EMBO reports

SWI/SNF chromatin remodeling controls Notch-responsive enhancer accessibility

Zoe Pillidge and Sarah J Bray

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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 28 September 2018

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. I am sorry for the slight delay in getting back to you. As the referee comments are not in agreement, I was waiting for referee cross-comments. All reports are pasted below.

As you will see, while the referees acknowledge that the findings are potentially interesting, referees 2 and 3 raise concerns about the overall conceptual novelty/general significance and the strength of the data. Importantly, it seems to be unclear whether the BRM complex generally affects chromatin, or whether it has a specific effect on the Notch signaling pathway. Based on these comments it is clear that we cannot consider the publication of the manuscript at this stage. However, I would like to give you the opportunity to respond to and to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board.

Should you decide to embark on such a revision, 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 major revision only and acceptance or rejection of the manuscript will 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. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. 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

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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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:

Pillidge and Bray examine the role of chromatin remodeling in the transcriptional output of Notch signaling. Previous research from the Bray lab and others established that enhancer accessibility is a central level of regulation in Notch-driven transcription. Here, they set out to analyze the role of nucleosome dynamics and the responsible ATP-dependent remodeler(s) during transcriptional activation by Notch. First, in vivo in salivary gland nuclei, they show that Notch signaling controls nucleosome turnover at the E(spl)-C enhancers, and promotes histone H3.3 incorporation. Next, they examined the effects of depletion of a wide variety of remodelers, and established that the BRM remodeling complex is required for Notch-induced accessibility (using in vivo imaging and ATAC-qPCR). Using Kc167 cells, they found that the BRM complex is crucial for the chromatin/transcriptional response to Notch signaling. Finally, using CATCH-IT, it is shown that (primarily) H3.3 turnover is increased by Notch signaling in a BRM-dependent manner.

This manuscript is of great interest because it firmly established the crucial function of SWI/SNF-driven nucleosome turnover in enhancer response to Notch signaling. Thus, linking developmental signaling to chromatin, enhancer function and transcriptional output. This work is of general importance for our

understanding of enhancer function. Moreover, it utilizes an impressive array of independent techniques to buttress its conclusions. I found this an outstanding, experimentally excellent and compelling manuscript that will be of great interest to the readership of EMBO Reports. I have only a few minor suggestion for the author's consideration.

Comments

1) In the CATCH-IT assays, I wonder what the BRM dependency for H3.3. turnover is under Notch-ON versus OFF conditions.
2) What is behind the difference in scale between Fig.4A and B?
3) The paper is written very succinctly, requiring a back-and-forth between main text, legends and methods. The readability would improve if more experimental details (e.g. times of induction etc.) were provided in the main text.
4) Likewise, what is the estimated turnover of histones at different genomic locations? E.g. enhancer induced/uninduced vs gene body vs unrelated region.

Referee #2:

Pillidge and Bray investigated the role of the BRM chromatin remodeling complex in the chromatin accessibility of E(spl) C enhancers that are bound by Su(H) and responsive to Notch activation. First, the authors used live imaging of Drosophila larval salivary gland nuclei and found that the levels of histone H3.3, but not H3, are increased upon constitutively active Notch activation. The authors then performed imaging of Su(H)-GFP in the presence of RNAi constructs in order to determine whether known remodelers and chaperones were required for Su(H) binding. They identified several components of the BRM SWI/SNF remodeling complex, i.e. Moira, Snr1 and Brm, to be important for locating Su(H) to E(spl)-C. Furthermore, they used ATAC to measure changes in chromatin accessibility and found that chromatin accessibility of the locus increases upon Notch activation. Expression of a dominant-negative Brm (BrmK804R) led to decreased chromatin accessibility independent of Notch activity (albeit with a more distinct effect upon Notch activation at the known enhancer regions mß and m3), confirming that the ATPase activity of Brm is important to prime the locus for activity as well as for increased accessibility upon Notch activation. The authors then turned to Kc cells and confirmed that knockdown of the components Snr1 and Brm leads to reduced recruitment of Su(H) at both enhancer regions (independent of the transcriptional activity), and that expression of dominant-negative Brm leads to diminished target gene expression upon Notch activation. Then, using CATCH-IT and ChIP of tagged histones H3 and H3.3, they showed that nucleosome turnover increases at enhancer regions upon Notch activation and decreases upon depletion of Brm, confirming a critical role of the BRM complex in nucleosome turnover.

Overall, this is an easy to follow manuscript and the performed experiments and shown results are logical and support the conclusions drawn by the authors. Unfortunately, the results are only novel for the system the authors studied and lack a general significance. The involvement of chromatin remodeling in enhancer activation is an important topic that is surprisingly poorly understood, especially given that the increased chromatin accessibility and nucleosome depletion at active enhancers was already discovered in the early 1980ies by Harold Weintraub, Carl Wu and Pierre Chambon. Subsequent biochemical studies have revealed a wealth of details on chromatin remodeling complexes and their activities in vitro, including the SWI/SNF complex in yeast and the BAF complex in mammals. More recently, genomics assays have allowed high-resolution information on chromatin accessibility and nucleosome occupancy at enhancers, and yeast studies have even provided nucleosome mappings at individual loci (Boeger lab). Yet, how exactly chromatin remodelers are recruited to active enhancers, what exactly happens to enhancer nucleosomes and how this leads to activation is still not clear.

For these reasons, I was hoping that this manuscript would have at least one small piece to contribute to this puzzle, but the scope, resolution and mechanistic insights of the manuscript are very limited in comparison to other studies. Essentially, the results are what one might expect based on general knowledge of enhancers and previously known genetic interactions between the Notch pathway and the BRM complex in Drosophila. They do not shed light on where the specificity between the Notch pathway and the BRM complex comes from - if there is any (see more below). Therefore, I struggle to see a clear novel finding here.

Specific suggestions for improvement:

It would be nice to obtain further insights into the mechanistic relationship between between Su(H) and the BRM complex. It seems that high binding of Su(H) binding in the presence of Notch activation requires the BRM complex, but how does the BRM complex get there? The fact that Su(H) knockdown increases the chromatin accessibility argues against Su(H) being the one that recruits the BRM complex. But then where does the implied specificity between the Notch signaling pathway and BRM complex come from? If it is a Notch signaling component upstream of Su(H), how can it be specific for Notch enhancers? Or are we simply seeing general principles of strong enhancer activation?

The introduction and discussion lack a more general outlook on enhancer activation and even within the field of the Drosophila Notch pathway, the references are incomplete. For example, several previous publications investigated and explored the link between the BRM SWI/SNF remodeling complex and Notch signaling, e.g.
Das et al., "SWI/SNF Chromatin Remodeling ATPase Brm Regulates the Differentiation of Early Retinal Stem Cells/Progenitors by Influencing Brn3b Expression and Notch Signaling", J Biol Chem (2007) or Xie et al., "The SWI/SNF Complex Protein Snr1 Is a Tumor Suppressor in Drosophila Imaginal Tissues", Cancer Res (2017).

Some technical details are either missing or not well described:
• Figure 2A: quantitative validation of RNAi knock-down efficiencies are missing
• Figure EV2: validation of Su(H) RNAi knock-down efficiency is missing
• Methods section on the live imaging in Figure 1 lacks details: it is not clear how measured values were processed and normalized between different fly lines expressing either H3-GFP, H3.3-GFP or H3.3core-GFP
• It is not mentioned how CATCH-IT differentiates between newly synthesized histones and other newly synthesized proteins. How do we know that nucleosome turnover is measured and not for instance newly synthesized Su(H) protein locating to the locus of interest? Since the authors also performed ChIP on histones, this is not a concern, but it would nevertheless be nice to comment on the possible limits of the CATCH-IT data.

Referee #3:

Pillidge and Bray present an interesting story examining recruitment of the Su(H) following N activation, and potential involvement of brm remodellers for Su(H) binding. However a number of additional controls and experiments would need to be performed to justify some of the conclusions made by the authors.

Specific points to address:

1) How many glands are analysed for Fig 1? The authors state that quantifications are Mean+/-SEM; n >= 5. This is single nuclei from five glands or a single gland with 5 nuclei analysed. Also the authors state that " with H3.3core-GFP we saw the same pattern as with H3.3-GFP". This does not seem to be the case where H3.3 overlaps ParB-mCherry but H3.3core-GFP flanks ParB-mCherry. Looking at the glands, my sense is that the time of E(spl) induction are not well-controlled and the H3.3core-GFP image is from much later after induction than H3.3core-GFP.

2) Could we get do a time-course of recruitment or get better control of the timings and number of glands used to assess inter-animal variability.

3) Brm knock-down blocks Su(H)-GFP incorporation following N activation. It would be nice to have image showing chromosome organisation (DAPI or H3-GFP) alongside the Su(H)-GFP image as a control to show that the Brm knock-down or inhibition does not have large-scale general effects on chromatin organisation. I guess the question is whether you can exclude the possibility that Brm knock-down has dramatic effects on chromosome morphology that would disrupt binding of transcription factors generally or disrupt chromosome morphology to impact imaging.

4)Likewise for ATAC as in point 2 above. Brm knockdown blocks chromatin accessibility at Su(H) sites. But is this a general or a specific effect. Are there regions like hsp promoters (hsp26 or hsp70) that can be used for control.

5) For CATCH-IT would like to understand how the turnover-values are calculated and what the treatment in 4B is? Is this Notch-on or Notch-off. I am comparing turnover-values in lacZ RNAi in 4B with values for WT in Notch-on or Notch-off and there is a 5-6 difference in value. What is the source of this variation. Surely they should be identical?

6) is it possible to do a time-course of recruitment of Su(H) and H3.3 following N activation. Not sure the availability of Fluor-tagged Su(H) and H3.3 constructs but would help to resolve the inter-relationship of H3.3 and Su(H) recruitment. My suspicion is that most of the H3.3 incorporation seen in polytenes is on the gene bodies during transcription.

7) Leading on from 5, a key conclusion the authors draw from the data is that Brm is mediating histone turnover. I am not sure that we can conclude that Brm is directly responsible for the histone turnover. Certainly Brm knock-down decreases turnover but it also decreases binding of TFs and activation of enhancers and mRNA transcription. Data presented do not allow one to exclude a pathway in which Brm facilities Su(H) binding which in turn recruits co-activators to drive enhancer activation and that this and associated transcription drives turnover. Possible option would be to treat salivary glands as in Fig 1A but co-treat with amanitin or DRB.

1st Revision - authors' response 23 January 2019

EMBOR-2018-46944V1 Response to Reviewers.
(comments from reviewers in grey, answers in black)

Referee #1:

Pillidge and Bray examine the role of chromatin remodeling in the transcriptional output of Notch signaling. Previous research from the Bray lab and others established that enhancer accessibility is a central level of regulation in Notch-driven transcription. Here, they set out to analyze the role of nucleosome dynamics and the responsible ATP-dependent remodeler(s) during transcriptional activation by Notch. First, in vivo in salivary gland nuclei, they show that Notch signaling controls nucleosome turnover at the E(spl)-C enhancers, and promotes histone H3.3 incorporation. Next, they examined the effects of depletion of a wide variety of remodelers, and established that the BRM remodeling complex is required for Notch-induced accessibility (using in vivo imaging and ATAC-qPCR). Using Kc167 cells, they found that the BRM complex is crucial for the chromatin/transcriptional response to Notch signaling. Finally, using CATCH-IT, it is shown that (primarily) H3.3 turnover is increased by Notch signaling in a BRM-dependent manner.

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We thank the referee for their comments and are delighted that they appreciated the importance of our study.

Comments
1) In the CATCH-IT assays, I wonder what the BRM dependency for H3.3. turnover is under Notch-ON versus OFF conditions.
We thank the referee for this suggestion. In light of our data showing that the BRM complex is crucial for various aspects of the Notch response in both salivary glands and Kc167 cells (chromatin accessibility, Su(H) recruitment and target gene expression), we anticipated that the Notch-responsive nucleosome turnover observed with CATCH-IT would also be dependent on the BRM complex. We have now tested this in a new set of CATCH-IT experiments where both an RNAi treatment and Notch activation were performed together. These new data (**new Figure 5C**) demonstrate that there is a drastic reduction in nucleosome turnover when Brm is depleted under Notch-ON conditions, confirming Brm is required for the large increase in turnover at Notch

responsive enhancers.

2) What is behind the difference in scale between Fig.4A and B?
We note that a similar comment was made by referee #3, and we thank both referees for bringing this to our attention. Although the relative amounts of DNA corresponding to different regions remained consistent between experiments, there were differences in the absolute levels because of variability in the efficiency of DNA recovery following micrococcal nuclease digestion and biotin-streptavidin purification. To circumvent this we have identified a region (Sec15) to use as an internal control to normalize for this variability; this region was found to have low to moderate accessibility in the previous genome-wide CATCH-IT experiments from the Henikoff lab. This normalization aids comparisons between the different experiments: values in **new Figure 5** are now given as a fold enrichment of turnover relative to the Sec15 control region (as explained in the legend and Methods section). We are grateful to the referees for prompting us to make this change.

Furthermore, we have now performed additional CATCH-IT experiments, to address the first point of referee #1, which demonstrate the reproducibility of our results. In these new experiments, Notch activation and *brm* RNAi (Notch-OFF) had the same effects (comparing **new Figure 5C with Figure 5A and B**). These results emphasize that the effects of *brm* RNAi are greater at Notchresponsive regions than control regions, strengthening our argument that the BRM complex has a particularly important effect at these regions.

3) The paper is written very succinctly, requiring a back-and-forth between main text, legends and methods. The readability would improve if more experimental details (e.g. times of induction etc.) were provided in the main text.
We thank the referee for suggesting this and have made several additions to the text to improve the readability. For example, we have including times of induction, highlighted the use of the GAL4/UAS system to activate Notch signaling in the salivary gland and added to our explanation of how Notch was activated for the CATCH-IT experiments.

4) Likewise, what is the estimated turnover of histones at different genomic locations? E.g. enhancer induced/uninduced vs gene body vs unrelated region.
We have now commented explicitly on the fold enrichment of nucleosome turnover and fold change with Notch at the enhancer regions in the text of the results section. In addition, we hope this information is more easily observed from the graphs in **new Figure 5**, with the revised approach for presenting the CATCH-IT data.

Referee #2:

Pillidge and Bray investigated the role of the BRM chromatin remodeling complex in the chromatin accessibility of E(spl) C enhancers that are bound by Su(H) and responsive to Notch activation. First, the authors used live imaging of Drosophila larval salivary gland nuclei and found that the levels of histone H3.3, but not H3, are increased upon constitutively active Notch activation. The authors then performed imaging of Su(H)-GFP in the presence of RNAi constructs in order to determine whether known remodelers and chaperones were required for Su(H) binding. They identified several components of the BRM SWI/SNF remodeling complex, i.e. Moira, Snr1 and Brm, to be important for locating Su(H) to E(spl)-C. Furthermore, they used ATAC to measure changes in chromatin accessibility and found that chromatin accessibility of the locus increases upon Notch activation. Expression of a dominant-negative Brm (BrmK804R) led to decreased chromatin accessibility independent of Notch activity (albeit with a more distinct effect upon Notch activation at the known enhancer regions mß and m3), confirming that the ATPase activity of Brm is important to prime the locus for activity as well as for increased accessibility upon Notch activation. The authors then turned to Kc cells and confirmed that knockdown of the components Snr1 and Brm leads to reduced recruitment of Su(H) at both enhancer regions (independent of the transcriptional activity), and that expression of dominant-negative Brm leads to diminished target gene expression upon Notch activation. Then, using CATCH-IT and ChIP of tagged histones H3 and H3.3, they showed that nucleosome turnover increases at enhancer regions upon Notch activation and decreases upon depletion of Brm, confirming a critical role of the BRM complex in nucleosome turnover.

Overall, this is an easy to follow manuscript and the performed experiments and shown results are logical and support the conclusions drawn by the authors. Unfortunately, the results are only novel for the system the authors studied and lack a general significance. The involvement of chromatin remodeling in enhancer activation is an important topic that is surprisingly poorly understood, especially given that the increased chromatin accessibility and nucleosome depletion at active enhancers was already discovered in the early 1980ies by Harold Weintraub, Carl Wu and Pierre Chambon. Subsequent biochemical studies have revealed a wealth of details on chromatin remodeling complexes and their activities in vitro, including the SWI/SNF complex in yeast and the BAF complex in mammals. More recently, genomics assays have allowed high-resolution information on chromatin accessibility and nucleosome occupancy at enhancers, and yeast studies have even provided nucleosome mappings at individual loci (Boeger lab). Yet, how exactly chromatin remodelers are recruited to active enhancers, what exactly happens to enhancer nucleosomes and how this leads to activation is still not clear.

For these reasons, I was hoping that this manuscript would have at least one small piece to contribute to this puzzle, but the scope, resolution and mechanistic insights of the manuscript are very limited in comparison to other studies. Essentially, the results are what one might expect based on general knowledge of enhancers and previously known genetic interactions between the Notch pathway and the BRM complex in Drosophila. They do not shed light on where the specificity between the Notch pathway and the BRM complex comes from - if there is any (see more below). Therefore, I struggle to see a clear novel finding here.

We are glad that the referee found the results logical and easy to follow and appreciated that this is an important topic. We also agree that the involvement of chromatin remodeling in enhancer activation is surprisingly poorly understood. As the reviewer highlights, there are extensive *in vitro* data showing how chromatin remodelers move and evict nucleosomes. However, there are remarkably few examples where their specific contribution to gene activation in multicellular organisms has been explored. We thus disagree that our results lack general significance. Notably, our discovery that signaling increases nucleosome turnover in a BRM complex-dependent way is important, firstly because it highlights that nucleosome dynamics contribute to the enhancer activation – something that is greatly overlooked in most ChIP studies for example – and secondly because it demonstrates a clear function for the BRM complex in this process. We therefore consider that our work makes an important contribution to the field, both in respect to Notch signaling regulation and in the field of enhancer activation more generally.

The referee is correct that some previous studies have made links between Brm and Notch signaling, both in flies and mammals. We consider that those data provide a valuable back-drop for our work, but many rely on genetic interactions and the interpretations are often diametrically opposite (for example, Brm causing inhibition of Notch signaling in retinal progenitors but promoting Notch signaling in sensory organs of Drosophila). We have now, as suggested by the referee, summarized some of these conflicting observations in the introduction, and we believe that the contrary nature of these results make our own study even more compelling. Our data provide strong and novel evidence for the mechanistic involvement of nucleosome turnover and Brm-mediated chromatin remodeling in the Notch response.

We have also added significant new data to strengthen the evidence that Brm regulates nucleosome turnover in a specific manner. Firstly we were inspired by recent studies from yeast, which showed that the actin related protein (ARP) components of the SWI/SNF complex were essential for efficient histone eviction (Clapier *et al.* 2016, Mol Cell). We therefore tested the role of BAP55, the Drosophila homolog of the yeast ARP, in our system. The results of these experiments, **new Figure 4F and G**, recapitulate the data with *brm* RNAi and demonstrate that BAP55 is an essential subunit for the Notch response. These data support the argument that a critical function of the BRM complex is to promote nucleosome turnover at Notch regulated enhancers. Furthermore, no previous molecular studies have been performed on BAP55 in *Drosophila*, adding further novelty to these results. Secondly, we have also performed additional experiments to test effects of Brm inhibition on the accessibility and expression of a range of different genes. These new data, incorporated in **new Figure EV3A-C**, show that the other, non-Notch-responsive, enhancers and genes are not similarly affected by the inhibition of Brm. Thus our work also reveals that the role of the BRM complex is not a generic one, but rather is deployed at a certain class of regulatory enhancers, epitomized by Notch targets. All of the new data

consolidate our model and we believe that they add significant and substantial new insights as highlighted by referee #1.

Specific suggestions for improvement:
It would be nice to obtain further insights into the mechanistic relationship between between Su(H) and the BRM complex. It seems that high binding of Su(H) binding in the presence of Notch activation requires the BRM complex, but how does the BRM complex get there? The fact that Su(H) knockdown increases the chromatin accessibility argues against Su(H) being the one that recruits the BRM complex. But then where does the implied specificity between the Notch signaling pathway and BRM complex come from? If it is a Notch signaling component upstream of Su(H), how can it be specific for Notch enhancers? Or are we simply seeing general principles of strong enhancer activation?
We agree with the referee that these are important questions. As they correctly note, our data argue against Su(H) being responsible for the initial recruitment of Brm in Notch-OFF conditions. We have now discussed this point explicitly in the discussion (**page 10**). As has been suggested for the Glucocorticoid receptor, we propose that tissue-specific factors are responsible for recruiting the BRM complex at Notch-responsive enhancers to permit Su(H) binding.

We have also considered the question of specificity, by expanding our analysis to several other classes of enhancers. These data highlight that Brm is not essential for basal accessibility of all enhancers as none of the other regions tested showed a similar decrease in accessibility when Brm was perturbed (**new Figure EV3A and B**). Nevertheless, we believe that Brm will be deployed in other contexts besides Notch, as suggested by work with Glucocorticoid receptor in mammalian cells. We have now added a separate Discussion to the manuscript, which has given us scope to discuss these issues in the context of the literature (**page 10/11**).

The introduction and discussion lack a more general outlook on enhancer activation and even within the field of the Drosophila Notch pathway, the references are incomplete. For example, several previous publications investigated and explored the link between the BRM SWI/SNF remodeling complex and Notch signaling, e.g.
Das et al., "SWI/SNF Chromatin Remodeling ATPase Brm Regulates the Differentiation of Early Retinal Stem Cells/Progenitors by Influencing Brn3b Expression and Notch Signaling", J Biol Chem (2007) or Xie et al., "The SWI/SNF Complex Protein Snr1 Is a Tumor Suppressor in Drosophila Imaginal Tissues", Cancer Res (2017).
As indicated above, we have now expanded the Introduction and added a separate Discussion so that we can incorporate these references as suggested by the referee. We note that many of the previous studies reach contradictory conclusions and indeed the Xie *et al.* paper proposes a nonnuclear function for Snr1 in vesicle trafficking. We hope however that the revised version has achieved a more general outlook.

Some technical details are either missing or not well described:
• Figure 2A: quantitative validation of RNAi knock-down efficiencies are missing
We have now performed reverse transcription-qPCR experiments on all genotypes used in Figure 2A, with the results shown in **new Figure EV2A**. We have also included details in **Table 1** of other papers where RNAi lines have been used and validated. As we were unable to show a clear knockdown of Brwd3 or His3.3B, those factors have not been included in the analysis.

• Figure EV2: validation of Su(H) RNAi knock-down efficiency is missing
We appreciate this comment and have added these data in **new Figure EV3D**. The approach we have taken, due to the low levels making it hard to accurately assess Su(H) RNA knockdown by RTqPCR, is to analyze the knockdown of Su(H)-GFP. The ATAC experiment was performed in a genotype containing a genomic Su(H)-GFP rescue construct so that we could analyze the knockdown by Su(H) RNAi under identical conditions. This also has the advantage that un-targeted tissues (e.g. fat body) serve as an internal control, showing that there is efficient knockdown specifically in the salivary glands where the RNAi is produced.

• Methods section on the live imaging in Figure 1 lacks details: it is not clear how measured values were processed and normalized between different fly lines expressing either H3-GFP, H3.3-GFP or H3.3core-GFP

We apologize for this omission and have now included details of how these quantifications were performed in the methods section.

• It is not mentioned how CATCH-IT differentiates between newly synthesized histones and other newly synthesized proteins. How do we know that nucleosome turnover is measured and not for instance newly synthesized Su(H) protein locating to the locus of interest? Since the authors also performed ChIP on histones, this is not a concern, but it would nevertheless be nice to comment on the possible limits of the CATCH-IT data.
We thank the referee for this suggestion and have added an explanation to the text on **page 8** explaining the steps that enrich specifically for histones.

Referee #3:

Pillidge and Bray present an interesting story examining recruitment of the Su(H) following N activation, and potential involvement of brm remodellers for Su(H) binding. However a number of additional controls and experiments would need to be performed to justify some of the conclusions made by the authors.
We thank referee #3 for taking an interest in our study.

Specific points to address:
1) How many glands are analysed for Fig 1? The authors state that quantifications are Mean+/-SEM; n >= 5. This is single nuclei from five glands or a single gland with 5 nuclei analysed. Also the authors state that " with H3.3core-GFP we saw the same pattern as with H3.3-GFP". This does not seem to be the case where H3.3 overlaps ParB-mCherry but H3.3core-GFP flanks ParB-mCherry. Looking at the glands, my sense is that the time of E(spl) induction are not well-controlled and the H3.3core-GFP image is from much later after induction than H3.3core-GFP.
We appreciate the referee's concerns here. We have now made clear both the number of glands and number of nuclei that were analyzed for each experiment. We would also like to reassure the referee that the timing of salivary gland imaging was carefully controlled, by taking timed collections and then selecting larvae at late wandering stage based on their morphology and behavior. It is possible that the plane of the section in the original figure led to the impression that the nuclei were of different sizes. To avoid this confusion, we have replaced the image shown in **Figure 1E** with another nucleus where the position of the imaging plane is similar. We also thank the reviewer for noticing that there was a slight difference in the graphs as originally in **Figure 1B and F**. This occurred due to a mis-alignment in the processing of 2 nuclei, which has now been corrected. We hope that it is now apparent in **Figure 1B and F** that the data are very comparable between H3.3-GFP and H3.3core-GFP.

2) Could we get do a time-course of recruitment or get better control of the timings and number of glands used to assess inter-animal variability.
We hope the response above addresses the concerns of the reviewer about inter-animal variability. Indeed in all cases the errors on each of the data points are remarkably small highlighting how reproducible the results are. While a more fine-scaled time course would be useful, as we rely on the GAL4/UAS system to control both ectopic Notch and histone-GFP expression, unfortunately the experiment would be confounded by effects on histone levels. However, we have taken an alternate strategy that allows us to determine whether newly synthesized histones are incorporated at the *E(spl)-C.* To achieve this we used heat shock-inducible FRT recombination to switch between histone-GFP and histone-mKO (an orange fluorophore) expression. While the experiment was technically challenging due to the use of multiple fluorophores, we were able to observe that newly-expressed H3.3-mKO was incorporated in a similar pattern to the prolonged expression of H3.3-GFP. These data are presented in **new Figure EV1**. We hope that they demonstrate the dynamic nature of histone H3.3 incorporation in the salivary gland.

3) Brm knock-down blocks Su(H)-GFP incorporation following N activation. It would be nice to have image showing chromosome organisation (DAPI or H3-GFP) alongside the Su(H)-GFP image as a control to show that the Brm knock-down or inhibition does not have large-scale general effects on chromatin organisation. I guess the question is whether you can exclude the possibility that Brm knock-down has dramatic effects on chromosome morphology that would disrupt binding of

transcription factors generally or disrupt chromosome morphology to impact imaging.
We thank the referee for this suggestion and have added a live Hoechst staining of nuclei in the presence of BrmK804R (**new Figure EV2C**) showing that the chromosomes retain a clear banding pattern. This illustrates that there is no overt disruption to the chromosome morphology that could have caused absence of Su(H) recruitment. We would also like to draw attention to the results with ATAC, which argue that there is not a very widespread effect of Brm perturbations on chromatin accessibility that would disrupt all transcription factor binding, but rather localized effects at specific enhancers.

4)Likewise for ATAC as in point 2 above. Brm knockdown blocks chromatin accessibility at Su(H) sites. But is this a general or a specific effect. Are there regions like hsp promoters (hsp26 or hsp70) that can be used for control.
We agree that this is important point and would like to highlight that we had already included several control regions in the ATAC analysis, namely a region within the intron of the Rab11 gene, which is highly accessible, and the ecdysone-binding enhancer of Eip78C, an active gene in the salivary gland at this stage of development. However, following the suggestions of the referee we have now added further control regions in **new Figure EV3A and B.** None of these regions showed a reduced accessibility with BrmK804R expression. Most show little change and, to our surprise, BrmK804R expression also appeared to increase the accessibility of some regions, notably the heat shock-responsive promoters. It is therefore possible that SWI/SNF chromatin remodeling plays a repressive role at these regions instead. While we cannot rule out the possibility that there are other genomic regions where the BRM complex plays the same role as it does at Notch-responsive regions, these data certainly strengthen our argument that not every enhancer is controlled by the same mechanisms and that SWI/SNF chromatin remodeling plays a role to promote Notchresponsive chromatin accessibility.

5) For CATCH-IT would like to understand how the turnover-values are calculated and what the treatment in 4B is? Is this Notch-on or Notch-off. I am comparing turnover-values in lacZ RNAi in 4B with values for WT in Notch-on or Notch-off and there is a 5-6 difference in value. What is the source of this variation. Surely they should be identical?
We thank the referee for this comment. A similar point was made by referee #1, and we thank both referees for bringing this to our attention. Although the relative amounts of DNA corresponding to different regions remained consistent between experiments, there were differences in the absolute levels because of variability in the efficiency of DNA recovery following microccocal nuclease digestion and biotin-streptavidin purification. To circumvent this we have identified a region (Sec15) to use as an internal control to normalize for this variability; this region was found to have low to moderate accessibility in the previous genome-wide CATCH-IT experiments from the Henikoff lab. This normalization aids comparisons between the different experiments: values in **new Figure 5** are now given as a fold enrichment of turnover relative to the Sec15 control region (as explained in the legends and Methods section).

6) is it possible to do a time-course of recruitment of Su(H) and H3.3 following N activation. Not sure the availability of Fluor-tagged Su(H) and H3.3 constructs but would help to resolve the interrelationship
of H3.3 and Su(H) recruitment. My suspicion is that most of the H3.3 incorporation seen in polytenes is on the gene bodies during transcription.
We appreciate the concerns highlighted here by the referee. Unfortunately we do not have the fine temporal control required for this experiment since both ectopic Notch and histone-GFP expression are controlled by the GAL4/UAS system. We have added an experiment, described above, which uses a heat shock method to flip on expression of H3.3-mKO. With this approach we have been able to demonstrate that the recruitment to the *E(spl)-C* of histone H3.3 involves newly synthesized protein and is ongoing during the Notch response (**new Figure EV1**). Although we can't determine whether histone H3.3 incorporation at the *E(spl)-C* in salivary glands is at enhancers or gene bodies, this resolution is provided in our CATCH-IT and histone ChIP experiments performed in Kc167 cells, which show there is histone turnover and recruitment at enhancers.

7) Leading on from 5, a key conclusion the authors draw from the data is that Brm is mediating histone turnover. I am not sure that we can conclude that Brm is directly responsible for the histone turnover. Certainly Brm knock-down decreases turnover but it also decreases binding of

TFs and activation of enhancers and mRNA transcription. Data presented do not allow one to exclude a pathway in which Brm facilities Su(H) binding which in turn recruits co-activators to drive enhancer activation and that this and associated transcription drives turnover. Possible option would be to treat salivary glands as in Fig 1A but co-treat with amanitin or DRB.
We agree with the referee that it is challenging to prove unequivocally that Brm is mediating histone turnover. We also agree that one role of Brm is to facilitate Su(H) binding. We would however like to highlight that this cannot explain the effects of Brm on the accessibility and histone turnover because our results show that Su(H) depletion has the opposite effects to Brm depletion on these parameters (**see Figure 3D**). To avoid this key experiment being overlooked, we have moved it into a main figure; it was originally presented in the expanded view figures. We note also that the increased nucleosome turnover observed with CATCH-IT in response to Notch activity occurs primarily at Notch-regulated enhancers rather than at gene bodies which argues against transcription itself being responsible for the turnover. Therefore we believe that our interpretation, that Brm directly alters accessibility and turnover, is the most parsimonious explanation.

The proposed experiment using a transcription inhibitor on the salivary glands is a potentially interesting way to de-convolute some of these issues. Following the suggestion of the referee, we incubated salivary glands with triptolide, a very effective transcription inhibitor, which we have used successfully on this tissue in the past (Gomez-Lamarca *et al.* 2018, Dev Cell). However, we did not detect any change in H3.3-GFP levels at *E(spl)-C* after an hour of triptolide treatment, which is sufficient to arrest transcription. This result is consistent with the turnover being independent of transcription as suggested by our model and argues against the alternate scenario proposed by the referee. However we have opted not to include these data or to pursue this experiment further as we are concerned about the technical short-comings – for example, we can't extend the time of triptolide treatment without then impacting on the expression of H3.3-GFP itself – and we believe that the other data discussed above already argue that there are specific effects independent of transcription.

2nd Editorial Decision 15 February 2019

Thank you for the submission of your revised manuscript. We have now received comments from all referees (pasted below) and I am happy to tell you that we can in principle accept your study for publication now.

Only a few minor changes are still needed:

Please address the comments from referee 3 in the manuscript text as you see fit.

In the Introduction you mention on top of page 4 that the SWI/SNF complex has been reported to have a positive and an inhibitory effect on Notch-dependent transcription, however, the following text highlights 2 positive roles of SWI/SNF in Notch target gene expression. Is this correct?

Fig 1C is called-out before 1B and Fig 4A is not called-out, please correct.

In Figs 1B,D,F and EV1B it is not clear on how many independent experiments the data are based on. Please add this information. If the experiment was repeated less than 3 times no error bars may be shown. In this case, the actual data points along with their mean can be shown in the graphs.

Please specify "n" the number of independently performed experiments also for Fig 2B,D. No statistics can be calculated if n<3.

For Fig 4A please specify "n". Please remove the error bars for Fig 4E where n=2.

Fig 5A states n=2, so the error bars and statistical calculations need to be removed.

Fig EV2 does not specify "n" nor the error bars. Please add this information.

Fig EV3 does not mention C in the legend but does list F although there is no panel F in the figure.

Fig EV4E and Fig EV5 state n=2 so the error bars need to be removed.

I would like to suggest a few changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Notch signaling plays a key role in many cell fate decisions during development by directing different gene expression programs via the transcription factor CSL, known as Su(H) in Drosophila. Which target genes are responsive to Notch signaling is influenced by the chromatin state of enhancers, yet how this is regulated is not fully known. Detecting an increase in the histone variant H3.3 in response to Notch signaling [Do you mean a general increase, or a specfiic one? Please clarify.], we tested which chromatin remodelers or histone chaperones are required for the changes in enhancer accessibility to Su(H) binding. We show a crucial role for the Brahma SWI/SNF chromatin remodeling complex, including the actin-related BAP55 subunit, in conferring enhancer accessibility and enabling the transcriptional response to Notch activity. The Notch-responsive regions have high levels of nucleosome turnover, which depend on the Brahma complex, increase with Notch signaling [Do you mean in number or in size?] and primarily involve histone H3.3. Together these results highlight the importance of SWI/SNF-mediated nucleosome turnover in rendering enhancers responsive to Notch.

I look forward to seeing a final manuscript as soon as possible. Please let me know if you have any questions.

REFEREE REPORTS

Referee #1:

the revised version of this manuscript is of an excellent technical quality and great general interest. Can be published as is.

Referee #2:

Pilllidge and Bray revised the paper based on all three reviewers' comments, performed the majority of the suggested additional experiments and made necessary improvements to the manuscript. Overall the manuscript now flows even better than before.

Most importantly, they addressed the problem of specificity - they performed additional experiments including BAP55 knock-downs in Figures 4F/G and ATAC/mRNA levels on several additional genes in Figures EV3A-C, and added a paragraph in the discussion that specifically addresses this point. Furthermore, they propose that upstream tissue-specific factors are necessary to recruit BRM at Notch genes to allow Su(H) binding, citing the glucocorticoid receptor as example. Although they did not add additional data, this addition still clarifies the point for the reader.

Referee #3:

The authors have made a significant number of corrections and additions to the original manuscript that go some way to addressing specific concerns raised by this author and are to be commended. The work is technically sound and merits publication.

However, the larger issue (raised more explicitly by reviewer 2 in the first round) still remains. Increased accessibility and turn-over at active enhancers is not surprising. Neither is the requirement for SWI/SNF remodelling activities to facilitate accessibility at enhancer elements. The combination of imaging, genetic and genomics approaches presented in the paper is certainly attractive but it does not get us much further on from the basic conclusion that enhancer activation is accompanied by increased accessibility and histone turnover and that SWI/SNF complexes mediate this.

There are still a number of unknowns not addressed in the manuscript which, for me, are the key questions. What are the mechanisms of recruitment of Brm to targets. For instance in Notch-off conditions Brm is required for accessibility. What is recruiting it? In Notch-on conditions Brm again is required for increased accessibility. What are the differences between Brm action/recruitment in Notch-Off and Notch-On. Are there any differences and, at the level of individual nucleosomes, what do these look like. What is the nature and dynamics of the association of SWI/SNF remodellers with target nucleosomes.

The assays used are inherently too coarse grained to provide more than a general overview that things are changing without providing the resolution required to reveal the mechanism by which these occur. Understanding what SWI/SNF type complexes actually do to nucleosomes in vivo is still very much a question for investigation given that most models for action are based on in vitro substrates that probably don't recapitulate all features of their normal targets in vivo.

2nd Revision - authors' response 18 February 2019

Please address the comments from referee 3 in the manuscript text as you see fit.
We have added statements in the Discussion noting the limitations raised by the reviewer.
End of first paragraph: "*In future, more fine-grained studies will be needed to determine precisely which nucleosomes are targeted by BRM complexes and what the dynamics of these interactions are."*
End of third paragraph: "*The final outcome therefore differs in in Notch-ON versus and Notch-OFF conditions, but it remains unclear how this is brought about. For example does BRM target different nucelosomes or interact with more prolonged dynamics in the Notch-ON state?*"

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: Sarah BRAY
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46944

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 χ2 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?	No power analysis was used. For salivary gland imaging experiments, no more than five nuclei were analysed per animal to ensure good quality images were obtained and datasets represented multiple animals. Differences in histone-GFP patterning were clear from the images observed by eye and collected on multiple days; thus, quantifications were used to exemplify the results observed by eye and no statistical tests were performed (Fig 1 and EV1). Likewise, for Su(H)-GFP imaging experiments (Fig 2), strong conclusions were only drawn from results that were very obvious by eye and quantifications with Fisher's exact statistical test used to exemplify the results. For experiments analysed by qPCR, internal controls were used to ensure differences observed were specific. Data were only incuded when the results were reproducible between replicates and sufficient replicates performed for the results to be convincing with the aid of statistical tests.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The number of biological replicates is given for every experiment in the figure legends, including details of nucleus vs. gland numbers in salivary gland experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from analysis. For salivary gland imaging experiments, only nuclei close to the surface were imaged to ensure good quality images were obtained.
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: animals/samples were not allocated to different treatments in our study. All genotypes and samples were treated equally and comparisons made between them.
For animal studies, include a statement about randomization even if no randomization was used.	NA: no animal experiment involved allocating different treatments.
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.	The investigator was not blinded. However, all samples were treated equally in molecular biology experiments. In scoring the band of Su(H)-GFP in salivary gland nuclei (Fig 2), strict criteria were used to assess the presence of a band (must be bright and sustained to be scored as a band), and strong conclusions have only been drawn from the clearest results.
4.b. For animal studies, include a statement about blinding even if no blinding was done	It is explicitly stated in the Methods section that Su(H)-GFP band scoring was not conducted blind, and an explanation is given about the steps taken to reduce subjective bias.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests are used to highlight the biggest differences in the data that are already obvious by eye. No justification is given.

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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The assumptions of independent and random sampling are valid for all experiments. No formal methods were used to assess normality or equality of variance. However, student's t-tests (equal variance) were used only for experiments where the scatter of data did not appear to change between samples and theoretically should not have changed (eg. ChIP and RT-qPCR experiments). Welch's t-tests (unequal variance) were used to analyse ATAC experiments, since the variance in this experiment is affected by the level of tagmentation and thus had the potential to vary between samples with large differences in accessibility (eg. Notch-OFF versus Notch-ON salivary glands).
Is there an estimate of variation within each group of data?	Standard error of the mean is shown in all graphs.
Is the variance similar between the groups that are being statistically compared?	Yes, variance was always similar between groups. As stated above, Welch's t-test was used for the ATAC experiments, since these experiments had the potential for unequal variances between samples.

C- Reagents

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).	Goat anti-Su(H): Skalska et al. 2015, EMBO J. Mouse anti-V5: Wirbelauer et al. 2005, Genes Dev.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Kc 167 cells were originally obtained from the authenticated source, the Drosophila Genomics Resource Center. These cells are not susceptible to mycoplasma.

* 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.	Drosophila melanogaster. Strains and origins are given in the Methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA: no vertebrate models used.
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.	We comply with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA: no human subjects.
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: no human subjects.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA: no human subjects.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA: no human subjects.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA: no human subjects.
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: no human subjects.
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: no human subjects.

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: No largescale data sets have been generated.
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: no largescale data sets have been generated or used.
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: No human clinical data has been used.
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: No computer models have been used.

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.	No, our study does not fall under dual use research restrictions.