EMBO reports

Ash1 counteracts Polycomb repression independent of histone H3 Lysine 36 methylation

Eshagh Dorafshan, Tatyana G. Kahn, Alexander Glotov, Mikhail Savitsky, Matthias Walther, Gunter Reuter and Yuri B. Schwartz

Review timeline:

Submission date:	17 July 2018
Editorial Decision:	3 September 2018
Revision received:	11 December 2018
Editorial Decision:	15 January 2019
Revision received:	31 January 2019
Accepted:	5 February 2019

Editor: Esther Schnapp/Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 3 September 2018

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We received the full set of referee reports last week but I was still waiting for referee cross-comments on referee 1's concern regarding the readout for counteracting polycomb repression. Unfortunately, the other referees did not reply, and I do not want to postpone the decision further.

As you will see, all referees acknowledge that the findings are potentially interesting and novel. However, they also all have suggestions for how the study could be improved and the findings strengthened. Given the number of comments and their relevance, I think that all should be addressed.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Supplementary figures, tables and movies can be provided as Expanded View (EV) files, and we can offer a maximum of 5 EV figures per manuscript, plus EV tables and movies. EV figures are

embedded in the main manuscript text and expand when clicked in the html version. Additional supplementary figures will need to be included in an Appendix file. Tables can either be provided as regular tables, as EV tables or as Datasets. Please see our guide to authors for more information.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution. In order to avoid delays later in the process, please read our figure guidelines before preparing your manuscript figures at: http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In vitro studies led to the well-known proposal that methylation of Histone H3 at both Lysine 4 (H3K4) by Trithorax and at Lysine 36 (H3K36) by Ash1 is inhibiting Polycomb silencing by blocking the methylation of Lysine 27 (H3K27). Here the authors perform an elegant in vivo analysis that do not support such model for ash1. In particular, they use the recently developed Drosophila strains in which it is possible to manipulate the multiple genes encoded within each histone cluster. With this system the authors generate embryos expressing only H3 variants in which K36 is replaced by Arginine and claim that they can still counteract Polycomb silencing at the Ubx and Abd-B loci. If the authors can argue with my concerns, I think that this in vivo analysis with provocative conclusions could be well suited for EMBO Reports.

Embryos lacking the histone cluster on the 2nd chromosome begin early embryogenesis thanks to the maternal load of histone mRNAs but die very quickly after nuclear division 15. Thanks to the φC31 integration system and recombineering, several laboratories have now shown that it is possible to fully complement this lethality by introducing 12 copies of the histone gene cluster. In 2015, McKay et al. have generated strains in which all H3.2 copies within a 12x complementing BAC carry an Arginine instead of K36 and observed that all animals die before the end of pupal stage. In the present study the authors have separated the growing 1st instar larvae of the complementing genotype from the heterozygous ΔHis sibling and observe that they all survive till pharate adult stage with even some adults escaping from the pupal case. Strikingly they display no homeotic transformations indicating that there is no erroneous repression of the Hox genes. There is however a caveat to this surprising result, because of the presence of 2 copies of the H3.3 variant genes that are transcribed throughout the whole cell cycle and that are known to incorporate into nucleosomes of transcribed regions. The Basler labs has shown that the H3.3 variants harbor all function of H3.2 and incorporation of H3.3 variant at active hox loci likely counteracts Polycomb silencing. The authors are fully aware of this caveat and go through the cumbersome procedure of generating animals in which the two H3.3 variants are also mutated. These animals die now as 1st instar larvae, a stage where it is unfortunately very difficult to assign segmental identities. As read out for failure of properly counteracting Polycomb repression the authors follow Abd-B expression in the embryonic central nervous system (CNS) and do not observe any difference with wild type embryos. To be honest I find that the lack of difference at such a young stage of embryogenesis is a weak argument. I feel a bit uncomfortable in complaining about the strength of the argument because I cannot readily propose a simple experiment that would help to solve the issue in a satisfactory fashion. It is a situation where one has reached the limits of the system and I do not feel comfortable enough to accept or reject the work. Simply, I am for example wondering if some remaining maternal contribution of H3.2 or H3.3 (what is actually known about H3.3 maternal contribution) could be responsible for the proper Abd-B regulation. Abd-B expression is known to be very tenacious and one need to remove both maternal and zygotic ash1 expression to see a decrease in Abd-B expression (Klymenko and Mueller, 2004). I am very curious to know the opinion and comments of the other referees.

Additional comments:
I was not aware of a trx-G abdominal phenotype in females. The black pigmentation on the 7th tergite is a variable feature that depends on the background. In figure 2A (top row) where the background is comparable between the 3 panels, there is not a spectacular difference between the FL and ΔPHD females. Authors should provide a reference where the female phenotype is reported as a reliable criterium.

Regarding the HR generated NSD mutant abortive events that lead into the insertion of the selectable marker without the deletion of the targeted gene can occur. The authors should provide evidence that they did check by PCR that the designed LSD deletion occurred.

I wonder if the authors have tried genetic interaction studies by looking at Set2(1)/+ in the background of as1(22)/ash1(21).

Same as above by adding NSD. NSD and ash1 are quite far from each other to generate easily recombinants with ash1(21) as well as with ash1(22), enabling to generate ash(22)/ash1(21 in the background of NSD knock out.

Regarding the ash1(-) male cuticle displayed in Figure 1, one can detect a small patch of 7th tergite, which is a feature of anterior transformation of A7 towards A6. I wonder if there are bristles on the ventral sternite of A6, which is also a sign of anterior transformation. As mentioned above and unlike the description of the male phenotype, there is no description in the Figure legend 1 or in the text of what we should be looking at on the female cuticles. The NSD halter shown in panel H of Figure 1 is not the very best example to document as WT looking halter, because it is slightly larger than the WT halter and the little depression gives the impression that there are little hairs.

I am a bit disturbed by the fact that the posterior side of the embryos displayed in Figure 4D are oriented up instead of down.

A reference to Allen Shearn's contribution to the trithorax-group of genes (1989, Genetics) should

be added just before the last paragraph in the introduction (https://www.ncbi.nlm.nih.gov/pubmed/2497049)

Referee #2:

Dorafshan et al investigate the mechanism by which the Trithorax group protein Ash1 antagonizes Polycomb repression in Drosophila. A large body of data has suggested that Ash1 carries out this role by methylating lysine 36 of histone H3. The authors present evidence that other H3K36 methyltransferases, including Set2 and NSD, are not required for the activation of Hox genes, highlighting the central role of Ash1 in this process. Consistent with previous reports, they show that the loss of Ash1 activity does not significantly reduce the bulk level of histone H3 methylation. The most provocative finding reported by the authors, however, is that the substitution of lysine 36 of histone H3 with an arginine does not lead to homeotic transformations or alter Hox expression. This surprising result suggests that Ash1 prevents Polycomb repression via a mechanism other than H3K36 methylation. If true, this finding would have a tremendous impact on the field.

Overall, the technical quality of the authors' work is strong and supports the main conclusions of the manuscript. Although my overall enthusiasm for this submission is high, several points need to be addressed prior to publication.

1. I am concerned by the chromatin immunoprecipitation data shown in figure 3 which suggest that the loss of Ash1 activity has only a slight effect on H3K36 monomethylation at specific target genes and no effect on H3K36 dimethylation. The authors' interpretation of these data is somewhat misleading since they examined whole larvae; previous studies by others using dissected imaginal discs showed that the loss of Ash1 activity significantly reduced H3K36 methylation at biologically relevant target genes. The authors should consider deleting this figure or providing a more cautious interpretation of their results. If these data are included in the final version of the manuscript, a supplemental figure showing the regions of Ubx (and other genes) analyzed in the ChIP experiments would be helpful.

2. In my opinion, authors' discovery that the substitution of K36 of histone H3 with an arginine does not affect Hox expression is the most important part of their submission. Given the provocative nature of their findings, additional controls should be provided, including an examination of the level of H3K36 methylation and Hox expression in the mutants. Verification that the complete loss of H3K36 methylation does not alter Hox expression would provide strong support for the authors' conclusions.

Referee #3:

This manuscript investigates the role of the Ash1 methyltransferase in counteracting Polycomb repression. It confirms using genetic evidence that Ash1 is the key histone H3 K36 methyltransferase that counters Pc repression. It also provides convincing in vivo evidence using transgenic histone H3 mutant constructs that mono-methylation (H3K36me1) is not the mechanism by which Ash1 counters Pc repression, which has been one of the leading ideas. This is an important discovery and adds to a growing list of examples in which the histone modification activity of a protein is not essential for its function.

The authors also show that the Set catalytic domain of Ash1 is needed to counter Pc repression, and that the PHD finger domain is not. It would greatly strengthen the paper if the authors provided western blots showing the expression of the various wild-type and mutant Ash1 proteins. This is particularly important for the delta Set1 protein that does not rescue. This is needed to establish that the lack of rescue does not reflect poor protein expression.

As a more minor point, it would be helpful if a diagram showing the locations of the ChIP PCR amplicons were shown in Figure 3.

Referee 1.

In vitro studies led to the well-known proposal that methylation of Histone H3 at both Lysine 4 (H3K4) by Trithorax and at Lysine 36 (H3K36) by Ash1 is inhibiting Polycomb silencing by blocking the methylation of Lysine 27 (H3K27). Here the authors perform an elegant in vivo analysis that do not support such model for ash1. In particular, they use the recently developed Drosophila strains in which it is possible to manipulate the multiple genes encoded within each histone cluster. With this system the authors generate embryos expressing only H3 variants in which K36 is replaced by Arginine and claim that they can still counteract Polycomb silencing at the Ubx and Abd-B loci. If the authors can argue with my concerns, I think that this in vivo analysis with provocative conclusions could be well suited for EMBO Reports.

We are grateful to the referee for appreciating our work.

Major concerns:

As read out for failure of properly counteracting Polycomb repression the authors follow Abd-B expression in the embryonic central nervous system (CNS) and do not observe any difference with wild type embryos. To be honest I find that the lack of difference at such a young stage of embryogenesis is a weak argument. I feel a bit uncomfortable in complaining about the strength of the argument because I cannot readily propose a simple experiment that would help to solve the issue in a satisfactory fashion. It is a situation where one has reached the limits of the system and I do not feel comfortable enough to accept or reject the work. Simply, I am for example wondering if some remaining maternal contribution of H3.2 or H3.3 (what is actually known about H3.3 maternal contribution) could be responsible for the proper Abd-B regulation. Abd-B expression is known to be very tenacious and one need to remove both maternal and zygotic ash1 expression to see a decrease in Abd-B expression (Klymenko and Mueller, 2004).

The referee raises two concerns which we will consider in sequence. Frist, she or he wonders whether it is at all possible to detect the stochastic loss of *Abd-B* expression after the loss of zygotic Ash1 function. Indeed, as the reviewer pointed out, in earlier study, Klymenko and Mueller saw the loss of *Abd-B* expression only in *ash1[22]* homozygous embryos derived from *ash1[22]* mutant germ cells. They interpreted it to indicate that maternally supplied Ash1 protein is always sufficient to maintain the expression of *Abd-B* in the embryo. We favour alternative explanation. We think that zygotic ash1 function is required for proper Abd-B expression at late embryonic stages and that homozygous *ash1[22]* embryos produced by heterozygous mothers maintain the expression of *Abd-B* because *ash1[22]* allele still supply a little bit of function (i.e. is a strong hypomorph but not a complete null). We base this conclusion on two lines of evidence. First, when *ash1[22]* is combined with *ash1[9011]* allele (a large deletion that removes the entire *ash1* gene) the loss of *Abd-B* expression in *ash1[22]/ ash1[9011]* embryos produced by heterozygous mothers is readily detectable. This positive control is now included as Figure 6B. Second, in a parallel study of individual Ash1 domains, which is being prepared for publication, we generated new *ash1* alleles using CRISPR/Cas9 genome editing. One of them is a deletion that introduces multiple successive stop codons in the middle of SET domain and a frame shift that persists till the end of the open reading frame. This allele (*ash1*M3) cannot produce any functional protein. Stage 16 homozygous *ash1*M3 embryos produced by heterozygous mothers show even more pronounced loss of *Abd-B* expression in the CNS (Figure 1 for the referee). We speculate that *ash1[22]* allele produces small amount of wild-type protein due to the read through its premature stop codon (to be surprised how commonly stop codons are read through in flies see Dunn et al., 2013 PMID: 24302569). To conclude, our system appears sensitive enough to detect the loss of *Abd-B* expression caused by zygotic disturbances.

The second question raised by the reviewer is whether, if methylated by Ash1, maternally contributed and K36-containing H3.2 or H3.3 could be sufficient to inhibit excessive Polycomb repression of *Abd-B*. While this is formally possible, we cannot come up with any realistic scenario of how this might work. As outlined in the Discussion (former Concluding remarks) section of the manuscript, after all maternal histone supply is cut off, CNS cells divide five times diluting the pool of maternal histones 32-fold. This means that, in the H3.3/H3.2-deficient, H3K36R transgenic CNS

cells, only 1 in 32 nucleosomes has an H3 tail that can be methylated at K36 and only 1 in 1024 nucleosomes has both H3 tails that can be methylated at K36. Even if we posit that K36 methylation completely prevents subsequent H3K27 methylation, which is an exaggeration (*in vitro* H3K36 di-methylated nucleosomes are just 5-fold poorer substrate for PRC2), the resulting gaps in H3K27 methylation will be extremely rare. To be counteracted by Ash1-mediated H3K36 methylation in our H3.3/H3.2-deficient, H3K36R replacement mutants, Polycomb repression must be blocked by a single nucleosome with one "K27-unmethylatable" H3 tail placed randomly within 6kb long stretch of the fully "K27-methylatable" chromatin. We think this is highly unlikely. For example, after DNA replication, every second H3 molecule within H3K27me3 domains of Polycomb-repressed genes becomes un-methylated and it takes hours until the H3K27me3 density is fully restored (Reveron-Gomez et al., 2018; PMID: 30146316). Yet, Polycomb repression copes with this just fine. We have expanded the Discussion section to articulate this argument.

Additional comments:

I was not aware of a trx-G abdominal phenotype in females. The black pigmentation on the 7th tergite is a variable feature that depends on the background. In figure 2A (top row) where the background is comparable between the 3 panels, there is not a spectacular difference between the FL and D*PHD females. Authors should provide a reference where the female phenotype is reported as a reliable criterium.*
We are grateful to the referee for pointing to this oversight. We are also not aware of *trxG* abdominal phenotypes in females and agree that the images of female abdomens shown on figures 1C, 2A and 4B contain no useful information. We removed them to avoid confusion.

Regarding the HR generated NSD mutant abortive events that lead into the insertion of the selectable marker without the deletion of the targeted gene can occur. The authors should provide evidence that they did check by PCR that the designed NSD deletion occurred.
We are well aware of the perils of homologous replacement strategy and thoroughly characterized our mutant before using it. To this end we have shown by PCR genotyping that the homozygous *NSDds46* mutants contain DsRed and do not contain *NSD* ORF. Using RT-qPCR we have also shown that the *NSDds46* flies produce no *NSD* mRNA. As suggested by the referee, this information is now included in Figure S1.

I wonder if the authors have tried genetic interaction studies by looking at Set2(1)/+ in the background of as1(22)/ash1(21).
As suggested by the referee we have generated the *Set21/+; ash122/ash121* flies and compared their phenotype to that of the *+/+; ash122/ash121* flies. We could see no difference (Figure 2 for the reviewer).

Same as above by adding NSD. NSD and ash1 are quite far from each other to generate easily recombinants with ash1(21) as well as with ash1(22), enabling to generate ash(22)/ash1(21 in the background of NSD knock out.
This is a good experiment, which we have already done and shown the results on Figure 1C.

Regarding the ash1(-) male cuticle displayed in Figure 1, one can detect a small patch of 7th tergite, which is a feature of anterior transformation of A7 towards A6. I wonder if there are bristles on the ventral sternite of A6, which is also a sign of anterior transformation. As mentioned above and unlike the description of the male phenotype, there is no description in the Figure legend 1 or in the text of what we should be looking at on the female cuticles. The NSD halter shown in panel H of Figure 1 is not the very best example to document as WT looking halter, because it is slightly larger than the WT halter and the little depression gives the impression that there are little hairs.
We thank the referee for helpful suggestions on how to best present our data. First, as explained above, we removed the images of female abdomens as these are not informative and may cause confusion. Second, the referee's guess is correct. The *ash1* mutant males do have bristles on the ventral sternite of A6 indicating that it is partially transformed to A5. Corresponding images are now presented on Figure 1C. Third, we replaced the image of the *NSD* haltere on Figure 1C with a better picture.

I am a bit disturbed by the fact that the posterior side of the embryos displayed in Figure 4D are oriented up instead of down.

As suggested by the referee we changed the presentation of embryonic images to more conventional: anterior side up, posterior side down.

A reference to Allen Shearn's contribution to the trithorax-group of genes (1989, Genetics) should be added just before the last paragraph in the introduction (https://www.ncbi.nlm.nih.gov/pubmed/2497049)
We appreciate the referee pointing to this oversight. The reference to Allen Shearn's paper is added where suggested.

Referee 2.

Dorafshan et al investigate the mechanism by which the Trithorax group protein Ash1 antagonizes Polycomb repression in Drosophila. A large body of data has suggested that Ash1 carries out this role by methylating lysine 36 of histone H3. The authors present evidence that other H3K36 methyltransferases, including Set2 and NSD, are not required for the activation of Hox genes, highlighting the central role of Ash1 in this process. Consistent with previous reports, they show that the loss of Ash1 activity does not significantly reduce the bulk level of histone H3 methylation. The most provocative finding reported by the authors, however, is that the substitution of lysine 36 of histone H3 with an arginine does not lead to homeotic transformations or alter Hox expression. This surprising result suggests that Ash1 prevents Polycomb repression via a mechanism other than H3K36 methylation. If true, this finding would have a tremendous impact on the field. Overall, the technical quality of the authors' work is strong and supports the main conclusions of the manuscript. Although my overall enthusiasm for this submission is high, several points need to be addressed prior to publication.

We are pleased that the reviewer finds our work interesting and technically sound.

Concerns:

1. I am concerned by the chromatin immunoprecipitation data shown in figure 3 which suggest that the loss of Ash1 activity has only a slight effect on H3K36 monomethylation at specific target genes and no effect on H3K36 dimethylation. The authors' interpretation of these data is somewhat misleading since they examined whole larvae; previous studies by others using dissected imaginal discs showed that the loss of Ash1 activity significantly reduced H3K36 methylation at biologically relevant target genes. The authors should consider deleting this figure or providing a more cautious interpretation of their results. If these data are included in the final version of the manuscript, a supplemental figure showing the regions of Ubx (and other genes) analyzed in the ChIP experiments would be helpful.

The referee brings up an interesting point although we do not fully agree with his/her arguments. To the best of our knowledge, all ChIP studies done to date concur that Polycomb target genes bind Ash1 and get methylated at H3K36 only when de-repressed. The referee is correct that in the larva only a fraction of cells has any given Polycomb target gene in the de-repressed state (actually, the same, just to a lesser extent, applies to imaginal discs). This should not, by itself, be problem. From all larval cells, only those, where selected genes are active, will contribute to the chromatin pool from where Polycomb target genes can be immunoprecipitated with antibodies against Ash1 and methylated H3K36. The cells, where selected genes are inactive, are simply excluded from our analysis. This may, potentially, make it hard to detect Ash1 and methylated H3K36. If the fraction of cells, where selected genes are active, is too small, the relative yield of the immunoprecipitation (% of input precipitated) may be too low to discriminate specific precipitation from the background. This, evidently, is not a problem in our case. For example, as seen on the former Figure 3C (now Figure 4C), we can reliably immunoprecipitate selected genes with anti-Ash1 antibodies. The ChIP yields are good (>1% of input), signals at the selected genes are 10-fold higher than at negative control regions and the signals drop down in the *ash1* mutant. As to the discrepancy with ChIP results of Schmähling et al., we think our results are actually not so different as they appear at the first glance. We think that the critical difference between the two sets of experiments is in the choice of genes interrogated by ChIP-qPCR. In our case all selected genes are direct targets of Ash1 and four out of five (*hth*, *Ubx*, *Su(z)2*, *emc*) genes are documented Polycomb targets. In contrast, Schmähling et al. assayed H3K36me2 at six genes (*Ubx*, *mth*, *CG6310*, *wg*, *tsh*, *lam*), three of which

Ubx, *mth*, *CG6310* were picked because they had reduced transcription in *ash1* mutants. From this set, *Ubx*, *wg*, *tsh* are known Polycomb targets yet only *Ubx* but not *wg* or *tsh* showed reduction of H3K36me2 in ash1 mutants. Conspicuously, the two other genes that showed reduced H3K36me2 were *mth* and *CG6310*. Neither of these genes is known to bind Polycomb or Ash1. In fact, in a preliminary ChIP-seq experiment, we have mapped genomic distribution of Ash1 within chromatin from the whole third instar larvae. While we see Ash1 binding at all our selected genes and also at *tsh*, selected by Schmähling et al (Figure 3 for the referee A), we did not detect any Ash1 binding at *mth* or *CG6310* (Figure 3 for the referee, B-C). It seems that, similar to our results, Schmähling et al. see no correlation between the presence/absence of Ash1 and the loss of H3K36me2. It appears that *mth* and *CG6310* are not direct targets of Ash1 and lose H3K36me2 simply because they become transcriptionally inactive, which applies to all active genes.

As suggested by the referee the schematic showing the regions of analysed in our ChIP experiments is now included as Figure S17

2. In my opinion, authors' discovery that the substitution of K36 of histone H3 with an arginine does not affect Hox expression is the most important part of their submission. Given the provocative nature of their findings, additional controls should be provided, including an examination of the level of H3K36 methylation and Hox expression in the mutants. Verification that the complete loss of H3K36 methylation does not alter Hox expression would provide strong support for the authors' conclusions.

To address the referee concerns, we repeated the cross described on Figure S8 and from its progeny hand-picked stage 16 YFP-positive embryos (*H3.2-, H3.3-, H3K36R* mutants) and their YFP-negative siblings (heterozygous for D*HisC* and *H3.3B* deletions, control). These were used to isolate RNA and measure the levels of the *Abd-B* mRNA by RT-qPCR. Consistent with results of our immunostaining experiments, two biologically independent replicate measurements show that the overall level of *Abd-B* RNA in the *H3.2-, H3.3-, H3K36R* mutants is not reduced compared to heterozygous control or stage 16 wild-type embryos. These results are added as panel B to Figure 6.

As further suggested by the referee, we have attempted to measure the fraction of H3K36 remaining in the *H3.2-, H3.3-, H3K36R* mutant embryos using the level of methylated H3K36 as a proxy. Unfortunately, despite a lot of effort, this turned out to be impossible. As outlined in the Discussion section and in the response to referee 1, after maternal histone supply becomes unavailable, most cells in the embryo undergo two rounds of cell division diluting the pool of maternal H3K36 four-fold, however the CNS cells, where we assay the loss of Abd-B expression, divide five times diluting the pool of maternal histones 32-fold. Thus, to measure the relevant H3K36 reduction, one has to look specifically in the CNS. This could only be done by immunostaining. Immunofluorescent detection of methylated H3K36 is generally a challenge because there is very little of it to begin with: H3K36me1=2.5% of total H3, H3K36me2 = 0.5% of total H3 and H3K36me3=1.5% of total H3. In our hands, immunostaining with anti-H3K36me2 antibodies was very weak to be practical, the embryo staining with anti-H3K36me1 antibodies gave high cytoplasmic background and only immunostaining with anti-H3K36me3 antibodies gave distinct nuclear signal. Using the anti-H3K36me3 antibodies, we did see approx. 4-fold reduction of immunofluorescent signal in the endoderm and ectoderm of the stage 16 YFP-positive embryos (*H3.2-, H3.3-, H3K36R* mutants) compared to their YFP-negative siblings (heterozygous for D*HisC* and *H3.3B* deletions, control). However, we could not measure the reduction in the CNS because we could detect very little H3K36me3 already in the CNS of the heterozygous control. This was clearly not the immunostaining problem *per se* because we got very nice CNS immunostaining with anti-Elav antibodies (pan neuronal marker). That said, we are confident that the 32-fold dilution of the maternal H3K36 in the CNS does happen, because it follows from genetics and we went through all the pain of thoroughly genotyping our embryos to make sure that they are exactly as expected from the crossing scheme.

Referee 3.

This manuscript investigates the role of the Ash1 methyltransferase in counteracting Polycomb repression. It confirms using genetic evidence that Ash1 is the key histone H3 K36 methyltransferase that counters Pc repression. It also provides convincing in vivo evidence using transgenic histone

H3 mutant constructs that mono-methylation (H3K36me1) is not the mechanism by which Ash1 counters Pc repression, which has been one of the leading ideas. This is an important discovery and adds to a growing list of examples in which the histone modification activity of a protein is not essential for its function.

We are happy that the reviewer appreciates our work.

The authors also show that the Set catalytic domain of Ash1 is needed to counter Pc repression, and that the PHD finger domain is not. It would greatly strengthen the paper if the authors provided western blots showing the expression of the various wild-type and mutant Ash1 proteins. This is particularly important for the delta Set1 protein that does not rescue. This is needed to establish that the lack of rescue does not reflect poor protein expression.

We appreciate the referee's suggestion although he/she may have misspoken when expressing his/her concerns. Figure 2A shows that flies where *ash1* mutation is complemented with transgenic protein lacking SET or PHD domains both have homeotic transformations. This is not the case for the control flies supplemented with the full-length Ash1. This observation suggests that SET and PHD domains are both required for Ash1 to counteract Polycomb repression. At the same time, ChIP experiments on Figure 4C show that, SET- or PHD-deficient transgenic Ash1 bind target genes equally well and to the same degree as endogenous Ash1 and even better than transgenic full-length Ash1 protein. This, by itself, makes it unlikely that SET- and PHD-deficient transgenic Ash1 proteins cannot counteract Polycomb repression because they are poorly expressed. As suggested by the referee, we further strengthen this argument by measuring the levels of the endogenous, full-length, SET- and PHD-deficient Ash1 in corresponding transgenic larvae with western-blot. The measurements indicate that their expression levels are indeed very similar. This result is shown on Figure 2B-C.

As a more minor point, it would be helpful if a diagram showing the locations of the ChIP PCR amplicons were shown in Figure 3.

This schematic is now included as Figure S17.

[Figures for referees not shown.]

2nd Editorial Decision 15 January 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your manuscript in EMBO reports. However, referee #1 has some final comments we ask you to address in a final revised version of your manuscript.

Further, I have these editorial requests:

- By journal policy, we do not allow titles phrased as a question. Please provide a different title (with not more than 100 characters including spaces).

- Please reduce the abstract to not more than 175 words, and also provide it written in present tense.

- Please add a conflict of interest statement, and a paragraph detailing the author contributions to the manuscript text (above the acknowledgements).

- Please call out the individual panels of each figure in a sequential manner, or change their order in the figure. Presently, it seems e.g. that panel Fig 6B is called-out after 6C+D.

- There is presently no callout for Fig. 6A. Please check.

- The Expanded View format, which will be displayed in the main HTML of the paper in a

collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Thus, please select up to 5 figures as EV figures, name them accordingly, upload these as single files, and provide their legend in the main manuscript text (after the main legends). The remaining supplementary material should then be supplied as a single pdf labeled Appendix (see below). For more details please refer to our guide to authors:
http://embor.embopress.org/authorguide#manuscriptpreparation

- Please add page numbers to the Appendix, and a TOC (table of contents with page numbers), and name the file 'Appendix'. Please use the nomenclature Appendix Figure Sx or Appendix Table Sx for Appendix items, and change the callouts accordingly throughout the manuscript text and the author checklist.

- Please check that all EV and Appendix figured are called out in the manuscript text (presently, it seems Figs S9, S12, S13, S14, S16 are not called out).

- Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. Please also add a paragraph to the methods section explaining the statistical testing used throughout the manuscript. See:
http://embor.embopress.org/authorguide#statisticalanalysis

- Several Western blot panels show splice lines. Could you therefore provide the source data for all the Western blots shown in the manuscript (main figures, EV figures and Appendix figures)? The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure. In case data shown in the manuscript figures was derived from different blots, and united for the figure panel, please indicate this by separating black lines in the final figure.

Finally, please format the references according to EMBO reports style. See:
http://embor.embopress.org/authorguide#referencesformat

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the final revised manuscript text
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels).
- The revised Appendix.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #1:

Not surprisingly this revision does not provide new data answering my concerns about a possible role of the remaining maternal contribution. Like I wrote in my 1st comment, it is a situation where the limits of the available techniques have been reached. Apparently the other referees did not have

any criticism regarding a possible role of the remaining maternal contribution. I will thus give the benefit of the doubt and a green light for publication.

Additional comments: I thank the authors for providing unpublished data about their new ash1 allele. The lack of effect in embryos when ash1 is only zygotically inactivated (as reported by Klymenco and Muller) seems indeed due to the fact that the mutant combinations used by these authors is not completely amorphic. But the outcome of ash1 mutations on Abd-B expression in embryos (having or not having an effect) was not really THE issue regarding my reluctance to accept the conclusions of the work.

At page 13 the authors write "the loss of Abd-B expression from CNS parasegments 10-12 of the as1 and trx mutants is well documented (Ingham, 1983; Klymenco and Muller, 2004)" and readily visible in the ash[22]/ash[9022] embryos (Fig6C). The authors are partisan (not to use a stronger adjective) in their argumentation because; 1st Ingham did not have antibodies, 2nd Klymenco/Muller write that they are unable to detect any difference with ash1, unless they inactivate the maternal contribution and 3rd the slight decrease in Abd-B staining in the embryo shown on the bottom panel of Fig6C, is likely due to the fact that the top embryo is slightly younger as judged from the fully extended nerve chord. In this respect, Abd-B is also slightly weaker in the 2 embryos displayed in panel D (which have a nerve chord similar to the bottom Fig6C embryo).

Finally to follow the logic of their argumentation, the authors should convince the reader that they can detect a decrease in RNA contents [by quantitative RT PCR (Fig6B] in ash[22]/ash[9022] embryos.

Referee #2:

The authors have satisfactorily addressed the concerns raised in the initial review of their manuscript. I am confident that their provocative findings will be of interest to a wide audience.

Referee #3:

The authors have addressed the previous concerns and the paper is ready for publication.

2nd Revision - authors' response 31 January 2019

As requested, we addressed the final comments of referee #1. To this end, we replaced the image of the heterozygous control embryo on Figure 6A (former Figure 6C, changed for sequential call out of individual panels) to one of the embryo whose nerve cord extends to exactly the same degree as the matching ash1mutant embryo. This does not change our conclusion but avoids ambiguous interpretation. We also amended the sentence that referee #1 deemed too partisan. It now reads as follows “The loss of Abd-Bexpression from CNS parasegments 10-12 of the ash122homozygous embryos derived from ash122mutant germ line cells was reported previously [9]. It is also readily visible in the ash122/ash19011embryos produced by heterozygous mothers (Figure 6A, C).”

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yuri Schwartz
Journal Submitted to: The EMBO Reports
Manuscript Number: EMBOR-2018-46762

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were chosen to include more than 150 flies per class, which satisfies asymptotic nature of the chi-square test employed (Appendix Figure S2).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Each phenotypic class included more than 150 flies
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was done
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All required information is listed in Appendix Table S2
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All work was done with Drosophila melanogaster and all strains and their genotypes listed in the Materials and methods section of the manuscript according to standard nomenclature (flybase.org)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA