Stuxnet fine-tunes Notch dose during development using a functional Polycomb response element

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MS TITLE: A functional Polycomb response element fine-tunes Notch dose in development

AUTHORS: Alan J Zhu, Min Liu, Tao He, Yu Fan, Mengyuan Yi, Juan Du, and Yajuan Li

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 BenchPressand 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the refereeâ€™s comments, and we will look over this and provide further guidance.

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

As stated in the title “A functional Polycomb Response Element fine-tunes Notch dose in development”- the main result the authors would like to emphasize is the description of a Polycomb Response Element (PRE) in the Notch locus, which by itself is an interesting result. However, to study the epigenetic control of Notch expression, the authors center the investigation on Stuxnet, a protein that promotes degradation of the epigenetic Polycomb (Pc) protein. In my opinion, focusing this investigation on Stuxnet undermines the value of the study and dilutes what the authors highlight to be the main result of the manuscript- the finding of a putative PRE in the Notch region.

This paper does not present significant scientific advance on the identification of a functional PRE in the Notch locus. A more complete characterization of the PRE is required. I believe this is not feasible within the timescale of a normal revision

Comments for the author

Review Development 2022_201297

In this manuscript, the authors investigate the epigenetic mechanisms involved in the regulation of Notch expression during Drosophila development. It is well established that the variation of levels of the epigenetic Polycomb Group of proteins leads to tumorogenesis mediated, in part, by the up-regulation of Notch expression. As stated in the title “A functional Polycomb Response Element fine-tunes Notch dose in development”- the main result the authors would like to emphasize is the description of a Polycomb Response Element (PRE) in the Notch locus, which by itself is an interesting result. However, as I will explain later in the review, I believe a more complete characterization of the PRE is required.

To study the epigenetic control of Notch expression, the authors center the investigation on Stuxnet, a protein that promotes degradation of the epigenetic Polycomb (Pc) protein. As such, elevated levels of Stuxnet reduce Polycomb cellular content while low levels of Stx promotes up-regulation of Polycomb. They first present the results showing the interaction of Stuxnet with Notch and, second, the experiments to show that this interaction is mediated by Polycomb. Finally, they present the Pc-Dam-ID–seq data indicating binding of PC in seven regions (E1-E7) in the Notch locus (Figure 4). The results of the Pc-Dam-ID–seq data prompt the investigations on the function of the E1-E7 regions as putative PREs.

In my opinion, focusing this investigation on Stuxnet undermines the value of the study and dilutes what the authors highlight to be the main result of the manuscript- the finding of a putative PRE in the Notch region (title of the manuscript). The way the manuscript is currently presented aims to relate that Stuxnet modifies Notch expression in a Pc-dependent manner (Figure 1, Figure 2, Figure 3, Figure S1) with the finding of a putative PRE (only Figure 4, Figure S2, Figure S3), resulting in false expectations. The results showing the Pc-dependent interaction of Stuxnet and Notch (Figure 1, Figure 2 and Figure 3) are not needed to support the existence of a PRE. They show that Stuxnet modulates Pc levels (previously reported by the authors, Du et al 2016) and, in turn, that Polycomb modifies Notch levels (previously established in the field). These results are dispensable for the focus of the manuscript. Also, the use of Stuxnet as a tool to modulate Pc levels seems to me a bit convoluted considering the numerous Polycomb tools available to modulate its expression. I would recommend removing Stuxnet from the manuscript, use the available tools to modulate Pc levels of expression and focus on the characterization of the PRE.

In the following, I review the manuscript data supporting the existence of a PRE in the Notch locus, mainly presented in Figure 4, Figure 5, Figure S2 and Figure S3.

Pc-Dam-ID–seq experiments identify 7 regions (E1-E7) of Pc binding (Figure 4, panel A) that match in some cases (for instance E7) with previously described Pc binding regions (panel A). However, the E5 region (the putative PRE) does not match with any previously described. Unfortunately, this

comparison with previous experiments is only briefly discussed (in the Discussion section) but it seems to me not relevant to support their findings. In the authors think that the data from other authors is important to support their findings, the comparison should be further elaborated or otherwise removed form the Results section. Also, little information is provided about the E5 fragment (size, genomic coordinates, overlapping with other Notch regulatory sequences, etc). Perhaps, the results presented in Figure S3, panel A-B-C will be more appropriate here and, also, an improved versi on of Figure S3 panel D. It would also be interesting to compare the E1 and the E5 sequences.

The vgOE system is used to study the repressor properties of the E1-E7 fragment. Among these, the E5 fragment shows Pc-dependent repression making it a good candidate for a PRE. A more complete characterization of the PRE is required. For example: a) Analysis of the expression of the PRE. Does E5 drive expression of GFP/lacZ in a simple TATA box-GFP/lacZ promoter? If so, where does it do it? Does the expression tell us something about the E5 as a putative PRE? b) Analysis of the E5 sequence. Does it contain transcription factors binding sites? Which ones? Does it contain PHO and GAGA binding sites? What does the sequence tell us about this fragment? c) Definition of a minimal PRE sequence within the E5 fragment, i.e obtain the minimal fragment required to confer Pc-dependent repression d) site–directed mutagenesis of the binding sites, d) pull-down experiments, etc. All together, the way the PRE is currently described is very poor.

I think a lot of work has to be done for the molecular characterization of the PRE.

In vivo deletion of the putative PRE- phenotypic analysis. Figure 5 shows the wing and notum phenotypes of NotchΔPRE, a deletion of the putative PRE. I believe, these phenotypes are not related with the deletion of a putative “functional PRE”. They show that NotchΔPRE produces some Notch-like phenotypes that can be modulated with mutations in the Notch gene. Thus, these results suggests that the E5 fragment regulates Notch expression, rather than the E5 fragment controls Notch expression in a Pc-dependent manner. If NotchΔPRE would be a deletion in a “functional PRE”, it is expected that the NotchΔPRE phenotypes be modulated by inactivation and overexpression of Polycomb group proteins. This is not shown. I would suggest studying the interactions of NotchΔPRE with Polycomb and other PcG proteins. This can be simply done using Polycomb mutations and Pc duplications or the use of the UAS/Gal4. Also, considering the weak phenotype of NotchΔPRE flies in the notum (lack of few bristles) and the wing (no phenotype), I find quite surprising that Notch mRNA levels are reduced 30% in either wing discs and/or whole larva (Figure 5, panel A and B). A 30% decrease would be possible to detect by immune-fluorescence - have the authors tried to do in situ and/or Ab staining? Do NotchΔPRE flies show additional phenotypes?

Other comments

- Notch-like phenotypes due to loss/gain of Notch function are very sensitive to growth conditions and temperature. Moreover, these phenotypes are usually variable in penetrance and expressivity/severity. It is surprising that this is not mentioned. Has this been taken in account when analyzing the wing phenotypes, for instance in Figure 5 M-O.

- Quantification of the fluorescence/area might be adequate to quantify GFP expression in the wing discs and, in particular when quantifying the number of cells in the lymph gland – have the authors check apoptosis?

- Shape of the wing disc in the posterior compartment in Figure 4 J-J’ seems not to be impacted- is UBX expressed in the posterior compartment of those discs?

- The manuscript is written in a rather spoken manner sometimes reaching the border of lacking scientific rigorousness. It should be revised. Also, it is expected that at least Figure legends (instead of a Table in Supplementary information) indicate the specific genotypes and experimental (temperature) econditions presented in the Figures.

- Which temperature was used when the ptcGal4, the hhGal4 and the enGal4 were used to modulate stx expression?

- The method to induce FRT recombination is not mentioned: when was the hs-flipase induced? Temperature? When were the imaginal discs dissected and stained?

- The authors use enGal4 to inactivate the expression of Pc (Figure 5J, J') at a quite low 18C temperature (Table S1). What is the Pc expression in those conditions? Did the authors do staining with anti-Pc Ab? The enGal4 line probably drives expression during embryogenesis- why not use other lines such as nubGal4 sdGal4 etc?

- Homozygous NotchΔPRE phenotypes should be shown.

- The authors explain the wing wild type phenotype of NotchΔPRE/+ (Figure 5, panel M, lines 338-353) due to developmental plasticity. What about the other wing phenotypes presented (for instance, Figure 5, panels M-P)? Are they not subjected to the same mechanisms?

- The nota shown in Figure 5 (for instance panel I and Q) seem of different sizes. Is there a size phenotype?
Or is it just simply the magnification (scale bars?).

- Does the GFP expression in panel C (Figure 4) correspond to vgOE-GFP wing discs or to FRT <>FRT-vgOE-GFP (empty construct) wing disc? Also, what is the GFP expression of vgOE-GFP (or the empty construct) in enGAl4; UAS-PcRNAi?

- Figure 4. As mentioned above, panel A should only be presented If discuss the relevance of the comparison to their finding in the Result section.

- Figure 5- Are the lymph glands dissected? Are the lymph glands bigger? Smaller? How is the quantification of the crystal cells done?

- Figure S2- I don't see the need to show panel A. It does not support the findings and, as mentioned by the authors, it has been already published (Du et al 2016).

-Figure S3, Panel A, B and C could perhaps be moved to main text. Also, if the authors would like to show panel E, it should at least be explained. Panel F should be removed.

Reviewer 2

Advance summary and potential significance to field

The discovery of a functional PRE in the Notch gene is novel. Unlike the best characterized Polycomb regulated genes (e.g. HOX genes), which are stably transcriptionally silenced by PRC1 through collaboration with the histone H3K27me3 modification made by PRC2, the PRC1 regulation of Notch does not appear to depend on PRC2/H3K27me3. This suggests that the PRE discovered in this study will be an important tool for understanding the mechanisms underlying the dynamic regulation by PRC1 of Notch and the growing class of PRC1 target genes that acquire little ofr no H3K27me3 do not undergo the stable silencing.

Comments for the author

This work is very suitable for publication in Development as is. It is a very well executed and the results are confirmed by multiple independent experiments and controls. The figure quality is excellent and supplemental figures and tables increase confidence in the conclusions. The Discussion is appropriately critical, balanced and nuanced and considers the behavior of the Notch E5 PRE in the context of the existing PRE literature as well as what is known about the variety of developmental mechanisms, some of which might be expected respond differently to disruption of PRC1 regulation. I highly recommend its publication in Development

Minor points

"bindings" (line 298) should be "binding sites"

"Hart" (lines 369, 449) should be "Harte"

Reviewer 3

Advance summary and potential significance to field

In this study, the authors identified Drosophila Stx as a positive regulator of Notch. They showed that Stx-mediated counteraction of Pc silencing enhances Notch mRNA expression. Furthermore, they found a new cis-element in the Notch locus, which is responsible for downregulation of Notch mRNA expression. Interestingly, the element recruits Pc in the absence of H3K27me3. If properly approved, these findings would provide a basic information for our understanding of the epigenetic regulation in developing tissues.

Comments for the author

Although this study is potentially interesting, further experiments are necessary to validate their claims. Followings are my suggestions to improve the manuscript.

Major concerns:

1. p2, lines 28-30, and p13, lines 253-254
They claimed identification of a new PRE at the Notch locus. However, they demonstrated occupancy of only Pc on the cis-element E5. To establish E5 as a bona fide PRE, they should show occupancies of at least two other PRC1 components such as Ph and Psc by ChIP qPCR.

2. Figure 4, p12, line 244, and p13, line 254
To claim E5 is PcG-dependent or E5 is regulated by PcG, they have to carryout functional tests of the E5 also under Ph-RNAi and Psc-RNAi conditions.

Minor comment:
p2, line 27, p10, lines 194 and 202, p11 line 214, p13, line 262 and so on
They used a term "Notch transcription" for many times in the manuscript. As they analyzed Notch mRNA levels by RT-qPCR and FISH assays, it is not clear whether the increase in Notch mRNA levels is due to transcriptional activation of Notch or stabilization of Notch mRNA. I recommend to use "Notch mRNA expression" in place of "Notch transcription".

First revision

Author response to reviewers' comments

A functional Polycomb response element fine-tunes *Notch* dose in development (MS ID#: DEVELOP/2022/201297)

Point-by-point response to reviewers (Reviewer's comments in black and our response in blue)

We thank all reviewers for the time and effort in evaluating our manuscript. We are very grateful for the highly constructive comments that helped us improve our manuscript during the revision process. Based on reviewers' recommendations, we conducted additional experiments and added new data to the revised manuscript, which are listed in this point-by-point response. For the convenience of the editor and reviewers, these new data are organized into 9 revision figures.

Responses to individual reviewers
Reviewer #1

1) Pc-Dam-ID-seq experiments identify 7 regions (E1-E7) of Pc binding (Figure 4, panel A) that match in some cases (for instance E7) with previously described Pc binding regions (panel A). However, the E5 region (the putative PRE) does not match with any previously described. Unfortunately, this comparison with previous experiments is only briefly discussed (in the Discussion section) but it seems to me not relevant to support their findings. If the authors think that the data from other authors is important to support their findings, the comparison should be further elaborated or otherwise removed from the Results section.

We thank Reviewer #1's comments and discuss accordingly the discrepancies between our Pc-Dam-ID-seq data and previously published data in the revised manuscript (lines 232-239).

2) Little information is provided about the E5 fragment (size, genomic coordinates, overlapping with other Notch regulatory sequences, etc). Perhaps, the results presented in Figure S3, panel A-B-C will be more appropriate here and, also, an improved version of Figure S3 panel D.

We thank Reviewer #1's comments and include detailed information of the E5 fragment in the revised manuscript (lines 261-265 and Figure S3).

3) A more complete characterization of the PRE is required. For example: a) Analysis of the expression of the PRE. Does E5 drive expression of GFP/lacZ in a simple TATA box-GFP/lacZ promoter? If so, where does it do it? Does the expression tell us something about the E5 as a putative PRE? b) Analysis of the E5 sequence. Does it contain transcription factors binding sites? Which ones? Does it contain PHO and GAGA binding sites? What does the sequence tell us about this fragment? c) Definition of a minimal PRE sequence within the E5 fragment, i.e, obtain the minimal fragment required to confer Pc-dependent repression d) site- directed mutagenesis of the binding sites, d) pull-down experiments, etc.

We appreciate the comments from Reviewer #1.
a) The expression of GFP reporters controlled by the *hsp70* basic promoter is too weak to examine the function of putative PREs, especially in cases where epigenetic repression leads to reduced GFP expression. To improve the sensitivity and robustness of GFP reporters, in addition to the *hsp70* basic promoter, we added the vg^{QE} element, which Dr. Jürg Müller's group has demonstrated to increase basal expression levels in GFP reporters (Sengupta et al., 2004, *Developmen*t). Another advantage of adding vg^{QE} to the control system is that it gives a unique expression pattern in the wing imaginal disc, so we can compare the GFP reporter expression manipulated by Stx, Pc or other members of PRC complexes within the same disc using the orthogonal Gal4 system.

b) Following the recommendations of Reviewer #1, we carefully analyzed the E5 sequence and found that it contains 5 putative Pho-binding sites, 13 putative GAGA-binding sites, and 14 putative DSP1-binding sites, the distribution of which is shown in **Revision Figure 1**. We have added this information to the revised manuscript (lines 261-265 and Figure S3).

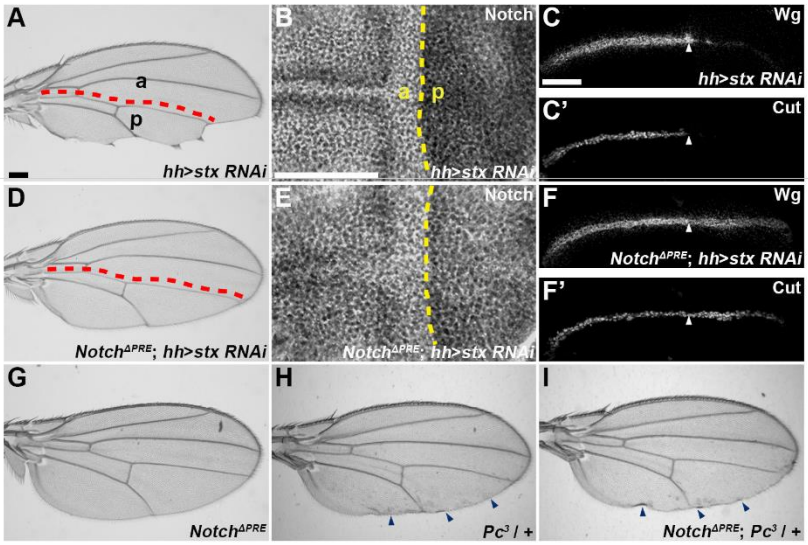
Revision Figure 1. A schematic showing the putative Pho-, GAGA- and DSP1-binding sites identified in the E5 sequence.

c and d) Reviewer #1 recommends further characterization of the E5 fragment using site- directed mutagenesis and pull-down. While these are good experiments for defining a minimal PRE sequence, these experiments cannot be completed within the typical timeframe of the revision cycle, so we believe these experiments are beyond the scope of the current manuscript. However, we will incorporate these experiments into our future studies.

4) If NotchΔPRE would be a deletion in a “functional PRE”, it is expected that the NotchΔPRE phenotypes be modulated by inactivation and overexpression of Polycomb group proteins. This is not shown. I would suggest studying the interactions of NotchΔPRE with Polycomb and other PcG proteins. This can be simply done using Polycomb mutations and Pc duplications or the use of the UAS/Gal4.

Our study shows that *Notch* expression acts downstream of Pc regulation. Therefore, in this case, *Notch* loss of function is epistatic to loss of *Pc* function. Contrary to Reviewer #1’s predictions, we anticipate that *Notch* activity should no longer be modulated by Polycomb group proteins after *in situ* deletion of the PRE from the *Notch* locus. Indeed, our data below support this view, as knocking down *stx*, known to increase Pc levels, did not result in the wing-notching phenotype in the genetic background of $Notch^{\Delta PRE}$ (**Revision Figure 2** D; cf. A), and consistently, there was no significant changes in the expression of Notch (N^{icd}) or in the activation of the target genes Wg and Cut (**Revision Figure 2** E-F’; cf. B-C’). Genetic interactions further support the notion, as the wing morphology of adult $Notch^{\Delta PRE}$ did not change in the background of Pc^{3} heterozygous (**Revision Figure 2** I; cf. G and H). These data have been included in the Figure S5 and described in the revised manuscript (lines 293-298).

To add the evidence that Notch PRE (*E5*) is functional, we tested whether E5-reporter activity was regulated by two other PcG components (Ph or Psc) in the wing disc, in addition to Pc that we showed in our originally submitted manuscript, and concluded that E5 does depend on PcG activity. Please see the response to Reviewer #3 (point 2) for more information.

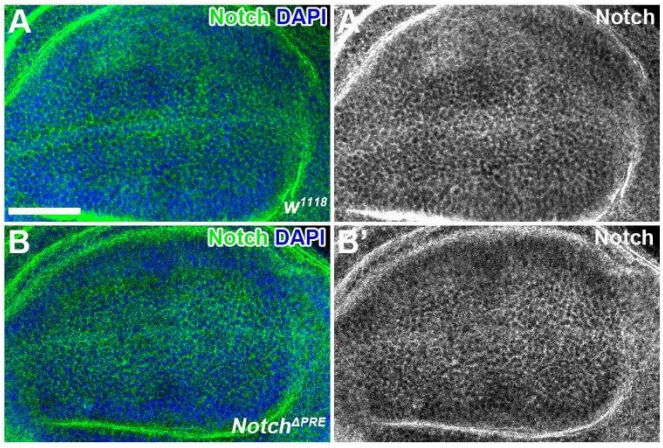


Revision Figure 2. *In situ* deletion of *Notch* PRE removes the PcG-mediated repression on *Notch* transcription. (A-F’) Adult wings and protein production of Notch (N^{icd}), Wg, and Cut in wing discs of the indicated genotypes are shown. RNAi knockdown of *stx* driven by *hh*-Gal4 resulted in a notched adult wing phenotype (A) and a corresponding decrease in the amounts of N^{icd} (B), Wg (C), and Cut (C’) protein in posterior compartment cells. These phenotypes were largely restored in the $Notch^{\Delta PRE}$ mutant (D-F’; cf. A-C’). (G-I) Adult $Notch^{\Delta PRE}$ wing phenotype was unchanged in the Pc^{3} heterozygous background. Arrowheads (C, C’, F and F’) mark the anterior/posterior boundary. The adult wings in all revision figures are shown proximal to the left, with anterior/posterior (a/p) boundaries marked with red dashed lines. The wing discs in all revision figures are shown anterior to the left and ventral at the top, with a/p boundaries marked with yellow dashed lines. Scale bars, A, D and G-I, 100 μm; B-C’ and E-F’, 50 μm.

5) Considering the weak phenotype of NotchΔPRE flies in the notum (lack of few bristles) and the

wing (no phenotype), I find quite surprising that Notch mRNA levels are reduced 30% in either wing discs and/or whole larva (Figure 5, panel A and B). A 30% decrease would be possible to detect by immune-fluorescence - have the authors tried to do in situ and/or Ab staining? Do NotchΔPRE flies show additional phenotypes?

Notch mRNA levels in the wing disc and whole larvae of the *Notch*$^{\Delta PRE}$ mutant increased rather than decreased by about 30%, as shown in Figure 5A and B. However, there was no significant changes in Notch protein levels in the *Notch*$^{\Delta PRE}$ wing discs, (**Revision Figure 3** B'; cf. A'), consistent with the lack of a distinct wing phenotype associated with *Notch*$^{\Delta PRE}$. This inconsistency between *Notch* mRNA and protein expression may reflect the developmental plasticity of the wing, but the underlying mechanism is unclear and may be due to the post- transcriptional buffering of the developing wing, which is worthy of future investigation.



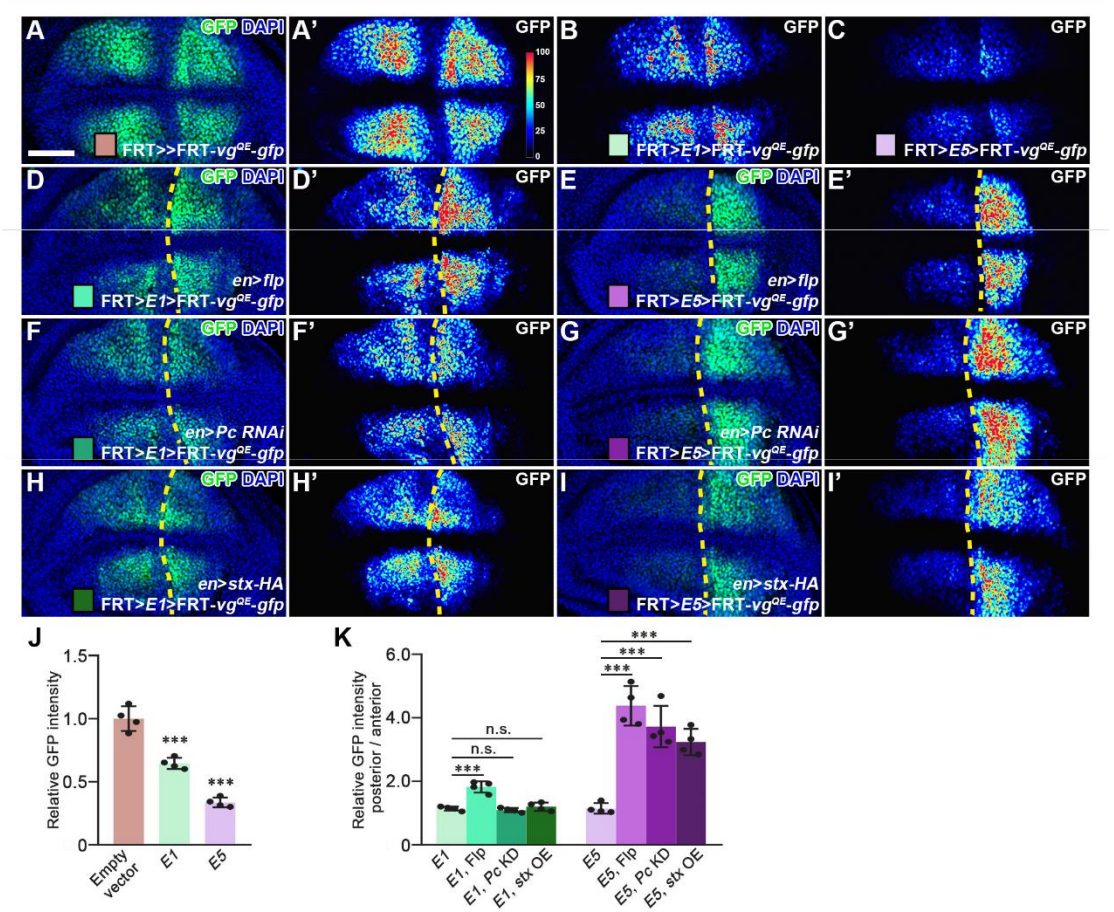
Revision Figure 3. Notch protein expression in wing discs of the indicated genotypes are shown. Notch protein levels did not increase in the *Notch*$^{\Delta PRE}$ wing discs. Scale bars, 50 μm.

6) Notch-like phenotypes due to loss/gain of Notch function are very sensitive to growth conditions and temperature. Moreover, these phenotypes are usually variable in penetrance and expressivity/severity. It is surprising that this is not mentioned. Has this been taken in account when analyzing the wing phenotypes, for instance in Figure 5 M-O.

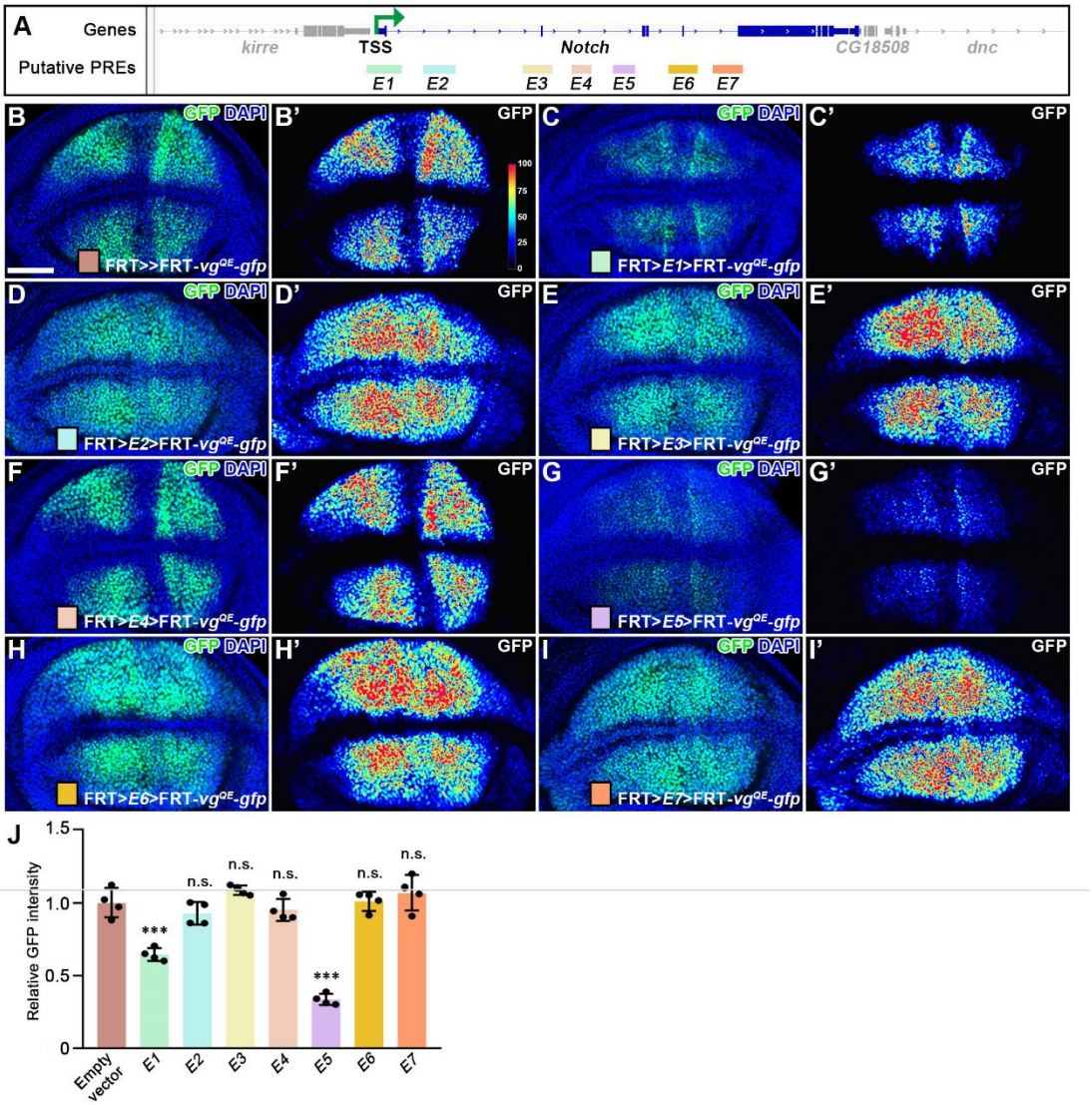
As noted by Reviewer #1, the wing and notum phenotypes exhibited by *Notch*$^{\Delta PRE}$ flies are indeed variable, therefore, we performed statistical analysis that are clearly shown in Figure 5 E and J of the revised manuscript. I would also like to point out that, in addition to the statistics on the adult *Notch*$^{\Delta PRE}$ phenotypes, we also performed statistical analyses of the genetic interactions between *Notch*$^{\Delta PRE}$ and various Notch signaling alleles, which are shown in Figure 5 N, R and V of the revised manuscript.

7) Quantification of the fluorescence/area might be adequate to quantify GFP expression in the wing discs and, in particular when quantifying the number of cells in the lymph gland – have the authors check apoptosis?

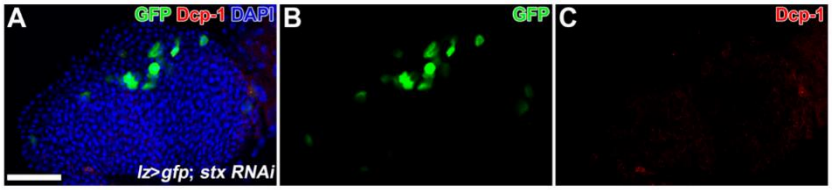
We thank Reviewer #1 for the suggestion. We quantified the the intensity of GFP fluorescence shown in Figure 4 and Figure S4 (**Revision Figure 4** J and K**; Revision Figure 5** J) with NIH ImageJ software. These data have been included in the revised manuscript (Figure 4 and Figure S4). We also examined the protein levels of cleaved Dcp-1 caspase, an indicator of apoptosis, in the lymph glands and found that knocking down *stx* expression did not induce the cleavage of Dcp-1 (**Revision Figure 6**). This point has been included in the revised manuscript (lines 167-171 and Figure S1).



Revision Figure 4. The GFP fluorescence intensity of wing pouch region (A-C), posterior and anterior compartment of the wing pouch (D-I') was measured using NIH ImageJ software. Statistical analyses of relative GFP fluorescence intensity in wing discs of the indicated genotype (*n*=4) are shown (J, K). Data were presented as mean±S.D, *** $p<0.001$, n.s. $p>0.05$ (One-way ANOVA, Dunnett's multiple comparison tests). Scale bars, 50 μm.



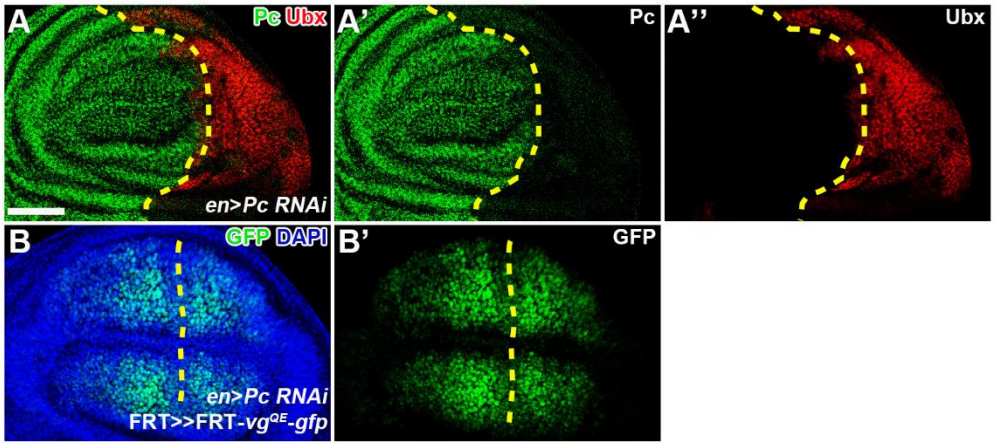
Revision Figure 5. The GFP fluorescence intensity of wing pouch region was measured using NIH ImageJ software. Statistical analyses of relative GFP fluorescence intensity in wing discs of the indicated genotype (*n*=4) are shown (J). Data were presented as mean±S.D, *** p<0.001, n.s. p>0.05 (One-way ANOVA, Dunnett's multiple comparison tests). Scale bars, 50 μm.



Revision Figure 6. The expression of *lz*>GFP and cleaved Dcp-1 in the lymph gland of the indicated genotype is shown. Knocking down *stx* did not induce the cleavage of Dcp-1. Scale bars, 50 μm.

8) Shape of the wing disc in the posterior compartment in Figure 4 J-J' seems not to be impacted- is UBX expressed in the posterior compartment of those discs? The authors use enGal4 to inactivate the expression of Pc (Figure 5J, J') at a quite low 18C temperature (Table S1). What is the Pc expression in those conditions? Did the authors do staining with anti-Pc Ab? The enGal4 line probably drives expression during embryogenesis- why not use other lines such as nubGal4, sdGal4 etc?

The reason we used the *en*-Gal4 driver to knock down the *Pc* expression in the posterior compartment of the wing disc by RNAi at 18 ^{O}C was because knocking down *Pc* at 25 ^{O}C can cause severe wing disc deformation. To demonstrate the effectiveness of *Pc* RNAi, we examined Pc expression in the wing disc by immunostaining when *Pc* RNAi is driven by *en*- Gal4 at 18 ^{O}C, and found that Pc protein levels were effectively reduced in posterior compartment cells (**Revision Figure 7** A') and, as a result, *Ubx* expression was derepressed (**Revision Figure 7** A''). Another reason we used *en*-Gal4 instead of *nub*-Gal4 or *sd*-Gal4 in Figure 4 F-K' of originally submitted manuscript was because *en*-Gal4 is only expressed in the posterior compartment of the wing disc, while the anterior half of the same wing disc can be directly compared as an internal control.



Revision Figure 7. Pc, Ubx and PRE reporter GFP expression in wing discs of the indicated genotypes are shown. When *Pc* was knocked down in posterior compartment cells of the wing disc by *en*-Gal4 at 18 ^{O}C, Pc protein levels were effectively reduced (A'), and Ubx expression was derepressed (A''). However, the FRT>>FRT-vg^{QE}-GFP expression was not affected by *Pc* RNAi in the posterior compartment (B'). Scale bars, 50 μm.

9) The manuscript is written in a rather spoken manner sometimes reaching the border of lacking scientific rigorousness. It should be revised. Also, it is expected that at least Figure legends (instead of a Table in Supplementary information) indicate the specific genotypes and experimental (temperature) conditions presented in the Figures.

In our initial submission, the genotype of all experiments was listed in the lower right corner of each panel in all figures. We have now included additional experimental details in Materials and Methods and all experimental conditions (including temperature) in Table S1.

10) Which temperature was used when the ptcGal4, the hhGal4 and the enGal4 were used to modulate stx expression?

All experimental conditions, including temperatures used for *ptc*-Gal4, *hh*-Gal4 and *en*-Gal4, are now described in Materials and Methods and in Table S1.

11) The method to induce FRT recombination is not mentioned: when was the hs-flipase induced? Temperature? When were the imaginal discs dissected and stained?

The method for inducing FRT recombination and dissection is now described in Table S1 in our originally submitted manuscript.

12) Homozygous NotchΔPRE phenotypes should be shown.

The homozygous $Notch^{\Delta PRE}$ phenotypes were shown in Figure 5 A-H and were described in lines 260-273 of our originally submitted manuscript (lines 300-311 in the revised manuscript).

13) The authors explain the wing wild type phenotype of NotchΔPRE/+ (Figure 5, panel M, lines 338-353) due to developmental plasticity. What about the other wing phenotypes presented (for instance, Figure 5, panels M-P)? Are they not subjected to the same mechanisms?

We believe that developmental plasticity also exists under the conditions of Figure 5 N and O of our originally submitted manuscript. However, given that *Notch*^{55e11} is a null allele (Rulifson and Blair, 1995, *Development*) and that *Notch* mRNA expression increased only slightly in *Notch*$^{\Delta PRE}$ heterozygotes, *Notch* expression in *Notch*^{55e11} / + and *Notch*^{55e11} / *Notch*$^{\Delta PRE}$ flies may be reduced beyond the buffering effect of developmental plasticity, resulting in wing-notching phenotype.

14) The nota shown in Figure 5 (for instance panel I and Q) seem of different sizes. Is there a size phenotype? Or is it just simply the magnification (scale bars?).

We used the same magnification when capturing notal images. After careful comparison of *Notch*$^{\Delta PRE}$ nota and wildtype nota, we conclude that there is no size phenotype in *Notch*$^{\Delta PRE}$ nota. The illusion displayed by panels I and Q may be caused by different focal planes when capturing images.

15) Does the GFP expression in panel C (Figure 4) correspond to vgOE-GFP wing discs or to FRT <>FRT-vgOE-GFP (empty construct) wing disc? Also, what is the GFP expression of vgOE- GFP (or the empty construct) in enGAl4; UAS-PcRNAi?

We thank Reviewer #1 for pointing out our oversight in the genotype description in Figure 4 C of our originally submitted manuscript. The correct genotype should be FRT>>FRT-*vg*^{QE}-GFP and has been corrected in the revised manuscript. The FRT>>FRT-*vg*^{QE}-GFP expression was unchanged in posterior compartment cells of the wing disc after reducing *Pc* levels by *en*-GAl4 (**Revision Figure 7** B').

16) Figure 5- Are the lymph glands dissected? Are the lymph glands bigger? Smaller? How is the quantification of the crystal cells done?

We performed lymph gland immunostaining and quantification of crystal cells following the protocol of Evans et al. (2014, *Methods*), which appears in lines 422-423 of the revised manuscript.

17) Figure S2- I don't see the need to show panel A. It does not support the findings and, as mentioned by the authors, it has been already published (Du et al 2016).

The DamID-seq on the *Hox* gene cluster shown in Figure S2 A (Figure S3 A in the revised manuscript) was used as quality control, indicating that the DamID-seq data in our manuscript are in good quality (lines 228-232). These data are consistent with ChIP-qPCR results shown in our previous published work (Du et al., 2016, *Dev. Cell*). However, DamID- seq was not performed in that paper.

18) Figure S3, Panel A, B and C could perhaps be moved to main text. Also, if the authors would like to show panel E, it should at least be explained. Panel F should be removed.

We thank Reviewer #1 for pointing out our negligence. Figure S3 F shows the Sanger sequencing result of two PCR products in Figure S3 E (Figure S5 B and C in the revised manuscript). Description of these two panels have been added in the revised manuscript (lines 289-290).

Reviewer#2

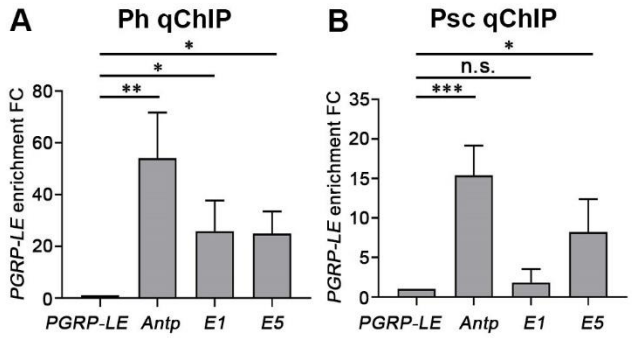
"bindings" (line 298) should be "binding sites", "Hart" (lines 369, 449) should be "Harte".

We apologize for the misspelling of Dr. Peter Harte's last name and have corrected them in the revised manuscript.

Reviewer#3

1) p2, lines 28-30, and p13, lines 253-254: They claimed identification of a new PRE at the Notch locus. However, they demonstrated occupancy of only Pc on the cis-element E5. To establish E5 as a bona fide PRE, they should show occupancies of at least two other PRC1 components such as Ph and Psc by ChIP qPCR.

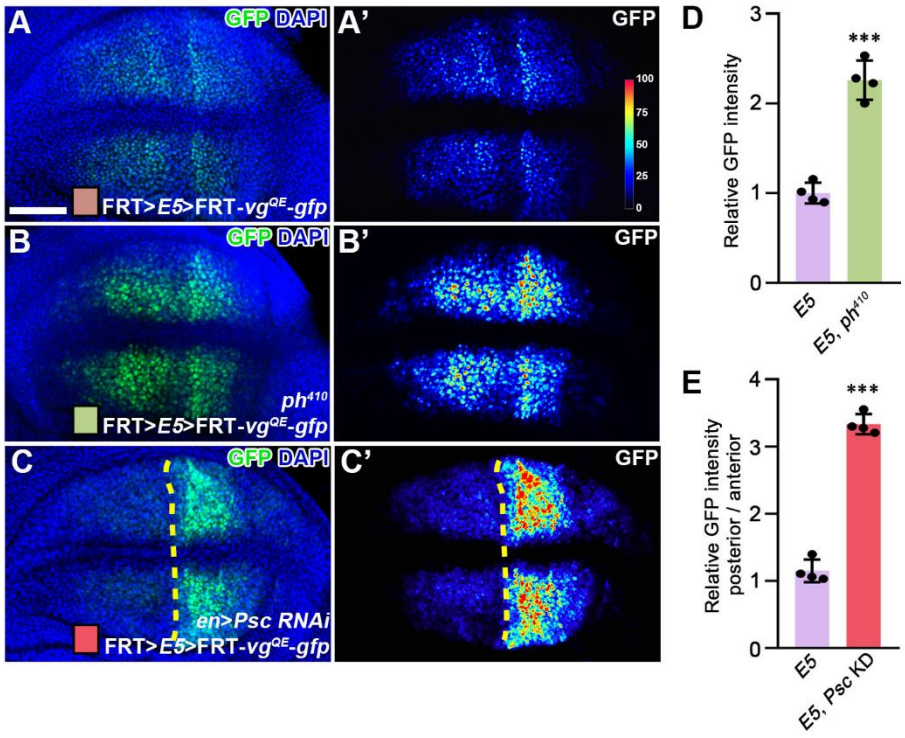
We thank the reviewer for his/her suggestions. We have performed qChIP in S2 cells expressing *ph-p-Flag* or *Psc-Flag* and detected their recruitment to the *E5* fragment (**Revision Figure 8**). These results have been included in Figure 4 and discussed in the revised manuscript (lines 274-276 and Figure 4).



Revision Figure 8. qChIP experiments for Ph and Psc were performed in S2 cells expressing *ph-p-Flag* (A) or *Psc-Flag* (B). *PGRP-LE* was used as a normalized standard. *Antp*, a widely studied PcG target gene, was used as a positive control. Ph was recruited to *E1* and *E5*, while Psc was recruited only to *E5* fragment. Experiments were performed in triplicate and data were presented as mean±S.D, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, n.s. $p>0.05$ (One-way ANOVA, Dunnett's multiple comparison tests).

2) Figure 4, p12, line 244, and p13, line 254: To claim E5 is PcG-dependent or E5 is regulated by PcG, they have to carry out functional tests of the E5 also under Ph-RNAi and Psc-RNAi conditions.

We thank the reviewer for his/her suggestions and performed the recommended experiments. We found that ph^{410} mutation (**Revision Figure 9** B'; cf. A') and *Psc* knockdown (**Revision Figure 9** C') both effectively increased GFP expression levels in the wing disc of FRT>E5>FRT-vg^{QE}-GFP reporter flies. These data are included in Figure S4 and described in the revised manuscript (lines 259-261).



Revision Figure 9. The E5 PRE-reporter activity is regulated by Psc and Ph. GFP expression images (A- C) and heatmap images (A'-C') of relative fluorescence intensities in the wing disc of the indicated genotype are shown. The vertical color bar on the right represents the intensity range. The *ph*^{410} mutation effectively increased the GFP expression in the wing disc (B'; cf. A'), while the *en*-Gal4- driven expression of *Psc* RNAi in the posterior compartment of the wing disc significantly increased GFP expression only in posterior cells (C'). The wing discs are outlined by DAPI stained nuclei (blue). The GFP fluorescence intensity of wing pouch region, posterior or anterior compartment of the wing pouch was measured using NIH ImageJ software. Statistical analyses of relative GFP fluorescence intensity in wing discs of the indicated genotype (*n*=4) are shown (D, E). Data were presented as mean±S.D, *** p<0.001 (One-way ANOVA, Dunnett's multiple comparison tests). Scale bars, 50 μm.

3) p2, line 27, p10, lines 194 and 202, p11 line 214, p13, line 262 and so on: They used a term "Notch transcription" for many times in the manuscript. As they analyzed Notch mRNA levels by RT-qPCR and FISH assays, it is not clear whether the increase in Notch mRNA levels is due to transcriptional activation of Notch or stabilization of Notch mRNA. I recommend to use "Notch mRNA expression" in place of "Notch transcription".

We appreciate the reviewer's suggestion. We have replaced "*Notch* transcription" with "*Notch* mRNA expression" in the revised manuscript.

Second decision letter

MS ID#: DEVELOP/2022/201297

MS TITLE: Stuxnet fine-tunes Notch dose during development with a functional Polycomb response element

AUTHORS: Alan J Zhu, Min Liu, Tao He, Yu Fan, Juan Du, Mengyuan Yi, and Yajuan Li

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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the refereeâ€™s comments, and we will look over this and provide further guidance.

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 finding of a novel PRE in the Notch locus could be relevant and therefore a thorough analysis needed before publishing in Development.

Comments for the author

No comments

Reviewer 3

Advance summary and potential significance to field

In this study, the authors identified Drosophila Stx as a positive regulator of Notch. They showed that Stx-mediated counteraction of Pc silencing enhances Notch mRNA expression. Furthermore, they found a new cis-element in the Notch locus, which is responsible for downregulation of Notch mRNA expression. Interestingly, the element recruits Pc in the absence of H3K27me3. If properly approved, these findings would provide a basic information for our understanding of the epigenetic regulation in developing tissues.

Comments for the author

They revised the manuscript according to reviewersʼ comments but there still remains a concern in the scientific context of the manuscript regarding my comment 1). They analyzed occupancies of Ph and Psc on E5 using cultured S2 cells (revised Figure 4Q and 4R). However, it is difficult to imagine that the chromatin states in S2 cells have something to do with the observed PcG-dependent phenotypes in the wing. I would like tosee the chromatin states in the wing disc. ChIP qPCR should be carried out using nuclear extracts from wing discs as they did to detect the Pc occupancy on E5 (revised Figure 4P). ChIP-grade antibodies against Ph, Psc or dRing will be available from other PcG researchers.

Second revision

Author response to reviewers' comments

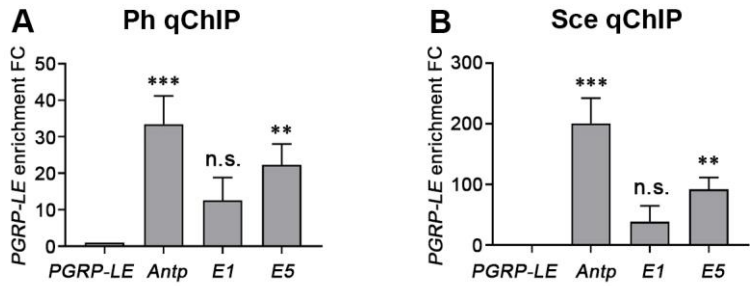
Stuxnet fine-tunes *Notch* dose during development with a functional Polycomb response element (MS ID#: DEVELOP/2022/201297)

Response to Reviewer #3 (Reviewer's comments in black and our response in blue)

Reviewer #3

They revised the manuscript according to reviewers' comments but there still remains a concern in the scientific context of the manuscript regarding my comment 1). They analyzed occupancies of Ph and Psc on E5 using cultured S2 cells (revised Figure 4Q and 4R). However, it is difficult to imagine that the chromatin states in S2 cells have something to do with the observed PcG-dependent phenotypes in the wing. I would like to see the chromatin states in the wing disc. ChIP qPCR should be carried out using nuclear extracts from wing discs as they did to detect the Pc occupancy on E5 (revised Figure 4P). ChIP-grade antibodies against Ph, Psc or dRing will be available from other PcG researchers.

We appreciate the reviewer's comments on the use of wing disc extracts instead of S2 cells in qChIP experiments, as well as the use of ChIP-grade antibodies against the PRC1 components, including Ph, Psc, or Sce (dRing). The Ph, Psc and Sce antibodies in our hands did not work in ChIP experiments. However, due to customs issues, we were unable to obtain ChIP-grade antibodies from overseas researchers. To circumvent this problem, we used transgenic flies expressing *ph-GFP* (Ficz et al., 2005) and *Sce-Flag* (Langlais et al., 2012) to detect the recruitment of Ph and Sce to the *E5* fragment in the wing disc, respectively (**Revision Figure 1**); approximately 900 pairs of wing discs dissected from third instar larvae with corresponding genotypes were used for each qChIP. These results have been incorporated into Figure 4 and discussed in the revised manuscript (lines 273-275 and Figure 4Q and R). Since we do not have transgenic flies expressing tagged *Psc*, we did not perform ChIP experiments on Psc. Nevertheless, the ChIP results for Ph and Sce shown in this revision, as well as the Pc results we originally submitted (Figure 4P), suggest that PRC1 is able to bind to *E5 in vivo* and that this binding is much stronger than *E1*.



Revision Figure 1. qChIP experiments for Ph and Psc were performed in wing discs expressing *ph-GFP* (A) or *Sce-Flag* (B) under *nub*-Gal4 control. *PGRP-LE* was used as a negative control for normalization. *Antp*, a widely studied PcG target gene, was used as a positive control. Ph and Sce were significantly recruited to *E5*. Experiments were performed in triplicate and data were presented as mean±S.D, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, n.s. $p>0.05$ (One-way ANOVA, Dunnett's multiple comparison tests).

Third decision letter

MS ID#: DEVELOP/2022/201297

MS TITLE: Stuxnet fine-tunes Notch dose during development with a functional Polycomb response element

AUTHORS: Alan J Zhu, Min Liu, Tao He, Yu Fan, Juan Du, Mengyuan Yi, and Yajuan Li
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 3

Advance summary and potential significance to field

Same as the previous one.

Comments for the author

The authors carried out additional experiments and satisfactorily revised the manuscript. I appreciate their efforts.