INO80 requires a polycomb subunit to regulate the establishment of poised chromatin in murine spermatocytes

Prabuddha Chakraborty and Terry Magnuson
DOI: 10.1242/dev.200089

Editor: Benoit Bruneau

Review timeline
Original submission: 7 August 2021
Editorial decision: 4 October 2021
First revision received: 22 November 2021
Accepted: 23 November 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/200089

MS TITLE: Polycomb subunit required for chromatin remodeler INO80 to regulate establishment of poised chromatin in murine spermatocytes

AUTHORS: Prabuddha Chakraborty and Terry Magnuson

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 have some significant criticisms and recommend a substantial revision of your 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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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 INO80 complex is a conserved ATP-dependent chromatin remodeling enzyme that is known to play key roles in transcriptional regulation by either mobilizing nucleosomes or by promoting exchange of the histone variant H2A.Z. Previous work has demonstrated that INO80 is important for ESC pluripotency, reprogramming, and transcriptional regulation; previous work from the authors' group has shown that germ cell depletion of INO80 leads to meiotic arrest of spermatocytes. Here the authors have investigated the impact of INO80 on spermatogenesis by performing comprehensive ChIP-seq, RNA-seq, and ATAC-seq studies. They find that INO80 is important for repression of poised, bivalent genes in during spermatogenesis, and that INO80 is required for both Suz12 and H2A.Z occupancy at this gene set. In general, the genome-wide studies are of high quality and their interpretation and presentation are excellent. The authors make a strong case for the majority of their conclusions and this appears to be a valuable study. There are several issues, however, that need to be addressed.

Comments for the author

1. Suz12/INO80 co-IPs have no controls for indirect DNA-mediated interactions. The washes are all below 350mM KCl, and thus both factors will retain nonspecific DNA binding. The standard control is to include 50-100 micrograms per ml ethidium bromide during the IP to eliminate potential artifacts (see Lai and Herr, 1992 PNAS 89:6958).

2. It is not clear which genes require INO80 for expression (down-regulated genes). The ChIP-seq dataset indicates that INO80 binds to nearly every active or poised gene, and the down-regulated gene set seems to reflect the major component of the DEGs, but they do not correspond to the C2 cluster. This needs better explanation. Does this mean that the C2 cluster have a high level of INO80, but it plays no obvious functional role? Are the down-regulated genes ones with low levels of H3-K4me3? This is also important in relation to the statement on line 355 which states that INO80 may play a broad role in spermatogenic gene expression.

3. Line 315. The authors state that depletion of INO80 does not impact chromatin accessibility or nucleosome positioning at bivalent genes. However, the only analysis is ATAC-seq data, and as currently depicted, it is not clear that the authors have rigorously analyzed nucleosome positioning (though overall accessibility looks normal). Note that in yeast, loss of INO80 leads to only a 2bp change in the positioning of the +1 nucleosome; changes that I do not think would be resolved by these ATAC-seq datasets. MNase-seq analyses I think would be required.

4. The authors need to note that previous work from the Boyer group (ref.59) showed that H2A.Z and Suz12 recruitment are interdependent in mouse ESCs. Given that depletion of INO80 has a dramatic impact on Suz12 recruitment in spermatocytes (this work), it is perhaps not surprising that a loss of H2A.Z also occurs. This does impact how the authors interpret these results. There is no biochemical evidence that INO80 can deposit H2A.Z, but rather this activity is performed by SRCAP and Tip60/p400 complexes in mammals. It would be appropriate for the authors to comment on whether roles for SRCAP or p400/Tip60 have been investigated in spermatocytes.

Minor points:
The references need some major editing. There are many examples where journal names are missing as well as other details.
Line 285, this statement should be softened to state that "… is consistent with loss of H3-K27me3" or "…is likely due to loss of H3-K27me3"
Line 270. Shouldn't this state "to determine if INO80 regulates…"?
Line 49. It is is no clear what "epigenetic shifts towards gene regulation" means?
This seems like too much epigenetic jargon. Do the authors simply mean "changes in transcriptional programs"?

Reviewer 2

Advance summary and potential significance to field

This study explores the mechanistic underpinning behind a previous finding from the same laboratory that germ cell-specific deletion of the chromatin remodeler Ino80, causes meiotic arrest in spermatocytes leading to DNA-damage and cell death. The biochemical function of Ino80 is commonly believed to involve histone variant exchange; however, the transcriptional effect in different cell types is varied. Therefore, how Ino80 facilitates meiotic progression is unclear. The approach used in this manuscript to define how Ino80 function leads to this specific developmental phenotype during meiotic progression was to pair gene expression analysis with genome-wide ChIP-Seq (Ino80, H3K27me, H3K4me3, H2AZ), Cut&Run (SUZ12) and ATAC-Seq analysis.

The major finding that result from this analysis is that Ino80 binding correlates with H3K4me3, a subset of which colocalizes with H3K27me3. Although Ino80cKO results in the increase in premeiotic genes and the decrease in meiotic gene expression during meiosis, Ino80 binding is primarily correlated to the premeiotic genes repressed and bound with H3K27me3 and H3K4me3. These sites require Ino80 for SUZ12 binding and H3K27me3. These results are robust, and they have performed necessary controls. These findings are a novel and significant insight into Ino80-mediated gene regulation.

To connect this finding to Ino80 mechanistically, the authors performed H2AZ ChIP-seq. At Ino80-bound and repressed genes, the authors observe a decrease in H2AZ and a decrease in H3K27me3 levels. In addition, they observe an association between SUZ12 and Ino80 at the protein level. These two pieces of data provide potential mechanistic insight connecting Ino80 to PRC2 activity at repressed developmental genes.

Comments for the author

The weakness in the manuscript at this point is the incomplete analysis to connect H2AZ levels with PRC2 in spermatocytes. H2AZ is proposed as the connection between Ino80 and PRC2 in the regulation of bivalent genes to repress premeiotic gene expression, but this is not fully examined to a satisfactory level. A decrease in H2AZ at Ino80-bound bivalent sites was established, but the general connection between a decrease in SUZ12 (or H3K27me3) and H2AZ levels isn't apparent. It is compelling that there is such a high concordance at the Ino80-cobound regions (Supp S7C), but is this specific to Ino80 sites or is there a significant correlation genome-wide between sites of H2AZ and SUZ12/H3K27me3? Some additional analysis of this data, with a panel in the main figure with the findings would help clarify whether H2AZ is a key component at Ino80-repressed genes or if direct recruitment of SUZ12 by Ino80 is a primarily component.
Minor comments:
A key component of the analysis is the comparison to the published dataset of gene expression changes in spermatocytes during development (ref 42). Additional details regarding this dataset (the time points use to define Pre, mei, late) would be helpful in assessing the comparisons to gene expression at P18.
Slightly more ATAC-Seq analysis in S6 would be useful in determining whether the lack of difference at poised sites is unique to these sites or generalizable. Is there any difference at all between Ino80WT and Ino80cKO? Is there any difference at activated Ino80 binding sites?
P15 line 327 has an incomplete sentence.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The INO80 complex is a conserved ATP-dependent chromatin remodeling enzyme that is known to play key roles in transcriptional regulation by either mobilizing nucleosomes or by promoting

exchange of the histone variant H2A.Z. Previous work has demonstrated that INO80 is important for ESC pluripotency, reprogramming, and transcriptional regulation; previous work from the authors' group has shown that germ cell depletion of INO80 leads to meiotic arrest of spermatocytes. Here the authors have investigated the impact of INO80 on spermatogenesis by performing comprehensive ChIP-seq, RNA-seq, and ATAC-seq studies. They find that INO80 is important for repression of poised, bivalent genes in during spermatogenesis, and that INO80 is required for both Suz12 and H2A.Z occupancy at this gene set. In general, the genome-wide studies are of high quality and their interpretation and presentation are excellent. The authors make a strong case for the majority of their conclusions and this appears to be a valuable study. There are several issues, however, that need to be addressed.

Reviewer 1 Comments for the Author:
1. Suz12/INO80 co-IPs have no controls for indirect DNA-mediated interactions. The washes are all below 350mM KCl, and thus both factors will retain nonspecific DNA binding. The standard control is to include 50-100 micrograms per ml ethidium bromide during the IP to eliminate potential artifacts (see Lai and Herr, 1992 PNAS 89:6958).

Response: We have repeated this experiment with an additional control group treated with ethidium bromide as suggested. Positive detection of INO80 in SUZ12 IP and SUZ12 in INO80 IP, both after ethidium bromide treatment, suggests that the detected interaction between INO80 and SUZ12 was not due to an indirect DNA-mediated interaction. The results are included as an updated panel in fig. 4E and described in the results section between lines 299-302. Supplementary methods section was also updated accordingly between lines 43-49.

2. It is not clear which genes require INO80 for expression (down-regulated genes). The ChIP-seq dataset indicates that INO80 binds to nearly every active or poised gene, and the down-regulated gene set seems to reflect the major component of the DEGs, but they do not correspond to the C2 cluster. This needs better explanation. Does this mean that the C2 cluster have a high level of INO80, but it plays no obvious functional role? Are the down-regulated genes ones with low levels of H3-K4me3? This is also important in relation to the statement on line 355 which states that INO80 may play a broad role in spermatogenic gene expression.

Response: Most of the genes in cluster C2 exhibit significant downregulation in *Ino80*^{cKO} (n=1369, <1.5fold), while a few genes show significant upregulation (n=89, >1.5fold). We have added an updated panel in Fig. S4A to illustrate this observation and changed the description accordingly between lines 253-254. The downregulated DEGs in Ino*80*^{cKO} (INO80 activated in WT) show relatively high level of H3K4me3 in *Ino80*^{WT}, which remains unchanged in *Ino80*^{cKO} (Fig. S4D). Therefore, although we postulate that INO80 may play a role in the downregulation of the DEGs, the mechanism is unclear and requires further investigation.

3. Line 315. The authors state that depletion of INO80 does not impact chromatin accessibility or nucleosome positioning at bivalent genes. However, the only analysis is ATAC-seq data, and as currently depicted, it is not clear that the authors have rigorously analyzed nucleosome positioning (though overall accessibility looks normal). Note that in yeast, loss of INO80 leads to only a 2bp change in the positioning of the +1 nucleosome; changes that I do not think would be resolved by these ATAC-seq datasets. MNase-seq analyses I think would be required.

Response: PRC2 is a large multiprotein complex and binds to approximately 2.5 kb DNA (Heenan P. R. *et al., Nucleic Acids Res.* 48, 2969–2981). Although INO80 plays a role in nucleosome shift, it is unlikely that a small shift contributes to the changes in the incorporation of a large multiprotein complex such as PRC2. Therefore, in this study, we determined whether there was an overall change in chromatin accessibility around promoter regions. To avoid confusion, we have removed 'nucleosome positioning' and modified the text between lines 307- 311.

4. The authors need to note that previous work from the Boyer group (ref.59) showed that H2A.Z and Suz12 recruitment are interdependent in mouse ESCs. Given that depletion of INO80 has a dramatic impact on Suz12 recruitment in spermatocytes (this work), it is perhaps not surprising that a loss of H2A.Z also occurs. This does impact how the authors interpret these results.

There is no biochemical evidence that INO80 can deposit H2A.Z, but rather this activity is performed by SRCAP and Tip60/p400 complexes in mammals. It would be appropriate for the authors to comment on whether roles for SRCAP or p400/Tip60 have been investigated in spermatocytes.

Response: Although several studies have demonstrated a role for INO80 in H2A.Z eviction and replacement, there are several recent reports suggesting a decrease in H2A.Z occupancy in absence of INO80 in several cell types. However, it is not clear how absence of INO80 is directly leading to the reduction of H2A.Z. It is possible that either SRCAP or TIP60/EP400 complex plays a role in INO80-mediated H2A.Z regulation. The role of either SRCAP or TIP60/EP400 complexes have not been studied in spermatogenesis, and therefore, requires further investigation. We have described this in the discussion section between lines 396-406.

In addition to the observed reduction in H2A.Z occupancy at selected genes in *Suz12*$^{-/-}$ ES cells reported by Creyghton *et al*. (2008), a later report by Illingworth *et al*. (2012) demonstrated little or no change in H2A.Z occupancy in *Eed*$^{-/-}$ or *Suz12*$^{-/-}$ ES cells, either by ChIP-qPCR or ChIP- on-chip studies, justifying further investigation to determine the interdependency between H2A.Z and SUZ12 or PRC2. Further, in the present study, in addition to the reduction of H2A.Z at the H3K27me3 bound promoters, SUZ12 free promoters showed a decreased in H2A.Z occupancy (Fig. S7E), suggesting an INO80-dependent H2A.Z incorporation that is independent of SUZ12 occupancy.

Minor points:

The references need some major editing. There are many examples where journal names are missing as well as other details.

Response: We have edited the references to correct these errors.

Line 285, this statement should be softened to state that "… is consistent with loss of H3-K27me3" or "…is likely due to loss of H3-K27me3"

Response: We have changed the sentence as suggested at line 274 in the revised manuscript.

Line 270. Shouldn't this state "to determine if INO80 regulates…"?

Response: We have changed the sentence as suggested at line 260 in the revised manuscript.

Line 49. It is is not clear what "epigenetic shifts towards gene regulation" means? This seems like too much epigenetic jargon. Do the authors simply mean "changes in transcriptional programs"?

Response: That is correct, however as this section (Significance) is not necessary under the prescribed format in Development, we have removed this section.

Reviewer 2 Advance Summary and Potential Significance to Field: This study explores the mechanistic underpinning behind a previous finding from the same laboratory that germ cell-specific deletion of the chromatin remodeler, Ino80, causes meiotic arrest in spermatocytes leading to DNA-damage and cell death. The biochemical function of Ino80 is commonly believed to involve histone variant exchange; however, the transcriptional effect in different cell types is varied. Therefore, how Ino80 facilitates meiotic progression is unclear.The approach used in this manuscript to define how Ino80 function leads to this specific developmental phenotype during meiotic progression was to pair gene expression analysis with genome-wide ChIP-Seq (Ino80, H3K27me, H3K4me3, H2AZ), Cut&Run (SUZ12) and ATAC-Seq analysis. The major finding that results from this analysis is that Ino80 binding correlates with H3K4me3, a subset of which colocalizes with H3K27me3. Although Ino80cKO results in the increase in premeiotic genes and the decrease in meiotic gene expression during meiosis, Ino80 binding is primarily correlated to the premeiotic genes repressed and bound with H3K27me3 and H3K4me3. These sites require Ino80 for SUZ12 binding and H3K27me3. These results are robust, and they have performed necessary

controls. These findings are a novel and significant insight into Ino80-mediated gene regulation. To connect this finding to Ino80 mechanistically, the authors performed H2AZ ChIP-seq. At Ino80-bound and repressed genes, the authors observe a decrease in H2AZ and a decrease in H3K27me3 levels. In addition, they observe an association between SUZ12 and Ino80 at the protein level. These two pieces of data provide potential mechanistic insight connecting Ino80 to PRC2 activity at repressed developmental genes.

Reviewer 2 Comments for the Author: The weakness in the manuscript at this point is the incomplete analysis to connect H2AZ levels with PRC2 in spermatocytes. H2AZ is proposed as the connection between Ino80 and PRC2 in the regulation of bivalent genes to repress premeiotic gene expression, but this is not fully examined to a satisfactory level. A decrease in H2AZ at Ino80-bound bivalent sites was established, but the general connection between a decrease in SUZ12 (or H3K27me3) and H2AZ levels isn't apparent. It is compelling that there is such a high concordance at the Ino80-cobound regions (Supp S7C), but is this specific to Ino80 sites or is there a significant correlation genome-wide between sites of H2AZ and SUZ12/H3K27me3? Some additional analysis of this data, with a panel in the main figure with the findings would help clarify whether H2AZ is a key component at Ino80-repressed genes or if direct recruitment of SUZ12 by Ino80 is a primarily component.

Response: We observed that all the promoters with substantial H3K27me3 enrichment were clustered in INO80 bound CL-1 (Fig 1A), thereby prompting us to determine whether the changes in H3K27me3 levels were in CL-1 only. Although a majority of the promoters with H3K27me3 peaks also showed H2A.Z occupancy (Fig. S7C), H2A.Z distribution is much wider. H2A.Z is present at promoters both from CL-1 and CL-2. We have observed a reduced H2A.Z occupancy in several promoters in *Ino80*^{cKO} (Fig. S7E) compared to *Ino80*^{WT} that are devoid of both H3K27me3 and SUZ12. In addition, an overall reduction in H2A.Z occupancy in H3K27me3-free CL-2 promoters in *Ino80*^{cKO} (Fig. 5H) suggests a role of INO80 in promoting H2A.Z occupancy in these cells, which is independent of either SUZ12 or H3K27me3 occupancy. Additionally, publications from Yu *et al*. (2021) and Wang et al. (2018) also concluded a direct role of H2A.Z in INO80-facilitated PRC2 binding and establishment of bivalency in ES cells, allowing us to speculate a similar role of H2A.Z in PRC2 binding and establishment of bivalency in spermatocytes as well. We have described the additional panels in results section (lines 331-341) and modified the discussion section between lines 407-416.

Minor comments:
A key component of the analysis is the comparison to the published dataset of gene expression changes in spermatocytes during development (ref 42). Additional details regarding this dataset (the time points use to define Pre, mei, late) would be helpful in assessing the comparisons to gene expression at P18.

Response: We have included additional details between lines 163-167.

Slightly more ATAC-Seq analysis in S6 would be useful in determining whether the lack of difference at poised sites is unique to these sites or generalizable. Is there any difference at all between Ino80WT and Ino80cKO? Is there any difference at activated Ino80 binding sites?

Response: The chromatin accessibility remains relatively unchanged in either all promoters or INO80-activated promoters, as well as all promoters in CL2. We have included an additional panel in Fig. S6 to illustrate this observation and described between lines 307-311 in results section.

P15 line 327 has an incomplete sentence.

Response: We have corrected this sentence, at line 321 in the revised manuscript.

Second decision letter

MS ID#: DEVELOP/2021/200089

MS TITLE: Polycomb subunit required for chromatin remodeler INO80 to regulate establishment of poised chromatin in murine spermatocytes

AUTHORS: Prabuddha Chakraborty and Terry Magnuson
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.