THE
EMBO
JOURNAL

Topologically Associating Domains and chromatin loops depend on cohesin and are regulated by CTCF, WAPL and PDS5 proteins

Gordana Wutz, Csilla Várnai, Kota Nagasaka, David A Cisneros, Roman R Stocsits, Wen Tang, Stefan Schoenfelder, Gregor Jessberger, Matthias Muhar, M Julius Hossain, Nike Walther, Birgit Koch, Moritz Kueblbeck, Jan Ellenberg, Johannes Zuber, Peter Fraser and Jan-Michael Peters

Review timeline:

Submission date:	14 August 2017
Editorial Decision:	27 September 2017
Revision received:	1 November 2017
Editorial Decision:	2 November 2017
Revision received:	7 November 2017
Accepted:	7 November 2017

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 27 September 2017

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, our referees all express interest in the findings reported in your manuscript and support publication here, pending relatively minor revision as outlined in the reports. While referees #1 and #3 are largely happy with the current version and only ask for textual clarifications, ref #2 raises a number of technical/clarification points to the analysis; however, these should all be addressable by elaborating on the existing dataset.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers.

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

REFEREE REPORTS

Referee #1:

The manuscript by Wutz et al investigates the role of the cohesin complex and its key regulators in 3D genome organization. The paper provides a range of important insights. First, the authors show

by using Auxin-mediated degradation of the cohesin subunit SCC1 that cohesin is essential for the vast bulk of chromatin loops and TADs. This by itself is a major finding that to my opinion already merits publication in a high-profile journal. The paper then goes on to show that CTCF loss reduces insulation between TADs, and that stabilization of cohesin on chromatin by depletion of WAPL and/or PDS5 proteins leads to extended loops. Both the WAPL and CTCF findings are supported by recent Cell papers. The PDS5 data however are both novel and intriguing. The current manuscript would suggest that PDS5 is important to delay cohesin at CTCF boundaries during the loop formation process.

I like this paper, and I would support publishing it virtually as is. I have a few textual points below.

1) The authors refer to the 'formation' of loops in the title. I expect that these factors do indeed regulate loop formation, but strictly speaking these factors could also rather regulate loop 'maintenance', as correctly noted in the main text.

2) After SCC1 depletion using Auxin for 15 minutes, 96% of the loops disappear, while only 53% of the TADs are undetectable. However, if TADs reflect collections of loops, how can there be such a difference in these numbers? Could it be that this is a consequence of the way loops and TADs are 'called', and that there in fact is a decrease in the intensity of the remaining TADs? In that case, loops would not be more sensitive than TADs to SCC1 depletion (as proposed on page 11).

3) In Figure S3a, the mild and moderate phenotypes display differences in SCC1 staining, while the DNA staining does not reveal evident differences. It could therefore be argued that these categories do not reflect 'chromosome compaction phenotypes', but merely differences in cohesin distribution. I presume however that this absence of a DNA staining phenotype simply is due to the limited resolution of the DAPI-stained images. Some extra explanation in the text could be beneficial here.

4) On page 19/20, the authors state that depletion of PDS5 leads to a similar result as does depletion of CTCF. The reduction in detectable loops could indeed indicate that PDS5 is important for the boundary function of CTCF. They substantiate this claim in the following paragraph, where they show that the orientation of the loops is as would be expected if CTCF sites were connected randomly. I am a bit confused here though, as cohesin still appears to be present at CTCF sites in PDS5 deficient cells. Isn't this finding contradictory to a loss in boundary function? Some more explanation would be helpful.

Referee #2:

In this manuscript, Wutz, Varnai and collaborators perform an exhaustive examination of the role of cohesin, CTCF, Wapl, and PDS5A/B in regulating three-dimensional chromatin conformation. To do so, the authors employ a combination of auxin-inducible degron (AID) and RNAi to deplete different combinations of factors. These samples are then examined using population based Hi-C analyses and cell biology approaches. By doing so, the authors evaluate how the loss of different proteins specifically affect the conformation of chromatin at the levels of loops, topologically associating domains (TADs) and compartments.

Overall the manuscript is very nicely written and provides compelling evidence for the role of these proteins in regulating 3D chromatin organisation. However, there are a number of issues that the authors need to address:

Major points:

1. The authors find that cohesin inactivation leads to the strengthening of compartments and the loss of TADs and loops (page 9). While I agree with this observation, a small level of insulation can still be observed at those TAD boundaries (characterised in Fig 1 Extended View B). To avoid future claims regarding the presence or not of TADs in these samples, it would be useful if the authors would perform aggregate TAD analysis (for example as in Gassler et al., bioRvix 2017) to examine and discuss how those plots look like.

2. In line with this observation, please provide total number of TADs detected in the SCC1 depleted samples. These numbers are not provided in the Supp. Table, although it is reported that a significant fraction of the genome is still covered by TADs (44%). The authors should reconcile both observations (TADs being not detectable by directly looking at the data vs. computational ability to detect insulation) and provide a more balanced description of their findings (I'd suggest to at least edit "destroys" in the section header).

3. There are conflicting results regarding the insulation score analysis in CTCF depleted samples. The authors report reduced insulation at TAD boundaries upon CTCF depletion, in agreement with a previous report (Nora et al., Cell 2017). However, a close examination of the insulation plots in comparison with the plots shown in Fig 1EVB, reveals that a different change in the distribution of insulation values (high values to low values after treatment in the SCC1 samples, and low values to high values after treatment in the CTCF samples) is interpreted similarly in both cases (loss of insulation). The details of how the insulation is calculated in these plots are not explained in the methods sections. Please include those details in the methods and explain the discrepancy between the interpretation of the two plots. Also, please include a description for the dotted lines on these plots and details for their calculation.

4. A similar issue with the insulation analysis is present in Fig 7D, when high to low levels of insulation are interpreted as a loss of insulation (similarly as in the SCC1 samples, but in disagreement with the CTCF samples).
5. In addition, there is a further discrepancy with the insulation analysis presented for Wapl RNAi samples in the context of the data presented in (Haarhuis et al., Cell 2017). Here, the authors report a loss of insulation upon Wapl depletion. However, the directionally index analysis in (Haarhuis et al., Cell 2017) seems to detect almost no change at the level of TAD insulation upon Wapl depletion. The authors should discuss about possible reasons leading to the differences between the two studies.

6. Figure 7 is very difficult to interpret in its current form. The allegedly reported longer TADs are not immediately visible from the selected region. In addition, I do not find convincing the interpretation based on "TAD fusions" (page 18). If TAD fusions would occur, it seems more plausible that those would manifest in specific gains of contacts in previous TAD boundaries. On the contrary, the bottom halves of the matrices presented in Fig 7 seem to indicate that there is a general loss of intra-TAD contacts (faint blue signal). Because of the matrix balancing approached used to normalise the data, which forces the sum of contacts for every region of the genome (sum of the columns on the Hi-C matrix) to be 1, a loss of intra-TAD contacts would be compensated by a gain of contacts elsewhere. Since TAD organisation seems to be hierarchical, with TAD structures being embedded into higher order structures (for example see structures around 76-77Mb on Fig 7A control panel), another interpretation for these data is that the depletion of Wapl would lead to the loss of local compaction, but unaffected higher order organisation of TADs. Could the authors please comment on this possibility? To make the interpretation of these data clearer, I'd also suggest that the authors mark the position of TADs in the top halves of the matrices in Fig 7a.

7. Also related to the Wapl depletion datasets, it seems that the Hi-C data produced in this manuscript display significant differences with those presented in (Haarhuis et al., Cell 2017). It could be just a coincidence of the regions chosen in Haarhuis et al., but it seems like all the regions of the genome explored there display the characteristic appearance of long-range contacts (similar as those presented in Fig 8 in this manuscript). In Haarhuis et al., this seems to be the case both for 'ordinary domains', which don't display a loop at the corner of the TAD, as well as for 'loop domains'. In this manuscript, 'ordinary domains', such as those presented in Fig. 7A, do not seem to display extended loop formation. It is very difficult to make general conclusions from just seeing the selected regions in these panels. I would therefore suggest that the authors perform a systematic characterisation of the differences between the two datasets so this is clarified. This is important since at the moment there is a discrepancy between the two Hi-C datasets despite both samples displaying similar vermicelli chromosomes.

8. The authors report ~1.500 TADs in prometaphase cells (Fig 7B), but these are not visible on the Hi-C plots (Fig 7A). In addition the level of insulation seems minimal (Fig 7D). This raises the question as to whether TADs are really present in mitotic chromatin. As mentioned above, it seems to me like the current methods used to call TADs lack a proper calibration that would allow the

© European Molecular Biology Organization

authors to distinguish between being able to detect fluctuations on the Hi-C signal and whether TADs are actually visible on the data. This has also been recently reported for interphase and mitotic chromatin in fly embryos (Hug et al., Cell 2017). The authors should discuss this limitation and/or provide examples of those TADs detected in prometaphase cells.

9. The authors state that 3C primers are available upon request. I'd request that the authors provide a suitable list of primers as part of the manuscript so the results can be easily reproduced.

10. The authors should also include a link to a public repository (eg. GEO) containing the raw and processed sequencing data produced here.

Referee #3:

The manuscript by Wutz et al. examines the effect of depletion of cohesin, WAPL, CTCF, PDS5A and PDS5B in genome architecture in HeLa cells. Cohesin and CTCF are removed acutely using auxin-inducible degrons, while other proteins are depleted by RNAi. The authors examine several levels of genome organisation based on Hi-C analysis: compartments, TADs and loops. They find that cohesin is required for the formation of TADs and loops and that compartments are increased in its absence. CTCF depletion causes distortion of TAD boundaries and reduced the number of TADs. WAPL or PDS5 depletion reduced the compartmentalization, increased the size but decreased the number of TADs, suggestion TAD fusion. WAPL depletion also resulted in the formation of more, longer loops. While PDS5 depletion resulted in many fewer loops. Both WAPL and, especially PDS5 are important for the CTCF convergence rule. Imaging and fractionation experiments showed that PDS5, like WAPL contributes to the turnover of cohesin on chromosomes. Based on these observations, the authors argue that their data is consistent with the idea that cohesin directly forms TADs and loops, that CTCF acts to enforce boundaries at the bases of these loops, that WAPL and PDS5 limit the extent of TADs and loops by decreasing the residence time of cohesin on chromosomes and that PDS5 helps to recognise CTCF to provide boundary function. These observations and their interpretation are consistent with the "loop extrusion" model for SMC protein function which is gathering strong support in the literature. Overall, the new findings add to our understanding of this rapidly developing field and are appropriately placed into context and the current literature. Conclusions are drawn with appropriate caution where technical limitations prevail. Experiments are well controlled and the manuscript is well written and presented. There are a few cases where the experimental design warrants further discussion, but this is a relatively minor criticism.

1. All experiments were conducted in HeLa cells, which are karyotypically abnormal. A consideration of how this affects the analysis and potentially also the conclusions should be mentioned, particularly as other published work has used cell types, which are expected to be karyotypically normal.
2. The effect of WAPL and PDS5 depletion on TADs is relatively modest (Figure 7B, C). Is this statistically significant? The authors should comment as to why this is so modest. Are particular TADs more affected that others and does this correlate with cohesin/CTCF density?
3. Some labelling in figures is not correctly reproduced in the online file (e.g. Figure 5D MAD2L-GFP + Hela).

1st Revision - authors' response 1 November 2017

Response to Reviewers' comments (Manuscript EMBOJ-2017-98004)

We would like to thank all three referees for their fast and constructive evaluation of our manuscript, which clearly helped to improve the paper.

Referee #1:

The manuscript by Wutz et al investigates the role of the cohesin complex and its key regulators in 3D genome organization. The paper provides a range of important insights. First, the authors show

by using Auxin-mediated degradation of the cohesin subunit SCC1 that cohesin is essential for the vast bulk of chromatin loops and TADs. This by itself is a major finding that to my opinion already merits publication in a high-profile journal. The paper then goes on to show that CTCF loss reduces insulation between TADs, and that stabilization of cohesin on chromatin by depletion of WAPL and/or PDS5 proteins leads to extended loops. Both the WAPL and CTCF findings are supported by recent Cell papers. The PDS5 data however are both novel and intriguing. The current manuscript would suggest that PDS5 is important to delay cohesin at CTCF boundaries during the loop formation process.

I like this paper, and I would support publishing it virtually as is. I have a few textual points below.

1) The authors refer to the 'formation' of loops in the title. I expect that these factors do indeed regulate loop formation, but strictly speaking these factors could also rather regulate loop 'maintenance', as correctly noted in the main text.

The Referee is correct. We have therefore changed the title to "TADs and chromatin loops depend on cohesin and are regulated by CTCF, WAPL and PDS5 proteins".

2) After SCC1 depletion using Auxin for 15 minutes, 96% of the loops disappear, while only 53% of the TADs are undetectable. However, if TADs reflect collections of loops, how can there be such a difference in these numbers? Could it be that this is a consequence of the way loops and TADs are 'called', and that there in fact is a decrease in the intensity of the remaining TADs? In that case, loops would not be more sensitive than TADs to SCC1 depletion (as proposed on page 11).

We thank the Referee for pointing out this discrepancy. Like the Referee, we now suspect that this phenomenon may reflect differences in TAD and loop calling algorithms rather than biological differences. For example, we found that TAD calling becomes more sensitive when TAD structures weaken (see points 2 and 8 in reply to Referee 2), which could contribute to the differences at which TADs and loops seem to disappear in this experiment. We have therefore amended the text and now only point out that TADs and loops become undetectable more rapidly than compartmentalization is detectably increased.

3) In Figure S3a, the mild and moderate phenotypes display differences in SCC1 staining, while the DNA staining does not reveal evident differences. It could therefore be argued that these categories do not reflect 'chromosome compaction phenotypes', but merely differences in cohesin distribution. I presume however that this absence of a DNA staining phenotype simply is due to the limited resolution of the DAPI-stained images. Some extra explanation in the text could be beneficial here.

The Referee is correct, that in some of the images of DAPI stained cells previously shown in FigS3A chromatin compaction was difficult to see. We have therefore now replaced these with more representative images in which these phenotypes can be seen more clearly.

4) On page 19/20, the authors state that depletion of PDS5 leads to a similar result as does depletion of CTCF. The reduction in detectable loops could indeed indicate that PDS5 is important for the boundary function of CTCF. They substantiate this claim in the following paragraph, where they show that the orientation of the loops is as would be expected if CTCF sites were connected randomly. I am a bit confused here though, as cohesin still appears to be present at CTCF sites in PDS5 deficient cells. Isn't this finding contradictory to a loss in boundary function? Some more explanation would be helpful.

This is an interesting question. To address it, we have now analyzed whether the disappearance of loops correlates with a decrease in cohesin binding in the affected loop anchors. Interestingly, we found that this is indeed the case, as one would predict if cohesin mediates long-range chromatin interactions. These new results are shown in Fig S6G and H.

Referee #2:

In this manuscript, Wutz, Varnai and collaborators perform an exhaustive examination of the role of cohesin, CTCF, Wapl, and PDS5A/B in regulating three-dimensional chromatin conformation. To

do so, the authors employ a combination of auxin-inducible degron (AID) and RNAi to deplete different combinations of factors. These samples are then examined using population based Hi-C analyses and cell biology approaches. By doing so, the authors evaluate how the loss of different proteins specifically affect the conformation of chromatin at the levels of loops, topologically associating domains (TADs) and compartments.

Overall the manuscript is very nicely written and provides compelling evidence for the role of these proteins in regulating 3D chromatin organisation. However, there are a number of issues that the authors need to address:

Major points:

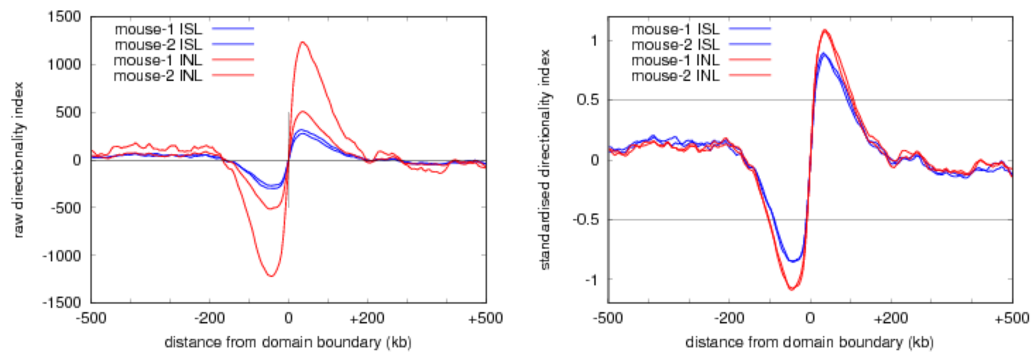
1. The authors find that cohesin inactivation leads to the strengthening of compartments and the loss of TADs and loops (page 9). While I agree with this observation, a small level of insulation can still be observed at those TAD boundaries (characterised in Fig 1 Extended View B). To avoid future claims regarding the presence or not of TADs in these samples, it would be useful if the authors would perform aggregate TAD analysis (for example as in Gassler et al., bioRvix 2017) to examine and discuss how those plots look like.

As suggested, we have performed aggregate TAD analysis, plotting the average Hi-C contact maps around control TADs in the different conditions. This analysis confirmed that TADs are greatly weakened upon SCC1 degradation (new Fig EV1B). We also used aggregate TAD analyses to confirm our observations made in cells depleted of CTCF, WAPL and PDS5 proteins (new Figs 2I, EV7A and S1D).

2. In line with this observation, please provide total number of TADs detected in the SCC1 depleted samples. These numbers are not provided in the Supp. Table, although it is reported that a significant fraction of the genome is still covered by TADs (44%). The authors should reconcile both observations (TADs being not detectable by directly looking at the data vs. computational ability to detect insulation) and provide a more balanced description of their findings (I'd suggest to at least edit "destroys" in the section header).

We have now added the total number of TADs that can be computationally called in SCC1 depleted cells. As can be seen in the revised Table S1, surprisingly, this number is higher than the number of TADs that can be called in Hi-C data from SCC1 containing cells, despite TAD structures being much weaker after SCC1 degradation, as has also been confirmed by our new aggregate TAD analysis (Fig EV1B). This phenomenon is an effect of the standardization of the directionality index by the TAD calling method we used, which generates reproducible results in noisy biological replicates. For the reasons explained below, this standardization increases the detectability of boundaries for samples with weakened domain structures, because weak raw directionality index signals are comparatively magnified by this standardization. We have now described this phenomenon in the Results section.
As suggested by the Referee, we have amended our statement that SCC1 degradation "destroys" TADs and loops, as weak domain structures are still detectable in these cells. The sub-heading has been changed to: "Cohesin inactivation strengthens compartments but weakens TADs and loops".

Explanation for why standardization of the directionality index increases the sensitivity with which TADs can be called:
Briefly, when calling TADs, we use a thresholding approach on standardized directionality index profiles. As is illustrated in the figure below based on data from Nagano et al., 2015, this has the advantage of being less sensitive to noise and coverage in the replicates.



Reviewer Figure 1.: Averaged raw (left) and standardised (right) directionality index profiles in the 1Mb region centered around TAD boundaries for the two replicates (mouse-1 and mouse-2) of the in-solution ligation (ISL, blue) and the in-nucleus ligation (INL, red) experiments. Data taken from GSE70181 (Nagano et al., 2015). TAD boundaries called in both ISL and INL datasets were used.

On the other hand, when there is weak directionality bias in the data, due to biological noise, such as the lack of strong domain structures in SCC1 depleted cells, the sensitivity of domain detection increases with standardization (by a relative strengthening of weak signal when it is scaled to the same normal distribution as a strong signal). TAD boundaries that pass the same threshold when detected this way are in general weaker in the original dataset. This is illustrated by the insulation score at the TAD boundaries, as well as the aggregate TAD analysis.
As a result, as long as there is a weak structure, some of this will still stand out of the noise, and with the increased sensitivity of detection it will show up as an unusually high genome coverage or number of TADs called, as is the case in our samples. However, as the biological signal decreases into the noise, TADs are detectable in less and less of the genome, and a trend in the genome coverage of TADs is a good indicator of decreasing structure strength (Pearson R=0.82 with average TAD boundary strength).

3. There are conflicting results regarding the insulation score analysis in CTCF depleted samples. The authors report reduced insulation at TAD boundaries upon CTCF depletion, in agreement with a previous report (Nora et al., Cell 2017). However, a close examination of the insulation plots in comparison with the plots shown in Fig 1EVB, reveals that a different change in the distribution of insulation values (high values to low values after treatment in the SCC1 samples, and low values to high values after treatment in the CTCF samples) is interpreted similarly in both cases (loss of insulation). The details of how the insulation is calculated in these plots are not explained in the methods sections. Please include those details in the methods and explain the discrepancy between the interpretation of the two plots. Also, please include a description for the dotted lines on these plots and details for their calculation.

We thank the Referee for noticing this discrepancy. It was caused by an accidental swap in the colors that were used in Fig 2H. We apologize for the mistake and have corrected it.
We have now added a description of how insulation scores were calculated to the Material and Methods section. In brief, the insulation score is the standardized -log enrichment of contacts between the downstream and upstream 300 kb regions ($I = -\log(a / (a+b1+b2))$ where a is the number of contacts between, and $b1$ and $b2$ the number of contacts within the upstream and downstream 300 kb regions). A high insulation score indicates a strong TAD boundary.
We also added an explanation of the dashed lines in the insulation score figures. These dotted lines represent negative controls, which are calculated as the insulation score profiles around a set of TAD boundaries that were artificially shifted by +1 Mb and defined as control genomic positions.

4. A similar issue with the insulation analysis is present in Fig 7D, when high to low levels of insulation are interpreted as a loss of insulation (similarly as in the SCC1 samples, but in disagreement with the CTCF samples).

Please see reply to point 3. above. All representations of insulation scores are now consistent.

5. In addition, there is a further discrepancy with the insulation analysis presented for Wapl RNAi samples in the context of the data presented in (Haarhuis et al., Cell 2017). Here, the authors report a loss of insulation upon Wapl depletion. However, the directionally index analysis in (Haarhuis et al., Cell 2017) seems to detect almost no change at the level of TAD insulation upon Wapl depletion. The authors should discuss about possible reasons leading to the differences between the two studies.

To understand the reasons for this discrepancy we have performed additional bioinformatic analyses and Hi-C experiments.

First, we perfomed aggregate TAD analyses. These confirmed that TAD boundaries were weakened in HeLa cells depleted of WAPL by RNAi (new Fig EV7A).

Second, to test the possibility that differences between our results and those published by Haarhuis et al., 2017, are due to differences in the algorithms used for data analysis, we analyzed TAD boundary strength in Hi-C data obtained by Haarhuis et al., 2017 for WAPL "knockout" HAP1 cells (replicates A and C) using the same algorithms used in our study. This revealed some weakening of TAD boundary strength also in the data published by Haarhuis et al., 2017, although not to the same extent as seen in our data, both using directionality index (Fig S8A) and insulation score measurements (Fig S8B). This implies that the results from both studies are consistent with each other and that apparent differences are caused at least in part by differences in the bioinformatic analyses.

Third, and in parallel to the bioinformatic analyses described above, we performed Hi-C experiments using mouse embryonic fibroblasts (MEFs) from which we had deleted the *Wapl* gene or not (Tedeschi et al., 2013), to address if differences in the experimental approaches used (RNAi in our case *versus* gene inactivation by Haarhuis et al., 2017) could have contributed to the discrepancy pointed out by the Referee. These experiments clearly confirmed the following observations that we made previously in HeLa cells depleted of WAPL by RNAi:

- **A genome-wide shift towards long-range interactions (Figs S7A and B)**
- **Weakening of compartments (Fig S7C)**
- **Increased local long-range interactions resulting in merging of TADs and detection of new loops (Fig S7D)**
- **Importantly, reduced TAD boundary strength as seen by changes in directionality index (Fig S7E) and insulation score (Fig S7F)**

These results indicate that apparent differences between observations reported by Haarhuis et al., 2017 and us were not caused by our usage of RNAi mediated WAPL depletion. Together with the results described above, our observations indicate instead that these differences are largely caused by differences in bioinformatic analyses. We have now discussed this possibility in the Results section.

6. Figure 7 is very difficult to interpret in its current form. The allegedly reported longer TADs are not immediately visible from the selected region. In addition, I do not find convincing the interpretation based on "TAD fusions" (page 18). If TAD fusions would occur, it seems more plausible that those would manifest in specific gains of contacts in previous TAD boundaries. On the contrary, the bottom halves of the matrices presented in Fig 7 seem to indicate that there is a general loss of intra-TAD contacts (faint blue signal). Because of the matrix balancing approached used to normalise the data, which forces the sum of contacts for every region of the genome (sum of the columns on the Hi-C matrix) to be 1, a loss of intra-TAD contacts would be compensated by a gain of contacts elsewhere. Since TAD organisation seems to be hierarchical, with TAD structures being embedded into higher order structures (for example see structures around 76-77Mb on Fig 7A control panel), another interpretation for these data is that the depletion of Wapl would lead to the loss of local compaction, but unaffected higher order organisation of TADs. Could the authors please comment on this possibility? To make the interpretation of these data clearer, I'd also suggest that the authors mark the position of TADs in the top halves of the matrices in Fig 7a.

First, the Referee is correct, that an increase in TAD size in cells depleted of WAPL and PDS5 proteins was not easily visible in the original version of Fig 7A. We have therefore selected a more representative region to show. The TADs, called in an unbiased manner, are visible as triangles in the upper right halves of the split Hi-C maps in the new version of Fig 7A. This labeling illustrates the results of our analysis more clearly by showing that TADs indeed increase in size after depletion of WAPL and PDS5 proteins.

Second, the Referee pointed out that another interpretation of our data could be that WAPL depletion leads to loss of local compaction without affecting higher-order organization of TADs. We agree with the Referee that this is a formal possibility because the observed contact frequencies are a reflection of their relative abundance, and an increase of long-range contacts from a particular locus would therefore also result in a decrease of short-range contacts, and *vice versa*. However, we consider this interpretation less plausible because it could not easily explain the strong chromatin compaction phenotypes we observed in these cells and the simulations reported by the Mirny lab (Fudenberg et al, 2016).

7. Also related to the Wapl depletion datasets, it seems that the Hi-C data produced in this manuscript display significant differences with those presented in (Haarhuis et al., Cell 2017). It could be just a coincidence of the regions chosen in Haarhuis et al., but it seems like all the regions of the genome explored there display the characteristic appearance of long-range contacts (similar as those presented in Fig 8 in this manuscript). In Haarhuis et al., this seems to be the case both for 'ordinary domains', which don't display a loop at the corner of the TAD, as well as for 'loop domains'. In this manuscript, 'ordinary domains', such as those presented in Fig. 7A, do not seem to display extended loop formation. It is very difficult to make general conclusions from just seeing the selected regions in these panels. I would therefore suggest that the authors perform a systematic characterisation of the differences between the two datasets so this is clarified. This is important since at the moment there is a discrepancy between the two Hi-C datasets despite both samples displaying similar vermicelli chromosomes.

The Referee is correct, that from the genomic regions shown in Fig 7A it is unclear if new long-range contacts also emerge from ordinary domains in WAPL depleted cells (please note that these regions were chosen to show that TADs tend to merge in WAPL depleted cells, and not to illustrate the appearance of new long-range contacts from ordinary domains). As suggested, we therefore now analyzed ordinary domains systematically for the appearance of such new long-range contacts. For this purpose, we identified among the TADs of HeLa control cells 826 that clearly represent ordinary domains and found that from 344 of these new long-range contacts emerged in WAPL depleted cells, consistent with the observations reported by Haarhuis et al., 2017.
We made similar observations in Hi-C data that we obtained in the meantime from Wapl depleted MEFs (new Fig S7; see point 5 above). In these cells, we identified 658 TADs that unambiguously represent ordinary domains. New long-range contacts emerged from 333 of these in WAPL depleted cells. These new analyses are described in the Results section.

8. The authors report ~1.500 TADs in prometaphase cells (Fig 7B), but these are not visible on the Hi-C plots (Fig 7A). In addition the level of insulation seems minimal (Fig 7D). This raises the question as to whether TADs are really present in mitotic chromatin. As mentioned above, it seems to me like the current methods used to call TADs lack a proper calibration that would allow the authors to distinguish between being able to detect fluctuations on the Hi-C signal and whether TADs are actually visible on the data. This has also been recently reported for interphase and mitotic chromatin in fly embryos (Hug et al., Cell 2017). The authors should discuss this limitation and/or provide examples of those TADs detected in prometaphase cells.

The Referee is correct, that the large number of TADs that can be called in prometaphase cells is largely due to the increased sensitivity of the TAD calling algorithm used for datasets with weaker boundary structure. We have now added an explanation of this issue to the manuscript (for further explanations, please see reply to point 2).
As suggested, we have now illustrated structures that are automatically called as TADs in prometaphase cells in the revised version of Fig 7A (see lower right panel).

9. The authors state that 3C primers are available upon request. I'd request that the authors provide a suitable list of primers as part of the manuscript so the results can be easily reproduced.

The primers used in this study are now listed in the Material and Methods section.

10. The authors should also include a link to a public repository (eg. GEO) containing the raw and processed sequencing data produced here.

© European Molecular Biology Organization

We uploaded all genomic data reported in our manuscript to the GEO database at NCBI and provided information about how the data can be accessed to the editorial office of EMBO Journal on 23 August. The data can be accessed via the following accession number together with a secure token, but will be made public upon publication of this manuscript.

Referee #3:

The manuscript by Wutz et al. examines the effect of depletion of cohesin, WAPL, CTCF, PDS5A and PDS5B in genome architecture in HeLa cells. Cohesin and CTCF are removed acutely using auxin-inducible degrons, while other proteins are depleted by RNAi. The authors examine several levels of genome organisation based on Hi-C analysis: compartments, TADs and loops. They find that cohesin is required for the formation of TADs and loops and that compartments are increased in its absence. CTCF depletion causes distortion of TAD boundaries and reduced the number of TADs. WAPL or PDS5 depletion reduced the compartmentalization, increased the size but decreased the number of TADs, suggestion TAD fusion. WAPL depletion also resulted in the formation of more, longer loops. While PDS5 depletion resulted in many fewer loops. Both WAPL and, especially PDS5 are important for the CTCF convergence rule. Imaging and fractionation experiments showed that PDS5, like WAPL contributes to the turnover of cohesin on chromosomes. Based on these observations, the authors argue that their data is consistent with the idea that cohesin directly forms TADs and loops, that CTCF acts to enforce boundaries at the bases of these loops, that WAPL and PDS5 limit the extent of TADs and loops by decreasing the residence time of cohesin on chromosomes and that PDS5 helps to recognise CTCF to provide boundary function. These observations and their interpretation are consistent with the "loop extrusion" model for SMC protein function which is gathering strong support in the literature. Overall, the new findings add to our understanding of this rapidly developing field and are appropriately placed into context and the current literature. Conclusions are drawn with appropriate caution where technical limitations prevail. Experiments are well controlled and the manuscript is well written and presented. There are a few cases where the experimental design warrants further discussion, but this is a relatively minor criticism.

1. All experiments were conducted in HeLa cells, which are karyotypically abnormal. A consideration of how this affects the analysis and potentially also the conclusions should be mentioned, particularly as other published work has used cell types, which are expected to be karyotypically normal.

This is an important question which we have now addressed in several ways.
We considered whether the three-dimensional organization of HeLa cells reflects principles that also operate in karyotypically normal cells. We assumed that this would be the case when we initiated our study because previous work from Rao et al., 2014 had indicated this, and because a large body of work performed in the cell cycle field and other areas of cell biology had implied that most basic processes observed in non-transformed diploid cells are fully operational in HeLa cells. Indeed, we observed the same vermicelli phenotype in HeLa cells upon Wapl depletion as in mouse embryonic fibroblasts (MEFs, Tedeschi et al., 2013).
Since then, we have also analyzed the effects of one of our experimental perturbations (Wapl depletion) in primary MEFs. The Hi-C results obtained from these experiments confirm our observations in HeLa cells and thus support the notion that the latter can be used for analyzing chromatin structure by Hi-C despite the karyotypic abnormalities. We have now mentioned these considerations in the Results section.

2. The effect of WAPL and PDS5 depletion on TADs is relatively modest (Figure 7B, C). Is this statistically significant? The authors should comment as to why this is so modest. Are particular TADs more affected that others and does this correlate with cohesin/CTCF density?

Our analysis of cells depleted of WAPL and PDS5 proteins did reveal clear and highly reproducible changes in the number and sizes of TADs (see Figs 7B and C). However, we cannot exclude that residual amounts of these proteins persisted under our experimental conditions, in which WAPL and PDS5 proteins were depleted by RNAi. This is known to lead

© European Molecular Biology Organization

to incomplete protein depletion and therefore to hypomorphic phenoytpes. To test this possibility, we have now performed Hi-C experiments in mouse embryonic fibroblasts (MEFs) from which the *Wapl* gene was deleted. Our previous analyses had indicated that most Wapl is depleted in these cells (Tedeschi et al., Nature 2013; Busslinger et al., Nature 2017). The results from these new Hi-C experiments confirmed what we had observed in HeLa cells (see new Fig S7).

3. Some labelling in figures is not correctly reproduced in the online file (e.g. Figure 5D MAD2L-GFP + Hela).

We thank the Referee for noticing this issue. We have now corrected the labeling in the figures.

2nd Editorial Decision 2 November 2017

Thank you for submitting a revised version of your manuscript. Based on your response to the referee concerns, your study is now in principle ready for acceptance in The EMBO Journal. However, before we can officially accept the manuscript and transfer it to production there are a few editorial issues concerning text and figures that I need you to address.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to receiving your final revision.

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: Jan -Michael Peters
Journal Submitted to: Embo j
Manuscript Number: EMBOJ-2017-98004

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were as high as was feasible given our experimental approach and were similar to those reported in experiments performed by colleagues.
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.	No randomisation was used in this study.
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.	Assessor blinding was not used in this study.
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?	S.E.M. is displayed on graphs in Fig 1D, 2D, 4B, 4E, 4F, 10B and EV3D.
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

Question	Answer
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).	Citations / product numbers provided for all antibodies used in this study.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	yes

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

D- Animal Models

Question	Answer
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

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
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

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'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Hi-C and ChIPseq data: Gene Expression Omnibus GSE102884
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).	
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

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