maps".*

**Our data do not allow us to create lattice maps of nucleosomes within the plates. As explained in our response to reviewer #3, the subtomogram average of the compact plate in the original manuscript was incorrect and has been removed. The compact plates are so densely packed that we cannot detect the positions of individual nucleosomes within the plates, and thus cannot map their organization within the plates. The simplified drawing of two plates in contact shown in Fig 6 schematically illustrates the dimensions obtained from our cryo-ET and SAXS experiments. In the revised version of the manuscript, the orientation of the nucleosomes shown in this scheme is based on the corrected measurements of the plate thickness (see Results, page 4); these measurements have been adjusted for the CTF fringes at the plate borders caused by defocus (new Fig EV2). The corrected thickness is ~7.5 nm (new Table 1). Considering the dimensions of the nucleosome core particle (cylinder of 5.7 nm height and 11 nm diameter), the observed thickness suggests that plates consist of a monolayer of slightly tilted nucleosomes. As described in Fig 6: "Our results suggest that the two turns of the nucleosomal DNA are oriented slightly tilted with respect to the axis normal to the plate surface, but they may have diverse orientations (not represented in this scheme) with respect to the other two axes of the plate."**

*• Fig 3C: If there were any other positions on the grid where the authors have found large structures formed by stacked layers, it would be nice to see several examples in Figure 3.*

**In the revised version we have included a new figure (Fig EV3) showing five additional large structures with stacked layers.**

*• The speculation "Therefore, the left (L) and right (R) regions of the multilayer structures shown in Fig 3C may correspond to a group of stacked layers of two sister chromatids that broke apart during the preparation and deposition procedures. " should be omitted. It seems more likely that the two "wings" of that structure are simply parts of the same chromatid disturbed by the preparation.*

**The dimensions of the stacked layers presented in the new Fig EV3 (~0.6 µm) indicate that they could correspond to fragments of single chromatids, but in Fig 3C the dimensions of the stacked layers are approximately 0.6+0.6 µm. We understand that the reviewer considers that our sentence "Therefore, the left (L) and right (R) regions…" seems too speculative. However, since successive slices through the tomogram in Fig 3C show that there is some kind of association between the left and right regions, and sister chromatids are often associated laterally (see for instance the two upper chromosomes in Fig 1A), we think that as authors it is reasonable to speculate that they could belong to two sister chromatids. In the revised version (see page 5) we have indicated that the dimensions of the structures presented in Fig EV3 suggest that they could be fragmented parts of single chromatids. In the case of Fig 3C, to indicate clearly that we are speculating, we have written: "In the particular case of the multilayered structures in Fig 3C, since the left and right regions are apparently in contact, it is tempting to speculate that these two regions could correspond to stacked layers of two sister chromatids that broke apart during the preparation and deposition procedures.". In addition, we have removed the letters R and L in Fig 3C and in the legend of this figure.**

*• Did the authors try focused refinement of the structures shown in the Figure 2F? It should be possible to get a better map of the nucleosome - comparable to the EM map shown in panel G of the Figure 2.*

**We tried tighter masking to exclude the connecting density, but unfortunately it was not possible for us to obtain a better averaged structure for nucleosomes decorating distorted plates (Fig 2F). Furthermore, during the preparation of the revised manuscript, we discovered an error that had caused and incorrect average of the compact plate in Fig 2G. Therefore, we removed Fig 2G from the revised version. Subtomogram averaging now plays a smaller role in the manuscript, with the average in Fig 2F simply confirming that the decorating particles have a size that is consistent with nucleosomes. Nevertheless, the central finding of our study**

 

**remains unchanged: hydrated chromatin forms planar layers even when not adsorbed to a surface. We therefore sought to improve the findings in our revised manuscript by other analysis. As discussed above, the measurement of plate thickness has been corrected to account for the CTF fringes that border the plates (see new Figs EV2 and EV4). The corrected thickness of monolayers and two layers in close contact (see new Table 1 and Results, pages 4 and 5) has allowed us to propose the model in Fig 6, which unifies our cryo-ET and SAXS results, and is consistent with many observations in different laboratories about interdigitation and face-to-face nucleosome interactions in higher-order chromatin structures (see Discussion, pages 7 and 8).**

*• In the methods section, please, add information about the masks used for subtomogram alignment and mention if the EM densities shown in the Figures EV2, EV3, Figure 2F,G were masked and what was the shape of the mask.*

**We performed reference-free subtomogram averaging of nucleosomes decorating distorted plates. As indicated in Materials and Methods of the revised version (pages 10 and 11), we did not apply a focused mask, only a standard spherical mask about the same size as the box to prevent hard box edges from influencing the alignment. We started from a weighted average structure obtained from random orientations of all particles. Fig EV1 shows the averaged maps of 15 iterations without filtering. The final density map was filtered to 25 Å (Fig 2F) and fitted with the molecular structure of the nucleosome core particle: the top region has the size of a nucleosome core particle and the lower region corresponds to the link of the particle to the plate; due to the large box size (50 pixels), part of the plate associated with decorative nucleosomes is also seen in the average.**

*• Please, label the main peaks not only in the Figure 5B, but also in the panels A and C.*

**In the revised version of Fig 5, panels A and C have arrows indicating the same distances than in panel B.**

**Reviewer #2:**
*In this manuscript authors look at metaphase chromosomes isolated from cells by cryo-ET. They observe that frozen hydrated chromatin from metaphase chromosomes is planar and forms multilayered plates. The study is important and timely and authors provide an interesting model about chromatin organization in metaphase chromosomes.*

**We thank this reviewer for the positive comments.**

*For a publication in EMBO I have two major concerns.*

*1) The model authors propose is very interesting, but I am not sure if it recapitulates in vivo conditions. It is possible that observed nucleosome organization might have been influenced by in vitro condition used to isolate chromatin. The authors should provide some kind of in vivo data that might support nucleosome organization they observe.*

**Under metaphase ionic conditions, chromosomes are very compact (see for instance Fig. 1A in our work) and it is not possible to directly study their internal structure using microscopy techniques. In early studies, to visualize the organization of chromatin, chromosomes were denatured by treatments with water without cations and were depleted of histones (see responses to reviewer #3). These far from in vivo conditions led to the proposal of several fibrillar models for the metaphase chromosome structure. In a more recent study, metaphase cells were studied in situ (Eltsov et al. 2008). As discussed in the manuscript (page 7), the analysis of the cryo-sections obtained by these authors showed that metaphase chromosomes are densely packed structures that are completely filled by nucleosomes without discernable order; it was not possible to directly visualize any higher-order organization in native chromosomes. In our work, we "opened" the internal structure of chromosomes using soft procedures that maintain the metaphase ionic conditions, and we used cryogenic conditions to preserve the uncrosslinked and unstained native structure of the emanated chromatin throughout the cryo-ET imaging. This procedure keeps our study as close as possible to the in**

 

**vivo conditions. Furthermore, in the second part of our work, we applied synchrotron SAXS to investigate the chromatin structure within whole intact chromosomes.**

**In order to highlight the biological significance of the multilayered organization of metaphase chromatin observed in this work, we have added a new paragraph in the revised manuscript (page 8):**
**"We have performed an in vitro study using conditions that approach as much as possible the structuring ionic concentrations of metaphase cells, but future in vivo research will be required to validate the observed multilayered organization of chromatin. However, the functional role of this chromatin organization can be inferred from its structural and physical properties. The mechanical strength of planar chromatin (Gállego *et al*, 2010) and the stability of the stacked chromatin layers in metaphase chromosomes (see above) suggest that its primary biological role is the maintenance of the integrity of genomic DNA during mitosis. Furthermore, it was shown that this chromatin organization avoids topological entanglements of the chromatin filament (Milla & Daban, 2012) and can justify the elongated cylindrical structure of chromosomes as well as their outstanding mechanical properties (Poirier *et al*, 2000; Daban, 2014). It was also shown that if chromosomes consist of many stacked layers of planar chromatin it is possible to explain many cytogenetic observations that were not previously understood (Daban, 2015). Presumably, the typical chromosome bands are produced by the preferential staining of several chromatin layers with different dyes, and the observed transverse orientation of the bands is due to the perpendicular orientation of the chromatin layers with respect to the chromosome axis. This also explains the splitting of broad bands (formed by several layers) observed in chromosome stretching experiments (Hliscs et al, 1997), and the maintenance of the orthogonal orientation of the split bands. According to the local concentration of DNA in metaphase chromosomes (~170 Mb/µm3; Daban, 2000 and 2014), each chromatin layer of a human chromosome is formed by ~0.5 Mb of DNA, which justifies the existence of very thin bands containing less than 1Mb (International Human Genome Sequencing Consortium, 2001). The multilayered structure of chromatin in metaphase chromosomes is also compatible with the orthogonal orientation and planar structure of the connection surfaces seen in sister chromatid exchanges, and in the translocations observed in cancer cells. It has been argued (Daban, 2015) that the fibrillar models proposed by other authors (Paulson and Laemmli, 1977; Poirier & Marko, 2002; Kireeva *et al*, 2004; Eltsov *et al*, 2008; Naumova et al, 2013) require large quantities of DNA to cover the chromosome cross-section and cannot justify the existence of very thin orthogonal bands and the orthogonal orientation of the connection surfaces in chromosome rearrangements."**

**To further increase the biological significance of our study, in the last part of the Discussion of the revised manuscript (page 9), we have integrated the results of our work with recent HiC results obtained from mitotic cells (this text is also included in the responses to reviewer #3):**
**"There are several chromosome conformation capture methods capable of identifying contacts between distant regions of the chromatin filament via chemical crosslinking (Sajan & Hawkins, 2012; Bonev & Cavalli, 2016). In the genome-wide HiC method the cross-linked contacting regions are identified by high-throughput sequencing (Lieberman-Aiden et al, 2009). Recently, HiC results obtained with mitotic cells were modeled using polymer-based simulations of chromatin structure, and it was proposed that chromatin in mitotic chromosomes is folded as a compact array of many loops having different sizes during mitosis (Gibcus et al, 2018). In the model proposed by these authors the final compact chromosomes are formed by loops of ~ 0.5 Mb (consisting of ~400-kb outer loops and ~80-kb inner loops) and have a linear density of ~60 Mb/µm. For chromatids with a radius of ~0.36 µm (Gibcus et al, 2018), this linear density corresponds to ~150 Mb/µm3. This local DNA concentration is similar to the value considered above for multilayered chromosomes (~170 Mb/µm3; Daban, 2000 and 2014), and is compatible with the high chromatin density observed for metaphase chromosomes by other authors (Eltsov et al, 2008; Ou et al, 2017). Obviously, to achieve this high density, the long chromatin filament in each 0.5-Mb loop cannot be extended and must be tightly packed. We propose that the chromatin in the loops detected in the HiC studies could be compacted into the multilayered plates observed in this work."**

*2) The resolution of the data is not great and single nucleosomes cannot be recognized in two interacting plates, weakening the significance of the manuscript. The authors should try to improve*

 

*the resolution to see the nucleosomes in interacting plates. Current data for interdigitated model are weak and this would make the finding much more interesting.*

**The main result of our study is that the frozen-hydrated metaphase chromatin (uncrosslinked, unstained, and not adsorbed to any substrate) is planar. Our tomographic volumes show many monolayer plates, two layers in close contact, and stacked multilayered plates. As suggested by the reviewer, it would be interesting to be able to visualize nucleosomes within the interacting plates. However, as indicated in Materials and Methods, we have already used the best instrumentation available at present to carry out cryo-ET: (i) Titan Krios microscope; (ii) field-emission gun operated at 300 kV; (iii) post-column energy filter; (iv) direct electron-detection camera; (v) Volta phase plate; (vi) automated acquisition of the tilt series under low-dose conditions; and (vii) correction of beam-induced motion. Thus, we cannot improve the resolution using other equipment. The resolution of the data is in fact very high (4.2 Å pixel size), and individual nucleosomes are readily visualized decorating distorted plates, but the dense structure of the compact plates precludes visualization of single nucleosomes. The issue is not resolution, but rather the extremely compact structure of the plate itself.**

**Nevertheless, to improve our analysis of the interacting plates, in the revised version, the measured plate thickness has been corrected to account for the CTF fringes caused by defocus (see new Fig EV2). The corrected thickness of a monolayer plate is ~7.5 nm (see new Table 1). Considering the dimensions of the nucleosome core particle (cylinder of 5.7 nm height and 11 nm diameter), the observed thickness suggests that plates consist of a monolayer of nucleosomes slightly tilted relative to the plate surface. The intensity profiles presented in the new Fig EV4 show that there is no empty space between interacting plates. Based on the measurements of monolayer plates, the thickness of two stacked layers is expected to be ~15 nm, but our measurements indicate that the corrected thickness of two layers in close contact is ~13 nm (new Table 1). These differences could be explained by a relatively low degree of interdigitation (~2 nm) between the two contacting layers (see new Fig 6). This structural solution is consistent with previous experimental and modeling studies performed in many laboratories that have demonstrated that interdigitation combined with face-to-face nucleosome association can stabilize diverse chromatin higher order structures (see Discussion, pages 7 and 8; in the revised manuscript we have added recent references about face-to-face nucleosome interactions). Our work gives biological significance to fundamental chromatin interactions that have been known for many years.**

*If the authors could address one of the two concerns, I would support the publication in the EMBO journal.*

**We think that the revised version makes clear the biological significance of our work. Although it is not possible to visualize individual nucleosomes inside compact plates by cryo-ET, our new measurements of plate thickness have improved the structural analysis of interacting plates.**

**Reviewer #3:**

*In this paper, the Chicano et al investigate the structure of denatured metaphase chromosomes using cryo electron tomography. They shear sheets of material from condensed isolated mitotic chromosomes. The cryo-ET images from this material shows thin, deformed sheets. Some sheets (relaxed plates) which exhibit nucleosome-like particles at the edges. Similarly, subtomogramm averaging of volume units within compact sheets show nucleosome-like shapes. The resulting data is interpreted that the sheets (or plates) are composed of tightly packed nucleosomes, that are tilted relative to the sheet plane. Further observations show multilayered plates, with layer separation of ~10 nm and a lateral expansion of roughly the dimension of a metaphase chromosome. Contacts between two layers appear thinner than expected from a direct layer stack. Interdigitation between nucleosomes is proposed as a mechanism for these contact points. To further analyze the molecular chromatin properties, the authors apply synchrotron SAXS to whole metaphase chromosomes. The observed scattering plots reveal peaks corresponding to individual nucleosomes and their expected face-to-face stacking distance (6 nm) and no indication of the presence of 30-nm chromatin fibers. In conclusion, this combined cryoET and SAXS analysis thus demonstrates that plates obtained from isolated mitotic chromosomes correspond to extended sheets of stacked nucleosomes.*

 

*This study is an extension of previous extensive work from these authors on the nature of chromatin plates. The potential significance of the study can thus be assessed based on the added value of this study and the biological relevance of the discoveries. With regards to the advancements, the authors investigate the previously observed phenomena with cryo-ET (as opposed to TEM & AFM). As a main advancement they obtain sub-tomogram reconstructions that exhibit nucleosome-like particles from the plates. I am not sure if that qualifies as a significant advancement over previous data (see also comment below).*

**We discovered chromatin plates over a decade ago (Caravaca et al. 2005; Gállego et al. 2009). Our studies were based primarily on conventional TEM and AFM techniques. Our findings have been continuously criticized because these techniques require the deposition of the sample on flat surfaces and, in the case of conventional TEM, the sample is crosslinked with glutaraldehyde and dehydrated. Now we have successfully applied cryo-ET techniques to study uncrosslinked and unstained metaphase chromatin suspended in aqueous media. Certainly, the results obtained are in agreement with our previous findings, but they are absolutely necessary to demonstrate convincingly that metaphase chromatin is planar.**

*Secondly, and more importantly, with regards to the biological relevance: It is not at all clear that the chromatin structure corresponding to the observed plates represents a physiological state of chromatin. Based on the reported methods, the extracted chromosomes are extensively manipulated, sheared, dialyzed into various buffers, conditions which might induced non-native clustering of nucleosomes over time.*

**It is known that metaphase chromosomes contain relatively high concentrations of Mg2+ distributed homogeneously within the chromatids (Strick et al. 2001). Under these conditions, chromosomes are very compact (see for instance Fig. 1b of Earnshaw & Laemmli, 1983 and Fig. 1A in our work) and it is not possible to directly visualize their internal ultrastructure. To know the organization of chromatin in the chromosomes, it is necessary to "open" this compact structure. Early results showed the emanation of fibrillar structures from chromosomes, when chromosomes were suspended in distilled water (DuPraw, 1966), and treated with the divalent cation chelator EDTA (Earnshaw & Laemmly, 1983). Although many studies have shown that the structure of chromatin is extremely dependent on the concentration of Mg2+, the current consensus in the chromatin literature is that chromosomes are formed by more or less regularly folded chromatin fibers that fill the chromatids. The well-known chromatin loops-scaffold model is based on the emanation of DNA observed when chromosomes were completely denatured by depletion of histones (Paulson & Laemmli, 1977). To avoid the strong denaturing conditions used in these studies, in our work, chromosomes were kept in the presence of Mg2+ throughout the entire procedure. Chromosomes purified on sucrose gradients containing PM buffer (5 mM Pipes, pH 7.2, 5 mM NaCl, and 5 mM MgCl2), were (1) diluted with the same buffer, (2) passed through a syringe needle, and (3) dialyzed for 2.5 h at 37ºC against the same buffer. Each one of these treatments applied separately favor the emanation of chromatin plates from chromosomes (Gállego et al. 2009; Castro-Hartmann et al. 2010), but we applied the three methods to obtain cryo-preparations with a high yield of plates (information included in the revised version; Materials and Methods, page 9). In the revised manuscript, we have also indicated that in order to preserve the native chromatin structure as much as possible, the concentration of Mg2+ was maintained throughout these treatments. Furthermore, to complement the cryo-ET study we used synchrotron SAXS to investigate the organization of nucleosomes within intact chromosomes (see Results, page 6, and Fig 5).**
**References described here but not included in the manuscript: DuPraw, 1966, Nature 209, 577-581; Earnshaw & Laemmli, 1983, J Cell Biol 96, 84-93.**

*At this point it would increase the significance of the study if the obtained models would be compared to Hi-C data from mitotic chromosomes, or corroborated with alternative less invasive methods.*

**Thank you for this suggestion. In the last part of the Discussion (page 9) we have added a commentary that compares the results of our work to recent HiC results obtained with mitotic cells (this text is also included in the responses to reviewer #2):**

 

**"There are several chromosome conformation capture methods capable of identifying contacts between distant regions of the chromatin filament via chemical crosslinking (Sajan & Hawkins, 2012; Bonev & Cavalli, 2016). In the genome-wide HiC method the cross-linked contacting regions are identified by high-throughput sequencing (Lieberman-Aiden et al, 2009). Recently, HiC results obtained with mitotic cells were modeled using polymer-based simulations of chromatin structure, and it was proposed that chromatin in mitotic chromosomes is folded as a compact array of many loops having different sizes during mitosis (Gibcus et al, 2018). In the model proposed by these authors the final compact chromosomes are formed by loops of ~ 0.5 Mb (consisting of ~400-kb outer loops and ~80-kb inner loops) and have a linear density of ~60 Mb/µm. For chromatids with a radius of ~0.36 µm (Gibcus et al, 2018), this linear density corresponds to ~150 Mb/µm3. This local DNA concentration is similar to the value considered above for multilayered chromosomes (~170 Mb/µm3; Daban, 2000 and 2014), and is compatible with the high chromatin density observed for metaphase chromosomes by other authors (Eltsov et al, 2008; Ou et al, 2017). Obviously, to achieve this high density, the long chromatin filament in each 0.5-Mb loop cannot be extended and must be tightly packed. We propose that the chromatin in the loops detected in the HiC studies could be compacted into the multilayered plates observed in this work."**
*With regards to the performed experiments: I was wondering how the sub-tomograms of the particles making up the plates were constructed. The authors write that 1243 particles were manually picked. From the figure 2a, structure P2, no particles can be discerned. It is thus unclear to me where these particle reconstructions come from and how they were obtained.*

**To perform our cryo-ET study, we used the best instruments presently available, including Volta phase plate and direct electron detection with correction of beam-induced motion. This enabled us to manually pick particles that were clearly seen decorating distorted plates, and produce subtomogram averaging of these particles (Fig 2F). The reviewer is correct that typical plates such as those shown in Fig. 2A (P2) are very compact, and individual particles cannot be discerned within the plates. To produce this subtomogram average, we simply picked 1243 small subvolumes from these plates and subjected them to reference-free alignment. Unfortunately, during the preparation of the revised version, we have realized an error that incorrectly lead to an average that resembles the size and shape of a nucleosome. With this error corrected, the average simply resembles the plate structure from which the particles were picked, without discernable nucleosome structure. Therefore, we do not include Fig 2G (and the related original Figs EV1 and EV3) in the revised manuscript. We apologize for this error and thank the reviewer for helping us correct it before publication. As discussed above in the responses to reviewers #1 and #2, we have taken a different approach to improve our analysis in the revised manuscript, with CTF-adjusted measurements of plate thickness enabling us to propose a modified model in Fig. 6 that integrates our cryo-ET and SAXS results.**

*With these reservations, I believe that the study is too preliminary for acceptance in EMBO J.*

**From our point of view, it is not correct that we had already convincingly demonstrated the planar structure of metaphase chromatin using conventional TEM and AFM and that, consequently, our cryo-ET results are not a significant advancement. Our earlier observations of planar chromatin structures are not widely accepted in the field; they have been heavily criticized due to the adsorption of chromatin to a support layer. Thus, it was absolutely necessary to investigate whether uncrosslinked and unstained frozen-hydrated chromatin from metaphase chromosomes is planar and can form multilayered structures when freely suspended in aqueous medium. We used state-of-the-art cryo-ET equipment to perform extensive work that cannot be considered a preliminary study (we analyzed 32 tomograms). Furthermore, in this work we have also included the results of our synchrotron SAXS analysis of whole metaphase chromosomes (see Results, page 6 and Fig 5). A significant motivation to submit our work to The EMBO journal was the fact that Nishino et al (2012) previously published a paper in this journal about metaphase chromosome structure based exclusively on synchrotron SAXS measurements. Nishino el al. interpreted the dominant scattering peak at 6 nm as face-to-face nucleosome interactions in densely packed irregularly folded chromatin fibers (polymer melt). Using the same technique, we also observed a dominant 6-nm scattering peak in different structuring conditions, but our cryo-ET results lead to a different interpretation; the face-to-face nucleosome interactions take place between nucleosomes**

 

**organized in interdigitating stacked layers of chromatin. In addition, this peak may also be related to the distance between stacked layers in condensed chromosomes (~6 nm; see Fig 6). This repeated distance strengthens the scattering peak at ~6 nm, and this can explain why the expected peak at ~11 nm (corresponding to edge-to-edge contacts between densely packed nucleosomes) has a low intensity in comparison to the 6-nm peak. The polymer melt model of Nishino et al. is different from the multilayer organization observed in our study using cryo-ET, and we would like to have the opportunity to publish this new perspective on chromatin organization in The EMBO journal.**

3rd Editorial Decision 28th November 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by all three original referees and their comments are shown below.

As you will see, the refs acknowledge that the manuscript has improved but refs #2 and #3 still point out that the data remains open to other interpretations and does not unequivocally support the model presented. However, at the same time they recognise the technical difficulties involved with such work and recommend that the data be published as a it for the general use in the field (and to spark further discussion). I would therefore invite you to submit a final revised version in which you include the clarification requested by ref #1 as well as the following editorial points:

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REFEREE REPORTS

Referee #1:

The authors have addressed our concerns and the paper can be accepted.

This is up to the authors to consider:

It would be easier for readers, if the authors explicitly mentioned in the materials and methods section "subtomogram averaging and classification", which particular dataset they have used for the subtomogram averaging: since they have acquired data with different settings (Krios/Polara, phase plate/no phase plate, different pixel sizes). If the data comes from tomograms with high defocus and no CTF-correction was applied - the subtomograms and final reconstruction should be low-pass-filtered according to the first zero of the CTF.

Referee #2:

I am still not convinced that the proposed model is correct and really supported by the data. However, this is a difficult problem and the model is interesting enough to be put forward. Despite many limitations of the study such as manipulation of the sample and the low resolution of the data, the study offers some interesting insights. Unfortunately, with the current data, the model the authors propose might be right, but might be equally likely wrong.

Nevertheless, I suggest to publish the study and let the time show if the authors are right or wrong.

Referee #3:

In the revised version of the paper, the authors have added additional explanations which clarify experimental issues as well as the connection to published data on mitotic chromatin. Moreover, an

 

error was corrected, which previously resulted in the detection of nucleosome-like particles in the obtained plate structures.
First, I recognize that the structural analysis of mitotic chromatin is an extremely hard problem, and of very high interest to the community. I also agree with the authors, that employing cryo-EM tomography of non-crosslinked, non-surface absorbed samples is important to exclude artifacts in their analysis.
Still, the model of mitotic chromatin as appearing as large dense plates strikes me a non-physiological state, perhaps induced by the preparation conditions. The lack of internal structural information is a problem (while not surprising due to the high molecular density). While the SAXS is informative, it is not performed on similarly treated samples but on separately performed non-sheared chromosomes, which may retain more native structure.
Therefore, the findings of the paper are still controversial, and a complete validation of this particular model will require more work (but this might beyond the scope of this article).

3rd Revision - authors' response 29th November 2018

**Frozen-hydrated chromatin from metaphase chromosomes has an interdigitated multilayer structure**
Andrea Chicano, Eva Crosas, Joaquín Otón, Roberto Melero, Benjamin D Engel, and Joan-Ramon Daban

**Response to the reviewers' comments on the revised version**
**Reviewer #1:**
*The authors have addressed our concerns and the paper can be accepted.*

*This is up to the authors to consider:*
*It would be easier for readers, if the authors explicitly mentioned in the materials and methods section "subtomogram averaging and classification", which particular dataset they have used for the subtomogram averaging: since they have acquired data with different settings (Krios/Polara, phase plate/no phase plate, different pixel sizes). If the data comes from tomograms with high defocus and no CTF-correction was applied - the subtomograms and final reconstruction should be low-pass-filtered according to the first zero of the CTF.*

**In the revised version we have indicated (page 10) that "The subtomogram average was generated from data acquired on the Titan Krios microscope using the Volta phase plate and 0.5-µm defocus." We did not apply the CTF correction because we used phase plate (Danev et al, 2014) data at close to focus instead of normal defocus (5-6 µm) data acquired with an objective aperture.**

*The authors made use of cryoEM and SAXS methods and report that metaphase chromosomes form planar structures consisting of stacked nucleosomes. The quality of the data is very good and supports a planar model of chromosome organization. Application of SAXS and CryoEM to isolated chromosomes is a good step toward understanding chromatin structural organization.*
*I recommend this paper for publication after minor revision.*

**We thank this reviewer for the positive comments.**

**Reviewer #2:**
*I am still not convinced that the proposed model is correct and really supported by the data. However, this is a difficult problem and the model is interesting enough to be put forward. Despite many limitations of the study such as manipulation of the sample and the low resolution of the data, the study offers some interesting insights. Unfortunately, with the current data, the model the authors propose might be right, but might be equally likely wrong.*
*Nevertheless, I suggest to publish the study and let the time show if the authors are right or wrong.*

**We thank this reviewer for the constructive comments.**

**Reviewer #3:**

*In the revised version of the paper, the authors have added additional explanations which clarify experimental issues as well as the connection to published data on mitotic chromatin. Moreover, an error was corrected, which previously resulted in the detection of nucleosome-like particles in the obtained plate structures.*
*First, I recognize that the structural analysis of mitotic chromatin is an extremely hard problem, and of very high interest to the community. I also agree with the authors, that employing cryo-EM tomography of non-crosslinked, non-surface absorbed samples is important to exclude artifacts in their analysis.*
*Still, the model of mitotic chromatin as appearing as large dense plates strikes me a non-physiological state, perhaps induced by the preparation conditions. The lack of internal structural information is a problem (while not surprising due to the high molecular density). While the SAXS is informative, it is not performed on similarly treated samples but on separately performed non-sheared chromosomes, which may retain more native structure.*
*Therefore, the findings of the paper are still controversial, and a complete validation of this particular model will require more work (but this might beyond the scope of this article).*

**We thank this reviewer for the constructive comments.**

Accepted 3rd December 2018

Thank you for submitting the final version of your manuscript here, I am pleased to inform you that your study has now been accepted for publication in the EMBO Journal.

 

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

| |
|---|
| Corresponding Author Name: Joan-Ramon Daban |
| Journal Submitted to: EMBO Journal |
| Manuscript Number: 2018-99769R1 |

**Reporting Checklist For Life Sciences Articles (Rev. June 2017)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

## B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

| | |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | We measured the thickness of monolayers and of two layers in contact from cryo-tomograms obatined with two microscopes (Table 1); the total number of measurements was 577 and 165, respectively. The thickness of single layers in multilaminar plates and the diameter of nucleosomes decorating distorted plates was obtained from 205 and 206 measurements (see Materials and Methods). |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | NA |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | NA |
| For animal studies, include a statement about randomization even if no randomization was used. | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | NA |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | NA |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | NA |
| Is there an estimate of variation within each group of data? | We calculated the standard deviation of each group of data. It has similar values in all groups. |

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| | |
|---|---|
| Is the variance similar between the groups that are being statistically compared? | NA |

## C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | NA |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | We obtained metaphase chromosomes from HeLa cells (American Type Culture Collection; ATCC number CCL-2). Cells used were negative for mycoplasma contamination; they were tested using the MycoAlert mycoplasma detection kit (Lonza). |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | NA |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | NA |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | NA |

## E- Human Subjects

| | |
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| 11. Identify the committee(s) approving the study protocol. | NA |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
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## F- Data Accessibility

| | |
|---|---|
| 18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | The subtomogram average of nucleosome particles decorating unstructured plates and a cryo-electron tomogram of chromatin plates emanated from metaphase chromosomes have been deposited in the Electron Microscopy Data Bank (EMDB; http://www.emdatabank.org) with accession numbers EMD-0117 and EMD-0119, respectively. |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | NA |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | NA |
| 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | NA |

## G- Dual use research of concern

| | |
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| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | NA |