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# Glycosphingolipid Metabolic Reprogramming Drives Neural Differentiation

Domenico Russo, Floriana Della Ragione, Riccardo Rizzo, Eiji Sugiyama, Francesco Scalabrì, Kei Hori, Serena Capasso, Lucia Sticco, Salvatore Fioriniello, Roberto De Gregorio, Ilaria Granata, Mario R. Guarracino, Vittorio Maglione, Ludger Johannes, Gian Carlo Bellenchi, Mikio Hoshino, Mitsutoshi Setou, Maurizio D'Esposito, Alberto Luini, and Giovanni D'Angelo

**Review timeline:**

| | |
|---|---|
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| Revision received: | 13 October 2017 |
| Editorial Decision: | 8 November 2017 |
| Revision received: | 17 November 2017 |
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Editor: Karin Dumstrei

## 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 26 July 2017

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by 2 referees and their comments are provided below.

As you can see from the comments, the referees find the analysis insightful, interesting and support publication here. However, revisions are also needed. Referee #1 rightfully points out that the majority of the work is carried out on HeLa cells and that it would therefore be good to extend the findings reported in figure 7. I also agree with the referee that the manuscript is at times difficult to read/follow and that some work is needed to improve the flow of the manuscript.

Given the comments raised by the referees I would like to invite you to submit a suitably revised manuscript for our consideration.

When preparing your letter of response to the referees' comments, please keep 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

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE REPORTS

Referee #1:

In this study, Russo et al pursue the mechanisms by which neural differentiation causes a major

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switch in the expression of glycosphingolipids (GSLs) and their synthetic enzymes from the Globo to the Ganglio series. They define a major component of a circuit that begins with loss of globosides which results in induction of the epigenetic modifier AUTS2 leading to induction of GMS3S and formation of gangliosides. This is an elaborate and impressive work. It should be noted however that the majority of the mechanistic work was conducted in HeLa cells and not in neuronal cells.

Major Points
1. It would add significantly to the study if the results in Figure 7 are developed further so as to emphasize the neuronal relevance.
2. Which globo GSL mediates the regulation of AUTS2? This can be approached by manipulating further enzymes in the globo pathway (as in fig 3A) and pharmacologically (as in Fig 3B).
3. Many of the results are presented cryptically in the Results section (see below for several examples). This renders the manuscript very hard to follow.

Specific Points
1. The text in the Results section should describe more clearly what GSLs are being evaluated in Fig 1D and why others were not.
2. Presentation of figure 2B and 2C in the text is very cryptic. What was done in these experiments?
3. Sentence on page 8:" In addition, we noted.." This is not a complete sentence. The subsequent sentence (When analyzed...) is also not clear: progenitor cell common to both types or specific progenitors to each type?
4. P 8 last paragraph: please indicate that this is done in HeLa cells. Same when presenting results in Figs 4-6.
5. Fig 3A: how come KD of FAPP2, Gb3S did not increase levels of GM3?
6. The bottom panel of figure 3A is not adequately labeled. Presumably these are the mRNA levels of the same genes shown in the upper panel (this needs its own key as the key for the upper panel is for KD). Also, on the bottom of the lower panel, indicate that these are KDs (presumably).
7. The dashed lines connecting the two panels of Fig 3A are not clear. They are not discussed in the text and they don't really correspond to each other. The upper panel is measuring Gb3 in response to KD of the various genes. The Lower panel is the effect of KD of GM3S (presumably this is the case, but not indicated) on the expression of the other genes (again, not clearly defined).
8. Figure 3E. please indicate which effects of Gb3 are statistically significant. Same for 4H. Also the statement on p 9 concerning these results in 3E is somewhat misleading as this is not a specific truncation of -432 to -324 but an inference from the progressive truncations.
9. Figure 6 is very difficult to follow and also has panels mis-identified in the Results section (but not the figure legend). The font is un readable for many of the panels.
10. How come AUTS2 does not show binding to the repressor region identified in Fig. 4?
11. Fig 7D needs quantitation.

Referee #2:

Russo and colleagues present a detailed, interesting - and potentially highly citable - manuscript describing new data on factors controlling GSL expression in developing cells/neurons. Given the long standing interest in the role of gangliosides in neuronal development and function, their study overall provides a new perspective on how this expression is controlled, and as such could stimulate additional investigations into this important but largely overlooked area of research. The key finding reported here is that globo series GSLs negatively modulate expression of an epigenetic regulator known as AUTS2 which itself binds to and activates a GM3S promoter inducing GM3 synthase expression along with downstream gangliosides. AUTS2 is intriguing in its own right, and has been linked to a rare form of intellectual disability (as well as autism and schizophrenia.) Thus the current work also should provide new ideas on how to best investigate AUTS2 gene defects and the intellectual disability syndrome in humans and as modeled in mice. Analysis of GLSs in the model comes immediately to mind. Such studies would have the possibility of dramatically advancing understand of this rare form of intellectual disability, in addition to making clearer how metabolic products can themselves drive neural differentiation. The underlying basic science findings in the current manuscript are a key to such advancement.

Specific comments on the manuscript.

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In the first Results section, studies to evaluate the role of GSLs in neural differentiation used NB-DNJ. While, as reported, there is evidence that this agent effects GSL synthesis, it is at best only a partial inhibitor of glucosylceramide synthase and may not be having dramatic effects on the levels of ganglio-series GSLs in this experiment. Data shown in Suppl fig 2 in fact shows only a reduction in expression as would be anticipated. It is somewhat surprising that the authors did not use more potent GSL synthesis inhibitors (e.g., PDMP compounds). Their conclusion that GSL production is a result not a prerequisite of neural differentiations may be correct but is not really proven by this experiment using NB-DNJ.

In the section called 'Gb3 represses GM3S expression' the text of the manuscript fails to say how the GSL perturbation was done, one has to go to the figure to find out. It really should be described in the text, with findings reported in the figure. Other sections appear more complete in this regard.
In the Discussion, while the hypothesized steps addressing neuronal differentiation are clear (though Suppl Fig 7 is not particularly clear), what exactly might cause the drop in globo-series GSLs was not evident. Perhaps the authors could further speculate. A summary figure with more clarity than that given in Suppl materials could also be useful summing up the important data revealed in the manuscript, and could be included as a new Fig. 8 (not in Suppl. Materials).

1st Revision - authors' response 13 October 2017

Response to Referees

Referee #1:

In this study, Russo et al pursue the mechanisms by which neural differentiation causes a major switch in the expression of glycosphingolipids (GSLs) and their synthetic enzymes from the Globo to the Ganglio series. They define a major component of a circuit that begins with loss of globosides which results in induction of the epigenetic modifier AUTS2 leading to induction of GMS3S and formation of gangliosides. This is an elaborate and impressive work. It should be noted however that the majority of the mechanistic work was conducted in HeLa cells and not in neuronal cells.
*We thank this Referee for her/ his appreciation of our work. We have now obtained new results in physiologically relevant systems (mouse brains and neurons) that we think will satisfy the Referee's concern.*

Major Points

1. It would add significantly to the study if the results in Figure 7 are developed further so as to emphasize the neuronal relevance.

*We thank this Referee for her/ his remark. We have now performed extensive ChIP experiments in mousebrain to profile the AUTS2 occupancy and histone modifications at mouse GM3S promoter (Fig 8A). According to our new results the GM3S promoter in mouse brain is bound by AUTS2 at the transcriptional starting site (TSS) where we also find Polycomb Repressive Complex 1 (PRC1) (i.e., RING1B) binding. Inspite of PRC1 binding, the mouse brains the GM3S promoter is not decorated by typical PRC-dependent histone modifications (i.e., Histone H3K27me3 or H2AK119Ub) that have gene repressive activity while it bears strong activatory histone acetylation signals (i.e., H3K27Ac, H3K9/14Ac, H4Ac) at its TSS. When these histone acetylation signals were followed during neural differentiation they were found to increase in parallel with AUTS2 expression (Fig 8D). This scenario is compatible with GM3S being a mechanistic AUTS2 target also in mouse neurons whereby the GM3S promoter (repressed by PRC1 in stem cells) is switched on following the induction and binding of the neural master regulator AUTS2 to PRC1.*

2. Which globo GSL mediates the regulation of AUTS2? This can be approached by manipulating further enzymes in the globo pathway (as in fig 3A) and pharmacologically (as in Fig 3B).

*Following this Referee suggestion we have now extended our analysis on the regulation of AUTS2*

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*expression to more complex Globo series GSLs. To this aim we have i) treated HeLa cells with siRNAs directed against three enzymes in Globo GSL synthesis, which act downstream Gb3S (i.e., Gb4S, Gb5S, and Forsmann synthase); ii) treated Gb3S-KD cells with exogenous Gb3 or Gb4 (these are the only commercially available pure globo series GSLs to the best of our knowledge). According to these experiments we can conclude that i) enzymes synthesizing globo GSLs more complex than Gb3 are barely expressed (and their products are undetectable by our lipid analytic techniques) in HeLa cells; ii) siRNAs directed against Gb4S, Gb5S, and Forsmann synthase do not induce any significant regulation on AUTS2 expression; iii) treatment of Gb3S-KD cells with either Gb3 or Gb4 induce a reduction in AUTS2 mRNA levels, suggesting that globo-series GSLs in general (and not specifically Gb3) repress AUTS2 expression (Fig EV5).*

*3. Many of the results are presented cryptically in the Results section (see below for several examples). This renders the manuscript very hard to follow.*

*We thank this Referee for having raised this point. We have now re-written the manuscript in the parts that were uneasy to follow (see below for a more detailed description).*

*Specific Points*

*1. The text in the Results section should describe more clearly what GSLs are being evaluated in Fig 1D and why others were not.*

*We have amended the text that now reads:*
*‘ To assess the impact of these transcriptional changes on GSL composition we used validated anti GSLantibodies (i.e., anti-Gb4 and anti-Forssman antigen for the globo series and anti-GT1b for the ganglio series) in cytofluorimetric assays. We observed that E14-mESCs expose globo-series GSLs at their cell surfaces (i.e., Gb4 and Forssman), while after neural differentiation, the globo-series GSLs are no longer detected and ganglio-series GSLs are produced (i.e., GT1b; Fig 2C).’*
*For clarity, we selected these anti-GSLs antibodies among the many we tested (including anti-GM3, anti- Gb3, anti-GM1, and anti-GD1a) for their better signal over noise ratio.*

*2. Presentation of figure 2B and 2C in the text is very cryptic. What was done in these experiments?*

*We apologize for the lack of clarity. We have now rephrased the paragraph describing these panels in the result section that now reads:*
*’As previously reported for other cell lines (Majoul et al. 2002), individual HeLa cells show mutual exclusion in the expression of either Gb3 or GM1 (Fig 3A). Nonetheless while a dependence of Gb3 or GM1 production on cell cycle-phase was found in other cell lines with GM1 being produced predominantly by cells in G0/G1 phase and Gb3 by cells in G2/M phase (Majoul et al. 2002), when the G2/M phase marker phosphor-Histone H3 (p-H3) was evaluated in ShTxB and ChTxB positive HeLa cells no significant enrichment was found (Fig 3B). Moreover, even though local crowding in cell populations (i.e., local cell density) impacts on lipid composition (Frechin et al. 2015) and GSL production (Snijder et al. 2009), when the distribution of ShTxB and ChTxB positive HeLa cells was considered along with cell crowding, local cell density failed to account for mutually exclusive toxin binding (Fig 3C). ‘*

*3. Sentence on page 8:" In addition, we noted.." This is not a complete sentence. The subsequent sentence (When analyzed...) is also not clear: progenitor cell common to both types or specific progenitors to each type?*

*We apologize again for this inconsistency. We have amended this part that now reads:*
*‘In addition, we noted that neighbouring cells tend to form Gb3-positive or GM1-positive cell colonies (Fig 3A). When analysed by correlative video-light microscopy for lineage tracking, each colony was found to derive in most cases from a single progenitor cell (Fig EV3B; Movie EV3) indicating that, once established, a specific GSL state (i.e., globo or ganglio positive) can be maintained through several cell generations.’*

*4. P 8 last paragraph: please indicate that this is done in HeLa cells. Same when presenting results in Figs 4-6.*

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*Done: the text now reads*

*'To study how this coordination is achieved, the GSL synthetic pathway was perturbed by silencing GSEs and factors involved in this metabolism (Fig 4A,B). We thus, systematically silenced HeLa cells for the expression of CERT; the sphingomyelin synthase 1 (SMS1); the glucosylceramide synthase (GCS), the LacCer synthase (LCS); FAPP2; Gb3S and GM3S and measured sphingolipid levels (Fig 4A).'*

*'Transcriptional changes were analysed by microarrays under conditions where the GSL synthetic pathways are perturbed in HeLa cells as in Fig 4A (Appendix Fig S2). By this procedure we isolated a group of six genes that were commonly and specifically up-regulated under conditions leading to decreased Gb3 (Fig 5A).'*

*'As shown in Fig 6A, in HeLa cells AUTS2 decorates discrete nuclear puncta that co-localize with the polycomb repressive complex 1 (PRC1) component RING1B (Satijn et al. 1997)'*
*'When AUTS2 occupancy on GM3S promoter was investigated in HeLa cells by chromatin immunoprecipitation (ChIP) AUTS2 was found to bind at 1600 to 400 bp upstream of GM3S transcriptional starting site (TSS; Fig 7A).'*

*5. Fig 3A: how come KD of FAPP2, Gb3S did not increase levels of GM3?*

*We thank this Referee to have raised this point. We indeed observe that reduced Gb3 levels, though consistently inducing GM3S mRNA up-regulation (□ 2 folds increase compared with control cells), results in minor changes in GM3 synthesis in HeLa cells. In line with these findings our data (see Appendix Figs S3 and 4) point to the lack of a strict correlation between GM3S levels and GM3 production in HeLa cells with a □10 folds increase in GM3S mRNA levels resulting in just a □ 2 folds increase in GM3 production. Similar results were obtained by overexpressing GM3S in HeLa cells where substantial GM3S upregulation resulted in modest increase in GM3 production (not shown). We interpret this evidence as an indication that in HeLa cells factors other than increased expression of GM3S are required to boost GM3 production. Nonetheless the control of GM3S expression by Globo GSLs is conserved in HeLa and in stem cells differentiating to neurons where the changes in GM3S expression better correlate with ganglioside production.*

*6. The bottom panel of figure 3A is not adequately labeled. Presumably these are the mRNA levels of the same genes shown in the upper panel (this needs its own key as the key for the upper panel is for KD). Also, on the bottom of the lower panel, indicate that these are KDs (presumably).*

*We apologize for the lack of clarity. We have now split Figure 3A in two independent panels (now Fig 4 A and B) each with its own key and labels.*

*7. The dashed lines connecting the two panels of Fig 3A are not clear. They are not discussed in the text and they don't really correspond to each other. The upper panel is measuring Gb3 in response to KD of the various genes. The Lower panel is the effect of KD of GM3S (presumably this is the case, but not indicated) on the expression of the other genes (again, not clearly defined).*

*We apologize again for the lack of clarity. The dashed lines were intended to highlight the fact that KDs inducing a reduction in Gb3 lipid levels (upper panel) also induced GM3S mRNA upregulation (lower panel). We have now split Figure 3A in two independent panels as discussed above and erased the dashed lines to increase readability.*

*8. Figure 3E. please indicate which effects of Gb3 are statistically significant. Same for 4H. Also the statement on p 9 concerning these results in 3E is somewhat misleading as this is not a specific truncation of -432 to -324 but an inference from the progressive truncations.*
*We have now indicated statistical significance in these figures.*
*As for the statement on p9 it before read*
*'... when truncated of the -432 to -324 region, the GM3S promoter activity was no longer sensitive to fluctuations in Gb3 levels (Fig 3E). '*
*Now we have changed it to*
*'... GM3S promoter fragments not containing the -432 to -324 region, were no longer sensitive to fluctuations in Gb3 levels (Fig 4F right panel).'*

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*9. Figure 6 is very difficult to follow and also has panels mis-identified in the Results section (but not the figure legend). The font is un readable for many of the panels.*
*We have now split this figure in two (Fig 7 and Fig EV7) to make the figure more immediately readable, increased the font and amended the panel identification in the Result section.*

*10. How come AUTS2 does not show binding to the repressor region identified in Fig. 4?*
*According to our results though AUTS2 occupancy on human GM3S promoter peaks at -1500 bp to the TSS, AUTS2 binds to the repressive region of GM3S promoter identified in Fig 4 significantly better than to the negative control (MyoD1 TSS) p=0,04 (Fig 7A).*

11. Fig 7D needs quantitation.
*We have now included quantitation for figure 7D (now Fig 8D).*

Referee #2:

Russo and colleagues present a detailed, interesting - and potentially highly citable - manuscript describing new data on factors controlling GSL expression in developing cells/neurons. Given the long standing interest in the role of gangliosides in neuronal development and function, their study overall provides a new perspective on how this expression is controlled, and as such could stimulate additional investigations into this important but largely overlooked area of research. The key finding reported here is that globo series GSLs negatively modulate expression of an epigenetic regulator known as AUTS2 which itself binds to and activates a GM3S promoter inducing GM3 synthase expression along with downstream gangliosides. AUTS2 is intriguing in its own right, and has been linked to a rare form of intellectual disability (as well as autism and schizophrenia.) Thus the current work also should provide new ideas on how to best investigate AUTS2 gene defects and the intellectual disability syndrome in humans and as modeled in mice. Analysis of GLSs in the model comes immediately to mind. Such studies would have the possibility of dramatically advancing understand of this rare form of intellectual disability, in addition to making clearer how metabolic products can themselves drive neural differentiation. The underlying basic science findings in the current manuscript are a key to such advancement.

*We thank this Referee for her/ his appreciation of our contribution.*

Specific comments on the manuscript.

In the first Results section, studies to evaluate the role of GSLs in neural differentiation used NB-DNJ. While, as reported, there is evidence that this agent effects GSL synthesis, it is at best only a partial inhibitor of glucosylceramide synthase and may not be having dramatic effects on the levels of ganglio-series GSLs in this experiment. Data shown in Suppl fig 2 in fact shows only a reduction in expression as would be anticipated. It is somewhat surprising that the authors did not use more potent GSL synthesis inhibitors (e.g., PDMP compounds). Their conclusion that GSL production is a result not a prerequisite of neural differentiations may be correct but is not really proven by this experiment using NB-DNJ.

*We have now expanded our analysis of the role of GSLs in neural differentiation to the use of different treatments aimed at inhibiting GSL synthesis (Fig EV2). These treatments include the use of the i) PDMPlike compound PPMP for the inhibition of glucosylceramide synthase; ii) Fuminisin B1 for the inhibition of ceramide syntahses; iii) Myriocin for the inhibition of the inhibition of serine palmitoyl transferase; iv) mouse GM3S directed siRNA. Each of these treatments induced a decrease in ganglioside (GT1b) content (ranging from 43% reduction in GM3S KD to 88% reduction in PPMP). Interestingly none of these treatments blocked neural differentiation apart from PPMP. Previous reports have shown that PDMP-like compounds inhibit neural differentiation independently on their effect on GSL synthesis inhibition (Liour et al. Neurochem. Res. 2002). Our results are in line with these data as Myriocin treatment that reduces cellular ganglioside content to a similar extent as PPMP (85%), does not impact on neural differentiation (Fig EV2). In the light of these findings we maintain that ganglioside production is a result and not a requisite of neural differentiation.*

*In the section called 'Gb3 represses GM3S expression' the text of the manuscript fails to say how the*

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*GSL perturbation was done, one has to go to the figure to find out. It really should be described in the text, with findings reported in the figure. Other sections appear more complete in this regard. We have now amended the text to include this description (see pages 9 and 10).*
*In the Discussion, while the hypothesized steps addressing neuronal differentiation are clear (though Suppl Fig 7 is not particularly clear), what exactly might cause the drop in globo-series GSLs was not evident. Perhaps the authors could further speculate. A summary figure with more clarity than that given in Suppl materials could also be useful summing up the important data revealed in the manuscript, and could be included as a new Fig. 8 (not in Suppl. Materials).*

*We thank this referee for having raised this point. We have now prepared a new and hopefully clearer summary figure (Fig 9) and included it among the main figures. We have also tried to speculate in the discussion about the possible events leading to the drop in globo series GSLs during loss of stemness and neural differentiation. This last issue is a matter of intense research in the lab at the moment and we hope to deliver some less speculative and more data based hypothesis in the next future.*

2nd Editorial Decision 8 November 2017

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the two referees and their comments are provided below. As you can see, both referees appreciate the added data and supports publication. I am therefore very pleased to accept the manuscript for publication here.

There are just a few things to sort out before we can pass it on to our publisher. I have provided a link below for you to upload the modified files. As soon as we get the final version in I will send you the formal acceptance letter.

That should be all - congratulations on a nice paper!

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REFEREE REPORTS

Referee #1:

The authors have done a remarkable job in dealing with all my comments. This is a very nice and meaningful study

Referee #2:

Reviewer concerns have been addressed satisfactorily.

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| |
|---|
| Corresponding Author Name: Giovanni D'Angelo |
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**Reporting Checklist For Life Sciences Articles (Rev. June 2017)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

## B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

| | |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | Most experiments were performed at least in quintuplicate and data are reported either as mean values or as representative images. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | Whole brains from C57BL/6 wild type, AUTS2 del8/+ and AUTS2 del8/del8 mouse embryos (E17.5) were obtained from Dr. Mikio Hoshino lab at the National Institute of Neuroscience, Tokyo, Japan. Whole brains from wild type adult (9weeks) C57BL/6 mice, processed for ChIP experiments, were obtained from Dr. Maurizio D'Esposito lab at the IGB-CNR, Naples, Italy. |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | All the experimentally produced samples were included in our analysis. |
| 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. | For fluorescence image analysis, random fields were chosen by observing samples on channels not relevant for the analysis (i.e., DAPI). Images were then acquired in all the channels and were applicable software based automated analysis was performed. For electron microscopy analysis field were selected based on the presence of a morphologically recognizable heterocromatin area surrounded by AUTS2 ring-like structures already characterized by super resolution nanoscopy. |
| For animal studies, include a statement about randomization even if no randomization was used. | No animal studies were carried out with randomization events. |
| 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. | When visual counting of phenotypes was performed, evaluation was done in 'single blind' where the experimentator was unaware of the exact identity of the samples he/ she was observing. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | No animal studies were carried out with blinding events. The brains we received were from mice with the genotype already known. We only measured the transcriptional differences of the genes of our interest by qPCR. |
| 5. For every figure, are statistical tests justified as appropriate? | Two-tailed Student's T-Test was used as a statistical test. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | When possible (for single cell analysis were the number of obsevation was >100) the values distribution of our measurements was evaluated and found to approximate to normal distribution. |
| Is there an estimate of variation within each group of data? | The statistical test used assumes unequal variance between groups so to keep the significant assessment more stringent |
| Is the variance similar between the groups that are being statistically compared? | The statistical test used assumes unequal variance between groups so to keep the significant assessment more stringent |

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## 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). | All the antibodies used in this study were previously used and profiled for the assay and species used in this study. References and sources (either commercial or academic are reported in Table S3). |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | HeLa cells were obtained from American Tissue Type Collection (ATTC, USA) (https://www.lgcstandards-atcc.org/products/all/CCL-2.aspx?geo_country=it). E14-mESCs were from Dr. Maurizio D'Esposito lab at IGB-CNR, (Fico A et al, 2008). |

* 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. | Whole brains from C57BL/6 wild type or AUTS2 del8/+ and AUTS2 del8/del8 mice were from embryo at E17.5 stage of development. Whole brains from wild type C57BL/6 mice, processed for ChIP experiments, were from adult male mice (9 weeks). |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | No experiment with live animal model was used in this study. |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | We have adequately reported all the informations about the animal studies we have performed. |

## E- Human Subjects

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

## F- Data Accessibility

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| 18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | No data of the kind refrred in this point are reported in this study. |
| 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). | We commit to provide all numerical row data central to this study once and if it is accepted by the journal. |
| 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). | No such data are reported in this study |
| 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | No computational models are reposted in this study |

## G- Dual use research of concern

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| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | We do not see room for dual use in our research. |