DOT1L promotes spermatid differentiation by regulating expression of genes required for histone-to-protamine replacement

Aushaq B. Malla, Shannon R. Rainsford, Zachary D. Smith and Bluma J. Lesch
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First decision letter

MS ID#: DEVELOP/2022/201497

MS TITLE: DOT1L promotes spermatid differentiation by regulating expression of genes required for the histone-to-protamine transition

AUTHORS: Aushaq B Malla, Shannon R Rainsford, Zachary D Smith, and Bluma J Lesch

I have now received two referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but also provide a significant number of recommendations to improve the rigor and clarity in the manuscript. In particular, as pointed out by reviewer 1, the single cell sequencing analysis needs to be analyzed (bioinformatically) with more rigor since the reduction in the number of spermatogenesis genes could well be a function of loss of those cells. As both reviewers point out, several figures need to be presented with better clarity, and the reviewers make additional recommendations. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns.

Please attend to all of the reviewers' comments and 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. 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

The paper by Malla et al entitled “DOT1L promotes spermatid differentiation by regulating expression of genes required for the histone-to-protamine transition” describes the establishment

and analysis of DOT1L-deficient mouse line. The authors report many pathophysiological changes and perform RNA-seq analyses comparing differences in gene expression as well as problems with transcript isoforms upon loss of Dot1L.

While it is an interesting phenotype, which adds to the understanding of the genes required for spermatogenesis the authors need to address some points before the ms should be considered for publication.

Comments for the author

Further points:
1) Fig. 2 E and 3 A . Images are of bad quality and should be replaced by better ones
2) Fig 2 F – the point the authors want to make about the nuclear morphology is not evident. The authors should consider an alternative method (morphometry) to substantiate the claim.
3) Fig 4 G. The pictures represent elongated spermatids from dark zone regions. Could the authors not use epidydimal sperm to substantiate their claim? Use of material from seminiferous tubules seems error prone since the spermatids shown could be of different stage.
4) Fig 4 H, (H) is missing. Also, the staining for gH2AX staining seems not really informative, maybe a 8OHdG staining would be more telling. The authors should at least comment on this, since abnormal protamination usually goes hand in hand with DNA damage.
5) Line 236-238 the view that this is a step by step process has been questioned by more recent datsets.
E.g. Barral et al showed that this is a more concerted action i.e. TNPs and Protamines interact instead of the one being replaced by the other.
6) Further to point 5) in line with the recent publications, the authors should consider looking at TNP1 and TNP2 using immunohistochemistry.
7) Fig 5 C – the approach to count "% tubules" seems rather crude – could the authors come up with a more precise way to analyze and display this datapoint.
8) Line 250-251 – could the authors provide data from epididymal sperm to validate the findings?
9) Line 295 ff the authors conducted a scRNAseq analysis to determine the role of DOT1L. They claim that elongating spermatids are substantially reduced in number in DOT1L ko males. However, and that is of importance to the interpretation of the data this finding must be considered when extracting differentially expressed genes in the following. For example, in line 312 they note, that Tnp1, Tnp1, Prm1 and Prm2 are the most significant downregulated – which is not surprising considering that this population of cells is substantially downregulated (see line 295). The authors must find a way to correct their data for this problem.
At present, it seems rather a secondary effect of the ko than a direct effect of Dot1l on expression of the respective genes.
10) Line 460 – this claim seems quite a mouthful. Even if the points raised by the reviewers could be addressed, the notion that a broadly expressed gene like Dot1l could be targeted for male contraceptive seems not realistic. The authors might consider watering down this claim.

Reviewer 2

Advance summary and potential significance to field

This manuscript reports a detailed phenotypic and molecular characterization of DOT1L function in spermatogenic cells. The main conclusion of this work is that DOL1L controls the expression of a series of critical genes in post-meiotic cells, including those driving histone replacement by protamines. DOT1L is therefore one of the essential factors ensuring functional sperm production and male fertility.

All experiments performed are appropriate, correctly interpreted, and strongly support the authors' conclusions.
However, the manuscript suffers from frequent citation errors, biased citations and a lack of reference to recent publications.

Before publication, the authors should pay attention to the following points: See below

Comments for the author

1 - In the introduction, the references cited regarding the factors involved in histone H4 hyperacetylation are not correct or complete. Berkovits and Wolgemuth, 2011, is not related to the topic, and GCN5 is not the only acetyltransferase system reported to be involved in H4 hyperacetylation. To present an unbiased view of the published works, the authors should at least cite here PMID: 28694333 and PMID: 30257209.

2 - Figure S1A is of poor quality with obvious problems in gel migration and/or transfer. Therefore, given the quality of this experiment, it is difficult to extract reliable information. To make their point, the author should either show a higher-quality image or delete this figure and refer to publicly available tissue-dependent gene expression data.

3 - Figure S1B is not appropriate. The authors should have shown in the same panel images of wild-type and Dot1L cKO testis sections taken under exactly the same conditions. As it is, the Dot1L cKO testis sections are shown in this figure, and the wild-type situation is in Fig. 1A.
As the authors have convincingly demonstrated the absence of DOT1L in the Dot1L cKO testis and the specificity of their antibody, this figure does not seem necessary.

4 - Figure 3B is not cited in the main text of the manuscript.

5 - Since 2014, there have been important findings on the molecular basis of chromatin reorganizations especially in post-meiotic spermatogenic cells. Therefore, Rathke et al. 2014, frequently cited in the manuscript in support of different statements, summarizes our knowledge until 2014. The authors should cite more recent reviews.

6 - A compelling example of more recent findings that were missed by the authors is the process of histone replacement by protamines. All the papers cited in this regard are prior to 2014 (page 9 top, and introduction). More recent work has indicated that transition proteins (TPs) do not replace histones but appear together with PRMs and mediate the proper assembly of PRMs (PMID: 28366643). This should also be mentioned in the introduction (lanes 61-63). Therefore, the old dogma based solely on descriptive work according to which histones are first replaced by TPs and TPs are in turn replaced by protamines, is seriously challenged. It is no longer possible to stick to the old dogma and ignore recent molecular work.

7 - The TUNEL+ cells in Figure 5F are barely visible.

8 - The authors considered the role of DOT1L in the expression of TP1/2 and PRM1/2, but not H2A.L.2. Knowledge of the more recent literature would have indicated to the authors that the incorporation of TPs and PRMs depends on the simultaneous expression of a histone H2A variant, H2A.L.2. Given this result, it is no longer possible to consider TPs and PRMs without paying attention to the expression of H2A.L.2. How is the expression of the mRNA coding for H2A.L.2 in this model?

9 - The authors incorrectly state that BRDT is expressed in elongating spermatids (page 12, lanes 340-341). BRDT is expressed earlier, is present in both meiotic and post-meiotic cells, and has stage-specific functions. The first bromodomain-dependent functions of BRDT become predominant in post-meiotic cells, most likely through the readout of H4K5acK8ac. The reference Berkovits and Wolgemuth 2011, is not appropriate here and should be replaced by Gaucher et al., 2012, already cited by the authors).

10 - Additionally, the references cited in support of BRDT's role in regulating chromocenter formation are incorrect. Shang et al. 2007 and Gaucher et al. 2012, did not present any data on the regulation of chromocenter formation by BRDT. The correct reference is Berkovits and Wolgemuth. However, this work shows that the effect of BRDT on the chromocenter is highly dependent on the genetic background of the mouse. Therefore, the author cannot draw a general conclusion about the role of BRDT in chromocenter formation as stated here. Please also correct the related misleading statements in the Discussion and the misquotes found in this section (lanes 412-413; 415-416).

11 - Lines 413-417, defective BRDT-dependent histone removal was first reported by Gaucher et al. 2012 while Shang et al. 2007, did not consider histone-related aspects, and neither of these publications reported chromocenter defects.

12 - References are missing for the statement "BRDT is known to bind acetylated H4 and facilitate histone eviction during spermiogenesis" (lanes 417-418). Here, the authors should cite Morinière et al, 2009; Gaucher et al, 2012; Goudarzi et al, 2016 (all three have already been cited by the authors), and possibly PMID 30257209.

13 - Regarding pericentric RNA expression in spermatogenic cells, there is more recent work that the authors could consider that is not cited.

First revision

Author response to reviewers' comments

Author responses to reviewers

Reviewer 1 Advance Summary and Potential Significance to Field:
The paper by Malla et al entitled “DOT1L promotes spermatid differentiation by regulating expression of genes required for the histone-to-protamine transition” describes the establishment and analysis of DOT1L-deficient mouse line. The authors report many pathophysiological changes and perform RNA- seq analyses comparing differences in gene expression as well as problems with transcript isoforms upon loss of Dot1L. While it is an interesting phenotype, which adds to the understanding of the genes required for spermatogenesis the authors need to address some points before the ms should be considered for publication.

We thank the reviewer for this positive assessment. We have addressed the specific points raised and we believe the paper is substantially improved.

Reviewer 1 Comments for the Author:
Further points:

1) Fig. 2 E and 3 A. Images are of bad quality and should be replaced by better ones

We have replaced the H&E images with higher quality ones.

2) Fig 2 F – the point the authors want to make about the nuclear morphology is not evident. The authors should consider an alternative method (morphometry) to substantiate the claim.

We have added new, higher-magnification images to **Figure 2F** to better illustrate the differences in morphology between control epididymal sperm and the abnormal sperm-like cells present in the epididymis of *Dot1L* cKO males. We also added DAPI images of epididymal germ cells co-stained for PRM1, where abnormal morphology is also evident, to **Figure 5E**. Because cKO animals have very few germ cells in the epididymis and even fewer that could be considered mature sperm, automated sperm morphometry measurements are challenging.

3) Fig 4 G. The pictures represent elongated spermatids from dark zone regions. Could the authors not use epididymal sperm to substantiate their claim? Use of material from seminiferous tubules seems error prone since the spermatids shown could be of different stage.

Spermatids from dark zone regions were used because we are not able to recover true sperm from the epididymis. We believe that the dark zone spermatids are useful in combination with our other data from epididymal germ cells (**Figures 2F** and **5E**) because they are more likely than epididymal germ cells to be at a comparable developmental stage. However, we acknowledge the possibility

that light/dark zone classification might be altered in cKO testis due to developmental defects, and we have added this caveat to the text (lines 232-236).

4) Fig 4 H, (H) is missing. Also, the staining for gH2AX staining seems not really informative, maybe a 8OHdG staining would be more telling. The authors should at least comment on this, since abnormal protamination usually goes hand in hand with DNA damage.

We have added (H) to the legend for Figure 4. We thank the reviewer for the excellent suggestion to assess 8OHdG. We performed this experiment and found that while 8-OHdG is not significantly elevated in testicular germ cells in the cKO, the epididymal sperm-like cells were strongly positive for 8- OHdG. This result is consistent with DNA damage occurring secondary to the sperm head morphology changes we observed. We have added this data to **Figure 4I-J**.

5) Line 236-238 the view that this is a step by step process has been questioned by more recent datsets. E.g. Barral et al showed that this is a more concerted action i.e. TNPs and Protamines interact instead of the one being replaced by the other.

We thank the reviewer for pointing this out. We have updated this description (now lines 251-256) as well as elsewhere throughout the manuscript to reflect the current state of the field regarding the dynamics of the histone-to-protamine transition, including that presented in Barral et al 2017.

6) Further to point 5) in line with the recent publications, the authors should consider looking at TNP1 and TNP2 using immunohistochemistry.

This is an important point. We performed immunofluorescence staining for TNP1 in testis sections and found that levels of TNP1 are substantially reduced in *Dot1L* cKO tubules. This is consistent with the proposed role for DOT1L in promoting expression of both transition proteins and protamines, as well as with the impaired protamine processing and deposition we observed. This immunofluorescence data has been added to **Figure 5F.**

7) Fig 5 C – the approach to count "% tubules" seems rather crude – could the authors come up with a more precise way to analyze and display this datapoint.

We reanalyzed these data by calculating the percentage of spermatids per tubule that are H3+, limiting this analysis to stage I-VIII tubules because spermatids in stage IX-XII tubules are still H3+ in normal spermatogenesis. We have added this analysis to **Figure 5C**. The combination of the two analyses supports the overall conclusion that H3 is abnormally retained in *Dot1L* cKO spermatids.

8) Line 250-251 – could the authors provide data from epididymal sperm to validate the findings?

We performed PRM1 staining in squash preparations of sperm-like cells isolated from the cauda epididymis and confirmed the findings shown in **Figure 5D**; these data are now shown in **Figure 5E**.

9) Line 295 ff the authors conducted a scRNAseq analysis to determine the role of DOT1L. They claim that elongating spermatids are substantially reduced in number in DOT1L ko males. However, and that is of importance to the interpretation of the data this finding must be considered when extracting differentially expressed genes in the following. For example, in line 312 they note, that Tnp1, Tnp1, Prm1 and Prm2 are the most significant downregulated – which is not surprising considering that this population of cells is substantially downregulated (see line 295). The authors must find a way to correct their data for this problem. At present, it seems rather a secondary effect of the ko than a direct effect of Dot1l on expression of the respective genes.

We thank the reviewer for raising this issue, which is an essential point to clarify. The analysis in Figure 6H shows changes in gene expression within each cell type cluster. This analysis in theory accounts for the different numbers of cells assigned to each cluster for each genotype, thus accounting for the reduced numbers of elongating spermatids in the *Dot1L* cKO. We have updated the main text (lines 325-327) to better specify this.

It is also possible that the disruption in developmental progression seen in the *Dot1L* cKO and related changes to transcriptional profiles could result in less accurate assignment of cells to clusters. To address this issue, we have performed a confidence analysis to quantify the probability that assignment of cells to a given cluster is accurate, for each cluster and each genotype. We found that there is very high support for cell type assignment, even for the late spermatogenic cells that are most strongly affected at the transcriptional level in the *Dot1L* cKO. We added **Figure S3A** showing the distributions of probabilities that each cell was assigned to the correct cluster, with clusters defined by our wild type dataset, and we discuss this analysis in the text (lines 330-334 and 479-482). A description of this analysis has been added to the "scRNA-seq library preparation and analysis" section of the Methods.

10) Line 460 – this claim seems quite a mouthful. Even if the points raised by the reviewers could be addressed, the notion that a broadly expressed gene like Dot1l could be targeted for male contraceptive seems not realistic. The authors might consider watering down this claim.

We have revised this sentence to make the more conservative statement that "Our results advance mechanistic understanding of sperm morphogenesis and suggest new directions for future studies of infertility and contraception."

Reviewer 2 Advance Summary and Potential Significance to Field:

This manuscript reports a detailed phenotypic and molecular characterization of DOT1L function in spermatogenic cells. The main conclusion of this work is that DOL1L controls the expression of a series of critical genes in post-meiotic cells, including those driving histone replacement by protamines.
DOT1L is therefore one of the essential factors ensuring functional sperm production and male fertility.

All experiments performed are appropriate, correctly interpreted, and strongly support the authors' conclusions. However, the manuscript suffers from frequent citation errors, biased citations and a lack of reference to recent publications.

We thank the reviewer for this positive assessment and for pointing out the citation errors. We have updated and corrected citations throughout the manuscript in keeping with the reviewer's comments below.

Before publication, the authors should pay attention to the following points: See below

Reviewer 2 Comments for the Author:

1) In the introduction, the references cited regarding the factors involved in histone H4 hyperacetylation are not correct or complete. Berkovits and Wolgemuth, 2011, is not related to the topic, and GCN5 is not the only acetyltransferase system reported to be involved in H4 hyperacetylation. To present an unbiased view of the published works, the authors should at least cite here PMID: 28694333 and PMID: 30257209.

We have updated this section to include the suggested citations and provide a more accurate summary of current literature.

2) Figure S1A is of poor quality with obvious problems in gel migration and/or transfer. Therefore, given the quality of this experiment, it is difficult to extract reliable information. To make their point, the author should either show a higher-quality image or delete this figure and refer to publicly available tissue-dependent gene expression data.

We have removed this Western blot and added references to three databases that provide support for the statement that DOT1L expression is highly abundant in testis tissue: the EMBL-EBI Expression Atlas (mouse and human), the GTEx database (human), and the Human Protein Atlas (human). We included human data among these references because of the greater availability of well-curated human pan-tissue expression data; note that the available human and mouse data are consistent in this respect.

3) Figure S1B is not appropriate. The authors should have shown in the same panel images of wild-type and Dot1L cKO testis sections taken under exactly the same conditions. As it is, the Dot1L cKO testis sections are shown in this figure, and the wild-type situation is in Fig. 1A. As the authors have convincingly demonstrated the absence of DOT1L in the Dot1L cKO testis and the specificity of the antibody, this figure does not seem necessary.

We have removed Figure S1B.

4) Figure 3B is not cited in the main text of the manuscript.

We now cite this panel (line 179).

5) Since 2014, there have been important findings on the molecular basis of chromatin reorganizations, especially in post-meiotic spermatogenic cells. Therefore, Rathke et al. 2014, frequently cited in the manuscript in support of different statements, summarizes our knowledge until 2014. The authors should cite more recent reviews.

We have replaced the Rathke et al. citation with references to the primary literature where appropriate, as well as with citations of more recent reviews.

6) A compelling example of more recent findings that were missed by the authors is the process of histone replacement by protamines. All the papers cited in this regard are prior to 2014 (page 9 top, and introduction). More recent work has indicated that transition proteins (TPs) do not replace histones but appear together with PRMs and mediate the proper assembly of PRMs (PMID: 28366643). This should also be mentioned in the introduction (lanes 61-63). Therefore, the old dogma based solely on descriptive work, according to which histones are first replaced by TPs and TPs are in turn replaced by protamines, is seriously challenged. It is no longer possible to stick to the old dogma and ignore recent molecular work.

We thank the reviewer for pointing out the current state of the field. We have made updates in the introduction and throughout the manuscript to reflect more up-to-date models for the dynamics of the histone-to-protamine transition, including that presented in Barral et al 2017 (see also our response to Reviewer 1, point 5).

7) The TUNEL+ cells in Figure 5F are barely visible.

We have enhanced the contrast in these images (now **Figure 5H**) and also provided zoomed-in images to better show the TUNEL+ cells.

8) The authors considered the role of DOT1L in the expression of TP1/2 and PRM1/2, but not H2A.L.2. Knowledge of the more recent literature would have indicated to the authors that the incorporation of TPs and PRMs depends on the simultaneous expression of a histone H2A variant, H2A.L.2. Given this result, it is no longer possible to consider TPs and PRMs without paying attention to the expression of H2A.L.2. How is the expression of the mRNA coding for H2A.L.2 in this model?

We thank the reviewer for pointing this out. We added an analysis of *H2al2a* expression (which does not change significantly in the *Dot1L* cKO) to **Figure S3C**. We have also included references and discussion of the important role of H2A.L.2 in protamine replacement throughout the manuscript.

9) The authors incorrectly state that BRDT is expressed in elongating spermatids (page 12, lanes 340-341). BRDT is expressed earlier, is present in both meiotic and post-meiotic cells, and has stage-specific functions. The first bromodomain-dependent functions of BRDT become predominant in post-meiotic cells, most likely through the readout of H4K5acK8ac. The reference Berkovits and Wolgemuth 2011, is not appropriate here and should be replaced by Gaucher et al., 2012, already cited by the authors).

We thank the reviewer for pointing this out. We have updated this part (now lines 374-377) to more accurately describe the link between BRDT and the observed *Dot1L* cKO phenotype. We have replaced the reference with the more appropriate citation of Gaucher et al. 2012.

10) Additionally, the references cited in support of BRDT's role in regulating chromocenter formation are incorrect. Shang et al. 2007 and Gaucher et al. 2012, did not present any data on the regulation of chromocenter formation by BRDT. The correct reference is Berkovits and Wolgemuth. However, this work shows that the effect of BRDT on the chromocenter is highly dependent on the genetic background of the mouse. Therefore, the author cannot draw a general conclusion about the role of BRDT in chromocenter formation as stated here. Please also correct the related misleading statements in the Discussion and the misquotes found in this section (lanes 412-413; 415-416).

We have corrected this reference as well as the corresponding statements (lines 409-411, 471-474).

11) Lines 413-417, defective BRDT-dependent histone removal was first reported by Gaucher et al. 2012, while Shang et al. 2007, did not consider histone-related aspects, and neither of these publications reported chromocenter defects.

We have updated this section and reference (lines 409-411).

12) References are missing for the statement "BRDT is known to bind acetylated H4 and facilitate histone eviction during spermiogenesis" (lanes 417-418). Here, the authors should cite Morinière et al, 2009; Gaucher et al, 2012; Goudarzi et al, 2016 (all three have already been cited by the authors), and possibly PMID 30257209.

We have added these citations (see lines 460-463).

13) Regarding pericentric RNA expression in spermatogenic cells, there is more recent work that the authors could consider that is not cited.

We have updated the references and text for this part of the manuscript (lines 395-403), including addition of both older and more recent work regarding the role of major satellite transcripts in the histone to protamine transition.

Second decision letter

MS ID#: DEVELOP/2022/201497

MS TITLE: DOT1L promotes spermatid differentiation by regulating expression of genes required for histone-to-protamine replacement

AUTHORS: Aushaq B Malla, Shannon R Rainsford, Zachary D Smith, and Bluma J Lesch
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

Done in previous assessment

Comments for the author

All my comments have been addressed, either by addition of experiments, amending the claims or extended explanation of experiments. I have no further comments or criticism - congratulations.

Reviewer 2

Advance summary and potential significance to field

Most of the concerns raised previously have been taken into account and the manuscript has now been much improved. I can therefore recommend this manuscript for publication.

Comments for the author

Most of the concerns raised previously have been taken into account and the manuscript has now been much improved. I can therefore recommend this manuscript for publication.