Manuscript EMBO-2017-96525

RBPJ/CBF1 interacts with L3MBTL3/MBT1 to promote repression of Notch signaling via histone demethylase KDM1A/LSD1

Tao Xu, Sung-Soo Park, Benedetto Daniele Giaimo, Daniel Hall, Francesca Ferrante, Diana M. Ho, Kazuya Hori, Lucas Anhezini, Iris Ertl, Marek Bartkuhn, Honglai Zhang, Eléna Milon, Kimberly Ha, Kevin P. Conlon, Rork Kuick, Brandon Govindarajoo, Yang Zhang, Yuqing Sun, Yali Dou, Venkatesha Basrur, Kojo S. J. Elenitoba-Johnson, Alexey I. Nesvizhskii, Julian Ceron, Cheng-Yu Lee, Tilman Borggrefe, Rhett A. Kovall & Jean-François Rual

Corresponding author: Jean-Francois Rual, University of Michigan

Review timeline:		
	Submission date:	13 January 2017
	Editorial Decision:	03 March 2017
	Revision received:	27 June 2017
	Editorial Decision:	07 August 2017
	Correspondence:	22 August 2017
	Revision received:	31 August 2017
	Accepted:	12 September 2017

Editor: Ieva Gailite

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   03 March 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received four referee reports on your manuscript, which I am copying below for your information.

As you can see from the comments, all three referees express interest in the work and the topic. However, they also raise several concerns that need to be addressed in order to consider publication here. I would like to invite you to submit your revised manuscript while addressing the comments of all referees, and focusing in particular on the following points:

- Include further description and characterisation of full mass-spectrometry and ChIP-seq data, as requested by referees #1 (point 1) and #4 (point 1)
- Address in more detail the physiological relevance of L3MBTL3/RBPJ interaction, as requested by referees #1, #2 (point 1) and #4 (point 6)
- Include genome-wide gene expression analysis of Notch versus total L3MBTL3 target genes upon L3MBTL3 loss (referee #4, point 2)

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. Please contact us in advance if you would need an additional extension. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work to discuss how to proceed.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

Xu et al. have used a range of complementary approaches to implicate L3MBTL3 in the transcriptional repression activity of RBPJ, specifically via a model in which L3MBTL3 serves to promote the association of RBPJ with KDM1A (LSD1), a histone demethylase. L3MBTL3 was initially identified as a RBPJ interacting factor through complementary yeast-2-hybrid and mass spectroscopic proteomic screens. The specificity of the interaction is bolstered by co-ip and GST-pulldown assays, as well as ITC experiments in a purified system. L3MBTL3 binds RBPJ (at least in part) via an N-terminal region with low micromolar affinity, substantially weaker than that with which NICD binds RBPJ, suggesting that NICD can outcompete L3MBTL3 for RBPJ following Notch activation, an idea in line with binding competition experiments. The experiments describing the discovery of L3MBTL3 as a RBPJ interacting factor, as well as its competition with NICD for binding to RBPJ, are straightforward and convincing. The remainder of the paper is focused on testing various predictions of the above model in cells and in model animals, specifically worms and flies. Overall, these data support the model and the idea that RBPJ is linked to KDM1A, at least in part, through its interaction with L3MBTL3.

What is left unclear is how important L3MBT3 in the grand scheme of Notch signaling. The germ line L3MBT3 knockout is an embryonic lethal, but the phenotype (failure of hematopoiesis) is not particularly "Notch-like". No CNS phenotype is mentioned, which might be expected if L3MBTL3 had a role in development of the cerebellum, a point relevant to the proposed link to medulloblastoma (discussed below). My overall sense is that the paper does merit publication after modification, as I agree with the authors' central premise: that L3MBTL3 is a novel, conserved RBPJ binding protein that contributes to RBPJ-dependent gene repression, and that at the end of the day it is likely to be important in modulation of Notch signaling tone under physiologic conditions, at least in some cellular contexts. More work will be needed to confirm this supposition; the current paper set the stage for such work.

Comments
1. A relative weakness is that RBPJ/L3MBTL3 colocalization in mammalian cell line genomes is confined to a relatively few, cherry-picked sites in gene promoters. This criticism is mitigated by the apparent overlap of Su(H) and dL(3)mbt binding in Drosophila genomes (Fig. 6b); however, these data are derived from 2 different cellular contexts (Kc167 cells and 3rd instar larval discs, respectively), and at a glance the ChIP-seq landscapes for both of these data sets appears to be pretty noisy (Fig. S9). The overall between data sets is described as "remarkable", but a more rigorous analysis, with statistic measures of significance, should be provided. Also, presumably de novo motif analysis of the Su(H) ChIP-Seq data would "discover" the Su(H)/RBPJ DNA binding motif; if a similar analysis is done with the dL(3)mbt binding regions, is the Su(H)/RBPJ motif also found? What about other motifs that could point to functions that are independent of Su(H)/RBPJ?

2. It is unclear on what basis the MT hybridoma cells are described as having "moderate" versus "high" signaling tone; presumably the point of comparison is a pre-T cell line like Jeko (also

described in the Oswald paper) or other lines with gain of function Notch mutations and "high" signaling tone. Were lines with high levels of activated Notch also tested? A prediction of the model is that in the presence of high levels of NICD, L3MBTL3 knockdown/knockout would have little effect due to swamping out of its activity.

3. In the discussion, the prior data linking L3MBTL3 to cancer, specifically medulloblastoma, is weak. To the best of this reviewer's knowledge, gain of function mutations in Notch receptors have never been described in medulloblastoma, and a review of genomic sequencing compendia (e.g., TumorPortal) shows a few loss of function mutations in L3MBTL3 in diverse cancers, which doesn't constitute a smoking gun. This situation differs from some other RBPJ binding factors, such as SPEN, which is recurrently mutated in certain cancers. The paragraph in question has a pro forma flavor and doesn't really add much to the paper. Unless more compelling data can be provided, it can be deleted.

Minor Comments
1. There is some imprecision of language that can be improved. For example, U87-MG cells are described as having "poorly active" Notch signaling; low levels or low signaling tone would seem more appropriate, given the data in figure S2. On page 5, it is stated that RBPJ and L3MBT3 co-localize on enhancers adjacent to HES1, HES4, HEY1 and HEY2; by convention, promoters are usually taken to lie +/- 2kb from transcription starts sites, and so all of the sites shown in figure 4 are promoter sites. These could be more accurately described here and elsewhere as promoter proximal Notch regulatory elements, or something to this effect.

Referee #2:

This paper reports on the identification of the L3MBTL3 protein, a methyl-lysine reader, as a critical molecular link between the RBPJ and KDM1A proteins, with KDM1A (a histone demethylase) being involved in RBPJ-dependent repression of transcription. While potentially interesting, there are some key issues that need to be addressed to make the paper of interest to a general audience :

1) It is important to assess whether endogenous RBPJ, L3MBTL3 and KDM1A - as opposed to one or more over-expressed proteins - bind to each other in biologically relevant cellular systems, such as the medulloblastoma cells mentioned at the end.

2) The mutually exclusive association of RBPJ with NIC versus L3MBTL3 needs to be validated in physiologically-relevant settings, with cells plus/minus activation of endogenous Notch signaling.

3) The relative levels of endogenous RBPJ, L3MBTL3 and KDM1A proteins in various cell types needs to be assessed, determining to which extent, in these various contexts, the RBPJ transcription repressive function is L3MBTL3- and/or KDM1A-dependent.

Referee #3:

This is a thorough and carefully executed study that makes a valuable contribution to our understanding of transcriptional repression by the Notch pathway-regulated CSL factors. The authors show convincingly that the mammalian L3MBTL3 protein interacts directly with the Notch TF RBPJ and recruits the KDM1A demethylase to repress Notch target genes. They go on to provide evidence that the physical and functional interaction of L3MBT and CSL also operates in Drosophila and C. elegans, indicating that this mechanism is deeply conserved evolutionarily. Following are several comments for the authors to consider.

(1) The MS repeatedly refers to amino acids 1-64 of L3MBTL3 as the "domain" that mediates direct binding between this protein and RBPJ. However, a stretch of amino acids found to be required for a particular protein-protein interaction (necessity) is not necessarily the same as the domain responsible for this interaction (sufficiency). Fig. 3a shows that aa 1-197 are sufficient, but this fragment is over three times the size of the aa 1-64 segment.

(2) The authors should comment on the ability of L3MBTL3-Δ(1-64) to partially rescue recruitment

of KDM1A to Notch target genes (Fig. 5c).

(3) Despite its direct relevance to the work reported here, the authors do not discuss or cite the prior literature on recruitment by the Drosophila Su(H)/H complex of chromatin-modifying complexes that include the LID demethylase (Goodfellow 2007, Moshkin 2009). Notably, the Moshkin paper reported that L3MBT is recovered by immunopurification of PF1, a component of both the LAF and RLAF complexes.

(4) The authors are strongly encouraged to move away from the use of the "RBPJ" designation for vertebrate CSLs. It's been known for over 20 years that the name "recombination signal-binding protein Jκ" reflects an experimental artefact [Henkel et al. (1994) Science 265: 92-95]. I urge the use of "CBF1" instead.

Referee #4:

Comments:

The authors of this manuscript identified L3MBTL3 as an RBPJ interacting protein using a yeast two-hybrid assay. After claiming confirmation using IP-MS, they report a series of experiments consistent with the interpretation that L3MBTL3 acts as a repressor of enhancer loci associated with RBPJ. Ultimately, what the authors are trying to claim is that the functional interaction (manifest as gene derepression, fly phenotype, etc.) of RBPJ with L3MBTL3 is a consequence of the binding interaction they have detected, which is relatively weak compared with NICD and other co-repressors known to bind at that site. The work is thorough, and the proposed role of L3MBTL3 is intriguing, but it is not clear from the data presented that the L3MBTL3 effect on transcription is specific to RBPJ-loaded genomic sites, and thus whether the functional connection is due to a direct binding interaction between L3MBTL3 and RBPJ.

Major points:

1) The authors report in the text that immunoprecipitation of HA-tagged RBPJ indeed recovers RBPJ and also results in detection of L3MBTL3 peptides (6 and 17 peptides in two IPs) upon mass spec analysis. HA-tagged L3MBTL3 likewise recovers itself and three RBPJ peptides. A widely accepted standard in the MS field is to provide the complete list of proteins identified in the IP, together with their abundance (number of unique peptides, peptide abundance, etc.) in the manuscript. Otherwise, how is the reader able to assess the specificity of the recovery of L3MBTL3 with RBPJ in cells? This information would also clarify whether KDM1A is found in association with L3MBTL3 in the unbiased MS experiment.

2) What happens globally to the transcriptome in response to L3MBTL3 withdrawal? Is the de-repression of transcription restricted to RBPJ-bound sites? Is it most evident at those sites? Or is the phenomenon of de-repression of expression more general across the transcriptome? A comparison of the transcriptomes in RBPJ and L3MBTL3 KO U87 lines (and ideally in a DKO) could clarify whether the apparent functional connection is specific, or if the effect of L3MBTL3 is more general (and thus detectable as a non-specific effect in the subset of sites bound by RBPJ).

3) The biochemistry of the protein-protein interaction is well documented (the mutational and competition studies presumably point to the RAM binding site as the site of L3MBTL3 binding), but affinity is 1-2 orders of magnitude lower than for NICD or some of the known co-repressors such as MINT, KyoT2, etc. How do the authors imagine that L3MBTL3 competes in the face of higher affinity competitors, especially if the other co-Rs are present in comparable abundance?

4) The idea that the RBPJ-binding region at the N-terminal end of the mammalian protein is positioned in an internal site (no longer N-terminal, and in a different part of the protein's domain organization) in the Drosophila protein is quite remarkable for a protein that otherwise has a completely conserved domain organization (and is a true ortholog). How do the authors imagine that the domains carry out a comparable function in the context of reorganized domain placement - and are there well-documented examples of such reshuffling from other similarly orthologous proteins?

It is unclear how the statistical analysis in SF 8a was carried out, but it seems that a pairwise statistical test, if used, would not be the right metric.

5) Some key statistical analyses are lacking - particularly with respect to the overlap of L3MBTL3 binding sites and RBPJ binding sites in Figure 6B. Is the overlap (which occurs at 10% of L3MBTL3 binding sites and fewer than 20% of the RBPJ sites) different for RBPJ than for other signal-dependent TFs?

6) Ultimately, what the authors are trying to claim is that the functional interaction (manifest as gene derepression, fly phenotype, etc.) of RBPJ with L3MBTL3 is a consequence of the binding interaction they have detected. Is there a synthetic phenotype between L3MBTL3 and RBPJ in a cellular or in vivo context? Such a true genetic interaction, if it exists, would make the claim that the direct binding interaction is of functional importance so much more compelling.

Minor comments:

1) dICD in 6B (according to the SI) corresponds to dRAM-ANK. Since the band is smaller than predicted for dNICD, this detail should be more explicitly stated, otherwise the reader will be confused.

2) Thermodynamic, not thermodynamical.

1st Revision - authors' response	27 June 2017

RESPONSE TO REFEREES' COMMENTS
We greatly appreciate the thoughtful comments and suggestions of the Referees and have revised the manuscript accordingly. The Referees' comments have been pasted one by one below (*italics*), followed by our responses (non-italics, blue font).

Referee #1:
Please note that, as requested by Referee #3, the "RBPJ" designation has been replaced by "CBF1" throughout the new manuscript as well as in our response to the referees.

Xu et al. have used a range of complementary approaches to implicate L3MBTL3 in the transcriptional repression activity of RBPJ, specifically via a model in which L3MBTL3 serves to promote the association of RBPJ with KDM1A (LSD1), a histone demethylase. L3MBTL3 was initially identified as a RBPJ interacting factor through complementary yeast-2-hybrid and mass spectroscopic proteomic screens. The specificity of the interaction is bolstered by co-ip and GST-pulldown assays, as well as ITC experiments in a purified system. L3MBTL3 binds RBPJ (at least in part) via an N-terminal region with low micromolar affinity, substantially weaker than that with which NICD binds RBPJ, suggesting that NICD can outcompete L3MBTL3 for RBPJ following Notch activation, an idea in line with binding competition experiments. The experiments describing the discovery of L3MBTL3 as a RBPJ interacting factor, as well as its competition with NICD for binding to RBPJ, are straightforward and convincing. The remainder of the paper is focused on testing various predictions of the above model in cells and in model animals, specifically worms and flies. Overall, these data support the model and the idea that RBPJ is linked to KDM1A, at least in part, through its interaction with L3MBTL3.

What is left unclear is how important L3MBT3 in the grand scheme of Notch signaling. The germ line L3MBT3 knockout is an embryonic lethal, but the phenotype (failure of hematopoiesis) is not particularly "Notch-like". No CNS phenotype is mentioned, which might be expected if L3MBTL3 had a role in development of the cerebellum, a point relevant to the proposed link to medulloblastoma (discussed below). My overall sense is that the paper does merit publication after modification, as I agree with the authors' central premise: that L3MBTL3 is a novel, conserved RBPJ binding protein that contributes to RBPJ-dependent gene repression, and that at the end of the day it is likely to be important in modulation of Notch signaling tone under physiologic conditions, at least in some cellular contexts. More work will be needed to confirm this supposition; the current paper set the stage for such work.

We thank Referee #1 for the nice appreciation of our work and its importance to the Notch field. We agree that assessing the potential link between the phenotype observed in *L3mbtl3* KO mouse embryos (Arai & Miyazaki, 2005) and the extent to which this phenotype is dependent on the regulation of CBF1 target genes, or not, is an important question that remains to be addressed. Our discovery indeed sets the stage for such complementary studies, which are currently ongoing in the Rual lab (study of the role of L3MBTL3 *in vivo* and its CBF1-dependence using a novel conditional *L3mbtl3* KO "floxed" mouse) and the Kovall lab (L3MBTL3/CBF1 structure analyses by X-ray crystallography).

Comments
1. A relative weakness is that RBPJ/L3MBTL3 colocalization in mammalian cell line genomes is confined to a relatively few, cherry-picked sites in gene promoters. This criticism is mitigated by the apparent overlap of Su(H) and dL(3)mbt binding in Drosophila genomes (Fig. 6b); however, these data are derived from 2 different cellular contexts (Kc167 cells and 3rd instar larval discs, respectively), and at a glance the ChIP-seq landscapes for both of these data sets appears to be pretty noisy (Fig. S9). The overall between data sets is described as "remarkable", but a more rigorous analysis, with statistic measures of significance, should be provided. Also, presumably de novo motif analysis of the Su(H) ChIP-Seq data would "discover" the Su(H)/RBPJ DNA binding motif; if a similar analysis is done with the dL(3)mbt binding regions, is the Su(H)/RBPJ motif also found? What about other motifs that could point to functions that are independent of Su(H)/RBPJ?

New ChIP-seq analyses of CBF1 and L3MBTL3 in the mammalian MDA-MB-231 cell line
The role of L3MBTL3 as a negative regulator of Notch target genes has been assessed extensively in both the U87-MG (Fig 4) and MDA-MB-231 (Appendix Fig S4) mammalian cell lines. To address the concern of Referee #1 regarding our CBF1/L3MBTL3 co-localization analysis in mammalian cells, we performed ChIP-seq experiments using the CBF1 and L3MBTL3 antibodies to investigate on a genome-wide scale the colocalization of the endogenous CBF1 and L3MBTL3 proteins on chromatin in MDA-MB-231 cells. The CBF1 and L3MBTL3 ChIP-seq experiments in MDA-MB-231 cells identified 2926 CBF1 binding sites associated with 2937 putative CBF1-bound genes and 444 L3MBTL3 binding sites associated with 411 putative L3MBTL3-bound genes, respectively. The 2937 CBF1-bound genes are enriched for genes associated with both the GO pathway terms "Notch pathway genes" (P = 7 x 10-12) and "Notch-mediated HES/HEY network" (P = 5 x 10-7), supporting the quality of the CBF1 ChIP-seq data set. Importantly, the 411 L3MBTL3-bound genes are also enriched for genes associated with both "Notch pathway genes" (P = 4 x 10-4) and "Notch-mediated HES/HEY network" (P = 6 x 10-5). Actually, these two GO pathway terms are the only ones (out of 1320 GO pathway terms tested) to show a significant enrichment in the set of 411 L3MBTL3-bound genes. This observation supports the hypothesis that L3MBTL3 specifically regulates the expression of Notch target genes in this cell line.

Overlap analyses of the CBF1 and L3MBTL3 ChIP-seq data sets revealed a substantial and significant genome-wide co-localization of the two proteins on chromatin. Indeed, 133 sites are bound by both CBF1 and L3MBTL3, i.e., 4.5% of the 2926 CBF1 binding sites and 30% of the 444 L3MBTL3 binding sites. Assuming that there are 50000 possible binding sites in the genome (a conservative assumption), this overlap is 5 times larger than expected; P = 2.4 x 10-57; two-sided Fisher Exact test; new Appendix Fig S4D). We note that as one assumes a higher number of possible binding sites, the significance of observing a larger than expected overlap becomes greater. Similarly, 252 genes are bound by both proteins, i.e., 8.6% of the 2937 CBF1-bound genes and 61% of the 411 L3MBTL3-bound genes. Given that there are ~20000 genes in the human genome, this overlap is 4 times larger than expected (P = 3.6 x 10-107; two-sided Fisher Exact test; new Appendix Fig S4D). Thus, in agreement with the observation that L3MBTL3's ability to co-localize with CBF1 on chromatin at the promoters of Notch target genes is dependent on both CBF1 (Fig 4D and Appendix Fig S4G) and the CBF1 interaction domain L3MBTL3-(1-64) (Fig 4F and Appendix Fig S4I), the fact that a large portion of the L3MBTL3 binding sites (30%) or L3MBTL3-bound genes (61%) are also bound by CBF1 supports the hypothesis that the recruitment of L3MBTL3 to chromatin is, for the most part, dependent on CBF1.

Additionally, we performed a CBF1 DNA binding motif enrichment analysis for the 2926 CBF1 binding sites and the 444 L3MBTL3 binding sites identified in our ChIP-seq analyses, as well as for the 133 overlapping sites. In brief, the analysis was performed by using the MEME-ChIP tool in the

MEME Suite (version 4.11.4) (Machanick & Bailey, 2011) to scan the DNA sequences corresponding to the CBF1 and L3MBTL3 peak intervals (100 bp). As expected, the consensus CBF1 DNA binding motif is enriched in the 2926 CBF1 binding sites (18% of the 2926 CBF1 binding sites contain a consensus CBF1 DNA binding motif; P = 3 x 10-63), further validating the quality of our CBF1 ChIP-seq dataset. In spite of the significant overlap observed between the CBF1 and L3MBTL3 binding sites, such a significant CBF1 DNA binding motif enrichment is not observed for the 444 L3MBTL3 binding sites (11% of the 444 L3MBTL3 binding sites contain a consensus CBF1 DNA binding motif). For the 133 overlapping sites bound by both CBF1 and L3MBTL3, we observed a significant enrichment for the CBF1 DNA binding motif (22%; P = 4 x 10-23). The lack of CBF1 DNA binding motif enrichment in the 444 L3MBTL3 binding sites could be due to the fact that L3MBTL3 may be recruited on chromatin by other factors, independently of CBF1.

The ChIP-Seq data have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE100375: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100375. Token for confidential access by peer-reviewers: sdoroomwnhehrub

Statistical analysis of the overlap between the Su(H) and the dL(3)mbt binding sites:
As suggested by Referee #1, we performed a statistical assessment of the overlap observed between the Su(H) and the dL(3)mbt binding sites. In addition, we performed an overlap analysis for the genes predicted to be bound by Su(H) and dL(3)mbt. The ChIP experiments identified 1764 Su(H) binding sites associated with 896 putative Su(H)-bound genes (GSE68614) and 2775 dL(3)mbt binding sites associated with 2380 putative dL(3)mbt-bound genes (GSE62904). There are 251 sites bound by both Su(H) and dL(3)mbt, i.e., 14.2% of the 1764 Su(H) binding sites and 9% of the 2775 dL(3)mbt binding sites. Assuming that there are 50000 possible binding sites in the genome (a conservative assumption), the overlap is 2.5 times larger than expected (P = 2 x 10-43; two-sided Fisher Exact test). We note that as one assumes a higher number of possible binding sites, the significance of observing a larger than expected overlap becomes greater. We also note that with the assumption of an even smaller number of possible binding sites, i.e., 25000 (a very conservative assumption), the P value remains significant (P = 2 x 10-5). There are 256 genes bound by both Su(H) and dL(3)mbt, i.e., 28.6% of the 896 Su(H)-bound genes and 10.8% of the 2380 dL(3)mbt-bound genes. Given that there are ~17000 genes in the *Drosophila* genome, this overlap is 2 times larger than expected (P = 1 x 10-31; two-sided Fisher Exact test). The results of these statistical analyses and a new Fig 6B showing the overlaps for both the binding sites and the bound genes have been added to the manuscript.

Su(H) DNA binding motif enrichment analysis:
We performed a Su(H) DNA binding motif enrichment analysis for the sites identified in GSE68614 [Su(H)] and GSE62904 [dL(3)mbt], as well as for the overlapping sites. In brief, we used the PWMEnrich package (Stojnic & Diez, 2014) to scan the DNA sequences corresponding to the Su(H) and the dL(3)mbt peak intervals for enrichment of known position-specific scoring matrices obtained from multiple databases. As expected, the Su(H) DNA binding motif is enriched in the 1764 Su(H) binding sites identified in GSE68614 (22.5% of the 1764 Su(H) binding sites contain a consensus Su(H) DNA binding motif; P = 2 x 10-51), validating the quality of the GSE68614 data set. Such a significant enrichment is not observed for the 2775 dL(3)mbt binding sites identified in GSE62904 (11.1%; P = 0.16). For the 251 sites bound by both Su(H) and dL(3)mbt, we observed a significant enrichment for the Su(H) DNA binding motif (28.2%; P = 2 x 10-12). As rightly inferred by Referee #1, it is probable that Su(H) is not the only protein interacting with dL(3)mbt and that dL(3)mbt is recruited on chromatin by other factors, independently of Su(H). These Su(H)-independent mechanisms could contribute a significant fraction of the dL(3)mbt binding sites identified in GSE62904, a potential reason for the absence of a significant enrichment for the Su(H) DNA binding motif across all 2775 dL(3)mbt binding sites. For example, our motif enrichment analysis revealed potential links to other transcription factors, e.g. Mad (31.9%; P = 2 x 10-180), underscoring the fact that dL(3)mbt, as a transcription co-regulator, may be used iteratively by other factors on chromatin. If verified, this observation would not be surprising as many other transcriptional coregulators collaborate with more than one transcription factor. In our opinion, the fact that dL(3)mbt could collaborate with other transcription factors is not incompatible with the hypothesis that Su(H) specifically recruits dL(3)mbt at Notch target genes.

ChIP snapshots shown in Appendix Fig S9
According to Referee #1, the snapshots shown in the original Appendix Fig S9 suggest that the GSE68614 [Su(H); ChIP-chip] and GSE62904 [dL(3)mbt; ChIP-seq] data sets may be noisy. In our opinion, the quality of these two data sets is reasonable in comparison to the one observed for ChIP-chip data sets obtained for other *Drosophila* transcription factors with direct DNA binding ability or to the one observed for ChIP-seq data sets obtained for other transcriptional co-regulators that are indirectly recruited to chromatin. As already mentioned above, the Su(H) DNA binding motif is enriched in the 1764 Su(H) binding sites identified in GSE68614 (P = 2 x 10-51), validating the quality of the GSE68614 data set. Accordingly, in Fig 6C, we observe specific, overlapping peaks at the *dNotch* locus. The fact that the snapshot shown in the original Appendix Fig S9A focused on a relatively small region of the *E(spl)* locus that is highly enriched for Su(H) binding sites was, admittedly, misleading. In the new Appendix Fig S9A, we now show a zoomed-out view of the *E(spl)* locus where the difference between specific binding signals and background can be better appreciated.

Altogether, the analyses of the *Drosophila* Su(H) and the dL(3)mbt ChIP data are in line with the analyses of our new ChIP-seq data for CBF1 and L3MBTL3 in the mammalian MDA-MB-231 cell line, i.e., a substantial and significant genome-wide co-localization of CBF1/Su(H) and L3MBTL3/dL(3)mbt on chromatin is observed. Furthermore, we want to note that the specific recruitment of L3MBTL3/dL(3)mbt by CBF1/Su(H) to Notch target genes is corroborated, beyond the analysis of these genome-wide ChIP analyses, by several, independent lines of evidence (molecular interaction, Notch target gene expression analyses, ChIP-qPCR for L3MBTL3 in presence or absence of CBF1, etc) both in *Drosophila* and in mammalian cells.

2. It is unclear on what basis the MT hybridoma cells are described as having "moderate" versus "high" signaling tone; presumably the point of comparison is a pre-T cell line like Jeko (also described in the Oswald paper) or other lines with gain of function Notch mutations and "high" signaling tone. Were lines with high levels of activated Notch also tested? A prediction of the model is that in the presence of high levels of NICD, L3MBTL3 knockdown/knockout would have little effect due to swamping out of its activity.

We have analyzed the level of expression of the cleaved NOTCH1 intracellular domain (NICD1) in whole cell extracts from both Beko, a mouse pre–T cell line that is characterized by constitutively active Notch signaling (Liefke *et al*, 2010) and the mouse hybridoma mature T-cells (MT) by Western blot (new Appendix Fig S5A). We observed that cleaved NICD1 is present in Beko cells but is not detectable in MT cells, suggesting that the Notch pathway is active in Beko but not in MT cells. Additionally, we have performed an expression analysis of Notch target genes upon *L3mbtl3* knockdown in Beko cells. In agreement with our model, we observed that the *Hey1* and *Hes1* Notch target genes are not de-repressed upon *L3mbtl3* knockdown in this mouse pre-T cell line that is characterized by high level of cleaved NOTCH1 ICD (new Appendix Fig S5F).

3. In the discussion, the prior data linking L3MBTL3 to cancer, specifically medulloblastoma, is weak. To the best of this reviewer's knowledge, gain of function mutations in Notch receptors have never been described in medulloblastoma, and a review of genomic sequencing compendia (e.g., TumorPortal) shows a few loss of function mutations in L3MBTL3 in diverse cancers, which doesn't constitute a smoking gun. This situation differs from some other RBPJ binding factors, such as SPEN, which is recurrently mutated in certain cancers. The paragraph in question has a pro forma flavor and doesn't really add much to the paper. Unless more compelling data can be provided, it can be deleted.

We have deleted this paragraph in the new manuscript, as suggested by Referee #1.

Minor Comments
1. There is some imprecision of language that can be improved. For example, U87-MG cells are described as having "poorly active" Notch signaling; low levels or low signaling tone would seem more appropriate, given the data in figure S2. On page 5, it is stated that RBPJ and L3MBT3 co-localize on enhancers adjacent to HES1, HES4, HEY1 and HEY2; by convention, promoters are usually taken to lie +/- 2kb from transcriptional starts sites, and so all of the sites shown in figure 4 are promoter sites. These could be more accurately described here and elsewhere as promoter

proximal Notch regulatory elements, or something to this effect.

We thank Referee #1 for the suggestions. We replaced "Notch signaling is poorly active" with "Notch signaling tone is low" in the new manuscript. Additionally, the CBF1-bound genomic sites used in the various ChIP experiments are now referred to as "proximal Notch-responsive elements" throughout the manuscript.

Referee #2:

Please note that, as requested by Referee #3, the "RBPJ" designation has been replaced by "CBF1" throughout the new manuscript as well as in our response to the referees.

This paper reports on the identification of the L3MBTL3 protein, a methyl-lysine reader, as a critical molecular link between the RBPJ and KDM1A proteins, with KDM1A (a histone demethylase) being involved in RBPJdependent repression of transcription. While potentially interesting, there are some key issues that need to be addressed to make the paper of interest to a general audience :

1) It is important to assess whether endogenous RBPJ, L3MBTL3 and KDM1A - as opposed to one or more over-expressed proteins - bind to each other in biologically relevant cellular systems, such as the medulloblastoma cells mentioned at the end.

As suggested by Referee #2, we performed IP of endogenous CBF1 in U87-MG or MDA-MB-231 cells followed by Western blot analyses using KDM1A, L3MBTL3 or CBF1 antibody (new Appendix Fig S6B). We observed that endogenous CBF1 interacts with both endogenous KDM1A and endogenous L3MBTL3.

The DAOY medulloblastoma cell line we mentioned at the end of the original manuscript is characterized by homozygous deletion of the *L3MBTL3* gene (Northcott *et al*, 2009). Therefore, the interaction between the endogenous CBF1, L3MBTL3 and KDM1A proteins could not be tested in this medulloblastoma cell line. Moreover, we have deleted the paragraph about medulloblastoma in the new manuscript, as suggested by Referee #1.

2) The mutually exclusive association of RBPJ with NIC versus L3MBTL3 needs to be validated in physiologically-relevant settings, with cells plus/minus activation of endogenous Notch signaling.

The existence of a direct molecular interaction between CBF1 and L3MBTL3 is supported by multiple observations, e.g., by Y2H (Fig 1A) and by ITC (Fig 3A). The hypothesis that NOTCH1 ICD and L3MBTL3 compete for binding to CBF1 was first inferred by the fact that both proteins bind the same interaction interface in the CBF1's BTD domain (Fig. 3E and Yuan *et al*, 2012). The hypothesis was validated by competition assays showing that NOTCH1 ICD outcompetes L3MBTL3 for binding to CBF1 (Fig. 3B). Moreover, in agreement with our Kd measurements, which suggest that NOTCH1 ICD has a significantly higher affinity (Fig 3A), the opposite is not observed, i.e., L3MBTL3 does not outcompete NOTCH1 ICD for binding to CBF1 (Fig 3C). As mentioned above in our answer to Comment #1, we performed IP of endogenous proteins and we observed that endogenous CBF1 interacts with endogenous L3MBTL3 in U87-MG or MDA-MB-231 cells (new Appendix Fig S6B). Upon EDTA treatment, which induces NOTCH ICD cleavage (Rand *et al*, 2000), we could not induce the expression of endogenous, cleaved NOTCH ICD proteins in U87-MG or MDA-MB-231 cells to levels high enough to compete with endogenous L3MBTL3. The absence of a detectable effect in the competition IP assay using endogenous proteins could be due to technical reasons (e.g., low level of expression of the full-length NOTCH receptors, partial activation or fast degradation of the cleaved NOTCH ICD proteins) and to the stoichiometric balance between the endogenous CBF1, L3MBTL3 and NOTCH ICD proteins in the U87-MG or MDA-MB-231 cells. Indeed, if endogenous CBF1 proteins are in large excess in comparison to the endogenous L3MBTL3 and NOTCH ICD proteins, then competition would be limited and both CBF1/L3MBTL3 and CBF1/NOTCH ICD protein complexes could co-occur.

3) The relative levels of endogenous RBPJ, L3MBTL3 and KDM1A proteins in various cell types needs to be assessed, determining to which extent, in these various contexts, the RBPJ transcription

repressive function is L3MBTL3- and/or KDM1A-dependent.
In the new Appendix Fig S6B (see input controls), the levels of expression of endogenous CBF1, L3MBTL3 and KDM1A were analyzed in both the U87-MG and MDA-MB-231 cell lines. The levels of expression of endogenous CBF1, L3MBTL3 and KDM1A were also investigated in other experiments throughout the manuscript: CBF1 in U87-MG cells (Fig 1C, 4A, 5A, S3E, S6B, S6C and S6E), CBF1 in MDA-MB-231 cells (Fig S4H and S6B), L3MBTL3 in U87-MG cells (Fig 1B, 4B, S3E, S6B and S6C), L3MBTL3 in MDA-MB-231 cells (Fig S4H and S6B), KDM1A in U87-MG cells (Fig S6B, S6C and S6D), KDM1A in MDA-MB-231 cells (Fig S6B).

Multiple lines of evidence support the hypothesis that the modulation of Notch signaling by L3MBTL3 is dependent on CBF1 and KDM1A:
-L3MBTL3's ability to co-localize with CBF1 on chromatin at the promoters of Notch target genes is dependent on both CBF1 (Fig 4D and Appendix Fig 4D) and the CBF1 interaction domain L3MBTL3-(1-64) (Fig 4F and Appendix Fig 4G).
-L3MBTL3's ability to repress Notch target genes is dependent on CBF1 (Fig 4E and Appendix Fig 4F), on the CBF1 interaction domain L3MBTL3-(1-64) (Fig 4G and Appendix Fig 4H) and on the KDM1A interaction domain L3MBTL3-(SAM) (Fig 5E).
-KDM1A's ability to co-localize with CBF1 on chromatin at the promoters of Notch target genes is dependent on L3MBTL3 (Fig 5B) and on L3MBTL3's ability to interact with both CBF1 and KDM1A (Fig 5C).
-L3MBTL3's ability to modulate the H3K4me2 mark at the *HES1* promoter is dependent on both the CBF1 interaction domain L3MBTL3-(1-64) and the KDM1A interaction domain L3MBTL3-(SAM) (Fig 5D).
-We also note that previous reports have described CBF1-dependent recruitment of KDM1A to chromatin as an important mechanism to modulate Notch signaling in various cell contexts (Mulligan *et al*, 2011, Wang *et al*, 2007, Yatim *et al*, 2012). Specifically, KDM1A contributes to the CBF1-mediated repression of Notch target genes via demethylation of H3K4me2 in U937 cells (Yatim *et al*, 2012).

Referee #3:
Please note that, as requested by Referee #3, the "RBPJ" designation has been replaced by "CBF1" throughout the new manuscript as well as in our response to the referees.

This is a thorough and carefully executed study that makes a valuable contribution to our understanding of transcriptional repression by the Notch pathway-regulated CSL factors. The authors show convincingly that the mammalian L3MBTL3 protein interacts directly with the Notch TF RBPJ and recruits the KDM1A demethylase to repress Notch target genes. They go on to provide evidence that the physical and functional interaction of L3MBT and CSL also operates in Drosophila and C. elegans, indicating that this mechanism is deeply conserved evolutionarily.

We thank Referee #3 for the nice appreciation of our work and its importance to the Notch field.

Following are several comments for the authors to consider.

(1) The MS repeatedly refers to amino acids 1-64 of L3MBTL3 as the "domain" that mediates direct binding between this protein and RBPJ. However, a stretch of amino acids found to be required for a particular proteinprotein interaction (necessity) is not necessarily the same as the domain responsible for this interaction (sufficiency). Fig. 3a shows that aa 1-197 are sufficient, but this fragment is over three times the size of the aa 1-64 segment.

We agree with Referee #3 that the "necessity" *versus* "sufficiency" distinction should have been made in the original manuscript. Please note that since the original submission, we have performed new ITC experiments using an L3MBTL3-(31-70) construct and we observe a moderate binding affinity (Kd = 0.45 μM) between L3MBTL3-(31-70) and CBF1 (new Fig 3A and new Table 1). The new ITC experiments demonstrate that the L3MBTL3-(31-70) domain is sufficient to mediate the CBF1/L3MBTL3 interaction, which corroborates our IP results with the L3MBTL3-Δ(1-64) construct (Fig 2A and B).

(2) The authors should comment on the ability of L3MBTL3-Δ(1-64) to partially rescue recruitment of KDM1A to Notch target genes (Fig. 5c).

This observation can be explained by the fact that other molecular mechanisms may contribute to the recruitment of L3MBTL3/KDM1A at Notch target genes independently of the CBF1/L3MBTL3 interaction and therefore independently of the L3MBTL3-(1-64) domain. For example, as noted in the discussion of the manuscript, the MBT domains of L3MBTL3 bind dimethylated histone marks (Nady *et al*, 2012), which could contribute to residual binding of L3MBTL3 at the Notch-responsive elements of Notch target genes. Residual binding of L3MBTL3 at the Notch-responsive elements of Notch target genes is observed in Fig 4F (compare the signals obtained for HA-L3MBTL3-Δ(1-64) at the Notch-responsive elements with the signals obtained for both the "NEG" and the IgG controls). This residual binding may contribute to the partial recruitment of KDM1A.

(3) Despite its direct relevance to the work reported here, the authors do not discuss or cite the prior literature on recruitment by the Drosophila Su(H)/H complex of chromatin-modifying complexes that include the LID demethylase (Goodfellow 2007, Moshkin 2009). Notably, the Moshkin paper reported that L3MBT is recovered by immunopurification of PF1, a component of both the LAF and RLAF complexes.

We thank Referee #3 for pointing this out. These observations provide an interesting parallel to our molecular model. The following sentences were added to the manuscript: "dL(3)mbt co-purifies with PF1, a PHD-finger protein that was previously linked to Notch signaling (Moshkin *et al*, 2009). It remains to be investigated if PF1 regulates Notch signaling as part of a dL(3)mbt-containing complex and/or as part of a complex containing ASF1 and the H3K4me2/3 demethylase LID (Goodfellow *et al*, 2007, Moshkin *et al*, 2009)."

We note though that co-purification of dL(3)mbt and Su(H) is not reported in these publications (Goodfellow *et al*, 2007, Moshkin *et al*, 2009). In the Moshkin paper, the authors observed that PF1 co-purifies with ASF1 in an a-ASF1 IP and, subsequently, that dL(3)mbt co-purifies with PF1 in an a-PF1 IP. These data do not allow to conclude whether ASF1 and dL(3)mbt are part of the same protein complex, or not. Additionally, we want to stress that *Drosophila* LID and human KDM1A do not share the same branch in the phylogenetic tree of lysinespecific demethylases [*Drosophila* LID is a JARID1-type H3K4me2/3 demethylase, which is orthologous to the human KDM5A-C proteins; human KDM1A is a flavin-dependent H3K4/9me1/2 demethylase, which is orthologous to *Drosophila* Su-var(3)3] (Cloos *et al*, 2008).

(4) The authors are strongly encouraged to move away from the use of the "RBPJ" designation for vertebrate CSLs. It's been known for over 20 years that the name "recombination signal-binding protein Jκ" reflects an experimental artefact [Henkel et al. (1994) Science 265: 92-95]. I urge the use of "CBF1" instead.

In the new manuscript, the "RBPJ" designation has been replaced by "CBF1", as requested by Referee #3. However, given that "RBPJ" remains the most commonly used designation and is the primary official name for this gene at the NCBI, to ensure maximum visibility of the publication, we will mention both the "CBF1" and the "RBPJ" designations in the abstract.

Referee #4:
Please note that, as requested by Referee #3, the "RBPJ" designation has been replaced by "CBF1" throughout the new manuscript as well as in our response to the referees.

Comments:
The authors of this manuscript identified L3MBTL3 as an RBPJ interacting protein using a yeast two-hybrid assay. After claiming confirmation using IP-MS, they report a series of experiments consistent with the interpretation that L3MBTL3 acts as a repressor of enhancer loci associated with RBPJ. Ultimately, what the authors are trying to claim is that the functional interaction (manifest as gene derepression, fly phenotype, etc.) of RBPJ with L3MBTL3 is a consequence of the binding interaction they have detected, which is relatively weak compared with NICD and other co-repressors known to bind at that site. The work is thorough, and the proposed role of L3MBTL3 is

intriguing, but it is not clear from the data presented that the L3MBTL3 effect on transcription is specific to RBPJ-loaded genomic sites, and thus whether the functional connection is due to a direct binding interaction between L3MBTL3 and RBPJ.

We thank Referee #4 for the nice appreciation of our work. Below, we describe in detail new ChIP-seq experiments using the CBF1 and L3MBTL3 antibodies to investigate on a genome-wide scale the colocalization of the endogenous CBF1 and L3MBTL3 proteins on chromatin in MDA-MB-231 cells. This experiment addresses the concern raised by Referee #4 regarding the specificity of the CBF1-L3MBTL3 connection on chromatin. For example, the 411 L3MBTL3-bound genes are enriched for genes associated with the both "Notch pathway genes" ($P = 4 \times 10^{-4}$) and "Notch-mediated HES/HEY network" ($P = 6 \times 10^{-5}$). Actually, these two GO pathway terms are the only ones (out of 1320 GO pathway terms tested) to show a significant enrichment in the set of 411 L3MBTL3-bound genes. This observation supports the hypothesis that L3MBTL3 specifically regulates the expression of Notch target genes in this cell line. Overlap analyses of the CBF1 and L3MBTL3 ChIP-seq data sets revealed a substantial and significant genome-wide co-localization of the two proteins on chromatin ($P = 2.4 \times 10^{-57}$; two-sided Fisher Exact test; new Appendix Fig S4D). Thus, in agreement with the observation that L3MBTL3's ability to co-localize with CBF1 on chromatin at the promoters of Notch target genes is dependent on both CBF1 (Fig 4D and Appendix Fig S4G) and the CBF1 interaction domain L3MBTL3-(1-64) (Fig 4F and Appendix Fig S4I), the fact that a large portion of the L3MBTL3 binding sites (30%) or L3MBTL3-bound genes (61%) are also bound by CBF1 supports the hypothesis that the recruitment of L3MBTL3 to chromatin is, for the most part, dependent on CBF1.

Major points:

1) The authors report in the text that immunoprecipitation of HA-tagged RBPJ indeed recovers RBPJ and also results in detection of L3MBTL3 peptides (6 and 17 peptides in two IPs) upon mass spec analysis. HA-tagged L3MBTL3 likewise recovers itself and three RBPJ peptides. A widely accepted standard in the MS field is to provide the complete list of proteins identified in the IP, together with their abundance (number of unique peptides, peptide abundance, etc.) in the manuscript. Otherwise, how is the reader able to assess the specificity of the recovery of L3MBTL3 with RBPJ in cells? This information would also clarify whether KDM1A is found in association with L3MBTL3 in the unbiased MS experiment.

The AP-MS data is now shown in Table EV1 and the raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino *et al*, 2016) partner repository with the data set identifier PXD004196.

During the peer review process the data can be accessed at:
Project accession: PXD004196
Submission date: 05/23/2016
Username: reviewer65849@ebi.ac.uk
Password: utJk1ZB0

KDM1A is not listed in Table EV1 but previous reports have described the CBF1-dependent recruitment of KDM1A to chromatin as an important mechanism to modulate Notch signaling in various cell contexts (Mulligan *et al*, 2011, Wang *et al*, 2007, Yatim *et al*, 2012), suggesting that the absence of signal for KDM1A in our APMS experiments is a false negative result. Indeed, we observed that endogenous KDM1A interacts with FLAGHA-tagged CBF1 and that endogenous CBF1 interacts with FLAG-HA-tagged KDM1A in reciprocal IP experiments in U87-MG cells followed by Western blot analyses, a more sensitive approach than AP-MS for the detection of co-purifying proteins (Appendix Fig S6D and S6E). Moreover, we obtained multiple, independent lines of evidence supporting a molecular interaction between L3MBTL3 and KDM1A. First, we identified the L3MBTL3/KDM1A interaction in a Y2H screen (Appendix Fig S6A). Second, we observed that endogenous KDM1A interacts with V5-tagged L3MBTL3 by IP in U87-MG cells followed by Western blot analysis (Appendix Fig S6C). Furthermore, the CBF1/KDM1A interaction, which is lost upon *L3MBTL3* KO (lane #5 in Fig 5A), is "rescued" in the presence of L3MBTL3 WT (lane #4) but not in the presence of L3MBTL3-Δ(1-64), the CBF1 interaction-defective mutant (lane #6), demonstrating the specificity of the interaction observed in these IPs and suggesting that the previously reported CBF1/KDM1A interaction (Mulligan *et al*, 2011, Wang *et*

al, 2007, Yatim *et al*, 2012) is indirect and occurs via L3MBTL3. Furthermore, the results shown in Fig 5B and C demonstrate that L3MBTL3 molecularly links KDM1A to CBF1 at the Notchresponsive elements of Notch target genes. Last but not least, in new IPs of endogenous CBF1 in U87-MG or MDA-MB-231 cells followed by Western blot analyses using KDM1A, L3MBTL3 or CBF1 antibody, we observed that endogenous CBF1 interacts with both endogenous KDM1A and endogenous L3MBTL3 (new Appendix Fig S6B).

2) What happens globally to the transcriptome in response to L3MBTL3 withdrawal? Is the de-repression of
transcription restricted to RBPJ-bound sites? Is it most evident at those sites? Or is the phenomenon of derepression
of expression more general across the transcriptome? A comparison of the transcriptomes in RBPJ and L3MBTL3 KO U87 lines (and ideally in a DKO) could clarify whether the apparent functional connection is
specific, or if the effect of L3MBTL3 is more general (and thus detectable as a non-specific effect in the subset
of sites bound by RBPJ).

If the repressive effect associated with L3MBTL3 on Notch target genes were indirect and only the result of a global effect on transcription, we would not expect it to be dependent on CBF1. This is not the case. Indeed, we provided multiple lines of evidence that demonstrate that the modulation of Notch signaling by L3MBTL3 is dependent on CBF1:
-L3MBTL3's ability to co-localize with CBF1 on chromatin at the promoters of Notch target genes is dependent on both CBF1 (Fig 4D and Appendix Fig 4D) and the CBF1 interaction domain L3MBTL3-(1-64) (Fig 4F and Appendix Fig 4G).
-L3MBTL3's ability to repress Notch target genes is dependent on CBF1 (Fig 4E and Appendix Fig 4F), on the CBF1 interaction domain L3MBTL3-(1-64) (Fig 4G and Appendix Fig 4H), and on the KDM1A interaction domain L3MBTL3-(SAM) (Fig 5E).
-KDM1A's ability to co-localize with CBF1 on chromatin at the promoters of Notch target genes is dependent on L3MBTL3 (Fig 5B) and on L3MBTL3's ability to interact with both CBF1 and KDM1A (Fig 5C).
-L3MBTL3's ability to modulate the H3K4me2 mark at the *HES1* promoter is dependent on both the CBF1 interaction domain L3MBTL3-(1-64) and the KDM1A interaction domain L3MBTL3-(SAM) (Fig 5D).

The role of L3MBTL3 as a negative regulator of Notch target genes has been assessed extensively in both the U87-MG (Fig 4) and MDA-MB-231 (Appendix Fig S4) mammalian cell lines. We performed ChIP-seq experiments using the CBF1 and L3MBTL3 antibodies to investigate on a genome-wide scale the colocalization of the endogenous CBF1 and L3MBTL3 proteins on chromatin in MDA-MB-231 cells. The CBF1 and L3MBTL3 ChIP-seq experiments in MDA-MB-231 cells identified 2926 CBF1 binding sites associated with 2937 putative CBF1-bound genes and 444 L3MBTL3 binding sites associated with 411 putative L3MBTL3-bound genes, respectively. The 2937 CBF1-bound genes are enriched for genes associated with both the GO pathway terms "Notch pathway genes" ($P = 7 \times 10^{-12}$) and "Notch-mediated HES/HEY network" ($P = 5 \times 10^{-7}$), supporting the quality of the CBF1 ChIP-seq data set. Importantly, the 411 L3MBTL3-bound genes are also enriched for genes associated with the both "Notch pathway genes" ($P = 4 \times 10^{-4}$) and "Notch-mediated HES/HEY network" ($P = 6 \times 10^{-5}$). Actually, these two GO pathway terms are the only ones (out of 1320 GO pathway terms tested) to show a significant enrichment in the set of 411 L3MBTL3-bound genes. This observation supports the hypothesis that L3MBTL3 specifically regulates the expression of Notch target genes in this cell line.

Overlap analyses of the CBF1 and L3MBTL3 ChIP-seq data sets revealed a substantial and significant genome-wide co-localization of the two proteins on chromatin. Indeed, 133 sites are bound by both CBF1 and L3MBTL3, i.e., 4.5% of the 2926 CBF1 binding sites and 30% of the 444 L3MBTL3 binding sites. Assuming that there are 50000 possible binding sites in the genome, this overlap is 5 times larger than expected ($P = 2.4 \times 10^{-57}$; two-sided Fisher Exact test; new Appendix Fig S4D). Similarly, 252 genes are bound by both proteins, i.e., 8.6% of the 2937 CBF1-bound genes and 61% of the 411 L3MBTL3-bound genes. Given that there are ~20000 genes in the human genome, this overlap is 4 times larger than expected ($P = 3.6 \times 10^{-107}$; two-sided Fisher Exact test; new Appendix Fig S4D). Thus, in agreement with the observation that L3MBTL3's ability to co-

localize with CBF1 on chromatin at the promoters of Notch target genes is dependent on both CBF1 (Fig 4D and Appendix Fig S4G) and the CBF1 interaction domain L3MBTL3-(1-64) (Fig 4F and Appendix Fig S4I), the fact that a large portion of the L3MBTL3 binding sites (30%) or L3MBTL3-bound genes (61%) are also bound by CBF1 supports the hypothesis that the recruitment of L3MBTL3 to chromatin is, for the most part, dependent on CBF1.

The ChIP-Seq data have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE100375: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100375. Token for confidential access by peer-reviewers: sdoroomwnhehrub

While our observations support a model where L3MBTL3 is specifically recruited by CBF1 to chromatin at Notch-responsive elements, we speculate that it is probable that CBF1 is not the only protein interacting with L3MBTL3 and that L3MBTL3, as a transcription co-regulator, could be recruited on chromatin by other factors, independently of CBF1. In other words, L3MBTL3 could be used iteratively by other factors and CBF1-independent mechanisms could contribute to the recruitment of L3MBTL3 to chromatin in other cellular contexts. This observation would not be surprising as many other transcriptional co-regulators collaborate with more than one transcription factors. In our opinion, the fact that L3MBTL3 could collaborate with other transcription factors, if verified, would not be incompatible with the hypothesis that RBPJ specifically recruits L3MBTL3 at Notch target genes.

3) The biochemistry of the protein-protein interaction is well documented (the mutational and competition studies presumably point to the RAM binding site as the site of L3MBTL3 binding), but affinity is 1-2 orders of magnitude lower than for NICD or some of the known co-repressors such as MINT, KyoT2, etc. How do the authors imagine that L3MBTL3 competes in the face of higher affinity competitors, especially if the other co-Rs are present in comparable abundance?

The results presented in Fig 1-3 and Appendix Fig S1 show that the CBF1/L3MBTL3 interaction is direct, specific and is characterized, under cell-free settings, by a moderate binding affinity (Kd = 0.45 μM). Please note that since the original submission, we have performed new ITC experiments using an L3MBTL3-(31-70) construct and we observe a moderate binding affinity (Kd = 0.45 μM) between L3MBTL3-(31-70) and CBF1. These new ITC experiments are now shown in the new Fig 3A and the new Table 1 as replacements of the previous ITC experiments with the L3MBTL3-(1-197) and L3MBTL3-(1-523) constructs (Kd = ~1.5 μM). The CBF1/L3MBTL3 Kd is thus one order of magnitude better than the affinity previously measured under identical conditions for the CBF1/EBNA2 interaction (Kd = 4.6 μM) (Johnson *et al*, 2010).

Although, the newly reported Kd has improved compared to the original submission (Kd = 0.45 μM *versus* 1.5 μM), the point made by Referee #4 stands as the affinity of the CBF1/L3MBTL3 interaction is about 20-fold weaker than the one previously observed for the CBF1/NOTCH ICD-RAM interaction (Kd = 22 nM) (Friedmann *et al*, 2008). Accordingly, NOTCH1 ICD displaces L3MBTL3 from CBF1 complexes (Fig 3B) but the reciprocal is not observed (Fig 3C). We speculate that the difference in CBF1 binding affinity between L3MBTL3 and NOTCH1 ICD is likely an important factor to ensure that, upon ligand-mediated release of NOTCH ICD, the L3MBTL3 co-repressor does not interfere with the CBF1/NOTCH ICD co-activator interaction and the resulting activation of Notch target genes.

We agree that other CBF1 co-repressors could compete with L3MBTL3 for binding to CBF1, e.g., KyoT2 (Kd = 12 nM) (Collins *et al*, 2014) and MINT (Kd = 11 nM) (VanderWielen *et al*, 2011). We note that competition would occur only when co-repressors are co-expressed with L3MBTL3 and when the level of expression of CBF1 is lower than the one of the co-repressors. The respective roles of L3MBTL3 and other CBF1 corepressors in the regulation of Notch target genes may be important in different contexts, e.g., when one but not the other protein is expressed. Of note, we would also like to emphasize the fact that these Kd measurements are obtained under cell-free settings and do not take into consideration various factors that may contribute to the dynamic regulation of the CBF1/L3MBTL3 interaction and of the other CBF1-associated protein complexes. Such factors include post-translational modifications or the potential contribution of other co-factors to protein complex stabilization. For example, as noted in the discussion of the manuscript, the MBT domains of L3MBTL3 bind dimethylated histone marks, e.g. H3K4me2 (Nady *et al*, 2012), which

could contribute to the stabilization of chromatin-bound CBF1/L3MBTL3 protein complexes. We speculate that, during the transition of CBF1-bound Notch-responsive elements from the "ON" to the "OFF" state, the preferential binding of L3MBTL3 to H3K4me2 may contribute to the preferential recruitment of KDM1A at sites where KDM1A's H3K4me2 demethylase activity is most needed to negatively regulate the chromatin landscape, i.e., at the hitherto active, yet-to-be inactivated, H3K4me2-rich CBF1-bound sites. As such, L3MBTL3 may play a crucial and unique role in the early transition of CBF1-bound sites from the active to the repressed state. In summary, L3MBTL3 may compete with other co-repressors for CBF1 binding but it may also play a different and complementary role by acting independently, at different Notch target genes, at different times and/or in different cellular contexts.

4) The idea that the RBPJ-binding region at the N-terminal end of the mammalian protein is positioned in an internal site (no longer N-terminal, and in a different part of the protein's domain organization) in the Drosophila protein is quite remarkable for a protein that otherwise has a completely conserved domain organization (and is a true ortholog). How do the authors imagine that the domains carry out a comparable function in the context of reorganized domain placement - and are there well-documented examples of such reshuffling from other similarly orthologous proteins? It is unclear how the statistical analysis in SF 8a was carried out, but it seems that a pairwise statistical test, if used, would not be the right metric.

A hidden Markov model (HMM) profile alignment approach (Soding, 2005) was used to study protein homology between human L3MBTL3 and *Drosophila* dL(3)mbt. The principle of this approach is to generate and compare the multiple sequence alignment (MSA) profile HMMs of both proteins (Soding, 2005). Profile HMMs are similar to simple sequence profiles but, in addition to the amino acid frequencies in the columns of a MSA, they contain the position-specific probabilities for inserts and deletions along the alignment. The power of the HMM approach to analyze sequence conservation of protein domains is well-established (~700 citations) and is much more efficient than single sequence based alignment methods such as the BLAST or the Needleman- Wunsch algorithm approaches to detect remote protein homology. HHM alignments can correctly identify homology in the "twilight zone", which refers to homology recognition below 30% sequence identity (Rost, 1999).

In our analysis of the L3MBTL3 and dL(3)mbt protein sequences, HMM profile alignment analyses were performed as previously described (Soding, 2005). With regard to the statistical analysis, in brief, E-value (Expected value) is a standard measure used in Bioinformatics to assess the significance of a sequence alignment, which is defined as the expected number of false positives ("wrong hits") with a score better than the one for the target when scanning the database. For instance, an E-value near 0 signifies a very reliable hit, while an E-value of 10 means that about 10 wrong hits are expected to be found in the database with a score at least this good. In HHM search, the P-value equals to the E-value divided by the number of sequences in the HHM search database. Mathematically, the P-value that we reported in Figure S8 is thus the probability that a wrong hit in a pairwise comparison will score at least this good.

As shown in Figure S8, using the HMM profile alignment approach, we identified a conserved region between the CBF1-interaction domain L3MBTL3-(M1-W64) (exact amino-acid position of the conserved region is Q11- N50) and a region in the *Drosophila* dL(3)mbt protein (amino-acid position S658-Q698), with a significant Pvalue (P = 6 x 10-19). The Q11-N50 domain accounts for most of the length of the M1-W64 domain, which we originally identified biochemically as the domain of interaction with CBF1 (Fig 2A,B and 3A). Interestingly, the dL(3)mbt-(S658-Q698) domain is 120 amino-acid away from the first MBT domain in *Drosophila* dL(3)mbt, a distance comparable to the one observed between L3MBTL3-(Q11-N50) and the first MBT domain in human L3MBTL3, i.e., 156 amino-acids. Thus, not only the CBF1-interaction motif and the major domains of human L3MBTL3 (MBT domains #1, #2 and #3, SAM domain and ZnF domains #1 and #2) are conserved in *Drosophila* dL(3)mbt, but the overall organization and spacing of these domains is essentially the same. Specifically, we note that there was no "reshuffling" of the conserved domains in the course of evolution of the two proteins. The only notable difference between the human L3MBTL3 and the *Drosophila* dL(3)mbt proteins is the presence of a large region at the N-terminal end of dL(3)mbt that is not present in L3MBTL3. The observation of both gains and losses of domains during proteome evolution is a well-documented phenomenon and different mechanisms have been described to explain the evolution of domain repertoires (Chothia *et*

al, 2003). Some of the most predominant events in the creation of novel multidomain proteins are genetic events, such as gene duplication, exon shuffling, gene fusion and gene fission, resulting in single domain insertion or deletion at either the N- or C- terminus of a protein (Moore *et al*, 2008).

5) Some key statistical analyses are lacking - particularly with respect to the overlap of L3MBTL3 binding sites and RBPJ binding sites in Figure 6B. Is the overlap (which occurs at 10% of L3MBTL3 binding sites and fewer than 20% of the RBPJ sites) different for RBPJ than for other signal-dependent TFs?

As suggested by Referee #4, we performed a statistical assessment of the overlap observed between the Su(H) and the dL(3)mbt binding sites. In addition, we performed an overlap analysis for the genes predicted to be bound by Su(H) and dL(3)mbt. The ChIP experiments identified 1764 Su(H) binding sites associated with 896 putative Su(H)-bound genes (GSE68614) and 2775 dL(3)mbt binding sites associated with 2380 putative dL(3)mbt-bound genes (GSE62904). There are 251 sites bound by both Su(H) and dL(3)mbt, i.e., 14.2% of the 1764 Su(H) binding sites and 9% of the 2775 dL(3)mbt binding sites. Assuming that there are 50000 possible binding sites in the genome (a conservative assumption), the overlap is 2.5 times larger than expected ($P = 2$ x 10-43; two-sided Fisher Exact test). We note that as one assumes a higher number of possible binding sites, the significance of observing a larger than expected overlap becomes greater. We also note that with the assumption of an even smaller number of possible binding sites, i.e., 25000 (a very conservative assumption), the P value remains significant ($P = 2$ x 10-5). There are 256 genes bound by both Su(H) and dL(3)mbt, i.e., 28.6% of the 896 Su(H)-bound genes and 10.8% of the 2380 dL(3)mbt-bound genes. Given that there are ~17000 genes in the *Drosophila* genome, this overlap is 2 times larger than expected ($P = 1$ x 10-31; two-sided Fisher Exact test). The results of these statistical analyses and a new Fig 6B showing the overlaps for both the binding sites and the bound genes have been added to the manuscript.

We performed a Su(H) DNA binding motif enrichment analysis for the sites identified in GSE68614 [Su(H)] and GSE62904 [dL(3)mbt], as well as for the overlapping sites. In brief, we used the PWMEnrich package (Stojnic & Diez, 2014) to scan the DNA sequences corresponding to the Su(H) and the dL(3)mbt peak intervals for enrichment of known position-specific scoring matrices obtained from multiple databases. As expected, the Su(H) DNA binding motif is enriched in the 1764 Su(H) binding sites identified in GSE68614 (22.5% of the 1764 Su(H) binding sites contain a consensus Su(H) DNA binding motif; $P = 2$ x 10-51), validating the quality of the GSE68614 data set. Such a significant enrichment is not observed for the 2775 dL(3)mbt binding sites identified in GSE62904 (11.1%; $P = 0.16$). For the 251 sites bound by both Su(H) and dL(3)mbt, we observed a significant enrichment for the Su(H) DNA binding motif (28.2%; $P = 2$ x 10-12). It is probable that Su(H) is not the only protein interacting with dL(3)mbt and that dL(3)mbt is recruited on chromatin by other factors, independently of Su(H). These Su(H)-independent mechanisms could contribute a significant fraction of the dL(3)mbt binding sites identified in GSE62904, a probable reason for the absence of a significant enrichment for the Su(H) DNA binding motif across all 2775 dL(3)mbt binding sites. Our motif enrichment analysis shows absence of enrichment for many transcription factor DNA binding motifs, e.g., cut, snail, daughterless, pannier, bicoid, pebbled, tramtrack, Ptx1, Dref, seven up, asense and extradenticle. Yet, it also revealed potential links to other transcription factors, e.g. Mad (31.9%; $P = 2$ x 10-180), underscoring the fact that dL(3)mbt, as a transcription co-regulator, may be used iteratively by other factors on chromatin. If verified, this observation would not be surprising as many other transcriptional co-regulators collaborate with more than one transcription factor. In our opinion, the fact that dL(3)mbt could collaborate with other transcription factors is not incompatible with the hypothesis that Su(H) specifically recruits dL(3)mbt at Notch target genes.

As described above in our answer to Comment #2, we performed ChIP-seq experiments to investigate on a genome-wide scale the co-localization of the endogenous CBF1 and L3MBTL3 proteins on chromatin in MDAMB-231 cells. Overlap analyses of the CBF1 and L3MBTL3 ChIP-seq data sets revealed a substantial and significant genome-wide co-localization of the two proteins on chromatin. Indeed, 133 sites are bound by both CBF1 and L3MBTL3, i.e., 4.5% of the 2926 CBF1 binding sites and 30% of the 444 L3MBTL3 binding sites (overlap is 5 times larger than expected; $P = 2.4$ x 10-57; two-sided Fisher Exact test; new Appendix Fig S4D). Similarly, 252 genes are bound by both proteins, i.e., 8.6% of the 2937 CBF1-bound genes and 61% of the 411 L3MBTL3-bound genes. Given that there are ~20000 genes in the human genome, this overlap is 4

times larger than expected (P = 3.6 x 10-107; two-sided Fisher Exact test; new Appendix Fig S4D).

Altogether, the analyses of the *Drosophila* Su(H) and the dL(3)mbt ChIP data are in line with the analyses of our new ChIP-seq data for CBF1 and L3MBTL3 in the mammalian MDA-MB-231 cell line, i.e., a substantial and significant genome-wide co-localization of CBF1/Su(H) and L3MBTL3/dL(3)mbt on chromatin is observed. Furthermore, we want to note that the specific recruitment of L3MBTL3/dL(3)mbt by CBF1/Su(H) to Notch target genes is corroborated, beyond the analysis of these genome-wide ChIP analyses, by several, independent lines of evidence (molecular interaction, Notch target gene expression analyses, ChIP-qPCR for L3MBTL3 in presence or absence of CBF1, etc) both in *Drosophila* and in mammalian cells.

6) Ultimately, what the authors are trying to claim is that the functional interaction (manifest as gene derepression, fly phenotype, etc.) of RBPJ with L3MBTL3 is a consequence of the binding interaction they have detected. Is there a synthetic phenotype between L3MBTL3 and RBPJ in a cellular or in vivo context? Such a true genetic interaction, if it exists, would make the claim that the direct binding interaction is of functional importance so much more compelling.

As stated above in our answer to Comment # 2, we provided multiple lines of evidence that demonstrate that the modulation of Notch signaling by L3MBTL3 is dependent on the CBF1/L3MBTL3 interaction. Our *in vivo* data in *Drosophila* also support a model in which *dL(3)mbt* represses Notch signaling and genetically interacts with *dNotch* in the eye imaginal disc. We agree with Referee #4 that assessing the functional cross-talk between L3MBTL3 and the CBF1/Notch pathway *in vivo* in mammals, *e.g.* during late embryonic hematopoiesis or medulloblastoma tumorigenesis, two contexts in which both L3MBTL3 and the CBF1/Notch pathway have been shown to have either a putative or an established role (Arai & Miyazaki, 2005, Fan *et al*, 2006, Gerhardt *et al*, 2014, Hallahan *et al*, 2004, Hui *et al*, 2005, Natarajan *et al*, 2013, Northcott *et al*, 2009, Souilhol *et al*, 2016), is an important question that remains to be addressed. This question is currently under investigation in the Rual lab using a novel conditional *L3mbtl3* KO "floxed" mouse but is out of the scope of the current manuscript.

Minor comments:
1) dICD in 6B (according to the SI) corresponds to dRAM-ANK. Since the band is smaller than predicted for dNICD, this detail should be more explicitly stated, otherwise the reader will be confused.

We thank Referee #4 for pointing this out. The legend of Fig 6A has been edited accordingly.

2) Thermodynamic, not thermodynamical.

We thank Referee #4 for pointing this out. The manuscript has been edited accordingly.

Arai S, Miyazaki T (2005) Impaired maturation of myeloid progenitors in mice lacking novel Polycomb group protein MBT-1. *Embo J* 24: 1863-73

Chothia C, Gough J, Vogel C, Teichmann SA (2003) Evolution of the protein repertoire. *Science* 300: 1701-3

Cloos PA, Christensen J, Agger K, Helin K (2008) Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes Dev* 22: 1115-40

Collins KJ, Yuan Z, Kovall RA (2014) Structure and function of the CSL-KyoT2 corepressor complex: a negative regulator of Notch signaling. *Structure* 22: 70-81

Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30: 207-10

Fan X, Matsui W, Khaki L, Stearns D, Chun J, Li YM, Eberhart CG (2006) Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. *Cancer Res* 66: 7445-52

Friedmann DR, Wilson JJ, Kovall RA (2008) RAM-induced allostery facilitates assembly of a notch pathway active transcription complex. *J Biol Chem* 283: 14781-91

Gerhardt DM, Pajcini KV, D'Altri T, Tu L, Jain R, Xu L, Chen MJ, Rentschler S, Shestova O, Wertheim GB, Tobias JW, Kluk M, Wood AW, Aster JC, Gimotty PA, Epstein JA, Speck N, Bigas A, Pear WS (2014) The Notch1 transcriptional activation domain is required for development and reveals a novel role for Notch1 signaling in fetal hematopoietic stem cells. *Genes Dev* 28: 576-93

Goodfellow H, Krejci A, Moshkin Y, Verrijzer CP, Karch F, Bray SJ (2007) Gene-specific targeting of the histone chaperone asf1 to mediate silencing. *Dev Cell* 13: 593-600

Hallahan AR, Pritchard JI, Hansen S, Benson M, Stoeck J, Hatton BA, Russell TL, Ellenbogen RG, Bernstein ID, Beachy PA, Olson JM (2004) The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. *Cancer Res* 64: 7794-800

Hui AB, Takano H, Lo KW, Kuo WL, Lam CN, Tong CY, Chang Q, Gray JW, Ng HK (2005) Identification of a novel homozygous deletion region at 6q23.1 in medulloblastomas using high-resolution array comparative genomic hybridization analysis. *Clin Cancer Res* 11: 4707-16

Johnson SE, Ilagan MX, Kopan R, Barrick D (2010) Thermodynamic analysis of the CSL x Notch interaction: distribution of binding energy of the Notch RAM region to the CSL beta-trefoil domain and the mode of competition with the viral transactivator EBNA2. *J Biol Chem* 285: 6681-92

Liefke R, Oswald F, Alvarado C, Ferres-Marco D, Mittler G, Rodriguez P, Dominguez M, Borggrefe T (2010) Histone demethylase KDM5A is an integral part of the core Notch-RBP-J repressor complex. *Genes Dev* 24: 590-601

Machanick P, Bailey TL (2011) MEME-ChIP: motif analysis of large DNA datasets. *Bioinformatics* 27: 1696-7
Moore AD, Bjorklund AK, Ekman D, Bornberg-Bauer E, Elofsson A (2008) Arrangements in the modular
evolution of proteins. *Trends Biochem Sci* 33: 444-51

Moshkin YM, Kan TW, Goodfellow H, Bezstarosti K, Maeda RK, Pilyugin M, Karch F, Bray SJ, Demmers JA, Verrijzer CP (2009) Histone chaperones ASF1 and NAP1 differentially modulate removal of active histone marks by LID-RPD3 complexes during NOTCH silencing. *Mol Cell* 35: 782-93

Mulligan P, Yang F, Di Stefano L, Ji JY, Ouyang J, Nishikawa JL, Toiber D, Kulkarni M, Wang Q, Najafi-Shoushtari SH, Mostoslavsky R, Gygi SP, Gill G, Dyson NJ, Naar AM (2011) A SIRT1-LSD1 corepressor complex regulates Notch target gene expression and development. *Mol Cell* 42: 689-99

Nady N, Krichevsky L, Zhong N, Duan S, Tempel W, Amaya MF, Ravichandran M, Arrowsmith CH (2012) Histone recognition by human malignant brain tumor domains. *J Mol Biol* 423: 702-18

Natarajan S, Li Y, Miller EE, Shih DJ, Taylor MD, Stearns TM, Bronson RT, Ackerman SL, Yoon J, Yun K (2013) Notch1 induced brain tumor models the sonic hedgehog subgroup of human medulloblastoma. *Cancer Res*

Northcott PA, Nakahara Y, Wu X, Feuk L, Ellison DW, Croul S, Mack S, Kongkham PN, Peacock J, Dubuc A, Ra YS, Zilberberg K, McLeod J, Scherer SW, Sunil Rao J, Eberhart CG, Grajkowska W, Gillespie Y, Lach B, Grundy R et al. (2009) Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma. *Nat Genet* 41: 465-72

Rand MD, Grimm LM, Artavanis-Tsakonas S, Patriub V, Blacklow SC, Sklar J, Aster JC (2000) Calcium depletion dissociates and activates heterodimeric notch receptors. *Mol Cell Biol* 20: 1825-35 Rost B (1999) Twilight zone of protein sequence alignments. *Protein Eng* 12: 85-94

Soding J (2005) Protein homology detection by HMM-HMM comparison. *Bioinformatics* 21: 951-60

Souilhol C, Lendinez JG, Rybtsov S, Murphy F, Wilson H, Hills D, Batsivari A, Binagui-Casas A McGarvey AC, MacDonald HR, Kageyama R, Siebel C, Zhao S, Medvinsky A (2016) Developing HSCs become Notch independent by the end of maturation in the AGM region. *Blood* 128: 1567-77

Stojnic R, Diez D (2014) PWMEnrich: PWM enrichment analysis. *R package version 420*
VanderWielen BD, Yuan Z, Friedmann DR, Kovall RA (2011) Transcriptional repression in the Notch pathway: thermodynamic characterization of CSL-MINT (Msx2-interacting nuclear target protein) complexes. *J Biol Chem* 286: 14892-902

Vizcaino JA, Csordas A, del-Toro N, Dianes JA, Griss J, Lavidas I, Mayer G, Perez-Riverol Y, Reisinger F, Ternent T, Xu QW, Wang R, Hermjakob H (2016) 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res* 44: D447-56

Wang J, Scully K, Zhu X, Cai L, Zhang J, Prefontaine GG, Krones A, Ohgi KA, Zhu P, Garcia-Bassets I, Liu F, Taylor H, Lozach J, Jayes FL, Korach KS, Glass CK, Fu XD, Rosenfeld MG (2007) Opposing LSD1 complexes function in developmental gene activation and repression programmes. *Nature* 446: 882-7

Yatim A, Benne C, Sobhian B, Laurent-Chabalier S, Deas O, Judde JG, Lelievre JD, Levy Y, Benkirane M (2012) NOTCH1 nuclear interactome reveals key regulators of its transcriptional activity and oncogenic function. *Mol Cell* 48: 445-58

Yuan Z, Friedmann DR, VanderWielen BD, Collins KJ, Kovall RA (2012) Characterization of CSL (CBF-1, Su(H), Lag-1) mutants reveals differences in signaling mediated by Notch1 and Notch2. *J Biol Chem* 287: 34904-16

2nd Editorial Decision   07 August 2017

Thank you for submitting a revised version of your manuscript. I apologise for the delay in communicating the decision due to traveling commitments and delayed referee reports. The manuscript has now been seen by all original referees, who find that their main concerns have been addressed. There remain only a few minor, mainly editorial issues that have to be resolved before formal acceptance of the manuscript.

1. If you have the data on transcription factor motif analysis requested by Reviewer #4, you are welcome to include this data in the manuscript, but this will not be essential for acceptance here.
2. The reviewers have expressed different views regarding the naming of CBF1/RBPJ/CSL. Since you have included all three naming options in the abstract and introduction, the use of CBF1 in the rest of the text can be retained.
3. Along the lines of the suggestion by Reviewer #4, I propose to change the title to "RBPJ/CBF1 interacts with L3MBTL3 to promote repression of Notch signaling via histone demethylase KDM1A".
4. Please provide tables EV1 and EV2 in the .doc or .xls format.
5. Western blot panels in the Figure S6C appear over-contrasted.
6. We are able to accommodate up to five Expanded View figures, which are then displayed together with the corresponding main figure in the online version of the manuscript, thus increasing the accessibility of supplemental data. I recommend to select five Appendix Figures that are more central to the message of the manuscript to display as Expanded View Figures. Please see our author guidelines on details about the content, purpose and preparation of Expanded View material (http://emboj.embopress.org/authorguide#expandedview). Please note that images in the Expanded View format have to fit onto one A4 page.
7. We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly

be useful but is not essential. These files will be published online with the article as supplementary "Source Data".

Finally, papers published in The EMBO Journal include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short introductory paragraph as well as 2-5 one-sentence bullet points that summarise the paper and are provided by the authors. Please send us your suggestions for bullet points and a synopsis image. This image should provide a rapid overview of the question addressed in the study, but still needs to be kept fairly modest, since the image size cannot exceed 550x400 pixels.

Please let me know if you have any further questions regarding this final revision. You can use the link below to upload the revised version.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I am looking forward to seeing the final version.

REFEREE COMMENTS

Referee #1:

Xu et al. have been responsive to the initial critiques. The additional analyses of RBPJ/L3MBTL3 binding overlap on chromatin and motif analysis of binding sites has fleshed out the extent of interaction between these factors in cells and further supports that idea that L3MBTL3 may have RBPJ-dependent and -independent functions. The existence and possible significance of RBPJ/L3MBTL3 interaction is clear and the paper in its current form should be accepted, pending potential minor revisions.

It also could be argued that the authors should not be forced to use the name CBF1. The official name for this factor is RBPJ, which people object to because it is a misnomer; for this reason, others in the field use CSL (CBF1/Su(H)/Lag1, an acronym for other names for this factor. The name CBF-1, by contrast, is used infrequently; to do so here will lower recognition and confuse potential readers.

Referee #2:

I am glad to report that I find the paper acceptable for publication, as the authors have addressed in a satisfactory manner my previous concerns.

Referee #3:

In this revised MS, the authors have conscientiously - and in my view, quite satisfactorily - addressed both my concerns and those of the other referees. Accordingly, I believe the paper is now fully suitable for publication in The EMBO Journal.

Referee #4:

The authors have comprehensively addressed the reviewer comments. The only analysis currently omitted that I would like to see are TF and de novo motif analysis of the authors' ChIP-seq studies of RBPJ and L3MBTL3, in order to clarify how strong the RBPJ "signal" is in both genomic ChIP studies. This analysis would take the form of a figure panel or table showing the TF motif logo(s) most highly enriched in the ChIPs, and the associated known factors that bind those sites for the TF analysis (de novo enriched motifs could be similarly listed, but without an associated known TF, of course).

I would also suggest retitling the manuscript to read something like:

"L3MBTL3 interacts with CBF1 in a complex that represses expression of Notch target genes"

To claim that there is a CBF1/L3MBTL3 "axis" (I am not even sure what a molecular "axis" is) supposes that this interaction is of broad functional importance. These studies, however, lay the groundwork for future and deeper exploration of that possibility, but do not yet establish such broad functional relevance.

Correspondence   22 August 2017

Author:
One more question.
Our "Answer" file (response to Referees' comments) will be published, correct?
Will it be published in its original form or should we edit it to reflect some of the latest changes, i.e., RBPJ/CBF1 nomenclature; Appendix Figures are now Expanded View figures.

Editor:
Regarding the nomenclature, you are welcome to use RBPJ throughout the manuscript. I have discussed the naming issue with the referees, and they do agree that RBPJ is the most widespread designation. Since you had modified the figure labels to CBF1 in the first revision, I didn't want to request a labour-intensive relabelling, but you are certainly welcome to do it as long as the other names of the protein are mentioned in the abstract and introduction.

2nd Revision - authors' response   31 August 2017

RESPONSE TO THE REFEREES' COMMENTS (ROUND #2)
We are pleased to note that we have satisfactorily addressed all the previous "Round #1" comments. Below are our answers (non-italics, blue font) to the new Referees' comments (*italics*).

Referee #1:
Xu et al. have been responsive to the initial critiques. The additional analyses of RBPJ/L3MBTL3 binding overlap on chromatin and motif analysis of binding sites has fleshed out the extent of interaction between these factors in cells and further supports that idea that L3MBTL3 may have RBPJ-dependent and –independent functions. The existence and possible significance of RBPJ/L3MBTL3 interaction is clear and the paper in its current form should be accepted, pending potential minor revisions.

We thank Referee #1 for the nice appreciation of our work and responses to the previous comments.

It also could be argued that the authors should not be forced to use the name CBF1. The official name for this factor is RBPJ, which people object to because it is a misnomer; for this reason, others in the field use CSL (CBF1/Su(H)/Lag1, an acronym for other names for this factor. The name CBF-1, by contrast, is used infrequently; to do so here will lower recognition and confuse potential readers.

Referee #3 was correct in pointing out the misnomer in the first round of review. Yet, as underlined by Referee #1 as well as in our original response, "RBPJ" is not only the official name but it is also the most frequently used and more recognizable designation in the literature for this gene. We share the concern raised by Referee #1 that the use of "CBF1" could potentially confuse some readers and could result in lower visibility of the publication. In the new submission, we now use the "RBPJ" designation throughout the manuscript. The "CBF1" designation will be mentioned in the title, abstract and introduction.

Referee #2:
I am glad to report that I find the paper acceptable for publication, as the authors have addressed in a satisfactory manner my previous concerns.

We thank Referee #2 for the nice appreciation of our work and responses to the previous comments.

Referee #3:
In this revised MS, the authors have conscientiously - and in my view, quite satisfactorily - addressed both my concerns and those of the other referees. Accordingly, I believe the paper is now fully suitable for publication in The EMBO Journal.

We thank Referee #3 for the nice appreciation of our work and responses to the previous comments.

Referee #4:
The authors have comprehensively addressed the reviewer comments.
We thank Referee #4 for the nice appreciation of our work and responses to the previous comments.

The only analysis currently omitted that I would like to see are TF and de novo motif analysis of the authors' ChIP-seq studies of RBPJ and L3MBTL3, in order to clarify how strong the RBPJ "signal" is in both genomic ChIP studies. This analysis would take the form of a figure panel or table showing the TF motif logo(s) most highly enriched in the ChIPs, and the associated known factors that bind those sites for the TF analysis (de novo enriched motifs could be similarly listed, but without an associated known TF, of course).

We think that a comprehensive description of the DNA binding motif enrichment analyses for the 2926 RBPJ binding sites and the 444 L3MBTL3 binding sites identified in our ChIP-seq analyses and a discussion about the putative factors co-bound at RBPJ- and L3MBTL3-bound sites are out of the focus of this manuscript, which focuses on the RBPJ-L3MBTL3 interaction. Moreover, we are limited by the length limit (55,000 characters). We note, though, that the DNA binding motif enrichment analyses are described in the "Response to referees' comments (round #1)" file, which is published alongside the manuscript as an online supplementary document.

I would also suggest retitling the manuscript to read something like: "L3MBTL3 interacts with CBF1 in a complex that represses expression of Notch target genes". To claim that there is a CBF1/L3MBTL3 "axis" (I am not even sure what a molecular "axis" is) supposes that this interaction is of broad functional importance. These studies, however, lay the groundwork for future and deeper exploration of that possibility, but do not yet establish such broad functional relevance.

We thank Referee #4 for the suggestion. The title has been changed and now reads: *"RBPJ/CBF1 interacts with L3MBTL3/MBT1 to promote repression of Notch signaling via histone demethylase KDM1A/LSD1".*

Additional changes since the previous submission:
We have selected 5 Appendix Figures to be shown as Expanded View figures in the new manuscript: Appendix Figures S4C and D, 6, 8, 11 and 16.
In summary:
-Appendix Figures 1, 2, 3 and 5 remain the same.
-Appendix Figures 4A, B, C, F, G, H, I and J are now Appendix Figures 4A, B, C, D, E, F, G and H respectively.
-Appendix Figures 4D and E are now Figures EV1A and B, respectively.
-Appendix Figures 7, 9, 10, 12, 13, 14 and 15 are now Appendix Figures 6, 7, 8, 9, 10, 11 and 12 respectively.
-Appendix Figures 6, 8, 11 and 16 are now Figures EV2, 3, 4 and 5, respectively.

EMBO PRESS

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jean-François Rual
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2017-96525

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. 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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The statistical methods and sample sizes are described in the figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For the fly and the worm studies, sample size estimates are described in the legends (Figs 6E, 6F and EV5).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	As described in the figures, figure legends or methods, animals were selected based on their genotype, age or gender. No additional criteria were used for inclusion/exclusion.
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.	No randomization was necessary.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was necessary.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	The statistical methods and sample sizes are described in the legend of the figures. Most of the tests we have employed, e.g., two-sample T-tests and ANOVA models, are very traditional. We model experiment effects, e.g. pairs of observations, when they are present. For abundance or size measurements, we consistently use log-transformation of the data. We do test the difference-of-differences (test interactions) when that is the appropriate statistical test. Given the absence of consensus in the community for the statistical analysis of the overlaps observed for the binding sites in ChIP experiments (see PMID: 23956260), we describe our procedure in detail.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We visually checked that assumptions of the tests (normal distribution, equal variances) are met. We note that the assumptions of ANOVA models are often better met after log-transforming, which we do for abundance or size measurements.

Is there an estimate of variation within each group of data?	All data is represented by error bars that depicts standard deviation of the mean.
Is the variance similar between the groups that are being statistically compared?	Yes. As mentioned above, we routinely log-transform abundance or size measurements to better insure the variances are similar between groups.

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).	The list of all the antibodies used in this study and their source/catalog number is described in the Appendix Supplementary Methods section (pages 1 and 2).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines is described in the Appendix Supplementary Methods section (page 2). Cell lines have been tested for mycoplasma contamination. Cell line authentications were performed by STR profiling.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	The mass spectrometry proteomics data from this publication have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al, 2016) partner repository with the data set identifier PXD004196. The ChIP-Seq data from this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al, 2002) and are accessible through GEO Series accession number GSE100375: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100375.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	The ChIP data sets used in our analysis are dL(3)mbt ChIP-seq in Kc167 cells (GSE62904) (Li et al, 2015) and Su(H) ChIP-chip in 3rd instar larval discs (GSE68614) (Zacharioudaki et al, 2016). They are cited in the Appendix Supplementary Methods section (page 7).
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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