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Chromatin signatures at Notch regulated enhancers reveal large-scale changes in H3K56ac upon activation

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(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.)

Editors: Alexander Kohlmaier and Anne Nielsen

1st Editorial Decision 21 October 2014

Thank you for submitting your manuscript "Chromatin states at Notch regulated enhancers reveal large scale changes in H3K56ac upon activation." for consideration to the EMBO Journal. We have now received the comments of two experts that you will find pasted below. We also have to apologize that we did not get back to you with a decision earlier - despite repeated requests the third referee has so far not submitted the assessment, which caused the delay. In the interest of your time, we have now decided to proceed to a decision:

I am glad to be able to inform you that the two referees concur that your work is of significance and of broader interest. Some major conceptual as well as technical issues remain, however, to be decisively addressed before publication can be offered. Therefore, we invite you to revise the manuscript according to the referees' suggestions, which we consider very constructive.

Please keep in mind that it is our policy to allow only a single round of major revision and that it is therefore important to carefully respond to all points at this stage. I will not repeat here in detail the referees' specific requests concerning mechanistic, and conceptual points and technical clarifications, but highlight only some key points for the revision:

a. Both referees point out that the re-definition of the new chromatin states to obtain a better definition (using multi-state models of different granularity) may not be fully conclusively supported by the data and as such would remain to be more decisively determined. In a subsequent

step of consultation, both referees let us know more specifically that such a detailed clarification of advance and of robustness of the state definitions would introduce a significant digression, and that leaving out the chromatin state definitions was advised. This is also our own understanding in reading the manuscript and we suggest leaving out the chromatin state definitions, unless the advanced predictive power could be very compellingly shown by direct tests of biological criteria. Please do contact me directly with a detailed provisional point-to-point response in the next weeks in case you want to bring up new evidence or strong additional points for keeping the state definitions in the revised manuscript.

b. A second line of criticism pertains to the experimental dynamic perturbations in the cell system and the comprehensiveness of their analysis. This relates on the one hand to the specificity of using EGTA for Notch activation. We understand that this technique has been used before to activate Notch signalling (for measuring specific and defined Notch output signalling) - But when addressing a global genomic response, as presented here, we agree with referee #3 that this technical approach should at least be supplemented or directly compared on a genomic level with more specific interventions of manipulating Notch activity.
Also, in specific point 4 referee #1 requests mapping of additional histone modifications after perturbations. Following consultation with referee #3 we do not request re-mapping a lot of different histone modifications after these perturbations for a possible final acceptance of your manuscript, as this endeavour could become quite open-ended. Yet we agree with referee #1 that it makes sense in this context to offer also a more comprehensive quantitative comparison of the range of effects triggered by the perturbations (local vs more systemic affects?, i.e. beyond looking at only a few selected local target sites).

c. Finally, we think that strong emphasis should also be put on referee #1's point 5 (using additional chemical and/or specific genetic approaches to substantiate the model, if possible). Regarding this point, we also think that the additional measurement of eRNA transcription in Fig. 6 could be interesting and rather straight-forward (i.e. testing any differential control of transcripts arising from TSS and enhancer regions): this could possibly be insightful from a conceptual point of view, as the inhibitor of the CBP bromodomain abolished transcripts from the TSS but did not abolish H3K56Ac.

Together, I am certain that the modifications requested by the referees would result in an improved study. I would, therefore, be pleased if you invested the necessary time and efforts to address the reviewers' concerns.

As indicated already above, should you have questions regarding the decision or should you have comments about extent or feasibility of a specific referee point, please do contact me within the next week or two.

I look forward to receiving your revision.

Referee #1:

In this manuscript, Skalska et al. use histone modification ChIP-chip data to identify chromatin states and study the characteristics and dynamics of defined chromatin states associated with Notch activation in Drosophila BG3 and KC167 cells. The authors found that Su(H)/CSL binding sites are enriched in enhancer or active promoter (TSS) chromatin states, and that Lz/Runx can act as a co-factor of Su(H) to help establish certain chromatin states. They also found that the activation of Notch enhancers result in a dynamic increase in the histone modification H3K56ac.

This work represents a systematic study of chromatin states and their relationship to Notch binding in Drosophila, and reveals a new association between a previously understudied histone modification, H3K56ac, and Notch activation. It provides a novel approach to study Notch signalling and related transcriptional activation in terms of chromatin context. The overall results are solid and will be of interest to both the genomics and Notch signalling research community. The manuscript is well written. However, there are several questions that need to be addressed by the authors.

1) Can the authors provide more compelling evidence to support the overall robustness and completeness of the Hidden Markov Model-defined chromatin state identification? For example, how distinct are different states according to histone modification pattern similarity? How sensitive is a chromatin state to a given histone modification (data availability and quality)? In other words, if the ChIP-chip dataset for one histone modification were significantly worse than the others, how would it change the chromatin state call?

2) It is interesting to see that Su(H) binding sites are mostly associated with an Enh state and a TSS state in both BG3 and Kc cells. How robust are the chromatin states associated with Su(H) or Notch binding? Do those Su(H) binding sites that are not associated with the Enh or TSS state have other specific functions, or (alternatively) are the chromatin states for these sites mis-assigned? Also, it looks like DHS can measure the open accessible chromatin very accurately, as DHS are high in only the Enh and TSS chromatin sites. Would DHS alone (or DHS + motif) be a better predictor of Su(H) binding than the 11 state chromatin HMM model?

3) The authors show differential Su(H) binding profiles in the BG3 and Kc cells (Fig. 1D). It would be helpful if authors present a comparative analysis of common Su(H) binding sites and cell-type specific Su(H) binding sites (according to Fig. 1D). What are the chromatin states of the shared binding sites (presumably the same) and the BG3 and Kc specific binding sites (presumably different)? These analyses could help reinforce some of the claimed relationships between chromatin states and functional binding events.

4) The authors present a few dynamic perturbations to the cell systems, including Runx overexpression, Su(H) knockdown, and Notch activation. For each of these perturbation analyses, it would be interesting to compare the changes in histone modification-defined chromatin states before and after the treatment. Do changes in regulators change chromatin states, either locally or systemically?

5) Some of the most interesting data pertain to the relationship between Notch recruitment to sites, H3K56ac, and CBP/p300 HAT activity and CBP/p300 bromodomain function, but a good part of it is inferred from CBP HAT and CBP bromodomain inhibitor drugs, which are low potency small molecules that undoubtedly have off-target effects. These data should be confirmed by additional controls with structurally related small molecules that lack these activities and/or genetic approaches.

Referee #3:

Major points:

This manuscript describes a detailed analysis of the genome-wide binding pattern of Su(H) following Notch activation in two types of cultured Drosophila cells. It also reports an interesting increase in H3K56ac upon Notch activation. Overall, this is a nice paper with interesting findings that will appeal to those working in signalling and chromatin fields.

Major comments:

1. The manuscript begins with a re-definition of chromatin states using an HMM approach similar to what several other labs have done. The state definition is based on modENCODE data plus a single additional ChIP track (H3K56ac). While I cannot judge the underlying math/stats of the approach in detail, it appears from reading the manuscript and Supp Methods that the authors think that their state definition is better than others. However, I propose that the authors remove this new chromatin state definition from the manuscript, for several reasons:

(i) New chromatin state definitions should be proposed sparingly. If every lab defines their own chromatin states, we end up with a mess that is even bigger than before the concept of chromatin states was proposed. State 12 of lab X may overlap by 70% with state 15 of lab Y, and by 40% with state 9 of lab Z, etc.... how will anyone be able to compare results from different papers?

(ii) If the authors really feel that their state definition is substantially better than previously published ones, then they should publish this separately. By tucking it into a paper that is primarily about Su(H) it will not be picked up by the community as a new standard.

(iii) The arguments why the new state definition is better than others are not entirely convincing, although it is clear that the authors have given it quite some thought. I tend to go along with the points about robustness and potential overfitting if there are too many parameters, yet the proof is really in the biological pudding: does the new state model predict biology better than the old ones? This should be rigorously investigated, using objective biological criteria, before the new state model is adopted as "the best".

Hence, I feel it would be better to use one of the previously published chromatin state definitions for the analysis of Su(H). Together with a comparison to the STARR-seq data this should suffice to make the point that Su(H) has a strong preference for active enhancer regions. This will also streamline the current manuscript.

2. I was unable to find GEO/ArrayExpress accession numbers for the new ChIP data. They should be provided to the reviewers and readers. How many replicate experiments were done? How reproducible were the results? Only <400 binding sites genome wide seems a very low number, most DNA-binding factors have thousands of sites; could there be a technical problem here? Related to this, the peaks in Fig 1C seem very broad (several kb) for a DNA-binding factor: how do the authors interpret this? How large were the sheared chromatin fragments?

3. Notch is activated by adding EGTA to the culture medium. This seems a very non-specific stimulus. If this is a standard method in the Notch field, please cite references that document the specificity.

Minor points:

4. The mixed use of "Su(H)" and "CSL" is confusing. Please adhere to official FlyBase name.

5. ChIP tracks of Su(H), H3K56ac and others in Figs 1C, 2, 5, 6: please add y-axis scale and units.

1st Revision - authors' response 27 February 2015

Reply to the Editors and the Reviewers.

Editors Comments:

a. Both referees point out that the re-definition of the new chromatin states to obtain a better definition (using multi-state models of different granularity) may not be fully conclusively supported by the data and as such would remain to be more decisively determined. In a subsequent step of consultation, both referees let us know more specifically that such a detailed clarification of advance and of robustness of the state definitions would introduce a significant digression, and that leaving out the chromatin state definitions was advised. This is also our own understanding in reading the manuscript and we suggest leaving out the chromatin state definitions, unless the advanced predictive power could be very compellingly shown by direct tests of biological criteria. Please do contact me directly with a detailed provisional point-to-point response in the next weeks in case you want to bring up new evidence or strong additional points for keeping the state definitions in the revised manuscript.

We have revised the manuscript along the lines suggested, by placing the chromatin analysis in the context of the existing framework of chromatin states. However, we did need to derive chromatin maps for the two specific cell lines we are working with. This has not been done in the previous

studies and is not straightforward using the published methods as they stand. By employing a variation of the published modENCODE method we were able to achieve reproducible maps for the cell types. We now refer to these as maps and we have avoided any claims about new chromatin states, instead focusing on the key similarities between our maps and those previously published. We have simplified Fig. 1, removed several of the supplementary figures and curtailed the text in the results and methods. We hope that the revised version achieves an appropriate balance, enabling us to derive the information that is essential for our analysis, while avoiding any explicit definitions/discussion of new chromatin states.

b. A second line of criticism pertains to the experimental dynamic perturbations in the cell system and the comprehensiveness of their analysis. This relates on the one hand to the specificity of using EGTA for Notch activation. We understand that this technique has been used before to activate Notch signalling (for measuring specific and defined Notch output signalling) - But when addressing a global genomic response, as presented here, we agree with referee #3 that this technical approach should at least be supplemented or directly compared on a genomic level with more specific interventions of manipulating Notch activity.

We appreciate the concerns, although there is substantial evidence that the EGTA treatment is an effective way to activate Notch (*e.g. Llagan et al, (2011) Sci Signal. 4(181):rs7; Krejci and Bray, (2007) Genes and Development 21:1322-7; Gupti-Rossi et al, (2001) J Biol Chem. 276(37):34371-8*). Please note also that we had used g-secretase inhibitors to block the activating Notch cleavage and thus demonstrated the specificity of the effects we measure. Nevertheless, taking on board this concern, we have generated a new cell line with copper-inducible NICD. These cells yield up-regulation of Notch targets within 2 hours (previously generated cell-lines with a similar construct were very inefficient), allowing us to compare the profile of H3K56ac when Notch (NICD) activity is manipulated. The results are fully consistent with those we reported originally, showing up-regulation of H3K56ac at several enhancers. These are discussed in the text (page 11, Table E1) and include the *E(spl)* region as depicted in new data added to Fig. 5 of the revised the manuscript

A*lso, in specific point 4 referee #1 requests mapping of additional histone modifications after perturbations. Following consultation with referee #3 we do not request re-mapping a lot of different histone modifications after these perturbations for a possible final acceptance of your manuscript, as this endeavour could become quite open-ended. Yet we agree with referee #1 that it makes sense in this context to offer also a more comprehensive quantitative comparison of the range of effects triggered by the perturbations (local vs more systemic affects?, i.e. beyond looking at only a few selected local target sites).*

The question asked by the reviewer is whether changes in regulators change chromatin states, either locally or systemically. Our results already argued strongly that perturbations to the regulators were sufficient to change chromatin signatures at specific enhancers. For example, rerunning the HMM with the genome-wide data from Notch activated cells showed that this was sufficient for the chromatin signature at enhancers to be changed (e.g. from competent to enhancer, Fig. 6A). Our more locus-specific analysis also showed that the manipulations were able to alter histone modifications indicative of changes in chromatin states at the positions where the regulators were known to bind but not at control sites.

Following the request we have now extended our analysis to include a genome-wide analysis of the H3K56ac changes following Runx/Lz overexpression. These new data, which have been added to Figure 6 (new Fig. 6G) and discussed in the text (page 8) demonstrate that Runx/ Lz expression is also sufficient to change chromatin at target enhancers.

c. Finally, we think that strong emphasis should also be put on referee #1's point 5 (using additional chemical and/or specific genetic approaches to substantiate the model, if possible). Regarding this point, we also think that the additional measurement of eRNA transcription in Fig. 6 could be interesting and rather straight-forward (i.e. testing any differential control of transcripts arising from TSS and enhancer regions): this could possibly be insightful from a conceptual point of view, as the inhibitor of the CBP bromodomain abolished transcripts from the TSS but did not abolish H3K56Ac.

We have taken on board the suggestion and have performed experiments with additional inhibitors

to substantiate the model (new Fig. E5). The results, using another CBP HAT inhibitor, another elongation inhibitor and another control inhibitor, are all consistent with the original findings. We have also tested the effects of CBP/nej knock-down in cells and in vivo and find a decrease in H3K56ac and reduced expression of a Notch regulated gene (new Fig. E5), although these data are more difficult to interpret as these are long-term effects. As suggested, we have extended the analysis of e-RNAs and tested the effects of inhibitors on their expression. The results are similar to these seen for the mRNAs, and argue that the H3K56ac precedes and may facilitate e-RNA expression. These data are added into new Fig. 6B.

Specific referees comments:

Referee #1:

1) Can the authors provide more compelling evidence to support the overall robustness and completeness of the Hidden Markov Model-defined chromatin state identification? For example, how distinct are different states according to histone modification pattern similarity? How sensitive is a chromatin state to a given histone modification (data availability and quality)? In other words, if the ChIP-chip dataset for one histone modification were significantly worse than the others, how would it change the chromatin state call?

We thank the reviewer for these suggestions. We have investigated how sensitive the chromatin states are to a given histone modification. Results show that some signatures are very sensitive to a given modification, for example the Polycomb state relies on H3K27me3, while many others are not reliant on a single modification. A new table summarizing the impact on the chromatin states by removing data sets is shown in the extended data (new Fig. E1F). Notably the enhancer signatures, which are the focus of our paper, withstand the removal of most single modifications. One exception is H2B ubiquitylation, which we now mention in the results (page 6).

Following the suggestions of the reviewers and the editor, we have toned down the consideration given to the chromatin states and their derivation. Instead we have focused on one or two key aspects, which are common between our analysis and the definitions made by others. We hope that the additional analysis and this shift in emphasis addresses the concerns of the reviewer

2) It is interesting to see that Su(H) binding sites are mostly associated with an Enh state and a TSS state in both BG3 and Kc cells. How robust are the chromatin states associated with Su(H) or Notch binding? Do those Su(H) binding sites that are not associated with the Enh or TSS state have other specific functions, or (alternatively) are the chromatin states for these sites mis-assigned? Also, it looks like DHS can measure the open accessible chromatin very accurately, as DHS are high in only the Enh and TSS chromatin sites. Would DHS alone (or DHS + motif) be a better predictor of Su(H) binding than the 11 state chromatin HMM model?

We thank the reviewer for this comment. Of the few Su(H) sites not associated with Enh and TSS many are in either Competent or Polycomb chromatin. Drilling more deeply into the Pc bound regions these have characteristics of bivalent chromatin. These sites are thus likely to represent loci that are inactive prior to Notch activation but that are poised for activation. The remainder constitutes less than 7% and it is certainly plausible that for these few positions the chromatin state is miss-assigned, or the peak is a false positive. We have added a statement discussing these points to the results (page 7, top) and to the discussion (page 15). We agree that there is a strong correlation between the DHS and the Enh and TSS signatures where Su(H) prefers to bind. However, DHS alone is not sufficient to predict these states nor is it a better predictor of Su(H) binding.

3) The authors show differential Su(H) binding profiles in the BG3 and Kc cells (Fig. 1D). It would be helpful if authors present a comparative analysis of common Su(H) binding sites and cell-type specific Su(H) binding sites (according to Fig. 1D). What are the chromatin states of the shared binding sites (presumably the same) and the BG3 and Kc specific binding sites (presumably different)? These analyses could help reinforce some of the claimed relationships between chromatin states and functional binding events.

As the reviewer highlights this is an important point. We had originally summarized the results from

such an analysis via a table that may not have been straightforward to interpret (old Fig. 2D). We have replaced this with a graph (new Fig. 2D) showing that, as the reviewer predicted, the chromatin states of the regions bound in both cell types are the same Enh chromatin in both. For those where they differ between cell types, the situation is not as simple as suggested. We do see a shift with 30-40% in less favorable chromatin when they are unbound. However, as we discuss, there are still a large proportion in the favorable Enh state even though they are unbound. We consider this to be an important observation as it shows that - while important - the chromatin environment is not sufficient to predict functional binding. We have revised this section of the results (page 7-8) to match the graph, and we hope that these points are now communicated more clearly.

4) The authors present a few dynamic perturbations to the cell systems, including Runx overexpression, Su(H) knockdown, and Notch activation. For each of these perturbation analyses, it would be interesting to compare the changes in histone modification-defined chromatin states before and after the treatment. Do changes in regulators change chromatin states, either locally or systemically?

We agree that the question of whether changes in regulators change chromatin states is a very relevant one. We note that we already examined local changes at specific loci to show that the manipulations were able to alter histone modifications indicative of changes in chromatin states at the positions where they were known to bind but not at control sites (Fig. 3). We had also analyzed global changes in H3K56ac following Notch activation and shown that, at some loci, these were sufficient for a switch in chromatin state (based on re-running the HMM; e.g. Fig. 6A).

Following the Reviewer's suggestion, we have now extended our analysis to include a genome-wide analysis of the changes following Runx/Lz overexpression. These data confirm that there are numerous regions across the genome with significant changes in H3K56ac, including *peb* and *Klu* (Notch responsive targets) and also *PPO1* and *PPO2* (known Lz targets). Many of the regions that change were defined as Competent or Polycomb states prior to Lz expression and a proportion were in basal state. These new data are discussed in the text (page 8) and representative examples have been added to Figure 3 (new Fig. 3G). They demonstrate that Runx/Lz expression is sufficient to change chromatin signatures. For Su(H) knockdown we have monitored effects at several different chromosomal regions, both bound and unbound, and shown that the changes are only detected at the specific bound loci (Fig. 3H).

We believe that together our data make a strong case for the fact that the regulators change chromatin states locally and that these local changes can, in some cases, encompass a larger region of the regulated locus

5) Some of the most interesting data pertain to the relationship between Notch recruitment to sites, H3K56ac, and CBP/p300 HAT activity and CBP/p300 bromodomain function, but a good part of it is inferred from CBP HAT and CBP bromodomain inhibitor drugs, which are low potency small molecules that undoubtedly have off-target effects. These data should be confirmed by additional controls with structurally related small molecules that lack these activities and/or genetic approaches.

Although we had taken care to use very-selective inhibitors that had been validated in other studies (e.g. Das et al, (2014) Proc Natl Acad Sci U S A. 111:E1072-81; Crump et al, (2011) Proc Natl Acad Sci U S A. 108:7814-9.), we appreciate the concerns of the reviewer. We have extended the inhibitor analysis to include: i. an additional CBP HAT domain inhibitor (curcumin; e.g. Das et al, (2009) Nature 459:113-7); ii. inhibitors that target other functions of CBP; iii. an additional elongation inhibitor (flavopiridol). These data are included in extended data (new Fig. E5) and are discussed in the text on page 12. The advantage of the inhibitor assays is that they allow us to assay effects after a very short period of inhibition, avoiding more indirect consequences from long-term loss of CBP. Nevertheless we have also taken a genetic strategy and shown that CBP-RNAi reduces H3K56ac levels in Kc cells and in *Drosophila* imaginal discs where it alters expression of a Notch regulated gene. These data are included in new Fig. E5, although these long-term experiments do not distinguish any close mechanistic relationship.

Referee #3:

Major comments:

1. The manuscript begins with a re-definition of chromatin states using an HMM approach similar to what several other labs have done. The state definition is based on modENCODE data plus a single additional ChIP track (H3K56ac). While I cannot judge the underlying math/stats of the approach in detail, it appears from reading the manuscript and Supp Methods that the authors think that their state definition is better than others. However, I propose that the authors remove this new chromatin state definition from the manuscript, for several reasons:

Following the suggestion of the reviewer we have removed the extensive discussion of the state definitions from the manuscript and have instead focused on the similarities between our signatures and the recent metazoan model. Because our analysis required a map of the chromatin states in the cell-types we have worked with (those are not available from other analyses), we still needed to use our own derivations (the published methods are very computationally intense and in our hands not very stable). But we have avoided any claims about new states or state definitions. We hope that, in revising the manuscript, we have achieved an appropriate balance.

(i) New chromatin state definitions should be proposed sparingly. If every lab defines their own chromatin states, we end up with a mess that is even bigger than before the concept of chromatin states was proposed. State 12 of lab X may overlap by 70% with state 15 of lab Y, and by 40% with state 9 of lab Z, etc.... how will anyone be able to compare results from different papers?

We appreciate the reviewers concerns. Indeed we had initially tried to derive states by replicating methods from the original modENCODE papers. However, we encountered some unexpected features and problems, which inspired us to take a modified approach and to generate a method that could be easily used by others.

Nevertheless, we accept that it can be confusing to have many iterations of this process, and so, as discussed above, we have removed the emphasis on new states and have instead related our analysis to the existing models as much as possible.

(ii) If the authors really feel that their state definition is substantially better than previously published ones, then they should publish this separately. By tucking it into a paper that is primarily about Su(H) it will not be picked up by the community as a new standard.

We thank the reviewer for this suggestion. We agree that the state definitions and the details of the approach are not necessary for the paper. We have referred to the strategy in the methods and provide access to the code. We will consider writing a more detailed discussion of the method as a separate paper.

(iii) The arguments why the new state definition is better than others are not entirely convincing, although it is clear that the authors have given it quite some thought. I tend to go along with the points about robustness and potential overfitting if there are too many parameters, yet the proof is really in the biological pudding: does the new state model predict biology better than the old ones? This should be rigorously investigated, using objective biological criteria, before the new state model is adopted as "the best".

We agree that it is difficult to assess which is the “best” model. One of our main goals was to adopt a method that was simple and reproducible (we tried it on several data sets and we have assessed the robustness by removing different combinations of histone modifications, new Fig. E1F). Based on these criteria, our method achieves a plausible chromatin distribution, but we agree that the outcome is not necessarily better than the more computationally complex models. To avoid confusion, we have now focused on the key similarities and common messages that emerge.

Hence, I feel it would be better to use one of the previously published chromatin state definitions for the analysis of Su(H). Together with a comparison to the STARR-seq data this should suffice to make the point that Su(H) has a strong preference for active enhancer regions. This will also streamline the current manuscript.

We appreciate the suggestion of the reviewer and have now placed our analysis in the context of

existing models. However, as none of the previous studies have generated a chromatin state-model for the two cell types we have used (likewise STARR-seq data) the derived states are not available and this is why we had embarked on a method to derive them ourselves. We note that the most recent method is computationally very intensive, for example their Bayesian model utilized only the data from one arm of the *Drosophila* chromosomes. This makes it problematic to try and reproduce this method on other data sets. We have instead related our method to those existing models to circumvent the confusions and to avoid over-stating the findings (new Fig. E1). We agree that the revised emphasis does help streamline the manuscript, and we hope that we have achieved an appropriate balance.

2. I was unable to find GEO/ArrayExpress accession numbers for the new ChIP data. They should be provided to the reviewers and readers. How many replicate experiments were done? How reproducible were the results? Only <400 binding sites genome wide seems a very low number, most DNA-binding factors have thousands of sites; could there be a technical problem here? Related to this, the peaks in Fig 1C seem very broad (several kb) for a DNA-binding factor: how do the authors interpret this? How large were the sheared chromatin fragments?

First, we apologise that the accession numbers were not provided in the original submission. The data have been submitted to GEO and the series accession number is now cited in the manuscript (methods, p17).

Second we stress that all of our original experiments, including the genome-wide binding profiles, were performed in triplicate (3 biological replicates). For the subsequent analysis of those data we used a stringent cut-off and focused on bound regions identified with a 1% FDR. The majority of sheared chromatin fragments were <500bp, but please note the data were from tiling arrays rather than from sequencing for historical reasons.

Finally, we do not believe there is a technical problem. The proportion of bound regions is not only consistent within an experiment but also across many different studies: we have performed ChIP examining Su(H) binding in many different cell types under many different conditions and consistently detect similar numbers of bound regions. Furthermore, in mammalian experiments the number of bound regions for CSL is also on the low side compared to other TFs (e.g. Castel et al. (2013) Genes Dev. 27: 1059-1071). In addition, please note that Su(H) binding appears to be enhanced by NICD and that most of our experiments are performed under conditions with no or low NICD.

3. Notch is activated by adding EGTA to the culture medium. This seems a very non-specific stimulus. If this is a standard method in the Notch field, please cite references that document the specificity.

EGTA is an effective means to activate Notch because it destabilizes the negative regulatory region, exposing the site for Adam10 and consequently results in g-secretase cleavage and release of NICD (e.g. Llagan et al, (2011) Sci Signal. 4:rs7; Krejci and Bray, (2007) Genes Dev. 21: 1322-7; Gupti-Rossi et al, (2001) J Biol Chem. 276:34371-8). Specificity can be demonstrated by the fact that the effects of EGTA can be blocked by g-secretase inhibitors, as was done in our experiments (e.g. Fig. 4F,G; Fig. 6B). The method has been quite extensively used in both *Drosophila* and mammalian cells because it has the advantage that the treatments rely on endogenous physiological levels of Notch receptor, and allow carefully timed Notch activation. This enables measurements of immediate changes. We note also that it is problematic to activate with Notch ligands, because they are membrane bound and require endocytosis in the ligand-presenting cell for full activity. The Fc-conjugated mammalian Dll is one of the few constructs that gives some activation. More explanation of the strategy and additional references have been included in the results (page 9) and methods (page 17).

However, we appreciate the concerns of the reviewer and have therefore verified the effects on H3K56ac using cells expressing a constitutively active form of Notch, NICD, under the control of a metallothienin promoter. Generating a new stable cell with this construct, we have been able to induce expression of target genes within 2 hours, and have now profiled the H3K56ac in these conditions. The results substantiate our previous findings with the EGTA treatment, demonstrating a significant increase in the histone modification at the 2 hour timepoint. These data have been

added to Figure. 5 and are discussed in the text (page 11, Table E2).

Minor points:
4. The mixed use of "Su(H)" and "CSL" is confusing. Please adhere to official FlyBase name.

We apologise for the confusion. We have adjusted the nomenclature as much as possible so that we only use Su(H) when referring to our own *Drosophila* results and to the *Drosophila* literature. However it is not appropriate to use that nomenclature when reporting results from mammalian systems, especially when referring to the published work of others. We have therefore retained the use of CSL when describing specific findings from mammalian studies. We hope that the usage is now less confusing.

5. ChIP tracks of Su(H), H3K56ac and others in Figs 1C, 2, 5, 6: please add y-axis scale and units.

Scales had been stated in the legends. They have now been added to the tracks also.

2nd Editorial Decision 26 March 2015

Thank you for submitting a revised version of your manuscript to The EMBO Journal and my apologies for the slight delay in our response to you. Your study has now been seen by the two original referees and their comments are shown below.

As you will see both referees find that the main criticisms have been addressed; however, they still both raise a few points regarding state definition and data presentation that will have to be discussed/clarified further before we can officially accept your manuscript for publication here. I will therefore invite you to submit a final revision in which you address the remaining points brought up by the referees.

In addition, and upon re-reading the revised manuscript, I noticed that the reduced initial focus on the new chromatin states leaves the origin and basis for the 11 state model a little unclear. For the sake of the general reader, I would therefore encourage you to include a few more sentences on the modelling work in the initial results section.

Please feel free to contact me with any questions concerning these points.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your final revision.

Referee #1:

The authors performed additional experiments following the editor and reviewers' suggestions and the manuscript has been improved. Some concerns remain:

1) The "leave out" analysis (Fig. E1F) is helpful in gauging the robustness of the 11 chromatin states and indicates that some chromatin states are determined or dominated by 1 or a few histone modifications, while other histone modifications contribute little to the chromatin states, e.g. without H3K9me1, 9 out of the 11 states are essentially unchanged and the other two states are at least 92% unchanged. Whether H3K9me1 contributes to any of the HMM-defined chromatin states is unclear, and by extension it remains questionable whether all 25 histone marks should be used to identify and define chromatin states. The limitations and potential caveats of overfitting need to be acknowledged more clearly in the text.

The caption to Fig. E1F is also confusing. Aren't the authors showing percent unchanged with perturbation, rather than % changed? This needs to be stated more clearly.

2) There is no call out in the text for Fig. 6 panels E-G, and there is a callout for Fig. 6H, which does not exist. There is also no label for panel 6G in Fig. 6, which is referred to at one point in the rebuttal letter mistakenly (the letter is actually referring to panel 3G). These errors are not helpful, but in piecing it together it appears that the new data do help to address the request for further analysis of changes in H3K56ac following Runx/Lz overexpression. But where is the genome-wide data that are referred to in the rebuttal letter? There are local data in Figure 3, presumably cherry-picked. How extensive or limited are these changes? Mention is made of 459 regions showing significant changes in H3K56ac following Lz expression in Kc cells, but how does this related to Su(H) bound regions and target genes more broadly. Some elaboration/clarification is needed.

3) The authors have performed additional experiments to firm up conclusions reached on the basis of experiments conducted with inhibitors, including a bit of work with siRNA approaches. Some uncertainties remain, but I am satisfied that the authors have been responsive and have done what is reasonably possible.

From my vantage point, once these lingering concerns are addressed, the paper will be suitable for publication in EMBO J.

Referee #3:

The manuscript has improved substantially, but I'm not entirely satisfied with the reply to my comments in point 2:

1. I understand from the rebuttal that there was no way around generating new state models. Indeed, in a quick literature search I could not find a pair of state models for BG3 and Kc cells that were generated with the same algorithm. The authors have shifted the emphasis in Results section towards the Su(H) analysis and I think that this is much better.

2. I'm happy to read that 3 independent replicates were done for the ChIP data. But my question "How reproducible were the results?" (particularly for Su(H)) is still not addressed in the manuscript. This could be done by showing some (supplementary) scatterplots (replicate 1 vs 2, etc), listing the number of overlapping peaks between replicates, etc.
I am also not satisfied by the hand-waving reply regarding the width of the Su(H) peaks, which is quite unusual for a DNA-binding factor. It could be technical, or a really interesting biological surprise! This must be discussed in the manuscript, not only in the rebuttal.
Furthermore, the authors argue in the rebuttal that they have done many Su(H) ChIP experiments in many different cell types under many conditions. This helps to boost confidence, but then it should be added to the manuscript; it is rather pointless to have this debate in the review process without seeing the outcome reflected in the manuscript.

3. OK.

4. OK.

5. OK.

2nd Revision - authors' response 03 May 2015

Reply to editors and reviewers comments

Editor’s comments;

In addition, and upon re-reading the revised manuscript, I noticed that the reduced initial focus on the new chromatin states leaves the origin and basis for the 11 state model a little unclear. For the sake of the general reader, I would therefore encourage you to include a few more sentences on the

modeling work in the initial results section.

We have added a few sentences as requested. These also discuss the issue of over-fitting as requested by reviewer 1.
"A single data matrix was therefore created, combining the H3K56ac and modENCODE ChIP data with DNase I accessibility, and then tied parameterisation was used to identify the maximum number of unique chromatin signatures that could be inferred before splitting a signature into two similar ones (see Extended view for more details). This strategy was used to minimize the risk of over-fitting, one potential drawback of this type of maximum likelihood HMM. The fact that we recovered similar signatures to those obtained through a more complex Bayesian model indicates the success of the strategy as did with results from a leave one out analysis, which demonstrates their robustness of the signatures (Fig. E1F)."

Reply to the reviewers' comments

Referee #1:

The authors performed additional experiments following the editor and reviewers' suggestions and the manuscript has been improved. Some concerns remain:

1) *The "leave out" analysis (Fig. E1F) is helpful in gauging the robustness of the 11 chromatin states and indicates that some chromatin states are determined or dominated by 1 or a few histone modifications, while other histone modifications contribute little to the chromatin states, e.g. without H3K9me1, 9 out of the 11 states are essentially unchanged and the other two states are at least 92% unchanged. Whether H3K9me1 contributes to any of the HMM-defined chromatin states is unclear, and by extension it remains questionable whether all 25 histone marks should be used to identify and define chromatin states. The limitations and potential caveats of overfitting need to be acknowledged more clearly in the text.*

We appreciate the concerns of the reviewer about the HMM, but we consider that our strategy has minimized the risks from over fitting. Specifically we tested with multiple iterations which number of unique signatures could be inferred before splitting a signature into two similar ones. We agree that it may not necessarily require all the histone modifications to generate these signatures, but we did not want to make assumptions beforehand so we opted to use all the available data. Indeed our model is more parsimonious than the metazoan state model generated by the Bayesian approach, which includes several closely related states. However, taking on board the reviewers concerns, we have acknowledged the potential caveats from over fitting in the text and have commented on the fact that some of the modifications contribute little to the signatures (Page 7).
"*A single data matrix was therefore created, combining the H3K56ac and modENCODE ChIP data with DNase I accessibility, and then tied parameterisation was used to identify the maximum number of unique chromatin signatures that could be inferred before splitting a signature into two similar ones (see Extended view for more details). This strategy was used to minimize the risk of over-fitting, one potential drawback of this type of maximum likelihood HMM. The fact that we recovered similar signatures to those obtained through a more complex Bayesian model indicates the success of the strategy as did results from a leave one out analysis, which demonstrates the robustness of the signatures (Fig. E1F). The latter also highlights that some histone modifications have more dominant roles, while others are less discriminatory for the chromatin signatures*."

The caption to Fig. E1F is also confusing. Aren't the authors showing percent unchanged with perturbation, rather than % changed? This needs to be stated more clearly.

We thank the reviewer for pointing this out, the legend has now been clarified "*Table showing how stable (% unchanged) the indicated chromatin signatures are to removing one of the data sets as in the top labels*. "

2) *There is no call out in the text for Fig. 6 panels E-G, and there is a callout for Fig. 6H, which does not exist. There is also no label for panel 6G in Fig. 6, which is referred to at one point in the rebuttal letter mistakenly (the letter is actually referring to panel 3G). These errors are not helpful, but in piecing it together it appears that the new data do help to address the request for further analysis of changes in H3K56ac following Runx/Lz overexpression. But where is the*

genome-wide data that are referred to in the rebuttal letter? There are local data in Figure 3, presumably cherry-picked. How extensive or limited are these changes? Mention is made of 459 regions showing significant changes in H3K56ac following Lz expression in Kc cells, but how does this related to Su(H) bound regions and target genes more broadly. Some elaboration/clarification is needed.

We apologise for the mistakes with the figure call-outs; these have now been corrected.
The genome-wide Lz data are uploaded in GEO and, as we stated in the results, a total of 459 regions with differential H3K56ac were identified. We have now added a pie chart, new Figure 3G, giving an overview of their relationship to Notch regulated genes and to previously characterized crystal cell genes. The enrichment for Notch regulated genes is highly significant (the others are also significantly enriched; p values are provided in the legend). We also summarize in the text some of the chromatin characteristics associated with the differential H3K56ac regions.

3) *The authors have performed additional experiments to firm up conclusions reached on the basis of experiments conducted with inhibitors, including a bit of work with siRNA approaches. Some uncertainties remain, but I am satisfied that the authors have been responsive and have done what is reasonably possible.*

We are glad the reviewer appreciates the additional experiments we have done to firm up the conclusions.

From my vantage point, once these lingering concerns are addressed, the paper will be suitable for publication in EMBO J.

Referee #3:

The manuscript has improved substantially, but I'm not entirely satisfied with the reply to my comments in point 2:

1. I understand from the rebuttal that there was no way around generating new state models. Indeed, in a quick literature search I could not find a pair of state models for BG3 and Kc cells that were generated with the same algorithm. The authors have shifted the emphasis in Results section towards the Su(H) analysis and I think that this is much better.

We are happy that the manuscript is improved by our revisions.

2. I'm happy to read that 3 independent replicates were done for the ChIP data. But my question "How reproducible were the results?" (particularly for Su(H)) is still not addressed in the manuscript. This could be done by showing some (supplementary) scatterplots (replicate 1 vs 2, etc), listing the number of overlapping peaks between replicates, etc.

First, we have shown in the results (Figure 4A) the overlap between the Su(H) ChIP peaks in BG3 cells before and after Notch, where 86% of peaks in the control are overlapping with peaks in the Notch activated experiment. This clearly demonstrates reproducibility between our data sets. In this context it is also important to note that we and others have shown that binding by Su(H) at many Notch-regulated enhancers is affected by the presence of NICD, with lower occupancy in the absence of Notch activity (e.g. Krejcí A, Bray S. (2007) Genes Dev. 21(11):1322-7; Wang et al, (2014). Proc Natl Acad Sci U S A. 111(2):705-10;). Thus it is not unexpected that there are relatively few bound regions detected in the control cells, we have added a sentence explaining this point in the results (page 8). *"Although relatively few peaks were identified in comparison to some other DNA binding proteins, the low number is consistent with the observation that CSL/Su(H) exhibits low occupancy in the absence of NICD at many Notch-regulated enhancers (Castel et al, 2013; Housden et al, 2013; Krejci & Bray, 2007; Wang et al, 2014) and the data were consistent across replicates (Fig E3A)."*
Second, we call peaks on the median of the replicates, not on individual replicates. Using the median ensures that the data are not skewed by region in a single replicate that is very high or low and we use a stringent threshold for peak calling (FDR 1%). The method uses a sliding window and for every window (600bp) the median signal of the values from all the probes in that region and from all the replicates is calculated. This approach smoothens the data across the genome and between

replicates simultaneously. The details are provided in the methods. To illustrate the reproducibility between replicates we have therefore added a Supplementary Figure showing the individual replicates along with the processed data (Ringo) and the peaks called from several genomic regions (Fig E3A).

I am also not satisfied by the hand-waving reply regarding the width of the Su(H) peaks, which is quite unusual for a DNA-binding factor. It could be technical, or a really interesting biological surprise! This must be discussed in the manuscript, not only in the rebuttal.

We apologise if our response seemed to be hand-waving. We are happy to discuss the peak widths, but they are not substantially different from other data-sets. In addition, as peak widths vary depending on the peak-calling algorithms used, they need to be taken with caution. The region in the *E(spl)* locus has three very close peaks that are sometimes called as a single peak, but elsewhere the average is circa 500bp. We have added a graph to the supplementary figures showing these data (Fig. E3B). We have added a sentence to the results stating that the median peak widths are ~500bp (page 8). "*In both cell types the mean peak width was similar (circa 500bp; Fig. E3B), but occasionally, in regions of high occupancy at the E(spl) locus, these peaks overlapped to generate a super-peak of several kb*."

Furthermore, the authors argue in the rebuttal that they have done many Su(H) ChIP experiments in many different cell types under many conditions. This helps to boost confidence, but then it should be added to the manuscript; it is rather pointless to have this debate in the review process without seeing the outcome reflected in the manuscript.

Several Su(H) ChIPs from other cell types, notably DmD8 cells, have already been published by others and us (Krejci et al, 2009 Sci Signal. 2009 Jan 27;2(55):ra1; Housden et al 2013, PLoS Genet. 2013;9(1):e1003162; Terriente-Felix et al, (2013) Development. 140(4):926-3). We note that we use well described analytical methods for the ChIP-array analysis that have been used in many published papers. We consider that other data are not relevant here since we have no chromatin maps to relate them to. As mentioned before, we do make a direct comparison between control and Notch activated for the BG3 cells, which show a very good relationship. We also note that there is significant overlap between the BG3 and Kc data from independently performed experiments.

3. OK.
4. OK.
5. OK.

