The DOT1L-MLLT10 complex regulates male fertility and promotes histone removal during spermiogenesis

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MS TITLE: The DOT1L-MLLT10 complex regulates male fertility and promotes histone removal during spermiogenesis

AUTHORS: Huijuan Lin, Isabella G Cossu, N. Adrian Leu, Kathrin M Bernt, Aniruddha J Deshpande, Mengcheng Luo, and Jeremy Wang

I have now received all the 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 provide recommendations to further improve the rigor of the manuscript before we can consider publication. 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 also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail.

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 Lin et al entitled “The DOT1L-MLLT10 complex regulates male fertility and promotes histone removal during spermiogenesis” describes the establishment and analysis of DOTL1L and

MLLT10 deficient mouse lines. The authors report many pathophysiological changes and demonstrate, that loss of either protein leads to problems with H3K79 methylation and subsequently, with histone to protamine exchange in sperm. The manuscript very nicely highlights the requirement for H3K79 modification by DOT1L/MLLT10 (i.e. methylation) for proper histone eviction. It extends on our knowledge of DOTL1L being essential for spermatogonial stem cells by the use of Stra8-Cre mice, which leave the gene intact in SSCs and initiate gene-deletion in differentiating spermatogonia prior to entry into meiosis. Further, in parallel, they take advantage of the Mllt10 floxed mouse line and, by mating to Ddx4-Cre mice, demonstrate loss of H3K79 dimethylation in germ cells.

Comments for the author

Further points:

1) Given the defects of the DOT1L males observed, the authors should consider looking at sperm motility using an appropriate assay, or at least add a comment.
2) Line 161 -167 seems repetitive – consider revising
3) Line 204 – Fig S6B-C should read S6B-D
4) Line 206-267 – please elaborate on discussing CDH5 and RNF8 in this context since it seems not connected to the story presented.
5) Line 380 – Please rephrase the way of sperm collection
6) Fig 6. – it appears that the upper row pictures are not the same magnification compared to the lower ones – please correct and add scale bar to upper row pictures.

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors knocked-in a 3x Flag tag at the C-terminal of Dot1L gene in mouse and immunoprecipitated protein complexes from the testes that contains DOT1L and MLLT10. The authors generated conditional knockout mice for Dot1L and Mllt10 in testes and found both conditional knockouts caused male subfertility and histone H3 retention in sperm. Using immunoprecipitation and Western blot, the authors verified the interaction between DOT1L and MLLT10. In immunofluorescence, the authors showed that DOT1L and MLLT10 colocalized on XY body and the localizations are interdependent.

Comments for the author

1. For Western blot and immunofluorescence, the authors used two different antibodies. The antibody used for immunofluorescence is home-made, and the authors referred to a previous G&D paper from the same lab. However, in the previous paper, the authors didn't provide validating data for the home-made antibody. As supplemental data, the authors should provide a full-length gel Western blot to show the antibody is specific to DOT1L.
2. In DOT1L Western blot, there is a lower band. The authors indicated it as non-specific, but didn't give any explanation. It is common that in testes, genes have alternatively spliced isoforms. How many bands the authors saw in the anti-Flag immunoprecipitated complexes, with both anti-DOT1L and anti-Flag antibodies?
The authors should provide full-length gel Western blots as supplemental data.
3. The authors showed DOT1L immunofluorescence staining in Mllt10 knockout testes, and MLLT10 immunofluorescence staining in Dot1L knockout testes, but didn't show DOT1L immunofluorescence staining in Dot1L knockout testes, and MLLT10 immunofluorescence staining in Mllt10 knockout testes. The authors should provide these data as supplement.
4. For the mass-spectrometry data, the authors may provide a diagram to show where these DOT1L and MLLT10 peptides matched on the genes as supplemental data.

First revision

Author response to reviewers' comments

Thank you for the opportunity to submit a revised manuscript. We thank both reviewers for their considerable interest in this study and thoughtful comments. We have addressed both reviewers' points with new data and text revisions. We have added three new supplementary figures (Fig. S7-9) and two new panels in Fig. S3. New or revised texts are shown in magenta.

Here are the main new and revised figures:
Fig. 6: The image in panel A was updated. In panel C, although not requested, we have added the data from a third pair of mice to the graph, resulting in a total of 3 mice per genotype. We have also added statistical analysis. The conclusion is the same.
Fig. S3. Immunofluorescence analysis of DOT1L, MLLT10, and H3K79me2 in germ cells from *Dot1l*^{cKO} and *Mllt10*^{cKO} testes. Panels A and C are new. (A) Immunofluorescence of DOT1L in *Dot1l*$^{fl/+}$ and *Dot1l*^{cKO} testes. (C) Immunofluorescence of MLLT10 in *Mllt10*$^{fl/+}$ and *Mllt10*^{cKO} testes.
Fig. S7. Full-length western blots of DOT1L in testes.
Fig. S8. Full-length Western blots of DOT1L, DOT1L-FLAG, and MLLT10 in testes.
Fig. S9. Peptide maps of mouse DOT1L and MLLT10 proteins.

Below is our point-by-point response to the reviewers' comments.

Reviewer 1 Advance Summary and Potential Significance to Field:
The paper by Lin et al entitled "The DOT1L-MLLT10 complex regulates male fertility and promotes histone removal during spermiogenesis" describes the establishment and analysis of DOTL1L and MLLT10 deficient mouse lines. The authors report many pathophysiological changes and demonstrate, that loss of either protein leads to problems with H3K79 methylation and subsequently, with histone to protamine exchange in sperm. The manuscript very nicely highlights the requirement for H3K79 modification by DOT1L/MLLT10 (i.e. methylation) for proper histone eviction. It extends on our knowledge of DOTL1L being essential for spermatogonial stem cells by the use of Stra8-Cre mice, which leave the gene intact in SSCs and initiate gene-deletion in differentiating spermatogonia prior to entry into meiosis. Further, in parallel, they take advantage of the Mllt10 floxed mouse line and, by mating to Ddx4-Cre mice, demonstrate loss of H3K79 dimethylation in germ cells.

Reviewer 1 Comments for the Author:
Further points:

1) Given the defects of the DOT1L males observed, the authors should consider looking at sperm motility using an appropriate assay, or at least add a comment.
Response: We appreciate reviewer 1's suggestion. We would like to perform CASA to quantify sperm motility. However, we have not been able to perform CASA for two reasons. First, unfortunately, no lab at UPenn campus and in Philadelphia has the equipment for CASA (Dr. George Gerton used to be able to do CASA. However, his equipment was too old and discarded when he retired). We could reach out to labs in other states for collaboration. However, this would require a collaborator to amend their IACUC mouse protocol in order for us to ship the mice. For these logistical reasons (lack of equipment and time constrains), this assay is difficult for us. Second, the KO males were subfertile.
The KO sperm appeared motile. Half of KO sperm had normal morphology but half had abnormal morphology (Fig. 6C). Therefore, sperm motility is expected to be lower at least for the abnormal looking sperm. CASA would provide additional information about motility but is not expected to change the conclusions. In advance communications, Dr. Arur, the editor, granted us permission not to perform CASA. We appreciate the reviewer's suggestion to add a comment. We have now added a comment on sperm motility in Results: "Under a dissecting microscope, all three types of sperm from the *Dot1l*^{cKO} males appeared motile."

2) Line 161 -167 seems repetitive – consider revising
Response: We have removed redundancy by deleting three sentences.

3) Line 204 – Fig S6B-C should read S6B-D
Response: corrected.

4) Line 260-267 – please elaborate on discussing CDH5 and RNF8 in this context since it seems not connected to the story presented.
Response: In this paragraph, we intended to discuss the known factors involved in the histone to protamine exchange process. To be coherent with the rest of discussion, we have added introductory and concluding sentences for this paragraph: "Two proteins were previously reported to play a role in histone removal: CHD5 and RNF8…… Here we report that the DOT1L-MLLT10 complex is important, highlighting the multi-factorial regulation of this complex process."

5) Line 380 – Please rephrase the way of sperm collection
Response: revised.

6) Fig 6. – it appears that the upper row pictures are not the same magnification compared to the lower ones – please correct and add scale bar to upper row pictures.
Response: We appreciate for pointing out this difference. We have replaced the upper row of images with the same magnification. In addition, there were only two pairs of mice in Fig. 6C at the time of submission. Now we have another pair of mice and added the additional data to this graph for a total of 3 pairs. The result is the same.

Reviewer 2 Advance Summary and Potential Significance to Field:

In this manuscript the authors knocked-in a 3x Flag tag at the C-terminal of Dot1L gene in mouse and immunoprecipitated protein complexes from the testes that contains DOT1L and MLLT10. The authors generated conditional knockout mice for Dot1L and Mllt10 in testes and found both conditional knockouts caused male subfertility and histone H3 retention in sperm. Using immunoprecipitation and Western blot, the authors verified the interaction between DOT1L and MLLT10. In immunofluorescence, the authors showed that DOT1L and MLLT10 colocalized on XY body and the localizations are interdependent.

Reviewer 2 Comments for the Author:
1. For Western blot and immunofluorescence, the authors used two different antibodies. The antibody used for immunofluorescence is home-made, and the authors referred to a previous G&D paper from the same lab. However, in the previous paper, the authors didn't provide validating data for the home- made antibody. As supplemental data, the authors should provide a full-length gel Western blot to show the antibody is specific to DOT1L.
Response: We have added the full-length Western blot in a new supplementary figure – Fig. S7. Both commercial (Abcam) and home made antibodies recognized the DOT1L protein in the wild type and heterozygous testes but the band intensity was substantially reduced in cKO testes (deletion only in germ cells), showing the specificity of this band. Both antibodies also gave smaller non-specific bands, whose abundance was similar in wild type and cKO testes.

2. In DOT1L Western blot, there is a lower band. The authors indicated it as non-specific, but didn't give any explanation. It is common that in testes, genes have alternatively spliced isoforms. How many bands the authors saw in the anti-Flag immunoprecipitated complexes, with both anti-DOT1L and anti-Flag antibodies? The authors should provide full-length gel Western blots as supplemental data.
Response: We have provided images of full-length Western blots used (DOT1L, FLAG, and MLLT10) in Fig. 1A, 1B, and 1C in a new supplementary figure – Fig. S8. In DOT1L-FLAG testis, anti-FLAG antibody produced only one small band slightly larger than 50 kDa, in addition to the DOT1L-FLAG-specific band (slightly smaller than 250 kDa). On the FLAG WB, we did not observe the ~200 kDa band that was present in WB with anti-DOT1L antibodies (both Abcam and home-made antibodies), showing that this ~200 kDa band was non-specific. In addition, the 200 kDa and other smaller bands on the WBs from anti-DOT1L antibodies were present in equal abundance between wild type and *Dot1*l^{cKO} testes, further supporting that these bands are non-specific (Fig. S7A and Fig. S7B).

The presence of these non-specific bands did not affect the WB results, as the specific DOT1L band (~250 kDa) was reduced in abundance in *Dot1l*^{cKO} testes as expected.

3. The authors showed DOT1L immunofluorescence staining in Mllt10 knockout testes, and MLLT10 immunofluorescence staining in Dot1L knockout testes, but didn't show DOT1L immunofluorescence staining in Dot1L knockout testes, and MLLT10 immunofluorescence staining in Mllt10 knockout testes. The authors should provide these data as supplement.
Response: In Fig. S3, we have now added DOT1L immunofluorescence staining in *Dot1l* knockout testes (Fig. S3A) and MLLT10 immunofluorescence staining in *Mllt10* knockout testes (Fig. S3B). The focal localization of DOT1L to XY chromatin in metaphase spermatocytes is specific, because this signal is absent in *Dot1l*^{cKO} metaphase spermatocytes (Fig. 3A). MLLT10 is mainly expressed in germ cells in testes and the immunofluorescence signals are absent in *Mllt10*^{cKO} testes (Fig. S3B).

4. For the mass-spectrometry data, the authors may provide a diagram to show where these DOT1L and MLLT10 peptides matched on the genes as supplemental data.
Response: We have now provided diagrams to show the positions of sequenced peptides on DOT1L and MLLT10 proteins in a new supplementary figure – Fig. S9.

Second decision letter

MS ID#: DEVELOP/2022/201501

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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

Aldready done

Comments for the author

All my concerns were appropriately answered. I have no further comments - Congratulations

Reviewer 2

Advance summary and potential significance to field

In the revised version,the authors have provided supplemental data about antibodies specificity and a diagram showing the distribution of DOT1L and MLLT10 peptides in mass-spectrometry.

Comments for the author

Since the specificity of the antibodies is not high, need to be cautious when interpreting the immunofluorescence data.