Journal of Cell Science

Review COMMONS

The histone methyltransferase NSD3 contributes to sister chromatid cohesion and to cohesin loading during mitotic exit

Grégory Eot-Houllier, Laura Magnaghi-Jaulin, Gaëlle Bourgine, Fatima Smagulova, Régis Giet, Erwan Watrin and Christian Jaulin
DOI: 10.1242/jcs.261014

Editor: David Glover

Review timeline

Submission to Review Commons:	17 December 2021
Submission to Journal of Cell Science:	25 January 2023
Editorial decision:	6 March 2023
First revision received:	14 April 2023
Accepted:	21 April 2023

Reviewer 1

Evidence, reproducibility and clarity

In the manuscript at hand the work by Eot-Houllier, Jaulin and coworkers begins with an RNAi screen depleting 14 different SET domain methyltransferases in HeLa-K cells. Only in case of a NSD3-depletion this resulted in premature loss of cohesion in 20-50% of nocodazole-arrested cells (depending on the siRNA used) as judged by chromosome spreading. Looking for a molecular explanation, the authors noted that less cohesin and Mau2 (a kollerin subunit) were loaded in telophase when the NSD3-methyltransferase is missing. Accoring to IFM data, this correlated with the recruitment of NSD3 to chromosomes in anaphase, i.e. slightly before the kollerin-mediated re-loading of cohesin. Finally, the authors show by isotype-specific depletion/rescue-experiments that proper cohesion requires presence of NSD3L, which - in contrast to the shorter NSD3s isoform - contains the SET methyltransferase domain. By inference, this could mean that the cohesion-supporting function of NSD3 is mediated by the methylation of histones, presumably at H3K36.

Significance

Frankly, I am not very excited about this manuscript because - as outlined in more detail below - 1) the conclusions are limited and 2) the data supporting the few claims made are neither exhaustive nor fully convincing.

Ad 1) The paper lacks mechanistic insight. Is the enzymatic activity of NSD3 needed or not and, if yes, what is the substrate? And how does the corresponding methylation result in the recruitment of kollerin? All these questions remain unanswered. At least whether the methyltransferase activity is needed could have been determined by easy rescue experiments making use of a catalytically inactive point mutant of NSD3L.

Ad 2) Localization of Bub1 to kinetochores is not an accepted method to show that the spindle assembly checkpoint (SAC) is still active. This is because of conflicting results regarding the requirement of Bub1 for the SAC and because significant amounts of Bub1 remain at kinetochores upon microtubule attachment (see R Soc Open Sci. 2021 Dec 15;8(12):210854.doi: 10.1098/rsos.210854, Semin Cell Dev Biol. 2021 Sep;117:86-98. doi: 10.1016/j.semcdb.2021.06.009 and references therein). The authors would have to look at Mad1 or Mad2 instead. Furthermore, they need to show that the mitotic index (and cyclin B1) goes up upon knock-down of NSD3L but not so if Mad2 is depleted at the same time.
Similarly, the presence of Sgo1 at kinetochores/centromeres does not prove that

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

(peri)centromeric cohesin is properly protected. Sgo1 shuttles from H2AT120p at inner kinetochores to (peri)centromeric cohesin and back (see Mol. Cell 2015 Aug 6;59(3):426-36. doi: 10.1016/j.molcel.2015.06.018 and references therein). Given the close juxtaposition of these sites, higher resolution imaging relative to marker proteins is required to conclude that Sgo1's localization to pericentromeres is undisturbed by the absence of NSD3.

The cohesion defect caused by the RNAi of NSD3 is rather small. Moreover, the efficiencies of the various siRNAs do not correlate with the effect on cohesion. For example, siRNA NSD3-beta gives the strongest effect but depletes NSD3 less well than the alpha and gamma siRNAs (Fig 5A/B). In their rescue-experiment, the authors already disregard all cells that are GFP-negative. Those that are GFP-positive overexpress LAP-NSD3L according to Western analysis (Fig. 5D). Why then is the rescue still incomplete (Fig. 5E)?

The effect of NSD3-depletion on the recruitment of kollerin and cohesin is small and hence, would need additional experimental support. Does overexpression of kollerin suppress the cohesion defect caused by NSD3 depletion? Alternatively, one could target kollerin to specific chromosomal regions. Would expression of Mau2 in fusion with Trf1 or Cenp-A rescue cohesion at telomers or centromeres, respectively, when NSD3 is depleted?

It appears that the distance between FISH- and CREST-signals in figures 2D and 5E, respectively, were determined without considering the z-axis. If so, then this would be an error-prone approach because it does not exclude a putative tilting of a pair of dots relative to the focal plane.

Minor points:

In figure 3B, why is the soluble cytosolic fraction not subjected to Western analyses as well and why is the decline of cyclin B1 (or securin) not used as a marker for mitotic exit, as the authors have done in a previous study of theirs?
Why were the cells in figure 4D/E not pre-extracted? The result would have been much clearer.

The fact that the paper is also poorly written does not help. To give just one example: Both the introduction and the beginning of the result sections imply that this study is about the identification of the methyltransferase responsible for the H3K4me2 mark that results as a consequence of HDAC3 depletion (as previously published by the same authors). The fact that it is actually not is confusing to the reader.

An unexpected link between the histone code and sister chromatid cohesion would of course be of great interest. Unfortunately, the work by Eot-Houllier, Jaulin and coworkers at the current stage fails to establish such a connection convincingly.

Reviewer 2

Evidence, reproducibility and clarity

Eot-Houllier et al. have previously published work implicating H3K4 acetylation/methylation in regulating chromosome cohesion in mitosis. In this study, they conduct a mini RNAi screen of histone methyltransferases in a human cell line looking for such modifiers that might regulate cohesion. They show that NSD3 localises to chromatin soon after mitotic exit, and just before the cohesin loader component MAU2 and cohesion subunit Rad21. They find that NSD3 knockdown compromises cohesion in G2 and mitosis and that it reduces MAU2 and Rad21 cohesin reloading in early G1. They conclude that the long form of NSD3 acts through MAU2 is regulate cohesin loading post-mitosis.

This is a careful and thoughtful study that is well-controlled. The conclusion that NSD3 loss alters chromosome cohesion is convincing, and novel as far as I know. However, the mechanism by which NSD3 affects cohesion remains speculative. Although NSD3 loss appears to alter MAU2 recruitment to chromosomes, no direct evidence is provided that the reduced MAU2 localisation is responsible

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

for the effect. Conceivably, there are many other proteins whose expression or recruitment to chromosomes are altered by NSD3 knockdown, so a main conclusion is rather shaky in my view.

Major Comments

1. There is a clear focus on MAU2 as the potential target of NSD3. However, the change in MAU2 association with chromosomes (Fig 3) is relatively modest compared to that of cohesin. Also, there are other cohesion regulators that are not examined that could alter cohesion in interphase and mitosis, such as WAPL, Sororin, ESCO1/2, and even Haspin (indeed, I do not believe that WAPL activity is restricted to G2 cells, as implied in the introduction). The conclusion that altered MAU2 is responsible for the observed cohesion changes is therefore not fully supported.
2. Related to point 1, an experimental approach to provide support for the role of MAU2 in the phenotype of NSD3 depletion would be very useful. Can cohesion be restored by forcing MAU2 back onto chromosomes, for example? Can direct or indirect physical interactions between NSD3 and MAU2 be demonstrated?
3. In Figure 2, Sgo1 and Bub1 presence is used to argue that cells with defective cohesion are still in mitosis (ie not anaphase). While useful (also showing that loss of these factors is likely not responsible for cohesion loss), both of these proteins can persist at normal anaphase chromosomes for a time. It might be more convincing to show that Cyclin B is still present, or that Cyclin B-Cdk1 substrates are still strongly phosphorylated.
4. In Figure 3, no protein that is newly re-recruited to chromatin during mitotic exit that is not altered by NSD3 depletion is shown (eg transcription factors, CTCF, nuclear envelope proteins?). This would be useful to show the specificity of the effect.
5. Fixation can alter protein localisation on/off chromatin. It would be useful to confirm NSD3 localisation studies in live cells using the LAP-NSD3 cell lines.

Minor Comments

1. Molecular weight markers should be shown on gels/blots.
2. The phrase "post-replicative" is used a few times. In my experience, this term is most often used for cells that have exited the cell cycle, and no longer engage in DNA replication and mitotic division, which is not the case here. Consider using a different term.
3. It would be useful to have a detailed scheme (as in Fig 3A etc) to show how the initial methyltransferase screen was done. Related to this, does the proportion of cells in prometaphase go up (ie essentially the mitotic index), as well as the proportion of prometaphase cells with defective cohesion? This was somewhat ambiguous.
4. Although NSD3 appears nuclear in prophase, colocalization with DNA staining is not very convincing. To my eye, NSD3 appears to leave chromosomes very rapidly in prophase.
5. Line 222-223. Should say "inactivation of NSD3 resulted in loss of cohesion and kollerin loading"?
6. Line 230. NSD3, not NDS3
7. Line 254. "consequently" here is too strong. Cause and effect are not established

Significance

Cohesion regulation is a topic of wide interest, as it is involved in gene regulation/genome compartmentalization as well as in sister chromosome cohesion. Indeed, it is quite intriguing that an H3K36-targeted enzyme might regulate cohesion, as this could suggest novel connections between euchromatin/gene bodies and cohesion loading. Therefore, this work has potential significance if the role of a new methyltransferase in cohesion loading can be substantiated.

Reviewer 3

Evidence, reproducibility and clarity

This manuscript describes a novel role for NSD3 in sister chromatid cohesion. In addition to the observed mitotic defects, the authors demonstrate that cohesion defects are observed prior to mitotic entry. Analysis of the dynamics of proteins association at mitotic exit led the authors to conclude that NSD3 impacts on sister chromatid cohesion by promoting the loading of the cohesin leader MAU2 at mitotic exit. Overall, I found the claim that NSD3 is required for sister chromatid

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

cohesion well supported, novel, interesting and with potential high reach. Less clear is exactly what is going on at the mechanistic level and whether or not this is required to report these interesting findings is at editorial discretion.

Major points:

1. The authors should clarify whether or not H3K4me2 at centromeres is affected in NSD3 depleted cells. This seemed as the initial premise for the entire study but the circle is not fully closed then. This is particularly confusing as Figure S2 show that global levels of H3K4me2 are not affected. Even if the answer is negative, and the authors wish to leave the molecular details open, closing this circle would be important.
2. Regarding the claim that cells do not exit mitosis prematurely and that cohesion protection remains unaffected, there are two major issues with the data provided (Fig. 2A):
- a. the choice for Bub1 to monitor if SAC is active is not the most straightforward to distinguish what the authors wish for (SAC activation vs cohesion protection), as Bub1 is also involved in cohesion protection. Hence, a more conventional SAC assay (e.g. Mad2 labelling or efficient arrest upon spindle poisons) should be used.
- b. In both single examples provided, both Bub1 and Sgo1 levels seem reduced relative to controls? Is this indeed the case? Can the authors back up their claims with a more thorough quantitative analysis on Bub1 (Mad2) and Sgot levels?
3. The data that best addresses what NDS3 may be doing at mitotic exit is the one presented in figure 3, where chromatin levels of different analyzed proteins are reported during mitotic exit/G1 entry. It would be important to state somewhere how many times these experiments were performed and replace the graphs by similar ones where each independent experiment is depicted.
4. The discussion is very interesting in speculating several potential mechanisms by which NSD2 could promote cohesion and MAU2 loading. Yet, I think the discussion should be more in line with the descriptive nature of the paper. In many cases it attempts to infers mechanistic links that are not experimentally addressed. For example, MAU2 loading is overemphasized as the "primary target", but looking at Figure 3 the reduction is rather mild. Also, what happens to NIPBL levels in this assay?

Minor points:

- The authors should have their manuscript corrected for English grammar.
- In the introduction (line 77) it states that NSDs are specific to H3K36 which is somehow contradicted in the subsequent sentence. This needs better clarification for non-experts.
- The entire section on the localization of NDS3 is somehow a bit odd, as the reported behaviour is what one would expect for most nuclear proteins (as most are evicted from mitotic chromatin). Hence, unless the authors wish to develop on the kinetics of association at mitotic exit with detailed mechanistic insights (e.g. labelling of the target), this section should be shortened. Otherwise, it attempts to provide a causal and temporal link that is not fully proven.
- the sentence in the discussion line 259 " the dynamic behaviour of NSD3 is similar to that of MAU2 and of cohesin" should be clarified to what "dynamics" the author refer to. The authors clearly state in this manuscript that with regard to cell cycle dynamics it is not exactly the same (NSD3 binds chromatin earlier in late mitosis), as stated in the subsequent sentence.

Significance

The mechanisms that regulate sister chromatid cohesion are of prime importance in the chromosome biology field as cohesin is required for correct cell division and also proper 3D genome architecture in interphase cells.

Although there is already a vast knowledge on cohesin regulation, mostly during replication and mitosis, not the entire cohesin cycle is fully understood and novel players are continuously emerging. Thus, uncovering a new regulator of sister chromatid cohesion is very important. Additionally, the mechanisms that regulate cohesin loading, during mitotic exit throughout the following cell cycle are comparatively less clear, particularly with regard to the direct interaction with chromatin.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Referees cross-commenting

Here the comments of the 3 reviewers are reported

Reviewer 1

I like the comments by the other two referees (#2 and #3) and fully agree with them. They are more positive in the overall tone than my comments but still raise a number of similar issues. I assume that the main purpose of this 'referee cross commenting' (which is new to me) is to agree upon a fair list of additional experiments that are doable within a reasonable time-frame and would strengthen the main claims of the paper. Reading all reviews, the smallest common denominator seems to be:

1. The statement that the absence of NSD3 results in a SAC-mediated mitotic arrest due to premature loss of cohesion needs substantiation beyond mere localization of Bub1 and Sgo1, for example by mitotic index measurements and determination of cyclin B levels.
2. The conclusion that NSD3 acts by recruiting Mau2 need further experimental proof. This could be a rescue of the NSD3-depletion phenotype by overexpression or forced chromosomal targeting of Mau2 and this could be the demonstration of a direct or indirect physical interaction between NSD3 and MAU2.

Reviewer 2

I agree that the reviews are pretty much in line with one another, and Reviewer 1's additional comment is very reasonable.
The extent to which the role of MAU2 needs to be consolidated may depend on the target journal but, in the absence of further work, the paper certainly needs to make clear the speculative nature of the connection to MAU2. My feeling is most journals in the Review Commons stable would prefer the role of MAU2 to be substantiated

Reviewer 3

I agree that despite differences in tone, we are all in line in our assessments, which is good :)
Without knowing for which journal we are reviewing, it is more difficult to access what really needs to be addressed. But I agree with the previous comment that most of the journals in review commons would like more depth in the study, particularly with regard to MAU2 chromosomal targeting. The finding is indeed novel and interesting, but in its current version it is still too descriptive.

Author response to reviewers' comments

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In the manuscript at hand the work by Eot-Houllier, Jaulin and coworkers begins with an RNAi screen depleting 14 different SET domain methyltransferases in HeLa-K cells. Only in case of a NSD3- depletion this resulted in premature loss of cohesion in 20-50% of nocodazole-arrested cells (depending on the siRNA used) as judged by chromosome spreading. Looking for a molecular explanation, the authors noted that less cohesin and Mau2 (a kollerin subunit) were loaded in telophase when the NSD3-methyltransferase is missing. Accoring to IFM data, this correlated with the recruitment of NSD3 to chromosomes in anaphase, i.e. slightly before the kollerin-mediated reloading of cohesin. Finally, the authors show by isotype-specific depletion/rescue-experiments that proper cohesion requires presence of NSD3L, which - in contrast to the shorter NSD3s isoform - contains the SET methyltransferase domain. By inference, this could mean that the cohesion-supporting function of NSD3 is mediated by the methylation of histones, presumably at H3K36.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Reviewer #1 (Significance (Required)):

Frankly, I am not very excited about this manuscript because - as outlined in more detail below - 1) the conclusions are limited and 2) the data supporting the few claims made are neither exhaustive nor fully convincing.

Ad 1) The paper lacks mechanistic insight. Is the enzymatic activity of NSD3 needed or not and, if yes, what is the substrate? And how does the corresponding methylation result in the recruitment of kollerin? All these questions remain unanswered.

- We share the reviewer's concern regarding the need for looking at NSD3 catalytic activity. We have established rescue experiments using new cell lines expressing either catalytically active or inactive NSD3. We unambiguously show in new figures 5E and 5F, that NSD3 activity is required to rescue mitotic cohesion defects. We have also shown in a new figure 5D that only depletion of the long Isoform carrying this activity decreases the amount of MAU2 and RAD21 on chromatin.

- We agree that the search for the methylation substrate and the identification of the mechanism of Kollerin recruitment would be a very important insight. However, we feel that the identification of NSD3 numerous substrates including those involved in sister chromatid cohesion mechanisms is beyond the scope of the current study. We have discussed potential recruitment mechanisms. These mechanisms could involve methylation of histone(s) that serve as a docking site for kollering. Histone methylation could create a favorable environment allowing Kollerin recruitment. It could also involve associated partner such as chromatin remodelers. Another possibility is that NSD3 would methylate Kollerin or another non-histone partner. Nevertheless, we describe here a new effector of kollerin recruitment, a key process required for cohesin loading and chromatin organization.

At least whether the methyltransferase activity is needed could have been determined by easy rescue experiments making use of a catalytically inactive point mutant of NSD3L.

- We demonstrated the requirement of the methyltransferase enzymatic activity to rescue the phenotype of sister chromatid cohesion. This result is now presented in the new figure 5E.

Ad 2) Localization of Bubl to kinetochores is not an accepted method to show that the spindle assembly checkpoint (SAC) is still active. This is because of conflicting results regarding the requirement of Bub1 for the SAC and because significant amounts of Bub1 remain at kinetochores upon microtubule attachment (see R Soc Open Sci. 2021 Dec 15;8(12):210854.doi: 10.1098/rsos.210854, Semin Cell Dev Biol. 2021 Sep;117:86-98. doi: 10.1016/j.semcdb.2021.06.009 and references therein). The authors would have to look at Mad1 or Mad2 instead. Furthermore, they need to show that the mitotic index (and cyclin B1) goes up upon knock-down of NSD3L but not so if Mad2 is depleted at the same time.

- We agree that the data concerning Bub1 and SAC activation is not completely convincing. We made a lot of work to unambiguously show that the mitotic arrest is a consequence of SAC activation.

  We have shown that depletion of NSD3 with various siRNA, significantly increases the mitotic index in the new figure 1D, and the amount of two mitotic markers (Cyclin B1 and of phosphorylated H3S10) in the new figure 1C. In our hands, siRNA-mediated depletion for MAD2 and NSD3 are achieved after 24h and 72h following siRNA transfection, respectively. This difference in depletion kinetics makes difficult to obtain clean co-depletions. To avoid early chromosome segregation defects and complex phenotype interpretation, we felt that the use of the MPS1/SAC inhibitor Reversine would be more straightforward to assay SAC activation in NSD3 depleted cells. Strikingly, the increase in Cyclin B1 and H3S10P levels that we observed in absence of NSD3 is abolished by a reversine treatment for 3 hours. The same exit from mitotic block was observed in cells treated with a siRNA specifically targeting NSD3- L (New figure 1E and figure 5C).
  In a distinct experiment, we have also shown that depletion of NSD3 has no effect on the accumulation of Cyclin B1 and phosphorylated H3S10 following cell treatment with nocodazole

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

for 6h, indicating that the spindle assembly checkpoint is fully functional in absence of NSD3 (new figure 1F).
Therefore, the most straightforward interpretation of these data is that precocious sister chromatid separation generated by NSD3 depletion leads to an SAC-dependent mitotic arrest.

Similarly, the presence of Sgo1 at kinetochores/centromeres does not prove that (peri)centromeric cohesin is properly protected. Sgo1 shuttles from H2AT120p at inner kinetochores to (peri)centromeric cohesin and back (see Mol. Cell 2015 Aug 6;59(3):426-36. doi: 10.1016/j.molcel.2015.06.018 and references therein). Given the close juxtaposition of these sites, higher resolution imaging relative to marker proteins is required to conclude that Sgo1's localization to pericentromeres is undisturbed by the absence of NSD3.

• There are two pools of SGO1 localized at inner and outer centromere, respectively. In the article cited by the reviewer, the authors have shown that the SGO1 pool that is localized at outer centromere is not sufficient to prevents cohesion dissociation at centromere. We have shown that, following NSD3 depletion, SGO1 is still present at centromeres on separated sister chromatids. Localization of SGO1 on separated sister chromatids in NSD3 depleted cells and associated chromatids in the control cannot be compared (the concept of inner and outer kinetochore does not apply to isolated chromatids) and, as a consequence, we cannot discriminate whether the SGO1 we observed on separated chromatids corresponds to the one localized at inner centromere (the one responsible for cohesion), at outer centromere or both.

• However, to determine a contribution of NSD3 in the mitotic cohesion regulating pathway, we performed co-depletion of WAPL and NSD3. SGO1 and Haspin protect centromeric cohesion from dissociation induced by WAPL. Even the cohesion fatigue that occurs following prolonged mitotic arrest with kinetochores under tension is a consequence of WAPL activity. We have shown (new Figure 2B) that depletion of WAPL do not rescue the PSCS induced by NSD3 (new Figure 2B) or RAD21 (new figure S3B) depletion. In contrast, PSCS induced by SGO1 depletion were rescued by WAPL depletion (new Figure S3A). This data show that the sister chromatid cohesion defect we observed in mitosis following NSD3 depletion is not a consequence of a defect in centromeric cohesion protection but takes its origin from an event occurring before mitotic entry.

The cohesion defect caused by the RNAi of NSD3 is rather small. Moreover, the efficiencies of the various siRNAs do not correlate with the effect on cohesion. For example, siRNA NSD3-beta gives the strongest effect but depletes NSD3 less well than the alpha and gamma siRNAs (Fig 5A/B).
In their rescue-experiment, the authors already disregard all cells that are GFP-negative. Those that are GFP-positive overexpress LAP-NSD3L according to Western analysis (Fig. 5D). Why then is the rescue still incomplete (Fig. 5E)?

• We agree that siRNA NSD3 have the strongest effect whereas it is less efficient in protein knock down. NSD3 is a protein that is involved in gene expression and genome organization. We also show in this work that NSD3 regulates cohesin loading, a complex also involved in gene expression and chromatin organization. We cannot rule out the possibility that a more efficient extinction of NSD3 leads to pleiotropic and indirect effect(s) that partially prevents sister chromatid dissociation.
• Rescue experiments frequently result in an incomplete rescued phenotype. The rescue seems better with the siRNA targeting specifically NSD3-L, but the phenotype is less penetrant is this case. Expression of the WT rescued 70% of the PSCS after depletion with the NSD3 siRNA targeting both isoforms compared to a 77% rescue after depletion with the siRNA specific for the long form of NSD3 (siRNA NSD3-L-2). This rather high level of rescued phenotype in two independent experimental setups - together with the fact that three different siRNAs against NSD3 give comparable phenotypes - make the hypothesis of an off- target phenomenon unlikely.
• In addition, the absence of rescue with the methyltransferase Y1261A inactive NSD3 mutant clearly demonstrate that the NSD3 long form contribute to sister chromatid cohesion (new Figure 5E). We modified the presentation of figure 5E to include results obtained with the NSD3 Y1261A mutant and expect that it renders this aspect of the results more convincing.

The effect of NSD3-depletion on the recruitment of kollerin and cohesin is small and hence, would need additional experimental support. Does overexpression of kollerin suppress the cohesion defect

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

caused by NSD3 depletion? Alternatively, one could target kollerin to specific chromosomal regions. Would expression of Mau2 in fusion with Trf1 or Cenp-A rescue cohesion at telomers or centromeres, respectively, when NSD3 is depleted?

- Because MAU2 expression level is not affected by NSD3 depletion, we are not fully convinced that kollerin overexpression would rescue the mitotic defects observed in absence of NSD3. Besides, the outcome of such overexpression experiments can be ambiguous. A rescued cohesion phenotype following kollerin overexpression (or targeting of a fusion protein) does not necessarily mean that NSD3 is involved in kollerin recruitment. Overexpressed kollerin may recruit more soluble cohesin (which is abundant in the nucleoplasm), independently of the putative function of NSD3 in this recruitment, and act as an unspecific "chromatid glue". Conversely, a lack of rescue may result from many experimental parameters, such as insufficient overexpression or targeting the fusion proteins to sites where essential partners are not present. Thus, the amount of work required for a result whose interpretation would be uncertain seems too elevated.
- However, to reinforce the data concerning the recruitment of cohesin and kollerin, we present a new figure 3B and 3C showing mean and standard deviation of the quantification of 2 independent experiments and comparison of the presence of those complexes on whole cell extracts and chromatin extracts. Thus, we can directly observe that the effects observed were not due to differences in global protein expression.
- In addition, we have also shown the existence of a physical interaction between NSD3 and the kollerin complex (new Figures 3D and 3E). This data confirmed the interaction between NSD3 and NIPBL recently published by Linares-Saldana & al. (Nature Genetics 2021).
- We have also shown that specific inhibition of NSD3-L, but not of NSD3-s, decreases the amount of Kollerin and cohesin on chromatin in the new figure 5D. For all these reasons we believe that the contribution of NSD3 to kollerin and cohesin recruitment on chromatin is a reliable interpretation of our data.

It appears that the distance between FISH- and CREST-signals in figures 2D and 5E, respectively, were determined without considering the z-axis. If so, then this would be an error-prone approach because it does not exclude a putative tilting of a pair of dots relative to the focal plane.

- In the past, we compared the data obtained from 2D images or by taking 3D into account and did not observed a significative difference in the results obtained. Moreover, the apparent distance between two dots in a 3D object following projection on a plane is smaller than the real distance in 3D. Thus, for figure 2D (FISH), if a difference is found in 2D analysis between NSD3-depleted and control cells, this difference would be even bigger if the real distance was measured in 3D. Thus, 2D analysis cannot overestimate the distance between two dots in a 3D object. However, when the two dots could not be clearly resolved, we removed them from the analysis. We modified the material and methods section concerning FISH experiments as follow (lane 494): "Analysis was only done on pairs for which the dots could be clearly resolved".
- The same line of reasoning applies to figure 5E.

Minor points:
In figure 3B, why is the soluble cytosolic fraction not subjected to Western analyses as well and why is the decline of cyclin B1 (or securin) not used as a marker for mitotic exit, as the authors have done in a previous study of theirs?

- We have modified figure 3B similarly to Watrin & al. (Curr Biol, 2006). The figure now shows a representative western blot of whole cell extracts versus chromatin cell extracts. Whole cell extracts allow to visualize the global level of proteins which would not be the case with cytoplasm only extracts. We also added mean and standard deviation of the quantification of two independent experiments. We used phosphorylated H3S10P as a mitotic marker instead of Cyclin B1 because Cyclin B1 is not bound to chromatin in our extraction protocol.

Why were the cells in figure 4D/E not pre-extracted? The result would have been much clearer.

- Actually, this was the case in figure S5 provided with the previous manuscript. In accordance with the reviewer suggestion, the previous figure 4D/E were removed from the manuscript and the previous figure S5 showing the results in pre-extraction condition is the new figure 4A and 4B

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

The fact that the paper is also poorly written does not help. To give just one example: Both the introduction and the beginning of the result sections imply that this study is about the identification of the methyltransferase responsible for the H3K4me2 mark that results as a consequence of HDAC3 depletion (as previously published by the same authors). The fact that it is actually not is confusing to the reader.

- We have now written a more focused introduction, largely modified the discussion and the manuscript has been edited by a professional edition service.

An unexpected link between the histone code and sister chromatid cohesion would of course be of great interest. Unfortunately, the work by Eot-Houllier, Jaulin and coworkers at the current stage fails to establish such a connection convincingly.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Eot-Houllier et al. have previously published work implicating H3K4 acetylation/methylation in regulating chromosome cohesion in mitosis. In this study, they conduct a mini RNAi screen of histone methyltransferases in a human cell line looking for such modifiers that might regulate cohesion. They show that NSD3 localises to chromatin soon after mitotic exit, and just before the cohesin loader component MAU2 and cohesion subunit Rad21. They find that NSD3 knockdown compromises cohesion in G2 and mitosis and that it reduces MAU2 and Rad21 cohesin reloading in early G1. They conclude that the long form of NSD3 acts through MAU2 is regulate cohesin loading post-mitosis.

This is a careful and thoughtful study that is well-controlled. The conclusion that NSD3 loss alters chromosome cohesion is convincing, and novel as far as I know. However, the mechanism by which NSD3 affects cohesion remains speculative. Although NSD3 loss appears to alter MAU2 recruitment to chromosomes, no direct evidence is provided that the reduced MAU2 localisation is responsible for the effect. Conceivably, there are many other proteins whose expression or recruitment to chromosomes are altered by NSD3 knockdown, so a main conclusion is rather shaky in my view.

We acknowledge this reviewer for its positive evaluation of our manuscript. Given the importance of chromosome cohesion in the cell division and the growing number of evidences of NSD3 involvement in multiple cancers, we believe that this study on NSD3 as a new crucial cohesion regulator will be of broad interest to our colleagues in the field.

Major Comments

1. There is a clear focus on MAU2 as the potential target of NSD3. However, the change in MAU2 association with chromosomes (Fig 3) is relatively modest compared to that of cohesin. Also, there are other cohesion regulators that are not examined that could alter cohesion in interphase and mitosis, such as WAPL, Sororin, ESCO1/2, and even Haspin (indeed, I do not believe that WAPL activity is restricted to G2 cells, as implied in the introduction). The conclusion that altered MAU2 is responsible for the observed cohesion changes is therefore not fully supported.

- In the new figure S2, we added analysis of the expression level of WAPL, Sororin and ESCO2. We showed that there is no obvious modification in the global expression level of these cohesion regulators. WAPL depletion does not rescue the sister chromatid cohesion defects induced by NSD3 depletion (new figure 2B) as after RAD21 depletion (new figure S3B) while it rescues SGO1 depletion-induced PSCS (new figure S3A). Therefore, it seems unlikely that WAPL, Sororin and Haspin are involved in the phenotype seen following NSD3 depletion.
- We also modified figure 3 by adding the mean and standard deviation of quantification from two independent experiments that gave the same profile of protein levels.
- We agree that WAPL activity is not restricted to G2 phase. In consequence, we modified the introduction to clarify this point as follows:
  - Lines 48-50:"Before DNA replication, cohesin and chromatin association is dynamic, and it is actively removed by the WAPL cohesin release factor (WAPL) aided by scaffolding proteins PDS5A and PDS5B."
  - Lines 54-56: "Acetylated SMC3 allows for the subsequent binding of sororin (also known as cell division cycle associated 5 or CDCA5) to cohesin complexes, where it antagonizes WAPL anti-cohesive activity until mitosis begins."

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

2. Related to point 1, an experimental approach to provide support for the role of MAU2 in the phenotype of NSD3 depletion would be very useful. Can cohesion be restored by forcing MAU2 back onto chromosomes, for example? Can direct or indirect physical interactions between NSD3 and MAU2 be demonstrated?

• The outcome of such forced localization experiments can be ambiguous. A rescued cohesion phenotype following the targeting of a fusion protein does not necessarily mean that NSD3 is involved in kollerin recruitment. Forced targeting of kollerin may recruit more soluble cohesin (which is abundant in the nucleoplasm), independently of the putative function of NSD3 in this recruitment, and act as an unspecific "chromatid glue". Conversely, a lack of rescue may result from many experimental parameters, such as targeting the fusion proteins to sites where essential partners are not present. Thus, the amount of work required for a result whose interpretation would be uncertain seems too elevated. In the new figure 3D-E, we now provide evidences for a direct interaction between endogenous and exogenous NSD3 and MAU2 and NIPBL, supporting the requirement of NSD3 for Kollerin recruitment.
This work is in agreement with a recently published study by Linares-Saldana & al. (Nature Genetics 2021).

3. In Figure 2, Sgo1 and Bub1 presence is used to argue that cells with defective cohesion are still in mitosis (ie not anaphase). While useful (also showing that loss of these factors is likely not responsible for cohesion loss), both of these proteins can persist at normal anaphase chromosomes for a time. It might be more convincing to show that Cyclin B is still present, or that Cyclin B-Cdk1 substrates are still strongly phosphorylated.

• In the new figure 1D, we show that the mitotic index is increased with the 3 siRNA tested. In the new figure 1C and 1E, we show that cyclin B1 is increased after depletion by the 2 siRNA that induce the strongest increase in mitotic index. Similar results were obtained with the H3S10P mitotic marker (new figure 1C and 1E). For both mitotic markers, the difference of signals observed after depletion of siRNA NSD3-a compared to the others correlates very well with the weak increase in mitotic index. We obtained the same results with other siRNA that targets specifically the long NSD3 isoform (new Figure 5C).
• In order to confirm that these observations came from the persistent activation of the spindle assembly checkpoint, we also analyzed the level of Cyclin B1 and phosphorylated H3S10 after abolition of the spindle checkpoint with the SAC inhibitor reversine. In this new experiment, we now show that the level of these two mitotic markers were strongly diminished following reversine treatment for 3 h (new figure 1E). We also show that NSD3 depletion do not inhibit the SAC after artificial activation with the microtubule polymerisation inhibitor nocodazole. In that case, both markers accumulate to the same extent in presence or in absence of NSD3 (new Figure 1 F).

4. In Figure 3, no protein that is newly re-recruited to chromatin during mitotic exit that is not altered by NSD3 depletion is shown (eg transcription factors, CTCF, nuclear envelope proteins?). This would be useful to show the specificity of the effect.

• We agree that we cannot rule out a global effect of the absence of NSD3 to the recruitment of multiple chromatin-associated factors during mitotic exit. However, even in this context, it does not change the fact that NSD3 depletion induces an alteration in cohesin loading and that this leads to a defect in sister chromatid cohesion, and thus the conclusions of this work. To take in consideration this open question, we add the following sentence in the discussion, line 347-350: " Future studies will also be necessary to precise the molecular mechanism of NSD3-dependent kollerin recruitment, and notably to confirm its specificity, to elucidate whether it occurs through direct interaction with the kollerin complex or by providing a chromatin context which is suitable for its binding."

5. Fixation can alter protein localisation on/off chromatin. It would be useful to confirm NSD3 localisation studies in live cells using the LAP-NSD3 cell lines.

• We share this reviewer concern. Therefore, we generated a new cell line to perform live imaging acquisition of NSD3 relative to that of histone H2A and show the dynamic localization

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

of NSD3 (new supplemental movie 1-3). This live cell imaging strongly supports the localization described on fixed preparations.

Minor Comments
1. Molecular weight markers should be shown on gels/blots.

• At this stage, due to possible rearrangement of the figures during an eventual future editing process, we will delay this finishing touch for the definitive figures.

2. The phrase "post-replicative" is used a few times. In my experience, this term is most often used for cells that have exited the cell cycle, and no longer engage in DNA replication and mitotic division, which is not the case here. Consider using a different term.

• Lines 98-99, the term "replicative cells" was replaced by the term "in G2 phase before mitotic entry"

3. It would be useful to have a detailed scheme (as in Fig 3A etc) to show how the initial methyltransferase screen was done. Related to this, does the proportion of cells in prometaphase go up (ie essentially the mitotic index), as well as the proportion of prometaphase cells with defective cohesion? This was somewhat ambiguous.

• Concerning the screen, we transfected cells for 72h with siRNA targeting some methyltransferase candidates and checked for induction of mitotic cohesion defects. We added this information on the new figure S1. We have not checked the mitotic index of all the siRNA tested because only NSD3 siRNAs gave a clear PSCS phenotype and our goal was to identify new methyltransferase involved in sister chromatid cohesion. Now, mitotic index of cells transfected with all NSD3 siRNA tested were presented in the new figure 1D.

4. Although NSD3 appears nuclear in prophase, colocalization with DNA staining is not very convincing. To my eye, NSD3 appears to leave chromosomes very rapidly in prophase.

• Lines 230-232, we have replaced the previous sentence with: "As is the case for many proteins that bind chromatin, we found that NSD3 localizes in the nucleus during interphase, is evicted from chromatin in prophase, then re-loads onto chromatin from anaphase onwards".

5. Line 222-223. Should say "inactivation of NSD3 resulted in loss of cohesion and kollerin loading"?

• This has been amended in the new version of this manuscript.

6. Line 230. NSD3, not NDS3

• This has been amended in the new version of this manuscript.

7. Line 254. "consequently" here is too strong. Cause and effect are not established.

• the discussion has been extensively rewritten and the term consequently has been removed. Line 308-310, the new corresponding sentence is: "Because of its involvement in kollerin recruitment and in cohesin loading onto chromatin, we propose that defective kollerin recruitment during mitotic exit is the cause of the PSCS observed in mitotic cells upon NSD3 inactivation.".

Reviewer #2 (Significance (Required)):

Cohesion regulation is a topic of wide interest, as it is involved in gene regulation/genome compartmentalization as well as in sister chromosome cohesion. Indeed, it is quite intriguing that an H3K36-targeted enzyme might regulate cohesion, as this could suggest novel connections between euchromatin/gene bodies and cohesion loading. Therefore, this work has potential significance if the role of a new methyltransferase in cohesion loading can be substantiated.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

This manuscript describes a novel role for NSD3 in sister chromatid cohesion. In addition to the observed mitotic defects, the authors demonstrate that cohesion defects are observed prior to mitotic entry. Analysis of the dynamics of proteins association at mitotic exit led the authors to conclude that NSD3 impacts on sister chromatid cohesion by promoting the loading of the cohesin leader MAU2 at mitotic exit. Overall, I found the claim that NSD3 is required for sister chromatid cohesion well supported, novel, interesting and with potential high reach. Less clear is exactly what is going on at the mechanistic level and whether or not this is required to report these interesting findings is at editorial discretion.

- We acknowledge this reviewer positive evaluation of our manuscript. We now clearly demonstrate that the role in sister chromatin cohesion require the methyltransferase activity (new figure 5E) and that NSD3 interacts with the kollerin complex onto chromatin (new figure 3D-E). We have also shown that the depletion of the NSD3 long isoform containing the enzymatic activity, but not that of the short one, affect the loading of kollerin and cohesin onto chromatin (new figure 5D). Therefore, we believe that these new data improve the mechanistic regulation aspects of sister chromatid cohesion and of kollerin recruitment on chromatin by NSD3.

Major points:
1) The authors should clarify whether or not H3K4me2 at centromeres is affected in NSD3 depleted cells. This seemed as the initial premise for the entire study but the circle is not fully closed then. This is particularly confusing as Figure S2 show that global levels of H3K4me2 are not affected. Even if the answer is negative, and the authors wish to leave the molecular details open, closing this circle would be important.

- After reading all the referees reviews, we understand that the reasons that led us to start this study generate a confusion. Because we have proposed a link between methylation and sister chromatid cohesion in a past publication, the goal of the work presented here was to screen for new methyltransferases involved in this process. However, we have no evidence that NSD3 is involved in H3K4 methylation.
- To make the manuscript more streamlined, we decided to remove most of the context concerning histone modifications. Related to this decision, we deeply rewrited the introduction and we considered that it would be better not to present data concerning H3K4 dimethylation.

2) Regarding the claim that cells do not exit mitosis prematurely and that cohesion protection remains unaffected, there are two major issues with the data provided (Fig. 2A):
a. the choice for Bub1 to monitor if SAC is active is not the most straightforward to distinguish what the authors wish for (SAC activation vs cohesion protection), as Bub1 is also involved in cohesion protection. Hence, a more conventional SAC assay (e.g. Mad2 labelling or efficient arrest upon spindle poisons) should be used.

- To provide more convincing data about the mitotic arrest, we added the data mentioned below:
- In figure 1D, we have shown that mitotic index is increased with the 3 siRNA tested. In the new figure 1C and 1E, we have shown that cyclin B1 is increased after depletion by the 2 siRNA that induce the strongest increase in mitotic index. Similar results were obtained with the H3S10P mitotic marker (new figure 1C and 1E). For both mitotic markers, the difference of signal observed after depletion of siRNA NSD3-1 compared to the others correlated very well with the weak increase in mitotic index. We obtained the same results with another siRNA that targets specifically the long NSD3 isoform (new Figure 5C).
- In order to confirm that these observations came from the persistent activation of the spindle assembly checkpoint, we also analyzed the level of Cyclin B1 and phosphorylated H3S10 after abolition of the spindle checkpoint with the SAC inhibitor reversine. In this new experiment, we now show that level these two mitotic markers were strongly diminished after reversine treatment for 3 h (new figure 1E). We have also shown that NSD3 depletion did not inhibit the spindle checkpoint after artificial activation with the microtubule polymerisation inhibitor

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

nocodazole. In that case, both markers accumulate to the same extent in presence or in absence of NSD3 (new Figure 1 F).

b. In both single examples provided, both Bub1 and Sgo1 levels seem reduced relative to controls? Is this indeed the case? Can the authors back up their claims with a more thorough quantitative analysis on Bub1 (Mad2) and Sgo levels?

• We added to figure 2A the quantification SGO1 intensity normalized against intensity of the centromere marker CREST on the right of each panel, showing no difference between NSD3 siRNA treated cells and control cells. We removed the data concerning Bub1 because SGO1 centromeric localization implies that Bub 1 is functional in recruiting SGO1, and we replaced it by a new figure showing that depletion of WAPL did not rescue NSD3 depletion-induced PSCS. This new data excluded any contribution of NSD3 in mitotic processes regulating sister chromatid cohesion, as explained in details in the new version of the manuscript.

3) The data that best addresses what NDS3 may be doing at mitotic exit is the one presented in figure 3, where chromatin levels of different analyzed proteins are reported during mitotic exit/G1 entry. It would be important to state somewhere how many times these experiments were performed and replace the graphs by similar ones where each independent experiment is depicted.

• To reinforce the data shown on figure 3 concerning the recruitment of cohesin and kollerin, we modified the presentation of the figure by showing mean and standard deviations of the quantification of 2 independent experiments and comparison of the presence of those complex on whole cell extracts against chromatin extracts.

4) The discussion is very interesting in speculating several potential mechanisms by which NSD2 could promote cohesion and MAU2 loading. Yet, I think the discussion should be more in line with the descriptive nature of the paper. In many cases it attempts to infers mechanistic links that are not experimentally addressed. For example, MAU2 loading is overemphasized as the "primary target", but looking at Figure 3 the reduction is rather mild. Also, what happens to NIPBL levels in this assay?

• We have strongly modified the discussion and hope that it will more straightforward and in accordance with the new results presented in this revised manuscript. We believed that some points of the discussion are more relevant with the new provided data, especially the physical interaction between the kollerin complex and NSD3, the effect of the long isoform on the presence of MAU2 and RAD21 on chromatin but not that of the short isoform and the requirement of the methyltransferase enzymatic activity to rescue the mitotic cohesion defects. We could not use the NIPBL antibody in the figure 3 assay because, in our hands, this antibody performs poorly in western-blot unless NIPL has been previously pre-purified by immunoprecipitation. Because of the deep link between NIPBL and MAU2 as a complex, we considered that we can extrapolate the results obtained with MAU2 to NIPBL.

Minor points:

The authors should have their manuscript corrected for English grammar.

• This version of the manuscript have been entirely rewritten and edited by a professional edition service.

In the introduction (line 77) it states that NSDs are specific to H3K36 which is somehow contradicted in the subsequent sentence. This needs better clarification for non-experts.

• The manuscript has been modified according to this reviewer's remark. As follow:
•Line 79-80: "NSD methyltransferases act as oncoproteins in different types of cancers 39,40, and they are considered to be specific for mono- or di-methylation of H3K36 ”

: The entire section on the localization of NDS3 is somehow a bit odd, as the reported behaviour is what one would expect for most nuclear proteins (as most are evicted from mitotic chromatin). Hence, unless the authors wish to develop on the kinetics of association at mitotic exit with detailed mechanistic insights (e.g. labelling of the target), this section should be shortened. Otherwise, it attempts to provide a causal and temporal link that is not fully proven.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

• As suggested, the text of this part was drastically reduced. Moreover, we suppressed figure 4C, 4D, 4E and S4. We removed figures 4A and 4B, and old figures S5A and S5B are the new figures 4A and 4B according to the recommendations of reviewer 1.

the sentence in the discussion line 259 " the dynamic behaviour of NSD3 is similar to that of MAU2 and of cohesin" should be clarified to what "dynamics" the author refer to. The authors clearly state in this manuscript that with regard to cell cycle dynamics it is not exactly the same (NSD3 binds chromatin earlier in late mitosis), as stated in the subsequent sentence.

• We replaced those 2 sentences as follows, line 325-327: " In full agreement with this function, we showed that NSD3 recruitment to chromatin occurs during early anaphase, slightly before those of MAU2 and RAD21."

Reviewer #3 (Significance (Required)):

The mechanisms that regulate sister chromatid cohesion are of prime importance in the chromosome biology field as cohesin is required for correct cell division and also proper 3D genome architecture in interphase cells.
Although there is already a vast knowledge on cohesin regulation, mostly during replication and mitosis, not the entire cohesin cycle is fully understood and novel players are continuously emerging. Thus, uncovering a new regulator of sister chromatid cohesion is very important. Additionally, the mechanisms that regulate cohesin loading, during mitotic exit throughout the following cell cycle are comparatively less clear, particularly with regard to the direct interaction with chromatin.

Referees cross-commenting

Here the comments of the 3 reviewers are reported

Reviewer 1

I like the comments by the other two referees (#2 and #3) and fully agree with them. They are more positive in the overall tone than my comments but still raise a number of similar issues. I assume that the main purpose of this 'referee cross commenting' (which is new to me) is to agree upon a fair list of additional experiments that are doable within a reasonable time-frame and would strengthen the main claims of the paper. Reading all reviews, the smallest common denominator seems to be:

1) The statement that the absence of NSD3 results in a SAC-mediated mitotic arrest due to premature loss of cohesion needs substantiation beyond mere localization of Bub1 and Sgo1, for example by mitotic index measurements and determination of cyclin B levels.
2) The conclusion that NSD3 acts by recruiting Mau2 need further experimental proof. This could be a rescue of the NSD3-depletion phenotype by overexpression or forced chromosomal targeting of Mau2 and this could be the demonstration of a direct or indirect physical interaction between NSD3 and MAU2.

Reviewer 2

I agree that the reviews are pretty much in line with one another, and Reviewer 1's additional comment is very reasonable.
The extent to which the role of MAU2 needs to be consolidated may depend on the target journal but, in the absence of further work, the paper certainly needs to make clear the speculative nature of the connection to MAU2. My feeling is most journals in the Review Commons stable would prefer the role of MAU2 to be substantiated

Reviewer 3

I agree that despite differences in tone, we are all in line in our assessments, which is good :)
Without knowing for which journal we are reviewing, it is more difficult to access what really needs to be addressed. But I agree with the previous comment that most of the journals in review

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

commons would like more depth in the study, particularly with regard to MAU2 chromosomal targeting. The finding is indeed novel and interesting, but in its current version it is still too descriptive.

Original submission

First decision letter

MS ID#: JOCES/2023/261014

MS TITLE: The histone methyltransferase NSD3 contributes to sister chromatid cohesion and to cohesin loading during mitotic exit

AUTHORS: Gregory Eot-Houllier, Laura Magnaghi-Jaulin, Gaelle Bourgine, Fatima Smagulova, Regis Giet, Erwan Watrin, and Christian Jaulin
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Eot-Houllier et al. have previously published work implicating H3K4 acetylation/methylation in regulating chromosome cohesion in mitosis. In this study, they conduct a mini RNAi screen of histone methyltransferases in a human cell line looking for such modifiers that might regulate cohesion. They show that NSD3 localises to chromatin soon after mitotic exit, and just before the cohesin loader component MAU2 and cohesion subunit Rad21. They find that NSD3 and MAU2 and NIPBL can co-immunoprecipitate, and that NSD3 knockdown compromises cohesion in G2 and mitosis and that it reduces MAU2 and Rad21 cohesin reloading in early G1. They conclude that the long form of NSD3 acts through MAU2 is regulate cohesin loading post-mitosis.

Cohesion regulation is a topic of wide interest, as it is involved in gene regulation/genome compartmentalization and as well as in sister chromosome cohesion. Indeed, it is significant that an H3K36-targeting enzyme might regulate cohesion, as this could suggest novel connections between euchromatin/gene bodies and cohesion loading.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Comments for the author

Overall, Eot-Houllier et al. have made a clear effort to address the comments of all three reviewers. While I do not fully agree with all their arguments (in particular, I think that an experiment showing that increasing MAU2 levels on chromatin can rescue NSD3 RNAi phenotypes in cohesion would be very valuable), they do now provide some molecular-level indication for a connection between NSD3 and the kollerin complex s(by co-ip in Fig 3). They also provide evidence that the methyltransferase activity of NSD3 is involved. In addition, the Introduction and Discussion are now more appropriately written given the results.

There are a few minor issues that should be addressed before publication in JCS:

1. Ensure molecular weight markers are added to blots. (It would be helpful for the reviewers to be able to see these too.)

2. It is not immediately obvious why NSD3 depletion causes a strong delay in mitosis in new Figure 1C-E, but not in the experiment in Figure 3. Because chromosomes need to align in mitosis following release from a nocodazole block, and this would not be possible if cohesion is lost between sisters, why is there no delay in entering anaphase in this experiment? Could this be related to the different synchronization protocol, and/or the shorter time of RNAi treatment? An explanation would be useful.

3. The co-knockdown experiments with WAPL vs Rad21, Sgo1 and NSD3 are a good addition to the paper, and important for interpreting the results. It is also good that these knockdowns were done sequentially, which minimizes the potential for one siRNA to dominate over the other, preventing effective knockdown of both proteins (an effect we have seen for WAPL siRNAs). It is not essential, but it would be useful to see immunoblots confirming the expected double knockdowns in these experiments.

4. As pointed out by Reviewer 1, the discrepancies between the extent of NSD3 knockdown and measured phenotypes (eg Cyclin B1 and H3S10ph in new figure 1C) for various siRNAs are sometimes striking. I wonder if this is worth discussing in the paper, as it is in the response to reviewers. I note that it is commendable that the authors include, rather than “hide”, such data.

5. Although mentioned in the response to reviewers, I couldn’t find any mention in the paper that unresolved double dots were removed from the analysis in Figure 2C, D. Moreover, although in this case removing such data is unlikely to make a huge difference to the phenotype measured (an increase in the distance between dots), I have reservations about this approach. It doesn’t seem a good idea to remove data from an experiment based on exactly the factor measured (ie distance between centromere probes), because this could skew the results.

6. In the Introduction, lines 88-89, the description of the NSD3-s and NSD3-L mRNAs is confusing as they are both described as having 24 exons. The word “induce” is also a strange choice here. Should this read “The NSD3 gene contains 24 exons that, in somatic cells, can produce alternatively spliced mRNAs encoding two protein isoforms…”?

Reviewer 2

Advance summary and potential significance to field

Review on the revised manuscript JOCES-2023-261014v1, entitled 'The histone methyltransferase NSD3 contributes to sister chromatid cohesion and to cohesin loading at mitotic exit' by GrÃ©gory Eot-Houllier Christian Jaulin and coworkers.

The revsised manuscript adequatedly addresses most of the previously raised issues. Importantly, the authors now demonstrate convincingly that the catalytic activity of the methyltransferase NSD3 is indeed needed for its pro-cohesive function and that the loss of cohesion upon depletion of NSD3 results in an SAC-mediated mitotic arrest.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Comments for the author

Based on its considerable improvement over its 1st version, I can now recommend publication of this manuscript in JOCES after the following minor additional revisions:
1) The co-IP of NIPBL and Mau2 with NSD3 is still not terribly convincing. Figures 3D, E would benefit from including as a negative control a constitutively chromatin-bound protein (e.g. topo II) in the Western analyses of the pull-downs to exclude that the weak interaction is merely DNA-bridged. (To meet this concern, the authors might also want to point out in the text that the chromatin fractions were nuclease-treated prior to IP.)
2) The authors should clarify in the method section whether the chromosome 11-specific FISH probe is complementary or identical to the sequence AgGgTtTcAgAgCtGcTc; the writing is ambiguous here.
3) Although the writing has much improved, the manuscript still needs editing in few places:

Line 136: Cyclin B1 levels rapidly degrade 'prior to' (not after) anaphase onset.

Line 146: It is thus clear that NSD3 depletion does not 'directly' affect SAC activity.

Line 154: Replace 'bivalent chromosome' with 'two-chromatid chromosome'.

Line 163f: Why is sororin not mentioned as an antagonist of WAPL?

Line 213: ...we could 'immunoprecipitate' (not immunoprecipitated) EmGFP-NSD3...

Line 240: ...EmGFP-NSD3 signals were detected on chromatin prior 'to' those of MAU2 and RAD21,...

Line 260: ...phosphorylated H3S10 signals 'accumulated' (not accumulate) strongly, and 'were' (not is) sensitive to a treatment with...

Line 261f: These results show that only depletion of the long form of NSD3 results in the accumulation of cells in mitosis as well as defects in sister chromatid cohesion.

Lane 264f: We therefore went on to compare by subcellular fractionation experiments the effect of depleting each NSD3 isoform on the levels of chromatin-bound MAU2 and cohesin.

Lane 272: ..., these were (not are) conversely increased in the soluble fractions (Figure 5D).

Lanes 279 & 284: Western blots (not western blots)

Lane 285f: As expected, since NSD3 is not associated with chromatin during prometaphase, EmGFP-NSD3-L was diffusely localized around chromosomes (Figure 5E).

Reviewer 3

Advance summary and potential significance to field

The modified version of the manuscript provides compelling evidence to support a novel role for NSD3 in sister chromatid cohesion. Considering how little is known about the determinants for kollerin targeting at the chromatin level, the novel findings reported here are of wide interest to chromosome biology. The study also raises several interesting questions to dissect further in subsequent studies.

Comments for the author

I congratulate the authors on their efforts to address most of the reviewers' points. The current version of the manuscript is presented in a more focused manner and the significant findings are more appropriately substantiated. Most of my comments have been properly addressed.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

The new data supporting a physical interaction between NDS3 and nipbl/MAU-2 although a good addition to the story, does not fully explain how exactly NDS3 ensures its chromatin loading. Much more interesting is the new data supporting that cohesin loading at mitotic exit requires NSD3 enzymatic activity. Dissecting what is the substrate that dictates kollerin loading at mitotic exit is probably beyond the scope of the current manuscript and may be an interesting follow-up. Despite these loose ends, the current manuscript provides novel and exciting findings that support the authors' central claim. I can therefore recommend its publication in JCS.

Minor issue:

. "Figure S3: WAPL prevents induction of mitotic cohesion defects when RAD21 is depleted but not when SGO1 is depleted."

The title of this figure is very convoluted and the logic appears reverted. It is very hard to extract the key message. I would suggest rephrasing this title

---

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
Eot-Houllier et al. have previously published work implicating H3K4 acetylation/methylation in regulating chromosome cohesion in mitosis. In this study, they conduct a mini RNAi screen of histone methyltransferases in a human cell line looking for such modifiers that might regulate cohesion. They show that NSD3 localises to chromatin soon after mitotic exit, and just before the cohesin loader component MAU2 and cohesion subunit Rad21. They find that NSD3 and MAU2 and NIPBL can co- immunoprecipitate, and that NSD3 knockdown compromises cohesion in G2 and mitosis and that it reduces MAU2 and Rad21 cohesin reloading in early G1. They conclude that the long form of NSD3 acts through MAU2 is regulate cohesin loading post-mitosis.
Cohesion regulation is a topic of wide interest, as it is involved in gene regulation/genome compartmentalization and as well as in sister chromosome cohesion. Indeed, it is significant that an H3K36-targeting enzyme might regulate cohesion, as this could suggest novel connections between euchromatin/gene bodies and cohesion loading.

Reviewer 1 Comments for the Author:
Overall, Eot-Houllier et al. have made a clear effort to address the comments of all three reviewers. While I do not fully agree with all their arguments (in particular, I think that an experiment showing that increasing MAU2 levels on chromatin can rescue NSD3 RNAi phenotypes in cohesion would be very valuable), they do now provide some molecular-level indication for a connection between NSD3 and the kollerin complex (by co-ip in Fig 3). They also provide evidence that the methyltransferase activity of NSD3 is involved. In addition, the Introduction and Discussion are now more appropriately written given the results.

There are a few minor issues that should be addressed before publication in JCS:

1.Ensure molecular weight markers are added to blots. (It would be helpful for the reviewers to be able to see these too.)

Molecular weight markers were added to each Western blot, except for the figure 5D in order to respect the size limitation of the figure. But, all the antibodies used for the blots in this figure were extensively presented in other figures.

2. It is not immediately obvious why NSD3 depletion causes a strong delay in mitosis in new Figure 1C-E, but not in the experiment in Figure 3. Because chromosomes need to align in mitosis following release from a nocodazole block, and this would not be possible if cohesion is lost between sisters, why is there no delay in entering anaphase in this experiment? Could this be

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

related to the different synchronization protocol, and/or the shorter time of RNAi treatment? An explanation would be useful.

In all experiments, except the one shown on figure 3, cells were transfected for 72h. Based on our data, the cohesion defects observed at that time is a consequence of the loading defects occurring at the exit of the previous cell division. In Figure 3, to determine an effect on cohesin loading, we harvested the cell only 48h after transfection. At this time point, the loading defect starts to occur but will be detectable as a premature sister chromatid separation only during the next mitosis. Thus, as expected, at 48h, we observed very few sister chromatid separation and the cells could exit mitosis.
We modify the text line 201-210 as follow:

"If so, the cohesion defects we observed 72h after transfection with siRNA NSD3 would be the consequence of a defect in cohesin loading occurring at the exit of the previous mitosis, approximatively 48h after NSD3 depletion. Thus, for these particular experiments, control and NSD3-depleted cells were synchronised by a single thymidine arrest-and-release, followed by mitotic arrest induced by nocodazole, and released from nocodazole-mediated arrest at 48h post-transfection. Then, cells were harvested at different time points and fractionated to obtain chromatin fractions (Figure 3A, see methods section for details). We next analysed these by SDS-PAGE and immunoblotting (Figure 3B). Under these experimental conditions, NSD3 inactivation did not impact kinetics of cell mitotic exit, "

3. The co-knockdown experiments with WAPL vs Rad21, Sgo1 and NSD3 are a good addition to the paper, and important for interpreting the results. It is also good that these knockdowns were done sequentially, which minimizes the potential for one siRNA to dominate over the other, preventing effective knockdown of both proteins (an effect we have seen for WAPL siRNAs). It is not essential, but it would be useful to see immunoblots confirming the expected double knockdowns in these experiments.

We modified figures 2B to add Western blots of the depletion of each factor involved in that experiment. The figure legends were amended accordingly.

4. As pointed out by Reviewer 1, the discrepancies between the extent of NSD3 knockdown and measured phenotypes (eg Cyclin B1 and H3S10ph in new figure 1C) for various siRNAs are sometimes striking. I wonder if this is worth discussing in the paper, as it is in the response to reviewers. I note that it is commendable that the authors include, rather than "hide", such data.

We have taken into account the comment and modified the discussion of the manuscript line 329-338 as follows:
In that regard, we noticed that the siRNA that induced the strongest phenotype is not the one leading to the most efficient NSD3 extinction (Figure 1C and 1E). However, given that NSD3 is involved in gene expression and genome organization and, in the regulation of cohesin (this study) we cannot rule out the possibility that a more efficient extinction of NSD3 leads to pleiotropic and indirect effects that would partially prevent sister chromatid dissociation. Nevertheless, the level of mitotic defects induced by all the NSD3 siRNAs used in this study are consistent with those usually observed in vertebrate cells upon depletion of kollerin or of other proteins known to be involved in cohesin loading during mitotic exit

5. Although mentioned in the response to reviewers, I couldn't find any mention in the paper that unresolved double dots were removed from the analysis in Figure 2C, D. Moreover, although in this case removing such data is unlikely to make a huge difference to the phenotype measured (an increase in the distance between dots), I have reservations about this approach. It doesn't seem a good idea to remove data from an experiment based on exactly the factor measured (ie distance between centromere probes), because this could skew the results.

In the material and methods section line 521-522: we amended the sentence as follows: "Analysis was only done on pairs for which the dots could be clearly resolved **in the same focal plan".**

6. In the Introduction, lines 88-89, the description of the NSD3-s and NSD3-L mRNAs is confusing as they are both described as having 24 exons. The word "induce" is also a strange choice here.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Should this read “The NSD3 gene contains 24 exons that, in somatic cells, can produce alternatively spliced mRNAs encoding two protein isoforms...”?

We changed the sentence in the introduction line 97-99 to:” In somatic cells, two alternative NSD3 messenger RNAs, composed of 24 and 10 exons, lead to the expression of two isoforms, NSD3-long (NSD3-L) and NSD3-short (NSD3-s) respectively”.

Reviewer 2 Advance Summary and Potential Significance to Field:
Review on the revised manuscript JOCES-2023-261014v1, entitled 'The histone methyltransferase NSD3 contributes to sister chromatid cohesion and to cohesin loading at mitotic exit' by Gregory Eot-Houllier, Christian Jaulin and coworkers.

The revised manuscript adequatedly addresses most of the previously raised issues. Importantly, the authors now demonstrate convincingly that the catalytic activity of the methyltransferase NSD3 is indeed needed for its pro-cohesive function and that the loss of cohesion upon depletion of NSD3 results in an SAC-mediated mitotic arrest.

Reviewer 2 Comments for the Author:
Based on its considerable improvement over its 1st version, I can now recommend publication of this manuscript in JOCES after the following minor additional revisions:
1) The co-IP of NIPBL and Mau2 with NSD3 is still not terribly convincing. Figures 3D, E would benefit from including as a negative control a constitutively chromatin-bound protein (e.g. topo II) in the Western analyses of the pull-downs to exclude that the weak interaction is merely DNA-bridged. (To meet this concern, the authors might also want to point out in the text that the chromatin fractions were nuclease-treated prior to IP.)

As suggested by the reviewer, we completed the sentence line 223-224 in the results section to make it more clear than when it was mentioned in the material and methods section only: **“Using HeLa cell chromatin extracts** treated with Turbonuclease, to limit indirect interaction due to protein-DNA bridging”.

2) The authors should clarify in the method section whether the chromosome 11-specific FISH probe is complementary or identical to the sequence AgGgTtTcAgAgCtGcTc; the writing is ambiguous here.

We clarified this point in the material and methods section line 522-524

3) Although the writing has much improved, the manuscript still needs editing in few places:

We corrected the following editing problem as requested by reviewer 2. For the question concerning the line 163 (now line 173), we explained below why we did not mention Sororin as an antagonist of WAPL in this part of the manuscript.

Line 136: Cyclin B1 levels rapidly degrade 'prior to' (not after) anaphase onset.Line 146: It is thus clear that NSD3 depletion does not 'directly' affect SAC activity.

Line 154: Replace 'bivalent chromosome' with 'two-chromatid chromosome'.

Line 163f: Why is sororin not mentioned as an antagonist of WAPL?

The role of sororin as an antagonist de WAPL is mentioned in lines 57-59 of the introduction. Line 163, we want specifically to point out the relationship between Haspin and WAPL to explain why the codepletion of WAPL with NSD3 should clarify a role of NSD3 in centromeric cohesion during prometaphase. Because WAPL do not rescue the phenotype, we think that adding more context, including Sororin, may be confusing. It would have been more appropriate if a rescue of the NSD3 phenotype by WAPL had been found, but it is not the case.

Line 213: .we could 'immunoprecipitate' (not immunoprecipitated) EmGFP-NSD3...
Line 240: ...EmGFP-NSD3 signals were detected on chromatin prior 'to' those of MAU2 and RAD21,... Line 260: ...phosphorylated H3S10 signals 'accumulated' (not accumulate) strongly, and

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

'were' (not is) sensitive to a treatment with...
Line 261f: These results show that only depletion of the long form of NSD3 results in the accumulation of cells in mitosis as well as defects in sister chromatid cohesion.
Lane 264f: We therefore went on to compare by subcellular fractionation experiments the effect of depleting
each NSD3 isoform on the levels of chromatin-bound MAU2 and cohesin.
Lane 272: ..., these were (not are) conversely increased in the soluble fractions (Figure 5D).
Lanes 279 & 284: Western blots (not western blots)
Lane 285f: As expected, since NSD3 is not associated with chromatin during prometaphase, EmGFP- NSD3-L was diffusely localized around chromosomes (Figure 5E).

Reviewer 3 Advance Summary and Potential Significance to Field:
The modified version of the manuscript provides compelling evidence to support a novel role for NSD3 in sister chromatid cohesion. Considering how little is known about the determinants for kollerin targeting at the chromatin level, the novel findings reported here are of wide interest to chromosome biology. The study also raises several interesting questions to dissect further in subsequent studies.

Reviewer 3 Comments for the Author:
I congratulate the authors on their efforts to address most of the reviewers' points. The current version of the manuscript is presented in a more focused manner and the significant findings are more appropriately substantiated. Most of my comments have been properly addressed.

The new data supporting a physical interaction between NDS3 and nipbl/MAU-2, although a good addition to the story, does not fully explain how exactly NDS3 ensures its chromatin loading. Much more interesting is the new data supporting that cohesin loading at mitotic exit requires NSD3 enzymatic activity. Dissecting what is the substrate that dictates kollerin loading at mitotic exit is probably beyond the scope of the current manuscript and may be an interesting follow-up. Despite these loose ends, the current manuscript provides novel and exciting findings that support the authors' central claim. I can therefore recommend its publication in JCS.

Minor issue:

“Figure S3: WAPL prevents induction of mitotic cohesion defects when RAD21 is depleted but not when SGO1 is depleted.”

The title of this figure is very convoluted and the logic appears reverted. It is very hard to extract the key message. I would suggest rephrasing this title

The Figure S3 is now included in the figure 2B to respect the rule to have no more supplemental figures than principal figures. Consequently, the title is removed.

The information contained in this message and any attachment is confidential, legally privileged and is intended for the addressee only. Any dissemination, distribution, copying, disclosure or use of this message/attachment or its contents is strictly prohibited and may be unlawful. No contract is intended or implied, unless confirmed by hard copy. If you have received this message in error, please inform the sender and delete it from your mailbox or any other storage mechanism.

The Company Of Biologists Ltd cannot accept liability for any statements made which are clearly the senders' own and not expressly made on behalf of the Company of Biologists Limited or one of their agents.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Second decision letter

MS ID#: JOCES/2023/261014

MS TITLE: The histone methyltransferase NSD3 contributes to sister chromatid cohesion and to cohesin loading during mitotic exit

AUTHORS: Gregory Eot-Houllier, Laura Magnaghi-Jaulin, Gaelle Bourgine, Fatima Smagulova, Regis Giet, Erwan Watrin, and Christian Jaulin
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

© 2023. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).