THE EMBO JOURNAL

Manuscript EMBO-2016-95235

let-7 regulates radial migration of new-born neurons through positive regulation of autophagy

Rebecca Petri, Karolina Pircs, Marie E Jönsson, Malin Åkerblom, Per Ludvik Brattås, Thies Klussendorf, Johan Jakobsson

Corresponding author: Johan Jakobsson, Lab of Molecular Neurogenetics, Wallenberg Neuroscience Center

Review timeline:

Submission date:	12 July 2016
Editorial Decision:	01 September 2016
Revision received:	04 January 2017
Editorial Decision:	07 February 2017
Revision received:	23 February 2017
Accepted:	01 March 2017

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 01 September 2016

Thanks for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision but I have now received input from the three referees. I am afraid that the overall recommendation is not very positive.

The referees appreciate the topic and find the link between miRNA, autophagy, and neuronal migration interesting. However, they also raise many concerns with the analysis that I am afraid preclude publication here at this stage. As you can see from the comments below the referees find the analysis too incomplete and not conclusive enough to support the key findings. Given these comments from good experts in the field, I am afraid that I can't offer to consider here.

I thank you for the opportunity to consider this manuscript. I am sorry that I cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

REFEREE REPORTS

Referee #1:

In this manuscript, the authors investigated miRNAs regulating adult neurogenesis. They used lenvirus-ARG2-GFP to target RMS and then performed RIP-seq of OB at 8-weeks later. They

identified 90 miRNAs that are enriched in RIP and about half of them belongs to let-7 family. They performed a series of experiments which led to the conclusion that let-7c regulates adult new neuron maturation and radial migration in OB through by regulating autophagy pathway and possibly through direct targeting of slc7a5. The story is very interesting and the discovery of the link between miRNA, autophagy, and radial migration of adult new neurons is novel. However there are missing links and the study is incomplete.

1) The strategy of using lentivirus targeting RMS and then perform RIP of OB is clever and logical. However RIP control should be the lentivirus-GFP (without ARG2) injected mice instead of sham-injected mice, because lentivirus infection may cause cellular changes and GFP antibody may also have non-specific binding. The more troublesome aspect of this experiment is the number of biological replicates used. It is unclear how many biological replicates were used to generate the data for the main figures (Figures 1 and 2). The authors describe another RIP replicate was performed and the data were included as supplemental data. Does it mean only 2 biological replicates were done with one replicate used for main figures and one for supplemental figures? If so, why did the author chose not to analyze the biological replicates together? In Fig 4E, the figures show miRNAs that are negatively enriched which is strange to- what is the biological meaning of these miRNAs?.

2) It is unclear why autophagy pathway was picked to study. There is no strong rationale.

3) Since radial migration is impaired, how about tangential migration in the RMS?

4) In Fig 4A-B, the authors aim to study the impact of let7c sponge on differentiation. However BrdU was given two hours before sacrificing the mice and authors conclude Let7c does not affect "cell cycle exit of neuroblasts. It is unclear where the analysis was performed. Neuroblast proliferation should happen in RMS or earlier. If differentiation is the goal of the assay, then 2 hours BrdU pulse is too short. This part of the manuscript is confusing and needs to be completely re-written.

5), The assessments of P62 and LC3 levels were solely based on fluorescent intensity, which is insufficient. The data shown in Fig 5 are far from convincing. Another method must be used to validate the changes in autophagy since this is a major point of the paper.

6). The last part of the manuscript attempts to link let-7c with autophagy by brining out slc7a5. However the analysis fell short. let-7c targeting of slc7a5 is only based on luciferase which is insufficient. How slc7a5 level changes affect the autophagy and new neurons in OB are not assessed.

7) In summary, uncover the role of autophagy on radial migration of new neurons would be a novel finding, however the manuscript did not provide sufficient data to support this conclusion.

Referee #2:

Petri et al find that the let-7 family of miRNAs is highly expressed in olfactory bulb (OB) newborn neurons and that reducing its activity inhibits neuronal migration and maturation. In parallel, they observe that reducing let-7 also inhibits autophagy, whose compensation by overexpression of autophagy-driving transcription factors compensate the let-7 phenotype. Hence, this work propose a new significance of let-7 and autophagy in adult neurogenesis. This is novel and interesting and of significance for the field.

Major points

1) The identification of let-7 upregulation is achieved by injection in the rostral migratory stream (RMS) of lentiviral particles encoding Ago-GFP followed by immunoprecipitation of GFP 8 weeks later. In this way, miRNAs expressed in 8 week old neurons can be assessed. Although a retrovirus would have been superior to birthdate neurons, it remains unclear what is the negative control here. The authors indicate that these are "sham injected mice", that to me suggests an injection with PBS. However, for the identification of newborn vs any other adult born neuron the authors should have

injected the really same Ago-GFP virus at a different time point as they do later to assess let-7 phenotypes. Even injection of GFP (or immunoprecipitation of endogenous Ago) would be superior to comparing any miRNA in the whole olfactory bulb as a way not only to normalize for unspecific pull down but also to distinguish adult born neurons (mostly granule cells) with any neuron (including mitral and others), glial, endothelial cells and so forth present in the OB. A sham injection is not appropriate to claim enrichment of let-7 in "newborn neurons" as it could equally indicate enrichment in any neuron relative to, say, macroglia. This point notwithstanding, it is clear that let-7 is highly expressed so I invite the authors to clearly explain what the "sham" is and revise their claims pointing out the limitation of this technical part of their study. Lastly, can the authors exclude that viruses follow up the RMS to reach the SVZ? Being these integrating viruses, newborn neurons will not be 8 week old and could be any younger than that.

2) As a follow up of the previous point, it is hard to claim that the in situ hybridizations in Fig 2 validate the upregulation of let-7 is newborn OB neurons. These figures show massive let-7 staining anywhere. It could equally be concluded that let-7 is highly expressed in any cell of the whole brain. What are the negative controls for this staining? Based on these data, I find the manuscript highly misleading on the definition of "newborn neuron". The fact that the authors can restrict their phenotypes by viral injection in the RMS it's OK, so the manuscript remains overall valid, but expression and role of let-7 cannot be claimed to be specific. This also asks for a substantial reconsideration of the conclusions and many links between adult neurogenesis and disease.

3) I do not understand why the authors restrict their phenotype in Fig 3 to migration only. How could they exclude that the reduction in GFP+ cells is not due to apoptosis, a block in neurogenesis, a block in migration within the RMS where the injection is performed? These cells are never quantified, nor the number of those in Fig. 4. I also find the pattern of GFP+ cells in the controls overtime very surprising... The rationale of this approach is that a wave of neuroblasts are birthdated that generate neurons that reach the OB. This should happen in short time, 1-2 weeks, since the injection is performed in close vicinity to the OB (Fig 1A). Hence, there should be a plateau reached very soon with no neuron being added later because the wave is over. Actually, there should be a decrease since most, about 50%, of newborn neurons die and do not integrate. The authors show completely different results in control injections in Fig 3, how can this be?

4) This work would profit a lot from the converse experiments by which overexpression of let-7 is shown to increase survival/migration/autophagy of newborn neurons. This is particularly relevant given that the authors emphasize so much a link to disease.

5) I am puzzled by the quantification in the rescue experiments. To my knowledge co-injection of two lentiviruses should give random infectivity with only about 30% of cells being co-transduced by both. As a result, among GFP+ cells, only about half should have also received the second virus resulting in a rescue in only half of the population. This seems not to reflect the variance and means of this experiment. Of note, only 1 out of 3 cells in Fig 6B have signal for p62, in the other 2 this is undetectable. How could this experiment be quantified given the extreme diversity in phenotype? Have the authors assessed efficiency of co-infectivity?

Minor points
1) The bars in Fig 1 are somehow fragmented, e.g. 1D miR-143

2) Second line, page 9. If the viruses were injected 8 weeks before giving BrdU, then no BrdU+ neuron should be detected within the GFP+ population since all neuroblast should have divided already and the population should be entirely made by posmitotic, BrdU- neurons (see also issues with this approach, major points above)

3) The first sentences of the 3rd paragraph in page 10 require citations for the role of LC3 in autophagy.

Referee #3:

This is an interesting article with potentially important implications at both the basic and the translational research levels. Rebecca Petri and co-workers performed a thorough, in-depth survey of the role of let-7 family members in radial migration of newly born neurons into the adult olfactory bulb; they identified this abundant microRNA family as potentially involved by a non-biased pull-down screen of AGO complexes, then manipulated the levels of let-7 in vivo and caused impaired migration. Enrichment analysis suggested relevance of autophagy and injecting the relevant constructs indicated that this impairment could be at least partially corrected by re-manipulating the beclin-1 and TFEB targets of let-7; consequently, the authors came to the conclusion that let-7 is pivotal for radial migration of newly born neurons in the adult brain and that its impact is mediated via controlling autophagy events, which are very likely to contribute to such neurons finding their way by migrating to their new position through solid tissue. While this may well be the case, the provided information is partial and not entirely conclusive. The authors may add to the already strong value of this study by addressing the comments below and adding the requested experiments.

Major comments

1 The experimental evidence referring to TFEB and beclin-1 may reflect indirect effect of the implemented changes in let-7, as is indeed noted by the authors; and the rescue achieved is partial at best. To directly prove the involvement of these two targets and their individual and joint impact on neuronal migration, the authors should inject GapMers targeting these transcripts into the brain of mice with let-7 knock-down, alone or together; and test the corrected neuronal migration processes.
2 Does the automated morphology analysis that was used in this study include stereology tests? If so, please detail those and in any case, refer to the affected three dimensional regions in volume terms.
3 To what extent did the migration distance get corrected in the rescue experiment, compared to healthy controls (page 11)? What would be the correction under individual and joint GapMers injections?
4 Migration of newly born olfactory neurons is notably impaired in patients with early phase Parkinson's disease, where the studied process is highly relevant. This should be cited. Also, modified microRNAs and coding and non-coding transcripts, and the inter-relationships between them were systematically analyzed in Parkinson's patients' blood leukocytes before and after deep-brain stimulation treatment. Interrogating the deposited datasets of those studies can add to the impact of the current one.

Additional comments

5 Since a large part of the brain microRNAs is primate-specific, it is advised to check if the TFEB and beclin-1 proteins are also targets of let-7 in the primate brain and cite the relevant studies of AGO2-driven precipitation of human brain microRNAs.
6 While the autophagy process is certainly of interest, it might be worthy to list the other processes targeted by let-7 and discuss their potential involvement in neuronal migration.
7 Let-7 has been reported to be functionally involved in regulating the expression of the RNA metabolism protein hnRNPA1/B2, shown to be impaired in Alzheimer's disease. This may imply relevance of the current observation to the brain RNA metabolism at large, which should be referred to as well.

1st Revision - authors' response 04 January 2017

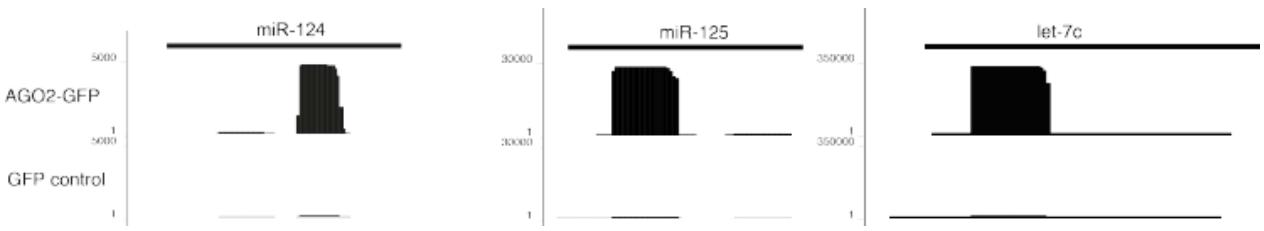
Referee #1:

In this manuscript, the authors investigated miRNAs regulating adult neurogenesis. They used lenvirus-ARG2-GFP to target RMS and then performed RIP-seq of OB at 8-weeks later. They identified 90 miRNAs that are enriched in RIP and about half of them belongs to let-7 family. They performed a series of experiments which led to the conclusion that let-7c regulates adult new neuron maturation and radial migration in OB through by regulating autophagy pathway and possibly through direct targeting of slc7a5. The story is very interesting and the discovery of the link between miRNA, autophagy, and radial migration of adult new neurons is novel. However there are missing links and the study is incomplete.

We thank the reviewer for the interest in our work and for the suggestions on how we should improve the manuscript. We honestly feel that his / her comments have greatly improved our manuscript. We have now modified our manuscript according to the reviewer's suggestions, and included additional experiments that strengthen the conclusions. The details of the changes we have made are outlined below in the point-by-point response. All changes are marked by red font in the manuscript.

1) The strategy of using lentivirus targeting RMS and then perform RIP of OB is clever and logical. However RIP control should be the lentivirus-GFP (without ARG2) injected mice instead of sham-injected mice, because lentivirus infection may cause cellular changes and GFP antibody may also have non-specific binding.

We and others (see e.g. He et al., Neuron, 2012 PMID: 22243745; Tan et al., Science, 2013 PMID: 24311694, Malmevik et al., Scientific Reports, 2015 PMID: 26219083) have used AGO2-GFP fusion proteins to pull out miRNAs in several previous experiments. In our lab we have been running such experiments for many years and we have during that time performed many different control experiments. We do not see any enrichment of miRNAs when performing IPs using a wild-type GFP protein or when using an alternative antibody (see figure below for a RIP-seq experiment using GFP as control). Thus, we are confident that the specificity for AGO-bound miRNAs is high in this experiment.



Genome-browser screen-shots of AGO2-GFP and GFP-ctrl RIP-seq experiments showing the lack of miRNA immunoprecipitation in the control setting.

However, we acknowledge that the choice of control is an important issue. Based on our previous experiences with RIP-seq we think that PBS sham-injected mice are the best option. By analyzing the GFP intensity following LV.GFP-AGO2 injection, we find a very low level of GFP-AGO2 protein expressed in adult-born neurons. This is in line with previous studies conducted in Drosophila and mouse, where any overexpression of tagged AGO proteins appears to be limited by a negative feedback loop (He et al., Neuron, 2012, Czech et al., Mol Cell, 2009 PMID: 19917252). This has two important implications: first the use of GFP-AGO2 is unlikely to affect the native miRNA profiles and their target mRNAs. Second, it means that non-fused GFP is a less efficient control since it is well established that GFP accumulates to high-level following lentiviral injections. Based on this, a sham injection is the best control in this experimental setup since lentiviral-based GFP expression is likely to influence the transcriptome in a different way than expression of GFP-AGO.

The more troublesome aspect of this experiment is the number of biological replicates used. It is unclear how many biological replicates were used to generate the data for the main figures (Figures 1 and 2). The authors describe another RIP replicate was performed and the data were included as supplemental data. Does it mean only 2 biological replicates were done with one replicate used for main figures and one for supplemental figures? If so, why did the author chose not to analyze the biological replicates together?

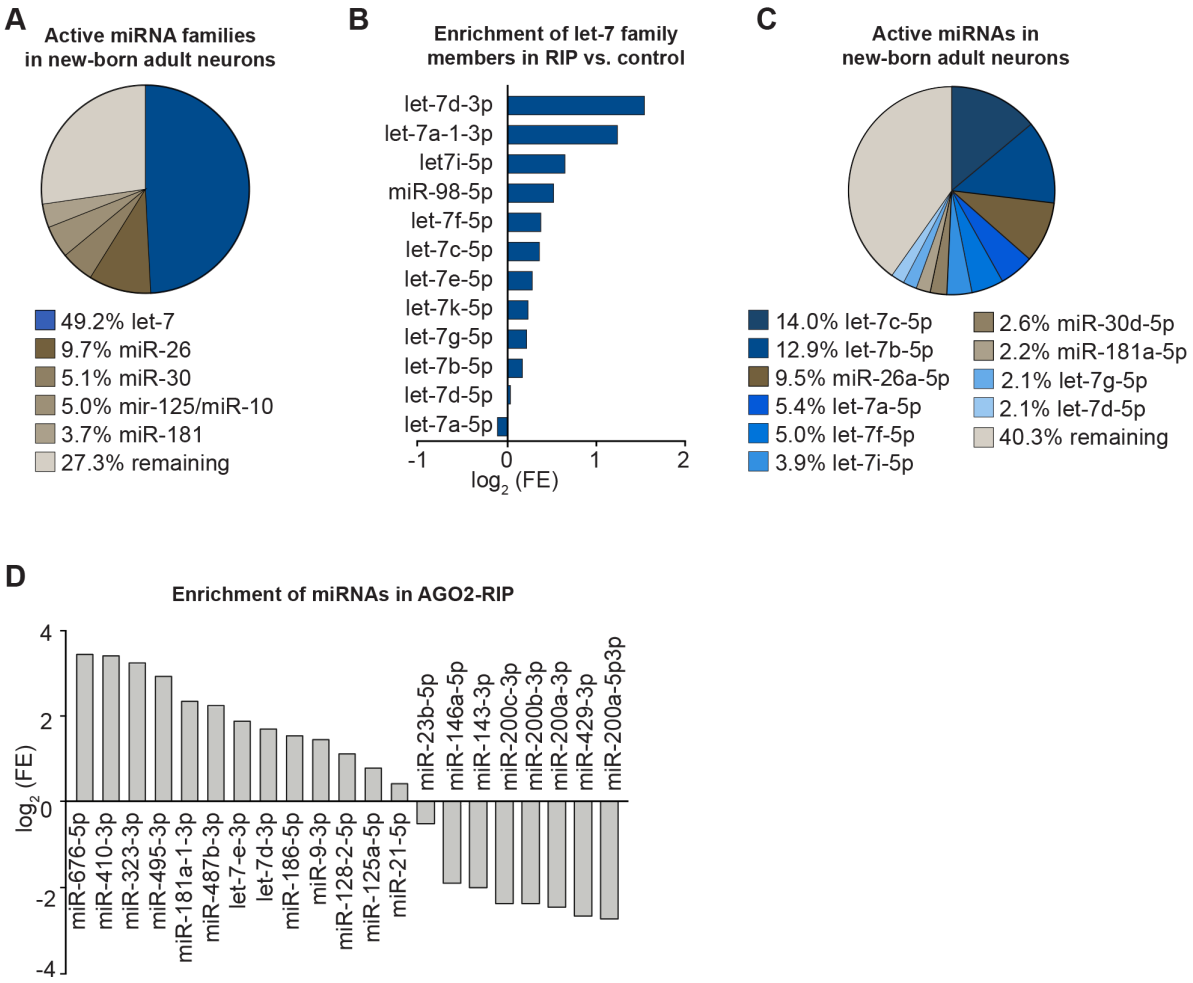
We agree with the reviewer that the experimental design in our previous version of the manuscript was poorly described. In the new version we have clarified this issue in the materials and methods section (p. 21).

We have generated two small RNA-seq libraries with AGO2-RIP-seq samples (and two libraries with ctrl-RIP-seq samples). Each AGO2-RIP-seq and ctrl-RIP-seq sample contains pooled tissue from three individual vector injected mice (the ctrl mice are PBS-injected).

This design has the advantage that it limits the influence of variation between different vector injections since tissue from three different mice was used for each sequencing library. We then performed a complete replication experiment (three other vector-injected mice to produce a second RNA-seq library) and analyzed this data set separately to confirm the robustness of the approach (Suppl Figure 1).

We have decided to use this type of data-presentation based on how we (and others) present ChIP-seq data (another IP-based next generation sequencing approach). This is common standard in the field. However, we have now also analysed the two replicates together (please see figure below) and see only

marginal effects on the results.



Analyses of the average of the two replicates of the AGO2-RIPseq data. Note the similarity to what is presented in Fig 1 and Supplementary Figure 1.

In Fig 4E, the figures show miRNAs that are negatively enriched which is strange to-what is the biological meaning of these miRNAs?.

We agree with the reviewer, that this was not properly explained in the previous version of our manuscript. The scale in Fig 1E is logarithmic and the miRNAs that appear to be "negatively-enriched" on the graph in Fig 1E rather have a very low enrichment score, since they show more reads in the ctrl samples (representing miRNAs expressed in non-GFP positive cells in the OB), than in the AGO2-RIP samples. These miRNAs represent miRNAs expressed in other cell-types such as astrocytes, microglia or other neuronal subtypes present in the olfactory bulb. The fact that some well-know glia-specific miRNAs turn up in this group serves as

an additional internal control of the specificity of the RIP-seq approach. We have clarified this part in the new version of the manuscript so that is easier to understand (p.6 and p.23) and changed the terminology to "enrichment score" (Fig 1E and Suppl Figure 1C).

2) It is unclear why autophagy pathway was picked to study. There is no strong rationale.

We acknowledge that in our previous version of the manuscript we have only poorly explained why we decided to study the autophagy pathway. In the new version of the manuscript we clarified this choice since we feel that there was a strong rational:

- **The phenotype we found after let-7 KD was associated with lack of radial migration. This is a process that is known to require a lot of energy of which autophagy is an essential source.**
- **There have been several recent studies showing that let-7 mediates a network-type of regulation of metabolism and autophagy in other cellular contexts (see e.g.: Dubinsky et al., 2014 (PMID: 25295787), Zhu et al., 2011 PMID: 21962509)**
- **A few emerging studies has shown that autophagy is important for neurogenesis (see e.g.: Li et al., 2016 (PMID: 26905199), Wu et al., 2016 (PMID: 26837467), Yazdankhah et al., 2014 (PMID: 25188513).**

Taken these facts together we think that there was strong rationale to investigate if impairments in autophagy underlie the let-7 phenotype we observed. We have modified our text accordingly (p. 10-11).

3) Since radial migration is impaired, how about tangential migration in the RMS?

We agree with the reviewer that this would be an interesting question to address. In the let-7 KD material (which is extensive, n>40) we do not see any obvious impairment in tangential migration and the let-7.sp-GFP expressing cells appear to accumulate in the OB. However, to analyze the role of let-7 in new-born adult-born neurons we decided to use lentiviral vector injections in the RMS since this is the best option in our hands (why is outlined in detail below). A drawback with this approach is that the genetic modification occurs in RMS when tangential migration has already started. Thus, we cannot robustly and conclusively assess impairments in tangential migration with this approach and have therefore decided not to comment on this issue in the manuscript.

4) In Fig 4A-B, the authors aim to study the impact of let7c sponge on differentiation.

However BrdU was given two hours before sacrificing the mice and authors conclude Let7c does not affect "cell cycle exit of neuroblasts. It is unclear where the analysis was performed. Neuroblast proliferation should happen in RMS or earlier. If differentiation is the goal of the assay, then 2 hours BrdU pulse is too short. This part of the manuscript is confusing and needs to be completely re-written.

We acknowledge that this experiment was poorly described and thank the reviewer for pointing this out. In the new version of our manuscript we have modified the text accordingly (p.9).

The rationale for this experiment is that let-7 has been found to be essential for terminal differentiation and exit from the cell-cycle (many studies in the cancer field e.g. PMID: 20607356, PMID: 25316189). Thus, we reasoned that inhibition of let-7 in dividing neuroblasts might interfere with differentiation and force the cells to remain proliferative. Thus, by injecting BrdU 2 hours before sacrificing the mice followed by GFP co-labelling we should be able to identify ectopic cell proliferation in let-7 KD cells.

The experiment clearly shows that there is no BrdU/GFP co-labelling in the let-7 KD animals showing that neuroblasts efficiently exit the cell-cycle.

5), The assessments of P62 and LC3 levels were solely based on fluorescent intensity, which is insufficient. The data shown in Fig 5 are far from convincing. Another method must be used to validate the changes in autophagy since this is a major point of the paper.

We agree with the reviewer that the assessment of autophagic activity using p62 and LC3 stainings was insufficient (although standard in the field Klionsky et al. 2016 PMID: 26799652 **). We have therefore performed transmission electron microscopy analysis on LV.let-7.sp and LV.GFP control animals. Our data show that let-7 KD cells display significantly fewer and smaller autophagic structures, demonstrating a decrease in autophagic activity, which is in line with increased p62 and decreased LC3 levels, seen by the stainings conducted. For the new version of the manuscript we have included an extensive EM-analysis including quantifications. The new data is added to Figures 4 D-G , Suppl. Figure 3 C-F, and described in p.11. We thank the referee for this comment and we are certain that the inclusion of these data is greatly strengthening the conclusion that let-7 KD impairs autophagy.**

6). The last part of the manuscript attempts to link let-7c with autophagy by brining out slc7a5. However the analysis fell short. let-7c targeting of slc7a5 is only based on luciferase which is insufficient. How slc7a5 level changes affect the autophagy and new neurons in OB are not assessed.

We agree with the reviewer that the description and analysis of let-7 targets regulating autophagy in the OB was inadequate in the previous version of the manuscript. In the new version of the manuscript we have extended this analysis. As shown by Dubinsky et al., 2014 (PMID: 25295787) let-7 regulates many genes related to autophagy in neurons: Slc7a5, Slc3a2, Slc1a5, Map4k3, RagA, RagB, RagC, RagD, LRS, Lamtor1, Lamtor2, Lamtor3, Lamtor4. We therefore think that the phenotype of the let-7 KD is due to an interplay between the effects on different let-7 target genes rather than on one single let-7 target gene. The study of Slc7a5 changes alone in new-born neurons would therefore not be sufficient and would not lead to conclusive data. In the new version of the manuscript we therefore

- **show that these genes in the amino acid sensing pathway are expressed in the OB using qRT-PCR (new data inserted in Figure 4A, Suppl. Figure 3B and p.11)**
- **performed additional RIP experiments followed by qRT-PCR and show that Slc7a5 and Slc3a2 are bound to AGO2 and therefore under miRNA-regulation in the OB (new data are inserted in Figure 4B and p.11)**
- **performed additional luciferase assays and demonstrate that both Slc7a5 and Slc3a2 are direct let-7 targets (new data inserted in Figure 4C and p.11)**

Together these experiments provide a more complete view in the autophagy-related network that is regulated by let-7 in the OB.

7) In summary, uncover the role of autophagy on radial migration of new neurons would be a novel finding, however the manuscript did not provide sufficient data to support this conclusion.

We thank the reviewer for his/her appreciation of the novelty of our findings. We hope that the referee agrees with us that the inclusion of the additional experiments, together with substantial modifications in the text to clarify the methodology shows that our findings are trustworthy and strongly strengthen our conclusion.

Referee #2:

Petri et al find that the let-7 family of miRNAs is highly expressed in olfactory bulb (OB) newborn neurons and that reducing its activity inhibits neuronal migration and maturation. In parallel, they observe that reducing let-7 also inhibits autophagy, whose compensation by overexpression of autophagy-driving transcription factors compensate

the let-7 phenotype. Hence, this work propose a new significance of let-7 and autophagy in adult neurogenesis. This is novel and interesting and of significance for the field.

We thank the reviewer for the interest in our study and for providing very useful comments that helped to improve our manuscript.
We have now modified our manuscript according to the suggested changes, and performed additional experiments. We have also clarified the text according to the reviewer's suggestions. The details of the changes we have made are outlined below in the point-by-point response. All changes are marked by red font in the manuscript.

Major points

1) The identification of let-7 upregulation is achieved by injection in the rostral migratory stream (RMS) of lentiviral particles encoding Ago-GFP followed by immunoprecipitation of GFP 8 weeks later. In this way, miRNAs expressed in 8 week old neurons can be assessed. Although a retrovirus would have been superior to birthdate neurons, it remains unclear what is the negative control here. The authors indicate that these are "sham injected mice", that to me suggests an injection with PBS.

We agree with the reviewer that the previous version of our manuscript was lacking a detailed description on why we chose lentiviral-based delivery of AGO-GFP to label adult-born neurons and thank the reviewer for pointing this out. Injection of lentiviral vectors into the RMS results in efficient transduction of both dividing and non-dividing cells resulting in a far higher transduction rate than injection of retrovirus (which certainly is more specific). However, the high number of transduced cells is absolutely necessary for the AGO2-GFP RIP technique to work. For an efficient RIP we need a certain number of cells (which we just reach by using a lentiviral vector). The sham-injected mice are indeed PBS-injected. These matters have been clarified in the new version of the manuscript (p.16).

However, for the identification of newborn vs any other adult born neuron the authors should have injected the really same Ago-GFP virus at a different time point as they do later to assess let-7 phenotypes. Even injection of GFP (or immunoprecipitation of endogenous Ago) would be superior to comparing any miRNA in the whole olfactory bulb as a way not only to normalize for unspecific pull down but also to distinguish adult born neurons (mostly granule cells) with any neuron (including mitral and others), glial, endothelial cells and so forth present in the OB. A sham injection is not appropriate to claim enrichment of let-7 in "newborn neurons" as it could equally indicate enrichment in any neuron relative to, say, macroglia. This point notwithstanding, it is clear that let-7 is highly expressed so I invite the authors to clearly explain what the "sham" is and revise their claims pointing out the limitation of this technical part of their study. Lastly, can the

authors exclude that viruses follow up the RMS to reach the SVZ? Being these integrating viruses, newborn neurons will not be 8 week old and could be any younger than that.

We agree that there are challenges with the RIP-approach when it comes to selecting an appropriate control. It is true that the composition of other cells in the OB (not expressing AGO-GFP) will influence which miRNAs are mostly enriched and one can certainly discuss which is the best option here.

As mentioned above (reviewer #1, comment#1) we and others (see e.g. He et al., Neuron, 2012 PMID: 22243745; Tan et al., Science, 2013 PMID: 24311694, Malmevik et al., Scientific Reports, 2015 PMID: 26219083) have used AGO2-GFP fusion proteins to immunoprecipiate miRNAs in several previous experiments. In our lab we have been running such experiments for many years and we have during that time performed many different control experiments to verify that our results are trustworthy. Based on our previous experiences with RIP-seq we think that PBS sham-injected mice are the best option (see comment to reviewer #1, comment#1).

We also agree with the reviewer that we cannot be sure that the labelled cells are 8 weeks old but we can rather only say that they are new-born, adult born neurons (that are less than 8 weeks old). We cannot exclude that a small amount of the virus would also target cells in the SVZ, although no GFP expression was observed in the SVZ of LV.GFP.let-7.sp animals.

Taking all this into account (and in agreements with the reviewers comment), it is still very clear from the data that let-7 is very highly expressed in adult-born neurons. The key finding that let-7 is very highly expressed in new-born neurons is a very solid and robust finding and the limitations with the RIP-seq approach definitely do not alter this interpretation in any way. We acknowledge that the previous version of the manuscript did not include a clear description of the sham injected controls, which is now stated in the 'Methods' section in the new version of the manuscript, p21.

2) As a follow up of the previous point, it is hard to claim that the in situ hybridizations in Fig 2 validate the upregulation of let-7 is newborn OB neurons. These figures show massive let-7 staining anywhere. It could equally be concluded that let-7 is highly expressed in any cell of the whole brain. What are the negative controls for this staining? Based on these data, I find the manuscript highly misleading on the definition of "newborn neuron". The fact that the authors can restrict their phenotypes by viral injection in the RMS it's OK, so the manuscript remains overall valid, but expression and role of let-7 cannot be claimed to be specific. This also asks for a substantial reconsideration of the conclusions and many links between adult neurogenesis and

disease.

We agree with the reviewer on this point and have modified the manuscript accordingly. This sentence now runs:

"Together these data demonstrate a high-level expression of several let-7 family members in new-born OB neurons." (p.8)

We have moreover removed the FISH analysis from the manuscript in response to the reviewers comment. We agree that this analysis does not add any conclusive information to the manuscript. Still, these modifications of the text does not take away anything from the finding that let-7 is very highly expressed in adult-born neurons (in agreement with the reviewers comment).

3) I do not understand why the authors restrict their phenotype in Fig 3 to migration only. How could they exclude that the reduction in GFP+ cells is not due to apoptosis, a block in neurogenesis, a block in migration within the RMS where the injection is performed? These cells are never quantified, nor the number of those in Fig. 4.

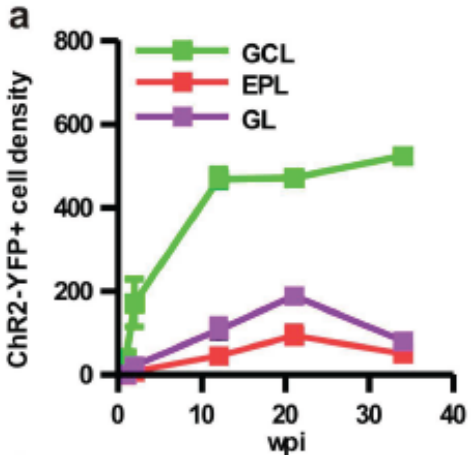
We agree with the reviewer that our previous version of the manuscript was lacking essential experiments addressing these issues. In the new version of the manuscript we have extended our analysis and included quantifications of GFP-expressing cells in the OB, showing no difference in the number of GFP-positive new-born neurons upon let-7 KD compared to LV.GFP injected animals (new data inserted as Suppl. Figure 2 A, p.9). We have also performed a Caspase-3 staining, which showed no sign for increased apoptosis upon let-7 KD (new data inserted in Suppl. Figure 2 B-C, p. 9) confirming that let-7 KD does not lead to a general block in neurogenesis or cell death.

I also find the pattern of GFP+ cells in the controls overtime very surprising... The rationale of this approach is that a wave of neuroblasts are birthdated that generate neurons that reach the OB. This should happen in short time, 1-2 weeks, since the injection is performed in close vicinity to the OB (Fig 1A). Hence, there should be a plateau reached very soon with no neuron being added later because the wave is over. Actually, there should be a decrease since most, about 50%, of newborn neurons die and do not integrate. The authors show completely different results in control injections in Fig 3, how can this be?

As mentioned above we have chosen to use lentiviral transduction since it targets many cells. It also allows a high-level of the transgene expression, which is crucial for the sponge construct to work.

We have chosen this technique based on a paper by the Lledo-group, which is one of the leading groups in the field (Alonso et al., Nat Neurosci, 2012 PMID:

22581183). In this study the authors inject lentiviral vectors into the RMS and quantify GFP expressing cells in the OB at different time-points after injections. They find a gradual increase over 12 weeks until the number reaches a plateu (see inserted figure below). These findings are very much in line with what we see. As mentioned above, this is likely due to the fact that we transduce both dividing and non-dividing neuroblasts in the RMS reaching a broader population of cells than with retrovirus transductions. In the paper by Alonso et al., they describe that only 3 % of transduced cells in the OB (after 12 weeks) are neuroblasts (DCX positive), while the rest are adult-born neurons. This is also very much in line with what we see in the ctrl LV.GFP injected mice (as well as in the LV-AGO2.GFP injected mice).



Sup Fig 1A from Alonso et al., Nature Neuroscience 2012.

4) This work would profit a lot from the converse experiments by which overexpression of let-7 is shown to increase survival/migration/autophagy of newborn neurons. This is particularly relevant given that the authors emphasize so much a link to disease.

We agree with the reviewer that this experiment would be very interesting. Unfortunately, it is technically not possible. As described in the manuscript, let-7 is very highly expressed in adult-born neurons and it would not be possible to overexpress let-7 with the technologies that are currently available for *in vivo* use.

5) I am puzzled by the quantification in the rescue experiments. To my knowledge co-injection of two lentiviruses should give random infectivity with only about 30% of cells being co-transduced by both. As a result, among GFP+ cells, only about half should have also received the second virus resulting in a rescue in only half of the population. This seems not to reflect the variance and means of this experiment. Of note, only 1 out of 3 cells in Fig 6B have signal for p62, in the other 2 this is undetectable. How could this experiment be quantified given the extreme diversity in phenotype? Have the authors

assessed efficiency of co-infectivity?

As mentioned above we achieve a very high-level transduction with lentiviral vectors injected in the RMS (much greater than 30%) hereby suggesting that we should get good rates of co-infection.

When it comes to p62 levels we agree with the reviewer, that the figures on the p62 staining were badly chosen and not representative for our data (due to the fact that the figure was a confocal scan). In fact, we do not see much variation between cells (see error-bars in Fig 4J & 5E). In the new version of the manuscript we have inserted more representative figures (Fig 4 H-I and Fig 5 B-D).

Minor points
1) The bars in Fig 1 are somehow fragmented, e.g. 1D miR-143

We thank the reviewer for pointing this out, we have corrected this in the new version of the manuscript

2) Second line, page 9. If the viruses were injected 8 weeks before giving BrdU, then no BrdU+ neuron should be detected within the GFP+ population since all neuroblast should have divided already and the population should be entirely made by posmitotic, BrdU- neurons (see also issues with this approach, major points above)

We would like to thank the reviewer for pointing this out and agree with her / him that our previous manuscript was lacking good explanations for these experiments. In the new version of our manuscript we have modified the text accordingly (p.9).

The rationale for this experiment is that let-7 has been found to be essential for terminal differentiation and exit from the cell-cycle (many studies in the cancer field e.g. PMID: 20607356, PMID: 25316189). Thus, we reasoned that inhibition of let-7 in dividing neuroblasts may interfere with differentiation and force the cells to remain proliferative. Thus, by injecting BrdU 2 hours before sacrificing the mice followed by GFP co-labelling we should be able to identify ectopic cell proliferation in let-7 KD cells.

The experiment clearly shows that there is no BrdU/GFP co-labelling in the let-7 KD animals showing that neuroblasts efficiently exit the cell-cycle (in spite of previous literature) (Figure 3 A-B).

3) The first sentences of the 3rd paragraph in page 10 require citations for the role of LC3 in autophagy.

We thank the reviewer for making us aware of this missing reference. We have added appropriate references (Klionsky DJ et al. PMID: 26799652) **to the new version of our manuscript.**

Referee #3:

This is an interesting article with potentially important implications at both the basic and the translational research levels. Rebecca Petri and co-workers performed a thorough, in-depth survey of the role of let-7 family members in radial migration of newly born neurons into the adult olfactory bulb; they identified this abundant microRNA family as potentially involved by a non-biased pull-down screen of AGO complexes, then manipulated the levels of let-7 in vivo and caused impaired migration. Enrichment analysis suggested relevance of autophagy and injecting the relevant constructs indicated that this impairment could be at least partially corrected by re-manipulating the beclin-1 and TFEB targets of let-7; consequently, the authors came to the conclusion that let-7 is pivotal for radial migration of newly born neurons in the adult brain and that its impact is mediated via controlling autophagy events, which are very likely to contribute to such neurons finding their way by migrating to their new position through solid tissue. While this may well be the case, the provided information is partial and not entirely conclusive. The authors may add to the already strong value of this study by addressing the comments below and adding the requested experiments.

We would like to thank the reviewer for the interest in our study and for providing some very useful comments that we believe greatly helped to improve our manuscript. We have now modified our manuscript according to the suggested changes, and included additional experiments. We have also clarified the text in line with the reviewer's suggestions. The details of the changes we have made are outlined below in the point-by-point response. All changes are marked by red font in the manuscript.

Major comments

1 The experimental evidence referring to TFEB and beclin-1 may reflect indirect effect of the implemented changes in let-7, as is indeed noted by the authors; and the rescue achieved is partial at best. To directly prove the involvement of these two targets and their individual and joint impact on neuronal migration, the authors should inject GapMers targeting these transcripts into the brain of mice with let-7 knock-down, alone or together; and test the corrected neuronal migration processes.

We thank the reviewer for this comment. We however feel that this comment is

due to a misunderstanding caused by insufficient description of the experiments in our manuscript. We do not think that TFEB or Beclin1 are direct targets of let-7. Rather we think and show in our new version of the manuscript (p. 10, Figure 4) that let-7 targets several genes related to autophagy hereby positively regulating this process. We chose to overexpress TFEB and Beclin-1 in order to mechanistically demonstrate that the phenotype observed after let-7 KD can be reversed by activating autophagy (both Beclin-1 and TFEB induce autophagic activity (Settembre et al. 2011 PMID: 21617040, He et al. 2010 PMID: 20097051)).

We have performed a more thorough examination of let-7 targets regulating autophagy that are expressed in the OB in the new version of our manuscript (p.10, Figure 4 and Suppl. Figure 3)

2 Does the automated morphology analysis that was used in this study include stereology tests? If so, please detail those and in any case, refer to the affected three dimensional regions in volume terms.

The automated morphology analysis does not include a stereology test. It is based on an automatic random sampling. It is done using the software HCS Studio – Cellomics Scan 6.6.0 from Thermo Scientific. All GFP-positive cells in an OB section are detected by the software and neurite length and number of branch points per cell are automatically measured. In that way hundreds of cells can be analysed. More details on this approach can be found in the materials and methods section p. 19-20.

3 To what extent did the migration distance get corrected in the rescue experiment, compared to healthy controls (page 11)? What would be the correction under individual and joint GapMers injections?

We have added new quantifications of the migrated distance to Figure 5, that show that the distance gets almost rescued to migrated distances of LV.GFP expressing cells after LV.Becn1 or LV.TFEB co-injection (new data inserted in Figure 5 I-J). For the reviewer's comment on GapMers we would like to refer to our response to comment #1. Since TFEB or Beclin1 are not direct targets of let-7 the use of Gapmers would not be conclusive in this analysis.

4 Migration of newly born olfactory neurons is notably impaired in patients with early phase Parkinson's disease, where the studied process is highly relevant. This should be cited. Also, modified microRNAs and coding and non-coding transcripts, and the inter-relationships between them were systematically analyzed in Parkinson's patients' blood leukocytes before and after deep-brain stimulation treatment. Interrogating the deposited

datasets of those studies can add to the impact of the current one.

In the new version of the manuscript we added a comment relating to PD in the discussion (p.15) and also cite (Nixon 2013, PMID: 23921753)**.**

Additional comments

5 Since a large part of the brain microRNAs is primate-specific, it is advised to check if the TFEB and beclin-1 proteins are also targets of let-7 in the primate brain and cite the relevant studies of AGO2-driven precipitation of human brain microRNAs.

We would like to thank the reviewer for this comment, however, as mentioned above, TFEB and Beclin-1 are not direct targets of let-7.

6 While the autophagy process is certainly of interest, it might be worthy to list the other processes targeted by let-7 and discuss their potential involvement in neuronal migration.

In the discussion (p. 15-16) we mention that let-7 likely targets hundreds of mRNAs in new-born neurons, and in that way controls multiple intracellular mechanisms. In the discussion (p. 15-16) we mention that other studies have found that let-7 regulates important processes in different steps of neurogenesis such as cell cycle progression (PMID: 20133835) and quiescence of NSCs (PMID: 25316189). We agree with the reviewer that let-7 also regulates other processes that are important for neurogenesis including migration of neuroblasts.

7 Let-7 has been reported to be functionally involved in regulating the expression of the RNA metabolism protein hnRNPA1/B2, shown to be impaired in Alzheimer's disease. This may imply relevance of the current observation to the brain RNA metabolism at large, which should be referred to as well.

We thank the reviewer for pointing this out. However, since no study exists (at least to our knowledge) that directly links let-7 and hnRNPA1/B2 dysregulation in Alzheimer's disease we feel that this topic should rather be discussed in a review. We have, however, added a comment on Alzheimer's disease to the discussion.

2nd Editorial Decision 07 February 2017

Thanks for submitting your manuscript to The EMBO Journal. This is a resubmission of a manuscript that was rejected post review last year. Given the significant revisions carried out, I did send the resubmission back to the referees.

I have now received the comments back from the referees and as you can see the referees appreciate that the analysis has been strengthened. However, they still raise concerns with the negative control used for the pull down analysis and the number of times the experiment was done.

I have looked at all the comments carefully and I see the issues raised. I also find your reasoning for using sham injected as control "because this is what we.others have done in the past" not very satisfying - this is not a good reasoning to keep doing so. I think it is a legitimate question to ask how valid the miRNA dataset is because of the control issue and the number of replicates used.

I also see that the strength on the manuscript lies in the follow up and functional analysis of the role of Let-7 and autophagy in the migration of neurons and that the miRNA profiling dataset is the entry point for this analysis.

After discussions with my colleagues, we have decided that we are interested in pursing the manuscript but we also find that we need a better validation of the miRNA dataset to see how good of a resource it is. If it turns out that it is not a good resource then we should think about how to present figure 1. I don't think anyone doubts that Let-7 is expressed a high levels, but the issue is more relevant for the miRNAs that are less abundant. So I would like to ask you to get back to me outlining how one can get a better idea of how good the miRNA profiling dataset is. I think it is also important to discuss these issues better in the manuscript

REFEREE REPORTS

Referee #1:

Overall the paper is interesting in identification of let-7b-autophagy-cell migration. However, I feel the methods from genome wide analysis to cell migration/morphological analysis were performed with sub-par standard. I have doubt in the results because of the data analysis methods. I was very interested in the title and abstract but very disappointed by the quality of data.
The previous R1 asked several really good questions.
First, the R1 indicated the sham injected is not a good control. The authors argued that Lenti-GFP alone is not a good control. I think the argument is a reasonable argument. However what is sham control? Did the mice underwent operation without injection or injection with saline? If overexpression of GFP is a concern, then another AGO2 alone or another gene with weaker promoter should be considered. Even though the authors have been using sham control as control in past papers, the research standard is higher now than before. I am concerned with this experimental design and its potential negative impact on the research field by publishing this method in a good journal.
Second, the R1 was concerned with the use of n=2 biological replicates. I do not agree with the argument made by the authors. There is a strong push and high standard by research community in terms of scientific rigor. I do not think this lack of replicates meets such standard of this journal.
Third, the previous R1 asked which regions of the brain the authors analyzed BrdU cells. The authors did not answer this question. I found similar lack of information in both brain regions and time points of analysis. For example, in Figure 2L and 2M, the legend and results did not describe which time points the authors analyzed GFP+ cell numbers. In addition it is unclear which region of the OB they analyzed. The method says all GFP-positive cells on an OB section were counted. However the OB sections change in size.
It is also unclear how morphological profiling was done. There seems many crossing between GFP+ cells, how do they quantify branches per cell? More precise morphological analysis uses tracing of individual neurons.

Referee #2:

The authors have considerably enriched their study and, for the most, satisfactorily addressed my points. About some remaining points, i maintain some reservations, but overall, these would not significantly change the major conclusion and finding of this work

Referee #3:

This revised article presents a strengthened and better controlled study of selected miRNA regulators of adult neurogenesis which function via modulating the autophagy pathway. The authors employed a GFP lentivirus vehicle for precipitation and sequencing of the selected miRNAs involved in neurogenesis in the adult brain, and found that many of those belong to the Let-7 family, known to be involved with neuronal development. They then validated their findings by a series of in vivo manipulation experiments that identified let-7c as functionally involved in adult neurogenesis and in the radial migration of newborn neurons in the mouse brain. Furthermore, their dataset analyses and manipulation tests point at the autophagy pathway as relevant to this process, with slc7a5 as a particular agent in this pathway. These observations are both innovative and sensible, and the authors performed much additional work in response to the first round of reviewers' comments; which substantially improved the impact of their study. Some issues that have not yet been corrected, however involve the use in this study of small animal numbers and of non-treated mice rather than mice injected with an 'empty' vehicle as controls, although the previous round of review commented on both of these issues as unsatisfactory. In our hands, the lentivirus itself induces an inflammatory reaction in the injected brains, such that its use as a control is pivotal for differentiating between the outcome of the attempted interference and global inflammation in the analyzed datasets.

2nd Revision - authors' response 23 February 2017

Referee #1:

Overall the paper is interesting in identification of let-7b-autophagy-cell migration. However, I feel the methods from genome wide analysis to cell migration/morphological analysis were performed with sub-par standard. I have doubt in the results because of the data analysis methods. I was very interested in the title and abstract but very disappointed by the quality of data.
The previous R1 asked several really good questions.
We thank the reviewer for the interest in our work and for the suggestions on how we should improve the manuscript. We have now modified and clarified parts in our manuscript according to the reviewer's suggestions. The details of the changes we have made are outlined below in the point-by-point response. All changes are marked by red font in the manuscript.

First, the R1 indicated the sham injected is not a good control. The authors argued that Lenti-GFP alone is not a good control. I think the argument is a reasonable argument. However what is sham control? Did the mice underwent operation without injection or injection with saline? If overexpression of GFP is a concern, then another AGO2 alone or another gene with weaker promoter should be considered. Even though the authors have been using sham control as control in past papers, the research standard is higher now than before. I am concerned with this experimental design and its potential negative impact on the research field by publishing this method in a good journal.
We agree with the reviewer that we described poorly how sham-injections were conducted. The sham-injected mice underwent the same surgery as viral-vector injected mice, however, instead of viral vectors, saline was injected. We have added a more detailed description of this procedure to the materials and methods section (p. 17).

Second, the R1 was concerned with the use of n=2 biological replicates. I do not agree with the argument made by the authors. There is a strong push and high standard by

research community in terms of scientific rigor. I do not think this lack of replicates meets such standard of this journal.
We agree with the reviewer that it is of high importance to replicate experiments and meet the high standard of current research. The RIPseq experiments are based on 6 animals each (= in total 12 animals) and the independent experiments show very similar results. We are therefore confident that the key finding of the RIPseq, that the let-7 family is highly expressed in new-born neurons, is true.
We agree that it is challenging to pick an appropriate control for this experiment, making the interpretation of the data problematic in particular when it comes to the lowly abundant miRNAs and the enrichment scores. We therefore decided to focus our manuscript on the finding that let-7 is highly expressed (which is a very solid finding) and accordingly re-structured Figure 1 and Figure EV1 in our revised version of the manuscript.

Third, the previous R1 asked which regions of the brain the authors analyzed BrdU cells. The authors did not answer this question. I found similar lack of information in both brain regions and time points of analysis. For example, in Figure 2L and 2M, the legend and results did not describe which time points the authors analyzed GFP+ cell numbers. In addition it is unclear which region of the OB they analyzed. The method says all GFPpositive cells on an OB section were counted. However the OB sections change in size.
We agree with the reviewer that the analysis was poorly described. In Figure 2L and 2M, brains of mice sacrificed four weeks after vector injection were analysed. We have added this information to materials and methods section (p. 19) and the Figure legend (p. 31 and p35). For the neuronal profiling analysis, olfactory bulb sections of similar sizes between animals were chosen. We have added a sentence to the materials and methods section for clarification (p19).

It is also unclear how morphological profiling was done. There seems many crossing between GFP+ cells, how do they quantify branches per cell? More precise morphological analysis uses tracing of individual neurons.
We agree with the reviewer, that this analysis was poorly explained. The neuronal profiling was conducted with the software HCS Studio – Cellomics Scan 6.6.0 from Thermo Scientific. The neuronal profiling software first detects the cell body and then traces the neurites from the specific cell to measure its length. Thereby it will also determine the number of branch points. Crossings of neurites of different cells were not counted. We have added additional details to the materials and methods section (p. 19)

Referee #2:

The authors have considerably enriched their study and, for the most, satisfactorily addressed my points. About some remaining points, i maintain some reservations, but overall, these would not significantly change the major conclusion and finding of this work
We thank the reviewer for his / her positive comments.

Referee #3:

This revised article presents a strengthened and better controlled study of selected miRNA regulators of adult neurogenesis which function via modulating the autophagy pathway. The authors employed a GFP lentivirus vehicle for precipitation and sequencing of the selected miRNAs involved in neurogenesis in the adult brain, and found that many of those belong to the Let-7 family, known to be involved with neuronal development. They then validated their findings by a series of in vivo manipulation experiments that identified let-7c as functionally involved in adult neurogenesis and in the radial migration of newborn neurons in the mouse brain. Furthermore, their dataset analyses and manipulation tests point at the autophagy pathway as relevant to this process, with slc7a5 as a particular agent in this pathway. These observations are both

innovative and sensible, and the authors performed much additional work in response to the first round of reviewers' comments; which substantially improved the impact of their study.

We thank the reviewer for the interest in our work and for the suggestions on how we should improve the manuscript. We honestly feel that his / her comments have greatly improved our manuscript. We have now modified our manuscript according to the reviewer's suggestions. The details of the changes we have made are outlined below in the point-by-point response. All changes are marked by red font in the manuscript.

Some issues that have not yet been corrected, however involve the use in this study of small animal numbers and of non-treated mice rather than mice injected with an 'empty' vehicle as controls, although the previous round of review commented on both of these issues as unsatisfactory. In our hands, the lentivirus itself induces an inflammatory reaction in the injected brains, such that its use as a control is pivotal for differentiating between the outcome of the attempted interference and global inflammation in the analyzed datasets.

As mentioned above (comment to reviewer #1) we agree that it is challenging to pick an appropriate control for this experiment, making the interpretation of the data problematic in particular when it comes to the lowly abundant miRNAs and the enrichment scores. We therefore decided to focus our manuscript on the finding that let-7 is highly expressed (which is a very solid finding) and accordingly re-structured Figure 1 and Figure EV1 in our revised version of the manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Johan Jakobsson
Journal Submitted to:	EMBO J.
Manuscript Number:	EMBOJ-2016-95235R

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	Sample size was chosen based on literature from the field and previous experiences with similar types of experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We chose the size of animal groups based on our experiences from previous experiments and success rates of vector injections into the RMS.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals were excluded from the analyses if the injection of the virus into the RMS was misplaced.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randolmy assigned to groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was conducted.
5. For every figure, are statistical tests justified as appropriate?	Yes, the statistical tests were justified as appropriate. Details to used statistical tests can be found in the materials and methods section and figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Depending on the analysed data, justified statistical tests were chosen for analyses.
Is there an estimate of variation within each group of data?	Weshow the Standard Error of Mean in our analyses.
Is the variance similar between the groups that are being statistically compared?	We have conducted F-test when suitable to determine if the variance was similar between groups. Details to used statistical tests can be found in the materials and methods section and figure legends.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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 antibodies were purchased commercially and catalogue numbers are given in the materials and methods section p. 17-18.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell lines used in this study were 293T cells and mycoplasma tests are regularily conducted in the lab.

* 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.	All animals used in the study were 10 week old female C57BL/6 mice (see p. 17) . All animal related procedures were approved by and conducted in accordance with the committee for use of laboratory animals at Lund University. Mice were ordered from Taconic.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal related procedures were approved by and conducted in accordance with the committee for use of laboratory animals at Lund University.
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 confirm the compliance.

E- Human Subjects

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

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The RNA sequencing data was submitted to the NCBI Gene Expression Omnibus database and assigned the GEO series accession number GSE83903.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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