Dissecting Hes-centred transcriptional networks in neural stem cell maintenance and tumorigenesis in *Drosophila*

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MS TITLE: Dissecting Hes-centered transcriptional networks in neural stem cell maintenance and tumorigenesis in Drosophila

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I have now received the reports of three referees on your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees express great interest in your work, but they recommend a substantial revision of your manuscript before we can consider publication and they make a number of specific suggestions to improve the manuscript. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

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how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This study examines the role of the HES genes in NSC maintenance in the developing fly brain. It first shows that overexpression of Dpn (D) and Espl (M) proteins interferes with the differentiation of NSC progeny cells and favors self-renewal. It also results in hyperplasia as demonstrated by an allograft assay. Transcriptome analysis further indicates that tumors produced by HES over-expression resemble those produced by Notch overactivation. These results are largely expected on the basis of the published literature, including studies by the same group :
Zacharioudaki, E., S. S. Magadi and C. Delidakis (2012). bHLH-O proteins are crucial for Drosophila neuroblast self-renewal and mediate Notch-induced overproliferation. Development 139: 1258-1269.
Zacharioudaki, E., B. Housden, G. Garinis, R. Stojnic, C. Delidakis and S. Bray (2016). Genes implicated in stem cell identity and temporal programme are directly targeted by Notch in neuroblast tumours. Development 143: 219-231.

The authors then characterize the direct HES targets involved in NSC maintenance using a combination of microarray and ChIP-seq. These approaches produced a list of 50 genes that are both located close to a Dpn peak and regulated by DM/MM. This list includes erm, a gene encoding a TF that promotes neural differentiation, confirming results from a previous study (Development 141:1036-1046) which identified erm as a direct Dpn target. Beyond this example, the possible HES-dependent repression of the putative Dpn targets identified here was not systematically studied.

In a last part, the authors examine the roles of two putative Dpn target genes, Zfh1 and Gcm in NSC maintenance. zfh1 is a direct Notch target (Development, 2010, 137, 2633–2642) that encodes a transcription factor. While the role of Zfh1 was examined using both loss and gain of function assays, no clear picture emerged. Gcm is a transcription factor promoting gliogenesis and is mostly not expressed in NSCs. Whether loss of Dpn+Espl results in a derepression of Gcm in NSCs was not examined and the relevance of the possible regulation of Gcm by HES remains unclear.

In sum, this study addresses an interesting topic and is technical sound. However, some of the results presented herein are expected while others provide insufficient advance to warrant publication in Development.

Comments for the author

Specific points
1. it is not fully clear whether expression of two HES genes, in the MM and DM combinations, has a stronger effect than expression of a single HES gene, M or D. If indeed MM or DM > M or D is observed, is this merely due to higher levels of global HES proteins? In other words, is 2x D more similar to DM than to 1x D? Or are there qualitative differences in the activities of D and M in this assay? The same questions apply for the different M proteins.

2. Given that HES proteins act as transcriptional repressors, it is somewhat puzzling that half of the genes located near Dpn binding sites and that are found to be regulated by HES by brain transcriptome analysis appear to be positively regulated by Dpn:
p. 7 'Although Hes proteins are thought to act as repressors, only half of these 50 Dpn-bound genes showed repression in the Hes-induced tumours. This is not surprising, because of the heterogeneity of the tissue analysed, which means that genes bound by Dpn in the NSCs may also be expressed in other cell types, so their behaviour in the microarray will reflect the average across all cell types in the tissue.'
If the heterogeneity argument is taken seriously, one should conclude that the results of the transcriptome should be considered inconclusive.
Thus, the reader is left considering that Dpn may act as a positive regulator at these target loci. This should be investigated. While the authors state: 'We do not exclude the possibility that Hes

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proteins might behave as activators in certain enhancer contexts, although we have no evidence in support of such a scenario' it is unclear if and how this possibility was actually explored.

3. p.3: 'it was not known whether the Notch-dependent hyperplasia also have a malignant potential' The authors could mention here the work by Caussinus and Gonzalez (2005) in Nature Genetics (37, 1125-29) showing that loss of the Notch inhibitor Numb produce hyperplasia with malignant potential in this assay. Accordingly, the allograft results are somewhat expected.

4. ChIP-seq profiles have previously been reported for various HES factors in the fly embryo (Genetics, 2018 vol. 208 no. 3 937-949). How do these profiles compare with the Dpn profile reported here?

5. Fig 4E: EsplM5 but not EsplMdelta appear to be up-regulated upon DM/MM overexpression. How do the authors interpret this result? Also, please explain why low EsplMgamma transcript levels are detected upon MM/DM (ie EsplMgamma) overexpression.

Reviewer 2

Advance summary and potential significance to field

In this very interesting paper, the authors examine the effects of Notch pathway activation on CNS tumorigenesis and describe a cross-regulatory transcriptional network that under normal developmental conditions ensures stem cell transition to neuronal differentiation and thus represses tumor formation.

The study is well conducted, the data convincing and the interpretations consistent with the evidence provided. We have only relatively minor Comments for the authors to consider for both text and figures.

Comments for the author

Comments on the text/data:

1- The authors refer to dedifferentiation when in fact what they observe can equally be could lack of differentiation or perturbed/delayed differentiation. The experiments where they express N, MM or DM in neurons and they don't observe dedifferentiation or hyperplasia would suggest support that this is not necessarily dedifferentiation.

2- The term DEG is not defined the first time it appears on the text.

3- The authors mention in the text the use of "RT-qPCR to show that RNA levels of several genes identified as differentially regulated in the microarray showed the same trend when individually tested". However, when we look at the figure 3 – supplemental 1, we see that MM and DM actually behave differently in most cases except in one (gcm) and in another one (zfh1) the tendency is the same but without a significant change in the expression. Because of this discrepancy, the authors should provide an explanation in the text and perhaps add additional genes to support their statement.

4- I would also suggest to add the effect on the selected genes in figure 3- supplemental 1 when creating N tumors as these genes belong to the overlapping genes between N MM and DM.

5- If they have such data or if generating them would not be a burden under the current circumstances, I would add the qRT-PCR profiles of the selected genes in figure 3-supplemental, especially gcm and zfh11, in allograft tumors after 20 days of transplantation. It is very exciting that the authors find a difference in the malignancy of the tumors when they force the expression of the Hes target genes, gmc and zhf1 (that would otherwise have been downregulated). Although that won't change the message of the paper, I think that showing what happens to these genes during the malignant transformation of N MM or DM tumors would add valuable and very interesting information to the paper.

6- The authors cross their data with available datasets of genes that are enriched in NBs vs neurons to show that the profile imposed by their tumor paradigm is not a simple consequence of an overrepresentation of NBs over neurons. I find this comparison very interesting however I believe that this lack of enrichment is in part due to the imposed overlap between the N and MM/DM datasets. MM and DM being very clearly similar and N being more divergent. The overlap of these

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datasets shows no enrichment in NBs genes however, judging from the figure, I believe that the N tumor could indeed be enriched in NBs. The authors may want to introduce a comment about this to justify the choice of N, MM/DM intersection.
7- After identifying Hes target genes the authors focus on two of them: gcm and zfh1. A better justification of the choice of these two genes should be added.
8- In Figure 5 and 6 the authors show the reduction of the expression of zfh1 and gcm in their tumor paradigms respectively. A quantification of this reduction should be added.
9- 348 genes assigned from 229 peaks. Is there any restriction to the closest gene or the distance to the peak to assign a peak to a gene? Perhaps I have missed this but otherwise it should be mentioned in the text because there are many genes out of the peaks the authors find.

Comments on the Figures:
1- In figure 3 the Venn diagram is hiding the text of the gene ontology graph in both the upregulated and downregulated genes
2- In figure 7 the labels on the diagrams G and H are not homogeneous. In one the expression of Dpn is labeled as Dpn +ve or Dpn-ve while in the other the expression of Elav is labeled as Elav –neg or Elav –pos (instead of elav +ve or elav –ve as in the rest of text).
3- Figure 10. The authors show an interesting reduction of the malignancy of tumors when imposing the expression of gcm or zfh1. I believe adding a control with and UAS-trangene (either GFP or LacZ or a neutral transgene) to show that this is not the effect of titration of gal4 would be necessary.
4- Figure 1-supplement 4. In C and D it seems that prospero is repressed outside of the clone as if some kind of competition is taking place. Do the authors have the same impression? Is it worth mentioning?
5- In Figure8-supplement1 at the end of this figure in the E' F' G' and H' the authors show the GFP channel. It would be more interesting to show the Repo channel.
6- Add a supplemental figure showing the expression domain of GrhNB-gal4.

Reviewer 3

Advance summary and potential significance to field

The submitted manuscript reports results obtained using chromatin occupancy and transcriptomics to identify genes regulated by Hes factors that are important to maintain both NSCs and NSC-derived tumours caused by overexpression of Notch or some of its targets in Drosophila. The authors conclude that Notch sets up a network of factors that, like Hes and Zfh1, exert opposite effects in maintaining stemness. Under normal conditions this network is balanced to ensure the normal course of differentiation and loss of such a balance can have tumorigenic effects.

These are interesting results that add new valuable insight into the complex mechanism involved in brain tumour growth in Drosophila.

Comments for the author

The experiments are well designed and the results are generally well documented. In general terms, the one less clear point is the sort of reciprocal regulation Dpn-Zfh1. ChIP data suggests that Dpn regulates zfh1, but I do not quite follow the conclusion that Zfh1 regules dpn. Dpn does not show up in Zfh1 overexpressing clones in brains, but it does in tumours. Perhaps a cartoon with the model would make the proposed network more amenable to readers that are not specialist in Notch signalling.

Listed below are all the important specific points (ordered by the corresponding figure) that should be addresses before publication.

FIGURE 1:
1) - "We imaged control and hyperplastic CNSs using NSC markers Dpn, Mira, and Wor…."
I could not find these images. Are they available?
2) - Please show grhNB-Gal4-driven GFP expression (GFP channel only) both in brain lobes ("superficial" and "deeper") and in VNCs.

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3) - “Hes overexpression was less disruptive than that of Necd…”.
It does not quite look like it in Figure 1: lobes in C and E seem identical (same goes for D and F). Could the “difference” not be due to that E(spl)m+Dpn overexpressing animals survive to pupation while Necd animals die as early larvae? Also the different survival rate could be due to systemic causes. Is there no expression of that Gal4 in other tissues? (salivary glands? ring glands?).
4) Panels G to J: please specify what these single confocal sections correspond to. Are they all taken at same depth?
5) Pros is referred to in this context, but not shown in the figure.

FIGURE 2:
1) “Either a brain hemisphere or a ventral nerve cord (VNC) was injected into the abdomen of a young female host. Out of the flies that survived the transplantation process, 60% developed tumours within 20 days (Figure 2).” This sentence contradicts Figure 2 legend: “single brain lobes”. What does 60% refer to? Lobes or lobes+VNC?
2) Figure 2A shows the percentage of tumours, 21 days after injection. Do these tumours kill the host? If so when? If not, why?
3) In Figure 2 C-E: magnification is different (C< D and E). That does not make sense.
4) The statement that N and Hes can drive malignancies from Type II and Type I lineages (data not shown) needs to be documented.
5) “These results indicate a NSC-like tumour phenotype with little differentiation”.
This conclusion could have been derived from Figure 1 that documents NSC hyperplasias. The question then is what allografts add? Do they demonstrate malignancy? I do not see such a conclusion in Results, but it appears in Abstract and Introduction. This needs to be clarified. Are these tumours lethal to the host?
6) Figure 2 legend: “ Single brain lobes containing…”. However in Results : “Either a brain hemisphere or a ventral nerve cord (VNC) was injected”.
Please, clarify.

FIGURE 3:
1) Panel 3A is not referred to in the text. The first reference to this figure is 3D. Is this a mistake, meaning 3A instead? References to B and C appear later in the text.
2) The following sentences are contradictory (i): “most N/Hes differentially regulated genes belonged to neither the neuron nor the NSC-enriched gene-sets”; (ii): “Upon GO term analysis of the N/Hes transcriptomes, few terms showed statistically significant enrichment (Figure 3B, C), mostly associated with neurogenesis”.
GOs related to the 573 most significantly upregulated genes include “neurogenesis” and “generation of neurons”. How does this fit with the interpretation that these are NSC tumours.
3) Comparing microarray data from different labs and obtained under very different conditions is extremely risky. This risk should be highlighted in the text. Moreover, Material and Methods should include a detailed explanation of effort made to normalise results from different sources.
4) Fig 3D legend: “Pearson correlation coefficients of the distributions of foldchange values for nine CNS tumour transcriptomes”.
I am not convinced that fold-change values from different experiments can be meaningfully compared. One thing is to compare lists of the top most up or down genes and a very different thing is to derive Pearson correlation coefficients.

FIGURE 4:
1) When comparing the lits of ChIP-seq Microarray genes the authors state:“ This is not surprising, because of the heterogeneity of the tissue analysed, which means that genes bound by Dpn in the NSCs may also be expressed in other cell types, so their behaviour in the microarray will reflect the average across all cell types in the tissue.”
This is correct, but sort of weakens the significance of their microarray data., Does it not?
2) More on this comparison: The crosses to obtain the samples to be analysed were made under rather different conditions: different genetic backgrounds; constitutive overexpression versus 24h only; temperature. Any of these factors can make huge differences in the result.

FIGURE 5:
1) “ Hyperplastic brains generated by overexpression of NΔecd or 2xHes during late larval stages using grhNB-Gal4 showed a severe reduction of zfh1 expression in the superficial layers, and a more

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moderate reduction of zfh1 positive neurons in deeper layers (Figure 5).” Figure 5 shows neither “superficial” nor “deeper” layers; it shows brain lobes (A, C, E) and VNC (B, D, F).
2) I do not appreciate any “severe reduction” of Zfh1.

FIGURE 6:
1) “ In deep areas (near the neuropils) LacZ was detected in some Repo positive glial cells”. Figure 6 does not show any Repo staining.
2) Very minor, but helpful: In FIGURES 5 and 6 Dpn and Elav are in red and blue and blue and red respectively. Please maintain consistent colour code when possible

FIGURE 7:
1) Most panels are not referred to in the text (only 7H). Makes it hard to follow
2) “Elav and Pros were also partially repressed in zfh1 overexpressing lineages: several cells lacked both Dpn and these differentiation markers in a large proportion of clones (Figure 7H).” Pros is not shown.
3) 7G must also include the corresponding plot of wild-type clones.
4) 7H: same as above (wild-type clones plot missing) plus it is unclear that this are UAS-zfh-1 clones (are they?).
5) It would be very helpful to see the Dpn channel alone

FIGURE 8:
1) “ zfh1 overexpression in a tumour background produced round clones”.
How does that compare to wild-type clones? Please show.
2) “Therefore, the ability of Zfh1 to repress dpn”.
It sounds as if it is a known fact that Zfh1 represses dpn. Does it? The loss of Dpn in N+Zfh1 and MM+Zfh1 clones could be due to the loss of NSC identity. It does not demonstrate that Zfh1 represses dpn. As a matter of fact if dpn was a Zhf1 target, should it not change in zhf1 LOF clones?
3) “More remarkable was the strong repression of Elav (Figure 8) and Pros (Figure 8 – supplement 1) in UAS- N (DM, MM) +zfh1 ” True, but the same goes for UAS-N only clones. Incidentally, what id the effect in brain lobes?
4) Please show Zfh1 clones in a wild-type background for comparison.
5) Likewise panel G should also show wilde-type as a control.
6) If Zfh1 overexpression in NSC-tumor-like clones (Notch y MM) abrogates Dpn and Elav expression, what is the identity of these cells.
7) Figure Legend does not specify wether “Only clones in the central brain regions were scored” (legend to Figure 9 does).
8) Please show brain lobe phenotypes in all instances in which brain lobes are injected. This figure shows only VNCs.

FIGURE 9:
1) It is not clear whether panels A-D show brain lobes or VNCs.

FIGURE 10:
1) UAS-N + UAS-Zfh1 brain lobe tumours present a large number of Elav positive cells while UAS-N + UAS-Zfh1 VNC tumours (Figure 8) showed a strong repression of Elav. Please elaborate on these seemingly contradictory results.
2) the injected UAS-N + UAS-Zfh1 tumours express Dpn, but not Dpn (shown in Figure 8). Why is this not commented upon? What does it mean? Does this not argue against Zfh1 repressing dpn?
3) There appears to be some inconsistencies regarding the injected tissue, here and in other figures. Please specify what the injected tissue was in each case i.e. brain lobes -one or the pair, VNCs, or the entire organ

DISCUSION:
1) “Earlier we showed that Su(H), the TF that tethers cleaved (activated) Notch to its target enhancers, binds near all genes specifically expressed in the larval NSC, indicatively klu, ase wor, pnt, dm and mira, besides the Hes genes in the dpn and E(spl) loci (Zacharioudaki et al. 2016).” The direct binding of Su(H) to the dpn promoter region was first reported by San-Juan and Baonza, 2011 (Dev Biol).
2) “We showed that this TF (zfh1) represses dpn and ase (Figure 7)”.

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This is not quite correct. The data show that zfh1 overexpression antagonizes both NSC and neuron cell fates which means that these cells may be in an undifferentiated intermediate state. Otherwise, one should also conclude that elav and pros too are repressed by Zfh.

First revision

Author response to reviewers' comments

As I mentioned in my previous email, we have just wrapped up all the requested experiments and have made the relevant changes to the manuscript, so we are resubmitting it. We hope that the changes that we have made have successfully addressed all reviewers' outstanding questions and comments, most of which we found very constructive. Before listing our changes one-by-one, I will make a summary:

We have added four new Supplementary Figures and have made substantial changes to three of the main figures (Figures 2, 7 and 10). We have performed two large series of tumour allograft experiments (close to 1000 flies injected) to quantify tumorigenicity as reduction of the host's lifespan, and not as time of detection of GFP at the stereoscope, which we were using before. This did not change our main conclusions and, in fact, has given us greater confidence on how to proceed in our analysis of these tumours in the future. These major changes, along with smaller additional experiments and textual modifications (the more extensive/ important ones are marked in red in the manuscript text), will be described in detail below. I will copy the reviewers' comments and reply to each of them (in blue) for easier reference.

REVIEWER 1:

… Gcm is a transcription factor promoting gliogenesis and is mostly not expressed in NSCs. Whether loss of Dpn+Espl results in a derepression of Gcm in NSCs was not examined and the relevance of the possible regulation of Gcm by HES remains unclear.

We could not do this experiment for technical reasons. Making double *dpn+E(spl)* loss clones is already challenging, since the two genes are on different chromosome arms; yet we have constructed fly strains for that purpose. Crossing *gcm-lacZ* into these strains would be next to impossible, so we had decided not to invest time and energy in such confirmation, already when we started this project several years ago.

1. it is not fully clear whether expression of two HES genes, in the MM and DM combinations, has a stronger effect than expression of a single HES gene, M or D. If indeed MM or DM > M or D is observed, is this merely due to higher levels of global HES proteins? In other words, is 2x D more similar to DM than to 1x D? Or are there qualitative differences in the activities of D and M in this assay? The same questions apply for the different M proteins.

Prompted by this comment, we performed a series of systematic comparisons of double transgenes and concluded that there are indeed some qualitative differences. These are described in the text (lines 143-145) and a new figure, Fig. S5.

2. Given that HES proteins act as transcriptional repressors, it is somewhat puzzling that half of the genes located near Dpn binding sites and that are found to be regulated by HES by brain transcriptome analysis appear to be positively regulated by Dpn:
p. 7 'Although Hes proteins are thought to act as repressors, only half of these 50 Dpn-bound genes showed repression in the Hes-induced tumours. This is not surprising, because of the heterogeneity of the tissue analysed, which means that genes bound by Dpn in the NSCs may also be expressed in other cell types, so their behaviour in the microarray will reflect the average across all cell types in the tissue.'
If the heterogeneity argument is taken seriously, one should conclude that the results of the

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transcriptome should be considered inconclusive.
Thus, the reader is left considering that Dpn may act as a positive regulator at these target loci. This should be investigated. While the authors state: 'We do not exclude the possibility that Hes proteins might behave as activators in certain enhancer contexts, although we have no evidence in support of such a scenario' it is unclear if and how this possibility was actually explored.

We made a more explicit presentation of the heterogeneity argument, which we believe is an inherent problem of all transcriptome studies in complex tissues (lines 243-245).
Moreover, it is not just tissue heterogeneity, but also the complexity of transcriptional networks, that could underlie the unexpected behaviour of some target genes. We also alluded to that in our text (lines 245-247). Finally, we added a new figure (Fig. S10) from old unpublished data, that lends support to the argument that Dpn, as well as the 7 E(spl) proteins, act as repressors.

3. p.3: 'it was not known whether the Notch-dependent hyperplasia also have a malignant potential'
The authors could mention here the work by Caussinus and Gonzalez (2005) in Nature Genetics (37, 1125-29) showing that loss of the Notch inhibitor Numb produce hyperplasia with malignant potential in this assay. Accordingly, the allograft results are somewhat expected.

We added a remark on the connection to *numb* lof tumours in the discussion (line 392-395)

4. ChIP-seq profiles have previously been reported for various HES factors in the fly embryo (Genetics, 2018 vol. 208 no. 3 937-949). How do these profiles compare with the Dpn profile reported here?

We downloaded two MODENCODE datasets from the above paper, ENCFF243RXN and ENCFF345SGB, containg embryo ChIP-seq data for Dpn and E(spl)mgamma, respectively. Only 14% (31) of our 229 larval Dpn binding events overlapped with these embryo peak sets. As the two tissues are vastly different we feel that comparing the Hes chromatin profiles would not add much information to our current paper, so we decided not to include this analysis (it is available to the reviewer, if requested).

5. Fig 4E: EsplM5 but not EsplMdelta appear to be up-regulated upon DM/MM overexpression. How do the authors interpret this result? Also, please explain why low EsplMgamma transcript levels are detected upon MM/DM (ie EsplMgamma) overexpression.

m5 and mdelta are examples of genes that are oppositely regulated, one (m5) down (expected) and the other (mdelta) up (unexpected) – see our reply to comment 2. In Fig. 4E, mgamma shows upregulation, as expected. Please note that the heatmap plots fold changes, not absolute transcript levels.

REVIEWER 2:

1-The authors refer to dedifferentiation when in fact what they observe can equally be could lack of differentiation or perturbed/delayed differentiation. The experiments where they express N, MM or DM in neurons and they don't observe dedifferentiation or hyperplasia would suggest support that this is not necessarily dedifferentiation.

We use "dedifferentiation" rather broadly to refer to reacquisition of the NSC fate by a postmitotic cell. Granted, this cell was not fully differentiated when we applied the insult; it was a young neuron that still expressed Pros (mature neurons do not). On the other hand, it was not a GMC progenitor, either. We think that most of the defects we see stem from a dedifferentiation of young neurons and not GMCs (or less so), since GMCs are attached to the NSC and we see most ectopic NSCs far from the main NSC (see also the live analysis of this process in Zacharioudaki et al, eLife 2019. which is in favour of this interpretation). We explain these issues in the text, lines 134-136. Indeed, fully differentiated mature neurons cannot be dedifferentiated by high N/Hes and we make this clear in the text, lines 148-152.

2-The term DEG is not defined the first time it appears on the text.

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We have corrected this, see line 185.

3-The authors mention in the text the use of “RT-qPCR to show that RNA levels of several genes identified as differentially regulated in the microarray showed the same trend when individually tested”. However, when we look at the figure 3 – supplemental 1, we see that MM and DM actually behave differently in most cases except in one (gcm) and in another one (zfh1) the tendency is the same but without a significant change in the expression. Because of this discrepancy, the authors should provide an explanation in the text and perhaps add additional genes to support their statement.

We repeated the RT-qPCR assays with fresh RNA samples and again we got very high inter- replicate variability, probably due to the low expression levels of the target genes, coupled with the low RNA yields from hand-dissected CNSs. We re-made the figure using only our newer results (Fig. S7). We excluded zfh1, because in a control UAS-zfh1 overexpressing sample our primer pair did not detect the expected mRNA increase. We are afraid that due to the technical limitations of this method, we can only use it as a peripheral confirmatory assay, and have therefore not given it much emphasis in the text. Note also that the MM samples have been removed, because in this series of experiments they gave extremely low RNA yields, not sufficient to get a good cDNA synthesis. We did add N samples, as requested in the next comment.

4-I would also suggest to add the effect on the selected genes in figure 3- supplemental 1 when creating N tumors as these genes belong to the overlapping genes between N MM and DM.

(see above)

5-If they have such data or if generating them would not be a burden under the current circumstances, I would add the qRT-PCR profiles of the selected genes in figure 3- supplemental, especially gcm and zfh11, in allograft tumors after 20 days of transplantation. It is very exciting that the authors find a difference in the malignancy of the tumors when they force the expression of the Hes target genes, gmc and zhf1 (that would otherwise have been downregulated). Although that won’t change the message of the paper, I think that showing what happens to these genes during the malignant transformation of N MM or DM tumors would add valuable and very interesting information to the paper.

We added N-T0 tumour samples to the RT-qPCR data in Fig. S7, which showed an interesting increase of stemness genes and a further decrease of differentiation genes. (We are currently in the process of analyzing RNA-seq data from such tumours. These are much more sensitive and reliable and will hopefully be the basis of a future publication on tumorigenesis. They agree with the trends shown in Fig. S7.)

6-The authors cross their data with available datasets of genes that are enriched in NBs vs neurons to show that the profile imposed by their tumor paradigm is not a simple consequence of an overrepresentation of NBs over neurons. I find this comparison very interesting however I believe that this lack of enrichment is in part due to the imposed overlap between the N and MM/DM datasets. MM and DM being very clearly similar and N being more divergent. The overlap of these datasets shows no enrichment in NBs genes however, judging from the figure, I believe that the N tumor could indeed be enriched in NBs. The authors may want to introduce a comment about this to justify the choice of N, MM/DM intersection.

We should clarify that we used the *union*, not the *overlap*, between N and DM/MM DEGs, so we had a broad collection of genes to compare with the external datasets – therefore, we do not think that we imposed a filtering bias. The lack of enrichment of genes expressed in normal NSCs, GMCs or neurons in our aberrant condition only confirms that we are indeed studying an aberrant condition.

7-After identifying Hes target genes the authors focus on two of them: gcm and zfh1. A better justification of the choice of these two genes should be added.

Besides being transcription factors that have been implicated in differentiation, this choice was also based on availability of reagents – we added a remark to that effect (line 266-267)

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8-In Figure 5 and 6 the authors show the reduction of the expression of zfh1 and gcm in their tumor paradigms respectively. A quantification of this reduction should be added.

As these genes, are expressed in widely separated areas within the complex brain/VNC, quantification is almost impossible. In Fig. 5, we tried to show clear examples of regions where downregulation is evident and to highlight them with arrows, wherever the surrounding complex pattern might obscure the effect.

9-348 genes assigned from 229 peaks. Is there any restriction to the closest gene or the distance to the peak to assign a peak to a gene? Perhaps I have missed this but otherwise it should be mentioned in the text because there are many genes out of the peaks the authors find.

The peak-to-gene assignment is explained in detail in the Methods (line 596-599) and briefly in the Results (line 236). The reason there are more genes than peaks is that a peak can be assigned to more than one genes, due to the relatively high gene density in the *Drosophila* genome.

(Reviewer 2/ Figure comments)

1-In figure 3 the Venn diagram is hiding the text of the gene ontology graph in both the upregulated and downregulated genes

We corrected this and made better ontology plots.

2-In figure 7 the labels on the diagrams G and H are not homogeneous. In one the expression of Dpn is labeled as Dpn +ve or Dpn-ve while in the other the expression of Elav is labeled as Elav –neg or Elav –pos (instead of elav +ve or elav –ve as in the rest of text).

In all clone scoring charts we used consistent notation, +ve for "positive" and –ve for "negative".

3-Figure 10. The authors show an interesting reduction of the malignancy of tumors when imposing the expression of gcm or zfh1. I believe adding a control with and UAS-trangene (either GFP or LacZ or a neutral transgene) to show that this is not the effect of titration of gal4 would be necessary.

We repeated this series of experiments making sure to equalize the dosage of UAS transgenes across genotypes. We also scored the severity of malignancy by measuring the lifespan of allografted hosts (as requested by Reviewer 3). Therefore Fig. 10A has been completely reconstructed with the new data.

4-Figure 1-supplement 4. In C and D it seems that prospero is repressed outside of the clone as if some kind of competition is taking place. Do the authors have the same impression? Is it worth mentioning?

Panels C,D show adult brains that would normally express no Pros. It is the persistence of Pros inside the clones, not its lack of expression outside, that we find noteworthy. We believe it shows that these regions are still proliferative (or were proliferative until recently), since Pros is an early differentiation marker, ie GMCs and young neurons. (In contrast, panels A,B come from early pupal brains where there is still extensive Pros expression in normal tissue). We have explained this in the legend of Figure S6.

5-In Figure8-supplement1 at the end of this figure in the E' F' G' and H' the authors show the GFP channel. It would be more interesting to show the Repo channel.

We added the Repo-only channel to Fig. S16

6-Add a supplemental figure showing the expression domain of GrhNB-gal4.

We made a new supplementary Figure (S2) showing the grhNB expression pattern at various magnifications.

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REVIEWER 3

….the one less clear point is the sort of reciprocal regulation Dpn-Zfh1. ChIP data suggests that Dpn regulates zfh1, but I do not quite follow the conclusion that Zfh1 regules dpn. …

We use the expression that Zfh1 "represses" *dpn* in an operational sense, based on the results of Fig. 7 (misexpression of zfh1 in the CNS results on loss of Dpn). We use this word (repress) in its operational sense in many other places throughout the text (repression of Elav by the tumour, etc). In the Discussion, where we speculate on the mechanisms of Hes- Zfh1 antagonism, we have added a disclaimer that dpn repression by Zfh1 may be direct or indirect (line 411-412) to avoid giving the wrong impression to the reader.

Having said that, we feel that the repression of dpn by Zfh1 could indeed be direct, since loss of Dpn is seen very early upon zfh1 overexpression, before the NSC becomes unrecognizable (e.g. see examples of large mitotic NSCs that are Dpn negative in Fig. S14F).

.. Dpn does not show up in Zfh1 overexpressing clones in brains, but it does in tumours. Perhaps a cartoon with the model would make the proposed network more amenable to readers that are not specialist in Notch signalling…

Indeed, despite the initial Dpn "rerpression" by Zfh1, it rebounds when we look at N+zfh1 allografted tumours – this is several days after allografting the larval tissue, so gene expression has plenty of time to change. We made a remark on this in the text (line 379). We really have no mechanistic insight to propose as to why this happens, so it is a little premature to make a summary cartoon.

FIGURE 1:
1) - “We imaged control and hyperplastic CNSs using NSC markers Dpn, Mira, and Wor….” I could not find these images. Are they available?

We added examples of these markers in Figures S4 and S5

2) - Please show grhNB-Gal4-driven GFP expression (GFP channel only) both in brain lobes (“superficial” and “deeper”) and in VNCs.

This was also a request of Reviewer 2, so we made a new supplementary figure (S2) showing the grhNB expression pattern at various magnifications.

3) - “Hes overexpression was less disruptive than that of Necd…”. It does not quite look like it in Figure 1: lobes in C and E seem identical (same goes for D and F). Could the “difference” not be due to that E(spl)m+Dpn overexpressing animals survive to pupation while Necd animals die as early larvae? Also, the different survival rate could be due to systemic causes. Is there no expression of that Gal4 in other tissues? (salivary glands? ring glands?).

This concern should be partly addressed by the expression pattern of grhNB-Gal4 (new Fig S2) and by the dosage comparison study that we added (new Fig S5). Could the increased lethality of grhNB>N vs DM be due to the (very strong) expression in the salivary glands? (Fig S2)

4) Panels G to J: please specify what these single confocal sections correspond to. Are they all taken at same depth?

We corrected this and all other instances where it was not clear what the Figure panels were depicting (Brain vs VNC, surface vs deep, single sections vs thicker projections). For the sake of brevity, I will skip subsequent comments that express similar queries.

5) Pros is referred to in this context, but not shown in the figure.
The effect of tumours on Pros are shown in Fig. S4 (citation in line 141).

FIGURE 2:
1) “Either a brain hemisphere or a ventral nerve cord (VNC) was injected into the abdomen of a

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young female host. Out of the flies that survived the transplantation process, 60% developed tumours within 20 days (Figure 2).” This sentence contradicts Figure 2 legend: “single brain lobes”. What does 60% refer to? Lobes or lobes+VNC?

This has been changed in both the text and the Figure to reflect new data that we obtained since the first submission (lines 156-161/ 639-645).

2) Figure 2A shows the percentage of tumours, 21 days after injection. Do these tumours kill the host? If so, when? If not, why?

We thank the reviewer for insisting that we focus on host viability. All our tumour data (here and in Fig. 10) are now presented in this way, which we agree is more meaningful.

3) In Figure 2 C-E: magnification is different (C< D and E). That does not make sense. We changed panel C to show an example at higher magnification.
4) The statement that N and Hes can drive malignancies from Type II and Type I lineages (data not shown) needs to be documented.

In the new experiments we included one series of VNC transplantations and these are shown separately in the last bar of Fig. 2A (labelled Nvnc).

FIGURE 3:
1) Panel 3A is not referred to in the text. The first reference to this figure is 3D. Is this a mistake, meaning 3A instead? References to B and C appear later in the text.

Corrected.

2) The following sentences are contradictory (i): “most N/Hes differentially regulated genes belonged to neither the neuron nor the NSC-enriched gene-sets”; (ii): “Upon GO term analysis of the N/Hes transcriptomes, few terms showed statistically significant enrichment (Figure 3B, C) mostly associated with neurogenesis”. GOs related to the 573 most significantly upregulated genes include “neurogenesis” and “generation of neurons”. How does this fit with the interpretation that these are NSC tumours.

We do not think that these are contradictory. Sentence (i) refers to the absolute percentage of our DEGs that belong to the neuron, NSC or GMC categories of Berger et al (2012) and Wissel et al (2018) (>50% of our DEGs don't belong to any of those categories). (ii) refers to the percentage of our DEGs associated with a given GO term *relative* to the percentage of that GO term in the entire genome - seen this way, terms referring to neurogenesis are enriched, even though they may be associated with only a fraction of our DEGs.

3) Comparing microarray data from different labs and obtained under very different conditions is extremely risky. This risk should be highlighted in the text. Moreover, Material and Methods should include a detailed explanation of effort made to normalise results from different sources.

4) Fig 3D legend: “Pearson correlation coefficients of the distributions of foldchange values for nine CNS tumour transcriptomes”. I am not convinced that fold-change values from different experiments can be meaningfully compared. One thing is to compare lists of the top most up or down genes and a very different thing is to derive Pearson correlation coefficients.

Both comments 3 & 4 express concern as to whether it is appropriate to compare our microarray data with those obtained in other settings/ labs. We have added a paragraph in the Methods explaining the way that external data was treated and why we think it is comparable to ours. We also changed the correlation test used from Pearson to Spearman rank, which is more appropriate when comparing disparate datasets. (See Methods, line 539-547).

FIGURE 4:
1) When comparing the lits of ChIP-seq Microarray genes the authors state:“ This is not surprising, because of the heterogeneity of the tissue analysed, which means that genes bound by Dpn in the

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NSCs may also be expressed in other cell types, so their behaviour in the microarray will reflect the average across all cell types in the tissue." This is correct, but sort of weakens the significance of their microarray data., Does it not?

(See our response to Reviewer 1/ point 2)

2) More on this comparison: The crosses to obtain the samples to be analysed were made under rather different conditions: different genetic backgrounds; constitutive overexpression versus 24h only; temperature. Any of these factors can make huge differences in the result.

Since the two experiments assay different parameters (RNA expression vs chromatin occupancy), we did not feel we had to use identical biological treatments. Within experiments we kept consistent procedures. That is, in the transcriptome analysis we made sure to use identical conditions for the N, DM and MM samples, even though the DM and MM animals would have survived to late larvae even if we hadn't included a Gal80ts to keep their expression off at early stages. In any case, we have explicitly described the expression regimens used in each experiment in the Material & Methods.

FIGURE 5:
1) " Hyperplastic brains generated by overexpression of NΔecd or 2xHes during late larval stages using grhNB-Gal4 showed a severe reduction of zfh1 expression in the superficial layers, and a more moderate reduction of zfh1 positive neurons in deeper layers (Figure 5)." Figure 5 shows neither "superficial" nor "deeper" layers; it shows brain lobes (A, C, E) and VNC (B, D, F). 2) I do not appreciate any "severe reduction" of Zfh1.

We clarified in the legend which subregions of the CNS are shown in each panel and we added arrows to point to regions of Zfh1 reduction.

FIGURE 6:
1) " In deep areas (near the neuropils) LacZ was detected in some Repo positive glial cells". Figure 6 does not show any Repo staining.

The reviewer is right, we had neglected this. We added a new Supplementary Figure (S12) with more details on gcm-lacZ expression, including Repo co-staining.

2) Very minor, but helpful: In FIGURES 5 and 6 Dpn and Elav are in red and blue and blue and red, respectively. Please maintain consistent colour code when possible

Corrected.

FIGURE 7:
1) Most panels are not referred to in the text (only 7H). Makes it hard to follow.

Corrected

2) "Elav and Pros were also partially repressed in zfh1 overexpressing lineages: several cells lacked both Dpn and these differentiation markers in a large proportion of clones (Figure 7H)." Pros is not shown.

The effect on Pros is shown in Fig. S14 (cited in the text, line 320)

3) 7G must also include the corresponding plot of wild-type clones.
4) 7H: same as above (wild-type clones plot missing) plus it is unclear that this are UAS-zfh-1 clones (are they?).
5) It would be very helpful to see the Dpn channel alone

We added a data-point for control clones in both G & H. We also added the Dpn-only channel.

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FIGURE 8:
1) " zfh1 overexpression in a tumour background produced round clones". How does that compare to wild-type clones? Please show.

It is very hard to describe the shape of NSC lineage clones, since the CNS is a complex 3-dimensional structure. The "roundness" of N/Hes+zfh1 clones can be appreciated by comparing the ones shown in this Figure (Fig. 8) with those in Fig. S4, S5 and 7 (which include examples of wt clones). Anyway, the clone shape is only mentioned in passing; we have not placed any emphasis on it.

2) "Therefore, the ability of Zfh1 to repress dpn". It sounds as if it is a known fact that Zfh1 represses dpn. Does it? The loss of Dpn in N+Zfh1 and MM+Zfh1 clones could be due to the loss of NSC identity. It does not demonstrate that Zfh1 represses dpn. As a matter of fact if dpn was a Zhf1 target, should it not change in zhf1 LOF clones?

As mentioned above (our first response to Reviewer 3's comments) we use the expression that Zfh1 "represses" *dpn* in a loose sense.

3) "More remarkable was the strong repression of Elav (Figure 8) and Pros (Figure 8 – supplement 1) in UAS-N (DM, MM) +zfh1 " True, but the same goes for UAS-N only clones. Incidentally, what id the effect in brain lobes?

The effect in brain lobes is exactly the same as in VNCs; we have added a statement to the legend. We only chose to show VNCs to be consistent across genotypes (to have uniformity in the Figure). We make it clear in the text (lines 345-347) that the repression of Elav is more conspicuous in N+zfh1 than in N alone (compare Fig. 8B with A) – we also added better references to the Figure panels regarding this comparison.

4) Please show Zfh1 clones in a wild-type background for comparison.

This is already shown in Fig. 7

5) Likewise panel G should also show wilde-type as a control.

We added a data-point for wt clones in panel 8G.

6) If Zfh1 overexpression in NSC-tumor-like clones (Notch y MM) abrogates Dpn and Elav expression, what is the identity of these cells.

This is indeed what we're wondering ourselves! Perhaps the conflicting N/Hes and Zfh1 signals "freeze" the cells in an aberrant progenitor state, which is neither NSC nor differentiated neuron.

FIGURE 10:
1) UAS-N + UAS-Zfh1 brain lobe tumours present a large number of Elav positive cells while UAS-N + UAS-Zfh1 VNC tumours (Figure 8) showed a strong repression of Elav. Please elaborate on these seemingly contradictory results.
2) the injected UAS-N + UAS-Zfh1 tumours express Dpn, but not Dpn (shown in Figure 8). Why is this not commented upon? What does it mean? Does this not argue against Zfh1 repressing dpn?

The Fig. 10 tumours are imaged after transplantation, whereas the Fig. 8 tumours are primary ones from larval CNSs; this is what makes the expression of Dpn and Elav different, not their anatomical difference (brain vs VNC). We don't know what happens after transplantation, but these results in themselves are an indication that many things change.

Note that the tumours have grown in the host for at least 5 days before dissecting out for histology. (See also our response to this Reviewer's second comment, further up). How the tumours progress is a current focus in the lab.

3) There appears to be some inconsistencies regarding the injected tissue, here and in other figures. Please specify what the injected tissue was in each case i.e. brain lobes -one or the pair,

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VNCs, or the entire organ.

We changed the Methods to explicitly state that we inject either single brain lobes or single VNCs (line…)

DISCUSION:

1) "Earlier we showed that Su(H), the TF that tethers cleaved (activated) Notch to its target enhancers, binds near all genes specifically expressed in the larval NSC, indicatively klu, ase, wor, pnt, dm and mira, besides the Hes genes in the dpn and E(spl) loci (Zacharioudaki et al. 2016)." The direct binding of Su(H) to the dpn promoter region was first reported by San-Juan and Baonza, 2011 (Dev Biol).

We corrected this omission by citing this paper already in the Introduction (line 56-57)

2) "We showed that this TF (zfh1) represses dpn and ase (Figure 7)". This is not quite correct. The data show that zfh1 overexpression antagonizes both NSC and neuron cell fates, which means that these cells may be in an undifferentiated intermediate state. Otherwise, one should also conclude that elav and pros too are repressed by Zfh.

As mentioned earlier (first response to Reviewer 3) we have added a remark about repression potentially being indirect (line 411-412) to avoid misleading the reader.

Second decision letter

MS ID#: DEVELOP/2020/191544

MS TITLE: Dissecting Hes-centered transcriptional networks in neural stem cell maintenance and tumorigenesis in Drosophila

AUTHORS: Srivathsa S Magadi, Chrysanthi Voutyraki, Gerasimos Anagnostopoulos, Evanthia Zacharioudaki, Ioanna K Poutakidou, Christina Efraimoglou, Margarita Stapountzi, Vasiliki Theodorou, Christoforos Nikolaou, Konstantinos A Koumbanakis, John F Fullard, and Christos Delidakis

ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 3

Advance summary and potential significance to field

The authors have convincingly addressed all standing issues. I recommend publication.

Comments for the author

The authors have convincingly addressed all standing issues. I recommend publication.

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