EMBO reports

Regulation of neuronal commitment in mouse embryonic stem cells by the Reno1/Bahcc1 locus

Hadas Hezroni, Rotem Ben-Tov Perry, Noa Gil, Neta Degani, and Igor Ulitsky
DOI: 10.15252/embr.202051264

Corresponding author(s): Igor Ulitsky (igor.ulitsky@weizmann.ac.il)

Review Timeline:	Submission with point-by-point response:	7th Jul 20
	Editorial Decision:	24th Aug 20
	Revision Received:	27th Aug 20
	Accepted:	1st Sep 20

Editor: Esther Schnapp

Transaction Report: This manuscript was transferred to EMBO reports following peer review at The EMBO Journal.

(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Point-by-point response to reviewers for *“Regulation of neuronal commitment in mouse embryonic stem cells by the Reno/Bahcc1 locus”* by Hezroni et al.

Referee #1:

The authors use lncRNA expression profiling and sequence conservation to identify new lncRNA regulators of neuronal differentiation. They perform loss of function studies for 7 conserved lncRNAs and report that knockdown of two of these (Reno and lnc-Nr2f1) using either shRNA or promoter deletion inhibits neuronal differentiation. The authors report that this leads to dysregulation of neuronal gene expression and that Reno loss of function remodels the chromatin landscape. They also show that the Reno locus loops onto the promoter of the nearby Bahcc1 gene suggesting co-regulation.

The experimental rationale and design is clear. In particular, the identification of conserved lncRNAs is important. However, this study does not constitute a major advance in our understanding of lncRNA function in neurogenesis. A number of lncRNAs have already been shown to alter neuronal differentiation using cells in culture. Moreover, I have concerns regarding the specificity of the loss of function studies.

(1) Reno levels in ES cells are only depleted by approx. 20% using shRNA. This induces a significant block in neuronal differentiation. In order to evaluate the specificity of the observed loss of function phenotype more analysis is needed. For example, it would be good to know how many molecules of Reno are expressed per cell. Is this level of knockdown then depleting a substantial amount of Reno transcript? Does shRNA mediated knockdown deplete the nuclear/chromatin enriched Reno pool? Rescue experiments would go a long way to validating specificity. In line with this, how does Reno work? Is its function transcript dependent or independent?

We agree with the reviewer that the significant effect of Reno knockdown on neuronal differentiation is surprising considering a ~20% depletion in mESCs. However, the expression level of Reno in ES cells is very low to begin with in these cells. During neuronal differentiation, when Reno levels are increased, we saw more substantial depletion: 50% depletion in day 2 (Figure S6A) and 30% depletion at day 8 (Figure 1C), at which point presumably some of the cells with the more substantial depletion have died. We now also show that removal of most of Reno sequence in *Reno*$^{m/m}$ cells, or depletion of Reno using GapmeRs, which also modestly yet significantly reduces its expression at day 2 of differentiation all lead to similar changes in cell viability and in gene expression, which are partially reversed by ectopic expression of Reno. This further solidifies the notion that mild depletion of Reno is sufficient for eliciting substantial and reproducible effects on neuronal differentiation.

To measure Reno copies per cell, as requested, we used the Reno plasmid to generate a standard curve for absolute quantification using qRT-PCR. These results are shown in Figure S3D (see below). In addition, we used single molecule RNA FISH (smFISH) to characterize Reno copy number and cellular localization. These results are shown in Figure 3D. Together, both of the assays suggest that cells at early stages of neuronal differentiation express ~5–10 copies of Reno on average, with high variability between cells suggested by smFISH.

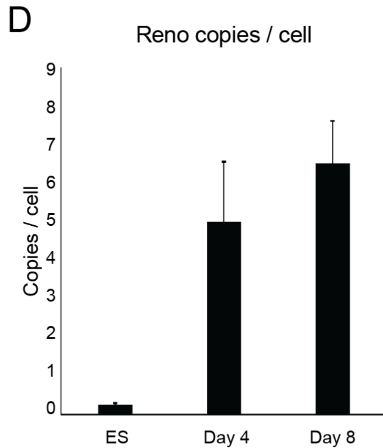


Fig. S3D: Absolute quantification of *Reno* RNA copy number per cell, estimated by qRT-PCR. Mean ± SEM is shown, n=4.

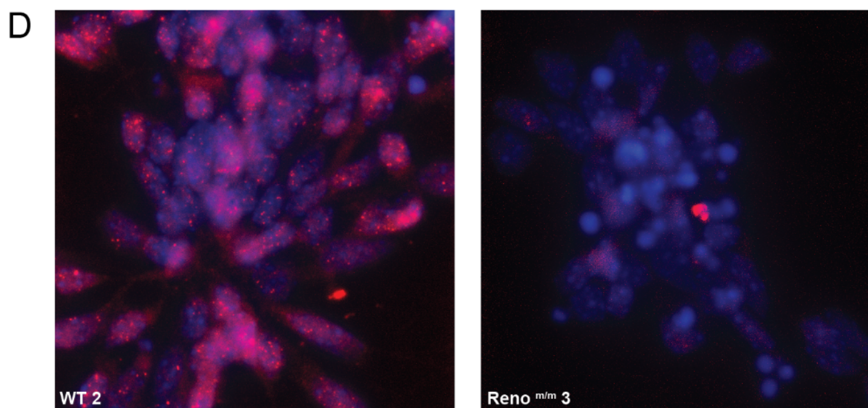


Fig. 3D: Reno smFISH signal (red) and DAPI staining (blue) in WT and *Reno*$^{m/m}$ cells at day 8 of neuronal differentiation, imaged using 100x objective.

In order to address the question of sufficiency of *Reno* transcript for its function, we have cloned Reno sequence to a mammalian expression vector, and used it to test whether ectopic expression of *Reno* can rescue the block of differentiation observed following Reno deletion in mESCs. Indeed, we find that *Reno* expression in *trans* can partially rescue the effects on cell proliferation during differentiation as well as the changes in gene expression (Page 17):

*In order to test whether expression of Reno produced in trans can rescue the phenotype observed in Reno depleted cells, we infected one WT and one Reno$^{m/m}$ line with lentivirus carrying a doxycycline (Dox)-inducible cDNA of the unspliced short isoform of Reno. We added Dox at the onset of differentiation and measured Reno levels at day 2. Reno was overexpressed by ~10-fold in the WT cells and ~70-fold in the Reno$^{m/m}$ cells following Dox addition (**Figure 5D)**. Dox addition increased the number of live Reno$^{m/m}$ cells at day 4 of differentiation (**Figure 5E)**. We also used RNA-seq to characterize gene expression at day 2 of differentiation, and found that Reno induction through Dox addition to either WT or Reno$^{m/m}$ cells upregulated the 168 gene signature that was significantly downregulated in Reno depleted cells, and downregulated the 94 gene signature that was significantly upregulated upon Reno loss-of-function (**Figure 5F-G)**. In Reno$^{m/m}$ cells, overexpression of Reno did not have a significant effect on Bahcc1 expression level (**Figure S6H**). These results demonstrate that ectopic expression of Reno can at least partially rescue the effect of Reno depletion on cell death and gene*

expression at early stages of differentiation and suggest that Reno might be able to carry its function when produced in trans to its regular site of transcription.

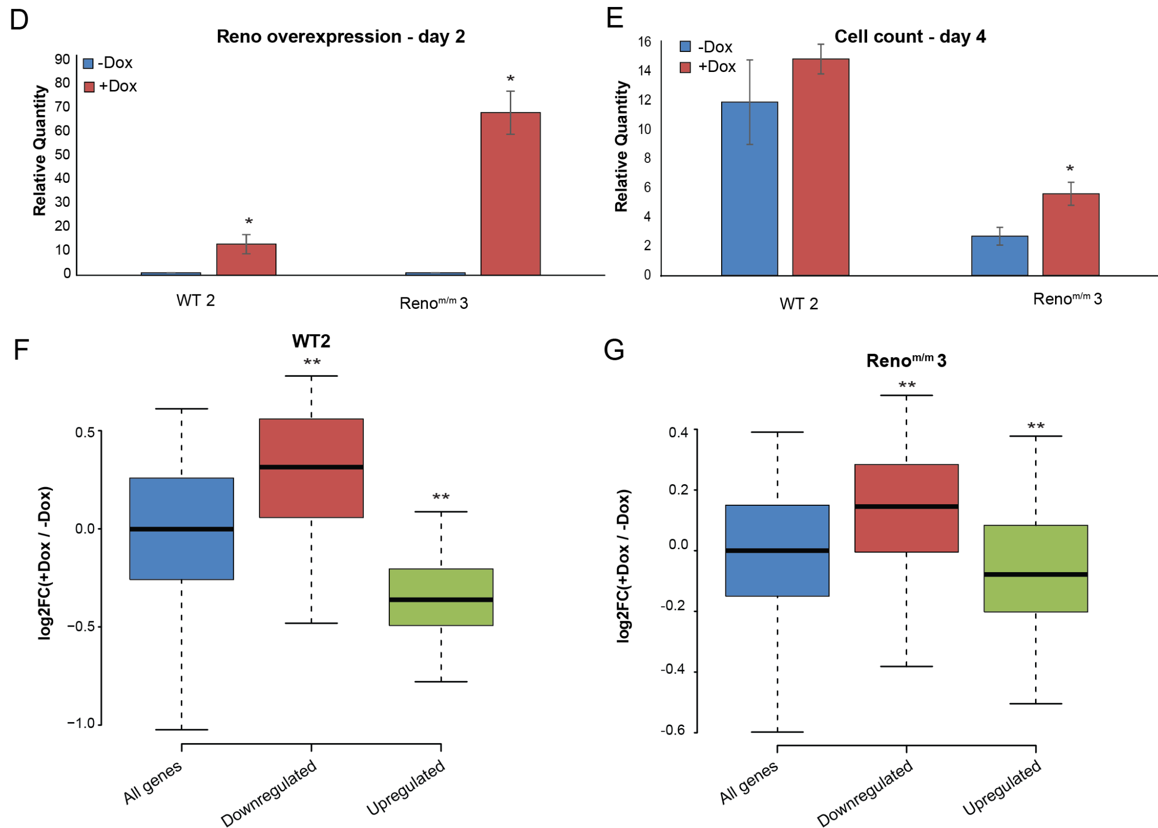


Figure 5: (D) Expression levels of *Reno* following Dox addition in WT and *Reno*$^{m/m}$ cells. Mean ± SEM is shown, n=3, * P<0.05 (unpaired two sample t-test). **(E)** Cell count of WT and *Reno*$^{m/m}$ cells following four days of differentiation, with or without Dox addition. Cells were harvested from one well of a 6-well plate, and counted using Orflo MOXI Z Mini Automated Cell Counter. Mean ± SEM is shown, n=3. * P<0.05 (unpaired two sample t-test). **(F-G)** Distributions of changes in expression levels of WT **(F)** or *Reno*$^{m/m}$ **(G)** cell treated with Dox for the first two days of differentiation compared to untreated cells, in genes which were significantly (P<0.05) dysregulated by at least two out of three *Reno* perturbations at day 2 in fig. 5A. ** P<0.01 (two-sided Wilcoxon rank sum test).

(2) The authors also use promoter deletion to deplete Reno expression. A detailed comparison with shRNA mediated analysis would improve the study. Expression changes are correlative and it is unclear which effects are direct or indirect or off target. How many genes are regulated when Reno is knocked down using both the shRNA and promoter deletion techniques? This might identify real targets and would help confirm specificity of action.

As suggested by the reviewer, we have now performed RNA-seq in all relevant conditions at day 2 following depletion of Reno (using shRNAs or GapmeRs), *Reno* deletion in *Reno*$^{m/m}$ cells, as well as following Reno ectopic expression in *Reno*$^{m/m}$ cells.

We now report a detailed comparison of these analyses using different methods. We note that it is difficult to directly select the "real" targets as this is sensitive to threshold selection. Nevertheless our results point to two signatures of genes that are up- and down- regulated following Reno depletion, the former of which are high-expressed in mESC and in early neurons. Furthermore, these signatures are changing in the opposite direction when Reno is expressed ectopically, strongly suggesting that they are indeed "on target" genes regulated by Reno, directly or indirectly:

*We used RNA-seq to characterize cells that experienced Reno knockdown by shRNAs (**Figure S6A**), Reno depletion by promoter deletion, or Reno knockdown by GapmeRs (**Figure S6B**). Transfection of GapmeRs targeting Reno led to a significant reduction in number of viable cells at an early stage of differentiation which resembled the outcome of Reno depletion by promoter deletion (**Figure S6C)**. Reno depletion did not have a significant effect on Bahcc1 mRNA expression level in any of the perturbation methods (**Figure S6D**). Hundreds of genes were dysregulated in Reno-depleted cells at day 2, and the gene expression pattern was highly concordant for the different perturbation methods (**Figures 5A, 5B, S6E**). The 168 genes which were downregulated in at least two of the three perturbations were enriched for GO terms related to cell migration and motility, and to developmental process, while the 94 genes which were upregulated in at least two of the three perturbations methods were enriched for GO terms related to metabolic processes related to translation (**Figure S6F)**. The upregulated genes are highly expressed in ES cells and their expression is reduced in early stages of neuronal differentiation, while downregulated genes are expressed at low levels in ES cells, and they are induced in early stages of differentiation (**Figures 5C, S6G).** These results suggest that appropriate levels of Reno transcript at an early stage of neuronal differentiation are required in order to acquire the transcriptional programs that allow proper differentiation.*

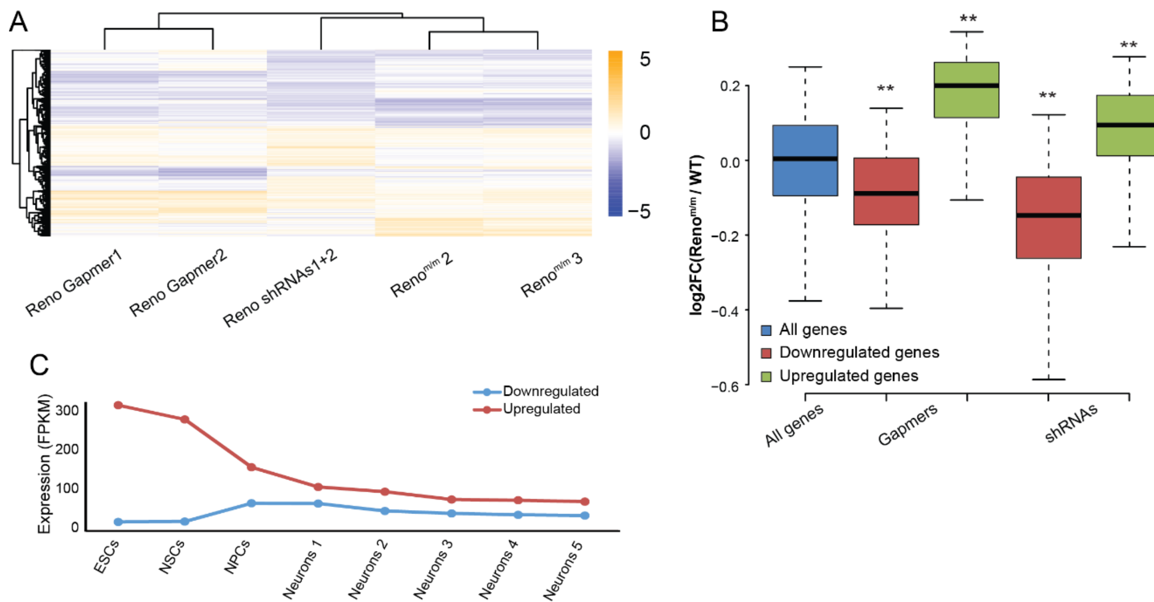
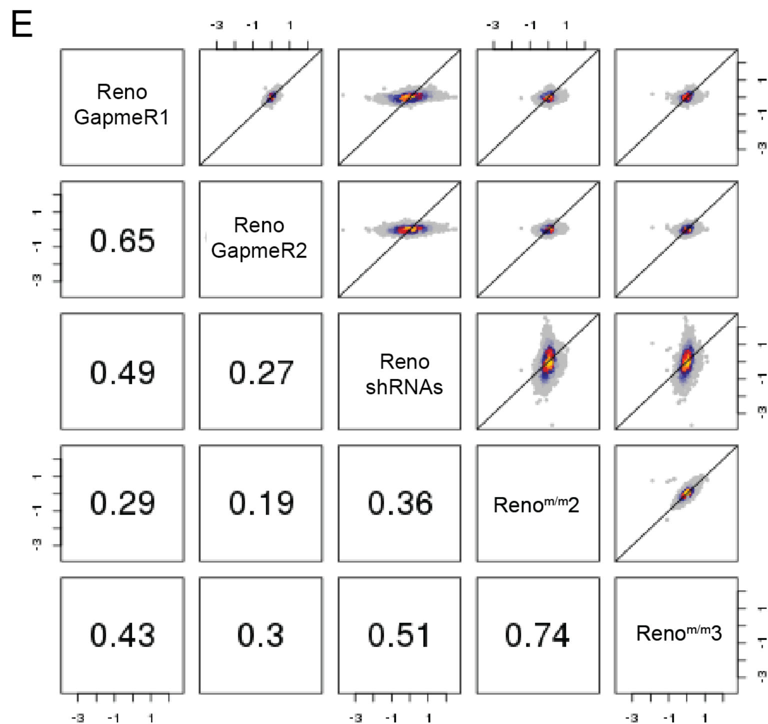


Fig. 5: (A). Heatmap showing $\log_2$-transformed fold changes of *Reno* depleted cells compared to their respective controls. All genes which were significantly differentially expressed (P<0.05) in any of the treatments are shown. **(B)** Distributions of changes in

expression levels of *Reno*$^{m/m}$ cells compared to WT cells, for genes with significant (P<0.05) change in cells where Reno was perturbed with the indicated method. ** P<0.01 (two-sided Wilcoxon rank sum test). **(C)** Mean expression levels of genes in genes which were significantly (P<0.05) dysregulated by at least two out of three *Reno* perturbations at day 2 in fig. 5A, during an eight time point neuronal differentiation and maturation of mouse ES cells: Neurons1 corresponds to day *in vitro* (DIV)1; Neurons2 – DIV7; Neurons3 – DIV16; Neurons4 – DIV21; and Neurons5 – DIV28 (Hubbard et al, 2013).

E



F

Downregulated genes

GO term	P-value
Positive Regulation of Cell Migration	2.26E-17
Positive Regulation of Cell Motility	5.36E-17
Regulation of Developmental Process	1.35E-16
Positive Regulation of Cellular Component Movement	1.39E-16
Regulation of Cell Motility	1.47E-16
Positive Regulation of Locomotion	2.61E-16
Regulation of Cell Migration	1.07E-15
Regulation of Cellular Component Movement	2.45E-15
Regulation of Locomotion	2.55E-15
Regulation of Multicellular Organismal Process	9.71E-15

Upregulated genes

GO term	P-value
Cellular Nitrogen Compound Metabolic Process	5.69E-08
Nucleobase -containing Compound Metabolic Process	1.99E-06
Nucleic Acid Metabolic Process	2.75E-06
Cellular Aromatic Compound Metabolic Process	3.04E-06
Organic Cyclic Compound Metabolic Process	3.86E-06
Heterocycle Metabolic Process	5.51E-06
Translation	9.38E-06
Peptide Biosynthetic Process	1.64E-05
ncRNA Processing	2.75E-05
ncRNA Metabolic Process	4.67E-05

Fig. S6: (E) Pairwise scatterplots showing $\log_2$-transformed fold changes of cells with the indicated *Reno* perturbation compared to their respective controls. Color indicates

local point density. Numbers indicate Spearman correlation coefficient between the fold changes **(F)** Biological Process GO categories most enriched in genes significantly down- or upregulated in at least two *Reno* perturbations, as identified using GOrilla (Eden et al, 2009).

(3) Reno loops onto the Bahcc1 promoter. However, the evidence suggests that Reno does not regulate Bachh1 expression. It would be important to further study the nature of this regulatory relationship and the mechanism of Reno action to improve the study. For example, does Reno bind Bachh1? Does Reno mediate the looping interaction?

We indeed previously found that perturbations of *Reno* do not affect *Bahcc1* expression. We now further report that expression of Reno in *trans* from the lentiviral vector is sufficient for partially rescuing the phenotype of *Reno*$^{m/m}$ cells, without apparent effect on *Bahcc1* mRNA levels, further arguing against a *cis*-acting function of *Reno*. These results argue that the looping between *Reno* locus and the *Bahcc1* promoter is related to their co-regulation by presumptive common enhancers, rather than to the requirement for regulation of *Bahcc1* by *Reno*. In this regard, as discussed in the text (now with an additional recent example), the *Reno*/*Bahcc1* circuit joins the ranks of other lncRNA/protein pairs that are co-located in the genome and act in the same or in related pathways, and yet there is not evidence that the lncRNA has cis-acting function (Page 22):

"These suggest that Bahcc1 and Reno may form part of the same pathway, but not in the direction that has been typically observed, in which the lncRNA regulates the expression of the protein-coding gene, but rather through co-regulation (supported by the high co-expression between Reno and Bahcc1, ***Fig. 3D****), and potential cooperation, reminiscent of that observed for the Six3os and Paupar lncRNAs and the TFs SIX3 and PAX6, respectively (Pavlaki et al., 2018; Rapicavoli et al., 2011; Vance et al., 2014), and the Pnky and POU3F2 lncRNA/TF pair that are co-located in the genome but appear to act independently in the developing brain (Andersen et al., 2019)."*

Further dissection of the mechanism of *Reno* action is of course now a top priority, but it is out of scope of the current manuscript.

(4) How does Reno directly regulate the observed genome wide H3K4me3 changes eg. does it directly target/decoy any chromatin remodelling proteins to these sites in the genome as suggested by the Fam60a depletion analysis. More mechanistic analysis is needed for publication in EMBO J.

The mechanism of Reno action is out of scope of the current manuscript.

Minor point, why did the authors choose to drop the analysis of lnc-Nr2f1 and focus the study on Reno. The lnc-Nr2f1 knockdown was more efficient and also inhibited neuronal differentiation.

A recent study (Ang et el., eLife, 2019) focused on the function of *lnc-Nr2f1* in the conversion of MEFs to neurons, and characterized the genes affected in ES cells differentiated into neurons. Their findings are in agreement to our data, and we preferred to focus on *Reno*, which has not been studied before.

Referee #2:

The study entitled "Regulation of neuronal commitment in mouse embryonic stem cells by the Reno/Bahcc1 locus" aims to use conservation to hone in on lncRNAs that are regulated during neuronal differentiation and mature neurons via RNA-sequencing. The authors identify a subset of 7 lncRNAs to test by shRNA loss of function (LOF) studies. This identifies two lncRNAs that are important for neuronal differentiation by sjRNA screening: Reno & lnc-Nr2f1. This followed by characterization of RNAi based LOF gene-expression changes which are described as "large scale". They further describe Reno's conserved position near BAHCC1 and move towards genetically defined LOF studies. The authors show a clear relationship between Reno and BAHCC1 but also major wide-spread gene-expression changes. Overall, this study suggests a cis regulatory relationship between Reno and BAHCC1 which are phenotypically similar. There are some key aspects that seem to be missing for compelling the readership of EMBO. I suggest that this study is better for EMBO reports in a format focused on cis regulation of BAHCC1 and or lnc-Nr2f1, or a more specialized neuroscience journal.

Overall,. there are many similar studies of ESC > neuronal differentiation RNA-seq and or functional studies there in, as well as eRAN and other cis regulatory roles of lncRNA loci similar to that reported here. Thus, it is unclear how this study is an advance in the lncRNA field that is of general interest to the Broad readership of EMBO and perhaps more fitting for a specialized neurobiology journal.

1) It is unclear from these studies if the role of Reno is simply a cis DNA regulatory element or requires the act of transcription. The authors use a combination of RNAi based and genetic mutants to show the regulation with BAHCC1. Yet it is unclear if this locus was silenced by RNAi based mechanisms (blockade of torpedo transcription) or CRISPR-I (K9me3). With HOTTIP and UMILIO it was possible to ectopically induce a lncRNA and in turn result in activation of nearby genes. Considering the context of this study's introduction, abstract and discussion is to do with lncRNA biology it maybe important to show in a conclusive manner that this mechanism is RNA based and or how it maybe different from other known examples.

We emphasize that the fact that we observe similar effects following RNAi and promoter KO of *Reno* point to the role of the RNA product of *Reno* in the function of the locus. We now further elaborate this point using GapmeR-mediated knockdown of *Reno*, which is not expected to affect the chromatin in the locus. More importantly, we now show that ectopic expression of *Reno* in *trans* using a lentiviral mammalian expression is sufficient for partially rescuing the phenotype of *Reno* loss and so *Reno* transcription from the endogenous locus is not essential for its function. Lastly, we now also refine the comparison between the RNAi, GapmeR, and promoter KO effects using RNA-seq on cells at matched stages, and comparing the changes in gene expression.

Suggested experiment:

Replacement of Reno by a reporter sequence to determine if any transcribed sequence

would be sufficient to activate BAHCC1. Place Reno in cis to other neuronal induction factors and determine if it is sufficient to induce gene expression in cis (which may point to a DNA based role or very local acting lncRNA).

We agree that this is a very interesting experiment, but it will be very challenging technically to obtain such cells and to induce them to differentiate, and given our new data on expression of *Reno* in *trans* we propose that it is out of scope of our current study.

2) RNA-FISH of Reno would be a major addition to this study. Is it stoichiometrically feasible for Reno to regulate so many genes? Also based on the implications in the study it would be hypothesized the Reno is nuclear. Either way RNA-FISH would contribute an important aspect to how, if at all, their maybe an RNA based mechanism.

We now performed single molecule RNA FISH in both cultured differentiated mESCs and *in vivo* in the mouse intestine. In both systems we see that Reno is enriched in the nucleus. Matching the quantifications from RNA-seq and from the calibration experiment we performed following the request of reviewer #1, we conclude that Reno is expressed on average at ~5-10 copies per cell, with non-negligible cell-to-cell variability, the sources of which are unclear at this point, and will require further study.

Page 10: *"Single-molecule fluorescence in situ hybridization (smFISH) in neurons differentiated from WT and Reno$^{m/m}$ ES cells (see below) and in mouse intestine confirmed the nuclear localization of Reno and suggested that 5-10 copies of Reno are found per cell on average, with substantial variability (**Figures 3D** and **S3C**)."*

D

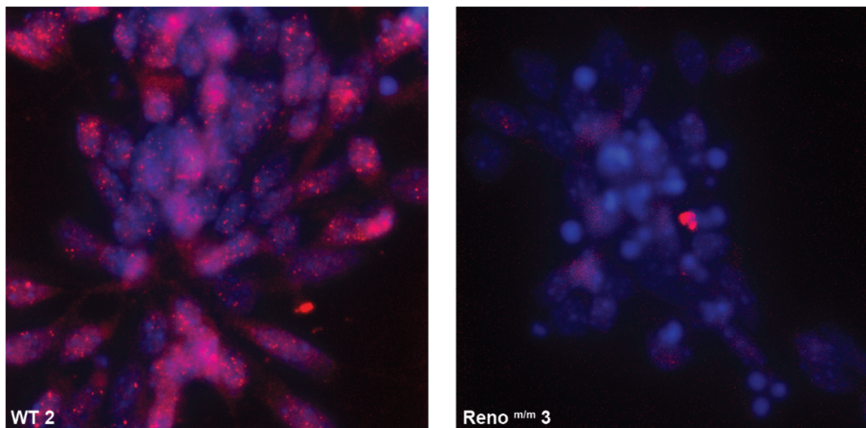


Fig 3D: Reno smFISH signal (red) and DAPI staining (blue) in WT and *Reno*$^{m/m}$ cells at day 8 of neuronal differentiation, imaged using 100x objective.

C

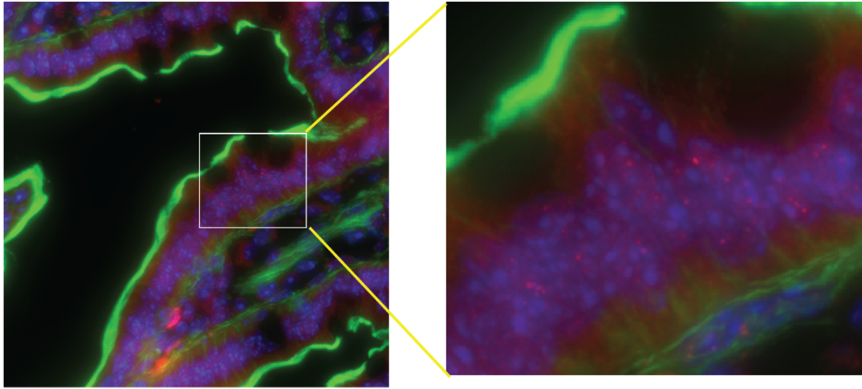


Fig S3C: Reno smFISH signal (red), DAPI staining (blue) and phall (green) in mouse intestine, imaged using 100x objective.

3) This study may benefit from a more focused approach of understanding cis regulatory elements of BAHCC1 rather than lncRNA biology. The numerous amount of RNA-sequencing data and evolutionary analysis resulted in a nearly unexplained rationale for 7 genes and found 2 that had some role. Thus it, is hard to see the context of this study as a resource in such a small scale of functional testing nor a study that aims to understand a RNA based mechanism of a lncRNA in cis. Overall, this study feels incomplete and peicemeal other than the clear relationship between the Reno locus and BAHCC1 -- which maybe of suitable interest to the general readership of EMBO.

We have now shortened the introduction and added additional experimental results that overall put more emphasis in the manuscript on the main findings are about the role of *Reno* (and *Bahcc1*) in neuronal differentiation. The additional results further show that the main function of Reno is not to regulate the mRNA levels of *Bahcc1*, but rather that it acts independently, likely in the same pathway as BAHCC1. The mechanism of how *Reno* and *Bahcc1* are connected is out of scope of the current manuscript.

4) What about lnc-Nr2f1?

A recent study (Ang et el., eLife, 2019) focused on the function of lnc-Nr2f1 in the conversion of MEFs to neurons, and characterized the genes affected in ES cells differentiated into neurons. Their findings are in agreement to our data, and we preferred to focus on Reno, which has not been studied before.

Minor:

1) Introduction reads similar to a review of a few selected lncRNAs that have some data relating to a function in the brain. However, the goal of the study or the need to address a missing knowledge gap is missing. There is a lot of information but doesn't really set up why this study is any different than many of those described in the introduction. The introduction should perhaps set up the need for conservation analyses and other aspects that start the results section. In short the introduction is very disconnected from motivating this study.

We now substantially focused introduction, citing a recent review about lncRNAs in neuronal differentiation and better setting the stage for our study.

2) The authors mention several examples where a lncRNA regulates the neighboring gene. However, it should be noted that for mRNAs and lncRNAs alike there are more long distance interactions for lncRNAs via RNA based mechanisms. For example HOTTIP and UMLILO have both been shown to be lncRNAs connected to neighboring regulation of key mRNA loci. It maybe useful to also show counter examples of how it is difficult to find the targets of "eRNAs" such as Peril that neighbors Sox2, but regulates genes in cis more distally.

We now discuss known mechanisms for cis regulation in the introduction, citing our recent review on the subject that discusses this in more detail. We note that the new results we now present further solidify the notion that *Reno* does not act in *cis*.

Referee #3:

In this paper, Hezroni et al. describes transcriptionally changed lncRNAs during mouse ES cells differentiation into neurons. After computational analysis, a few lncRNAs were prioritized for further study. RNAi-mediated knockdown of two lncRNAs Reno and Nr2f1 was investigated and authors found that neuronal markers were changed. Then a link between Reno and Bahcc1 was proposed where Reno and its adjacent protein-coding Bahcc1 leads to an early arrest in neuronal development. Overall, as major concerns outlined below, the study is quite preliminary to make a strong case for lncRNA function. Bioinformatics analyses of RNA seq and other experiments is quite solid but lacks the functional correlations with lncRNA deletion/expression data..

1) The characterization of the lncRNAs has not been performed. For example, the authors stated that Reno may have two isoforms. This has to be proved by RACE and Northern blot experiments. Are these lncRNA really noncoding? Their coding potential should be determined as described by many labs in the lncRNA field such as David Bartel and Howard Chang groups.

We now performed a 3' RACE experiment that supports the existence of the two major isoforms that were suggested by the RNA-seq data (Figure S3A shown below). We note that the expression levels of *Reno* are likely too low for it to be robustly detected by a Northern blot, which has limited sensitivity.

Page 10: *"In the RefSeq database it is annotated as a three-exon transcript, but based on RNA-seq data and 3' RACE in ES-derived neurons, we propose that there are two main Reno transcript variants: a 1.8 Kb single-exon variant, which is typically more abundant, and a spliced three-exon variant, which is expressed at lower levels and whose relative abundance increases in more mature neurons (**Figure 3B and S3A**)."*

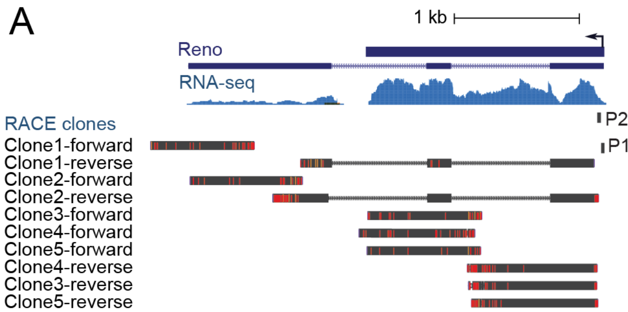


Fig. S3A: RNA-seq read coverage and 3' RACE on RNA extracted from cells following eight days of differentiation. BLAT alignments of Sanger sequencing results of five independent 3' RACE clones and the position of the nested primers used for 3' RACE (P1 and P2) are shown.

All lncRNAs studied in this paper have been identified using PLAR, a pipeline which filters out any transcript with coding potential (Hezroni et el., 2015). We now better emphasize this in the text, and include PhyloCSF tracks (an additional method for detecting coding potential that is not part of PLAR) for the studied lncRNAs (Page 5):

*"All seven lncRNAs have sequence-conserved orthologs in several other species (**Figure S1C**), are predicted to be noncoding by the three coding predictors in PLAR (Hezroni et al., 2015) and by PhyloCSF (Lin et al, 2011), which is not part of PLAR (**Figure S1D**)."*

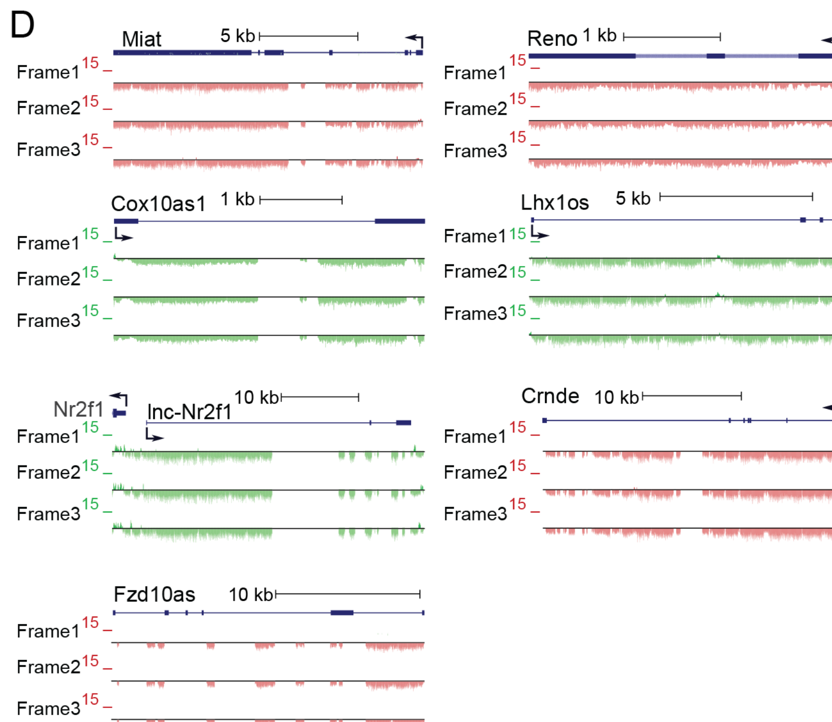


Fig. S1D: PhyloCSF scores (Lin et al, 2011) throughout the candidate lncRNAs loci in the three possible frames.

2) shRNA knockdown effects were quite mild and very low silencing was observed. But the phenotypes are quite strong. See Figs 2 and 4, S6. As shRNA has off-target effects, at least three shRNAs should be used here. Thus, 20% decrease of Reno expression

may not make sense for functional studies (Figure 2). Why not use antisense to enehance th KD and specificity for validation?

It is true that in ES cells we saw only 20% decrease in Reno expression levels. However, the expression level of Reno in ES cells is very low in mESCs to begin with. In cells during neuronal differentiation, where Reno levels are increased, we saw higher levels of depletion: 50% depletion at day 2 (Figure S6A) and 30% depletion at day 8 (Figure 1C). As suggested, we now introduced an additional perturbation method and used GapmeR LNAs to knock down *Reno* expression, and observed increased cell death at day 2 of differentiation, which was also observed in mESC clones where the promoter of Reno has been deleted. Since transfection of GapmeRs leads only to a transient knockdown of *Reno*, the phenotype observed at later time points in differentiation could not be examined using GapmeRs. Further validating specificity are our new data showing that expression of Reno in *trans* using lentiviral infection of a mammalian expression vector can rescue the phenotype. All these results together argue that the phenotype is the result of an on-target perturbation.

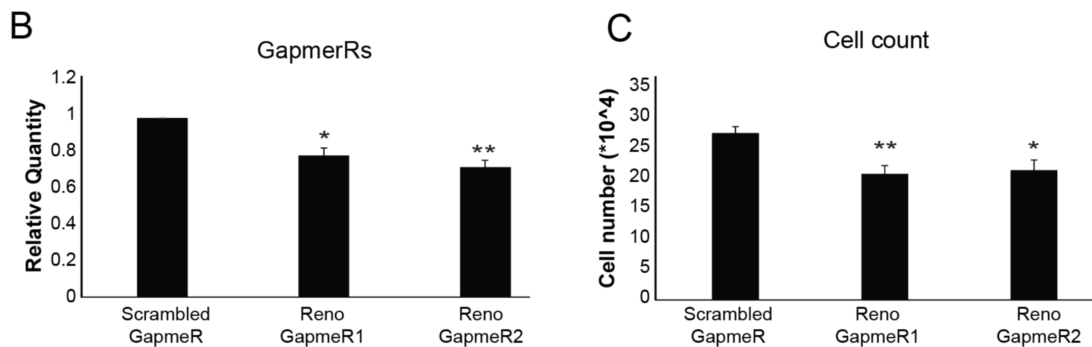


Figure S6: (B) qRT-PCR of *Reno* in ES cells transfected with LNA GapmeRs targeting *Reno* or a scrambled GapmeR that was used for normalization, at day 2 of differentiation. Mean ± SEM is shown, n=3, * P<0.05, ** P<0.01 (unpaired two sample t-test). **(C)** Cell counts for cells transfected with LNA GapmeRs targeting *Reno* or a scrambled GapmeR following four days of differentiation. Cells were harvested from one well of a 6-well plate, and counted using Orflo MOXI Z Mini Automated Cell Counter. Mean ± SEM is shown, n=3. * P<0.05, ** P<0.01 (unpaired two sample t-test).

3) The major claim of the paper in abstract states that "two highly conserved lncRNA..." but there is no information about the conservation analyses. How was the conservation defined and determined? Is this based on location (seems that way). How about their sequences? How much % of the sequences is conserved? According to the published work, very few lncRNAs are conserved at their sequence levels. How about the structure? Which region sof lncRNAs are functional and why?

Both Reno and lnc-Nr2f1 are deeply conserved on the sequence level and not just on the location level. We previously showed this schematically in Fig. S1 (which was based on sequence-similar clusters of lncRNAs from Hezroni et al. 2015. We now added a new Figure S8 that shows in detail the alignable regions between *Reno* and *lnc-Nr2f1* homologs in different species:

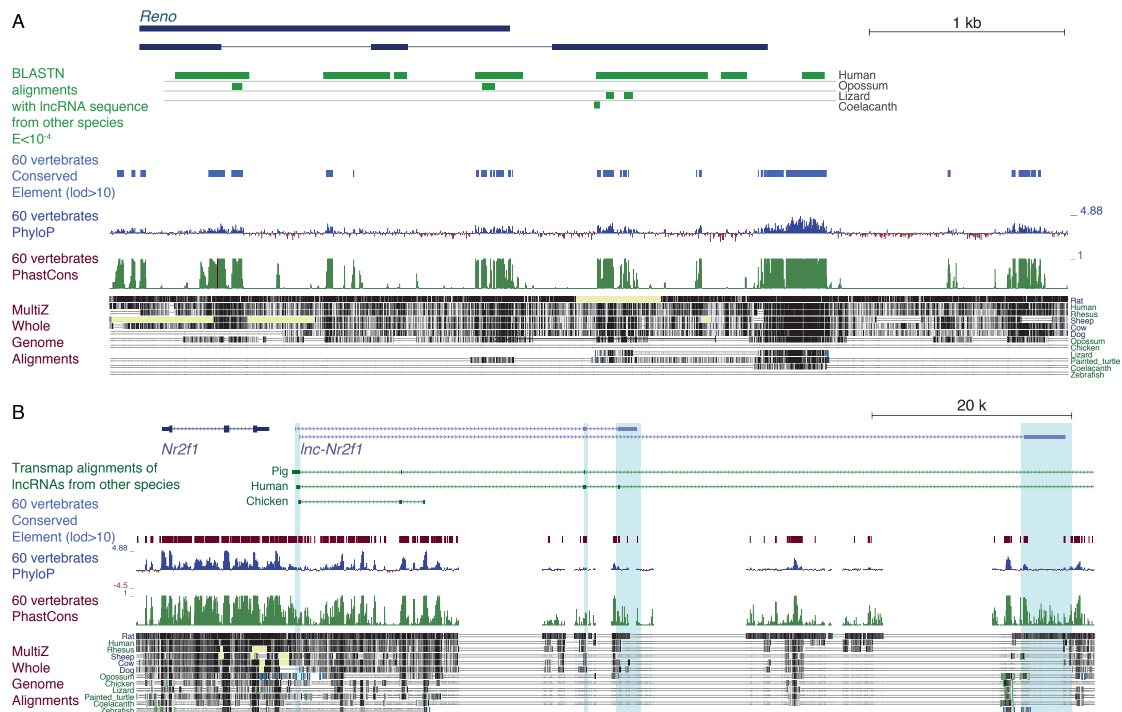


Figure S8. Sequence conservation of *Reno* and lnc-*Nr2f1*. (A) *Reno* lncRNA locus in human with the two isoforms show and with the regions alignable (E-value<10^{-4}) with lncRNAs from other species shown in Figure 3A, and conserved elements, conservation scores, and whole-genome alignments from the UCSC genome browser. **(B)** Transmap alignments of Ensembl transcripts from other species and genome conservation information from the UCSC genome browser for the *lnc-Nr2f1* lncRNA.

We also mention this in the text (Page 20):

*"Notably, Reno and lnc-Nr2f1 are also some of the most conserved lncRNAs in vertebrates – sequence-similar homologs of Reno are found in tetrapods and coelacanth (**Figures 3A, S1C, and S8A**). Inc-Nr2f1 belongs to a cluster of sequence-similar and syntenic lncRNAs that are found throughout vertebrates (**Figure S1C**) and regions alignable to the human lnc-Nr2f1 are found in amniotes (**Figure S8B**)."*

4) The expression of the Reno is not robust and convincing with sequencing data only in Figure 3. Confirming its expression in tissues and species by RT-qPCR is needed.

We show extensive evidence for *Reno* expression by RNA-seq in multiple tissues and species and using CAGE from FANTOM data (**Figure S4**), which is a complementary sequencing-based technique. We now also show Reno expression by smFISH in mESC-derived neurons and in the mouse intestine *in vivo*, and characterize the transcript by 3'RACE. We believe that RT-qPCR is less specific than these techniques for characterizing gene expression.

5) From the Figure 4, the authors found that the adjacent protein-coding Bahcc1 is regulating Reno. How and what is mechanism for this regulation?

We argue that the mechanism of *Reno* and *Bahcc1* actions is out of scope of the current manuscript.

6) Interestingly, both RNA-seq in Reno knock-down and knock-out cells was performed. Why not compare these results to conform and narrow down the targets for further validation.

We now performed RNAseq analysis for promoter deletion, shRNA-mediated and GapmeR-mediated knockdowns of *Reno*, at two days of differentiation, prior to the massive cell death observed in *Reno* loss-of-function lines. This experimental setup enabled us to directly compare the genes affected in each condition at an early stage of differentiation. We find that the three perturbation methods result in similar changes in gene expression, which correspond to signatures of up- and down-regulated genes that are partially averted when *Reno* RNA is ectopically expressed.

Page 15: "*We used RNA-seq to characterize cells that experienced Reno knockdown by shRNAs (**Figure S6A**), Reno depletion by promoter deletion, or Reno knockdown by GapmeRs (**Figure S6B**). Transfection of GapmeRs targeting Reno led to a significant reduction in number of viable cells at an early stage of differentiation which resembled the outcome of Reno depletion by promoter deletion (**Figure S6C)**. Reno depletion did not have a significant effect on Bahcc1 mRNA expression level in any of the perturbation methods (**Figure S6D**). Hundreds of genes were dysregulated in Reno-depleted cells at day 2, and the gene expression pattern was highly concordant for the different perturbation methods (**Figures 5A, 5B, S6E**). The 168 genes which were downregulated in at least two of the three perturbations were enriched for GO terms related to cell migration and motility, and to developmental process, while the 94 genes which were upregulated in at least two of the three perturbations methods were enriched for GO terms related to metabolic processes related to translation (**Figure S6F)**. The upregulated genes are highly expressed in ES cells and their expression is reduced in early stages of neuronal differentiation, while downregulated genes are expressed at low levels in ES cells, and they are induced in early stages of differentiation (**Figures 5C, S6G).** These results suggest that appropriate levels of Reno transcript at an early stage of neuronal differentiation are required in order to acquire the transcriptional programs that allow proper differentiation."*

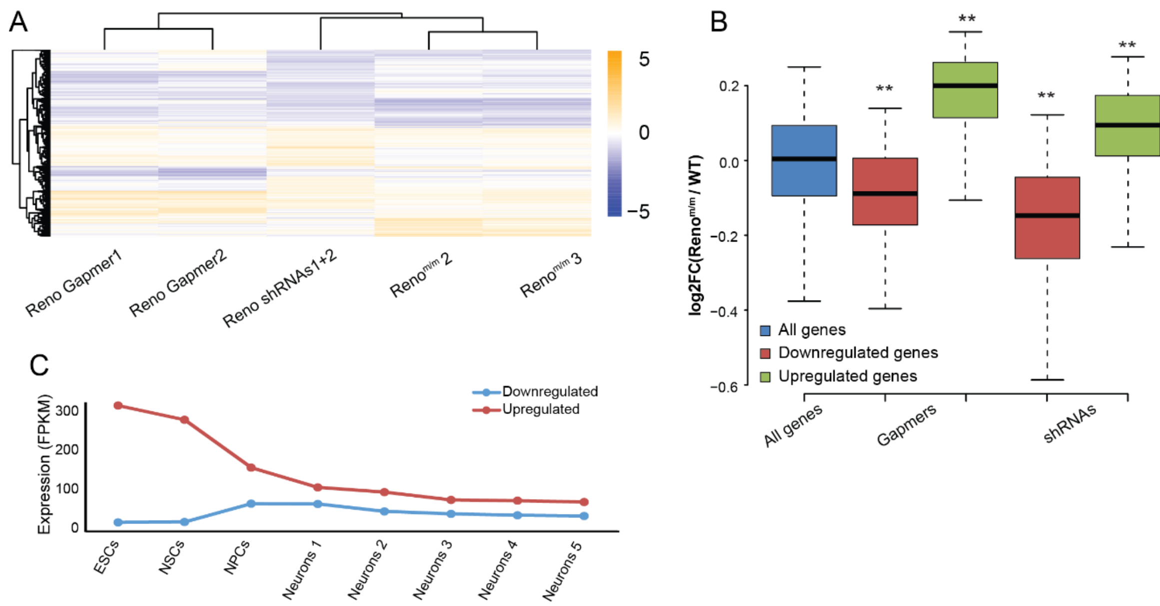
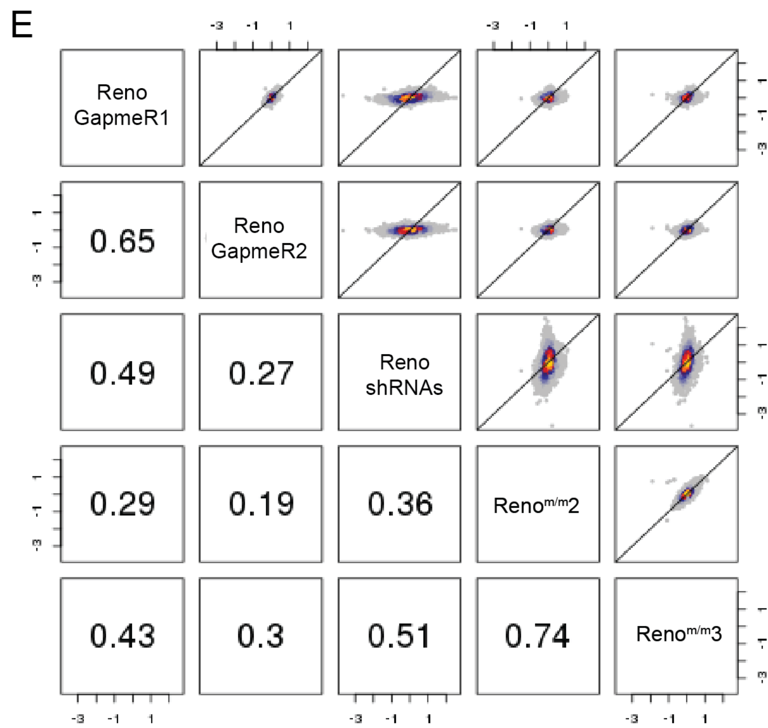


Figure 5: (A). Heatmap showing log_2-transformed fold changes of *Reno* depleted cells compared to their respective controls. All genes which were significantly differentially expressed ($P<0.05$) in any of the treatments are shown. **(B)** Distributions of changes in expression levels of *Reno*$^{m/m}$ cells compared to WT cells, for genes with significant ($P<0.05$) change in cells where Reno was perturbed with the indicated method. ** $P<0.01$ (two-sided Wilcoxon rank sum test). **(C)** Mean expression levels of genes in genes which were significantly ($P<0.05$) dysregulated by at least two out of three *Reno* perturbations at day 2 in fig. 5A, during an eight time point neuronal differentiation and maturation of mouse ES cells: Neurons1 corresponds to day *in vitro* (DIV)1; Neurons2 – DIV7; Neurons3 – DIV16; Neurons4 – DIV21; and Neurons5 – DIV28 (Hubbard et al, 2013).



F

Downregulated genes

GO term	P-value
Positive Regulation of Cell Migration	2.26E-17
Positive Regulation of Cell Motility	5.36E-17
Regulation of Developmental Process	1.35E-16
Positive Regulation of Cellular Component Movement	1.39E-16
Regulation of Cell Motility	1.47E-16
Positive Regulation of Locomotion	2.61E-16
Regulation of Cell Migration	1.07E-15
Regulation of Cellular Component Movement	2.45E-15
Regulation of Locomotion	2.55E-15
Regulation of Multicellular Organismal Process	9.71E-15

Upregulated genes

GO term	P-value
Cellular Nitrogen Compound Metabolic Process	5.69E-08
Nucleobase -containing Compound Metabolic Process	1.99E-06
Nucleic Acid Metabolic Process	2.75E-06
Cellular Aromatic Compound Metabolic Process	3.04E-06
Organic Cyclic Compound Metabolic Process	3.86E-06
Heterocycle Metabolic Process	5.51E-06
Translation	9.38E-06
Peptide Biosynthetic Process	1.64E-05
ncRNA Processing	2.75E-05
ncRNA Metabolic Process	4.67E-05

Figure S6: (E) Pairwise scatterplots showing log_2-transformed fold changes of cells with the indicated *Reno* perturbation compared to their respective controls. Color indicates local point density. Numbers indicate Spearman correlation coefficient between the fold changes **(F)** Biological Process GO categories most enriched in genes significantly down- or upregulated in at least two *Reno* perturbations, as identified using GOrilla (Eden et al, 2009).

7) The mechanistic insight of Reno and Bahcc1's functions on ES differentiation is very limited. The real targets for their function is missing, especially for Reno. Knock-out of Reno did not regulate Bahcc1, which is confirmed in Figure 4. Then how can Reno functions like Bahcc1?

In the revised manuscript we now show that *Reno* expression in *trans* is sufficient for its function, and does not substantially affect the expression of *Bahcc1* mRNA, further showing that Reno does not have a substantial *cis*-regulatory function. The substantially expanded analysis of the RNA-seq data now also allowed us to show which genes are the most likely to be regulated directly by *Reno*. The full mechanism of *Reno* action is out of scope of the current manuscript.

8) A key paper on lincRNAs that regulated ES differentiation should be cited and discussed. Cell Stem Cell. 2016 May 5;18(5):637-52. doi: 10.1016/j.stem.2016.01.024. Epub 2016 Mar 17.

We now cited and discussed this reference in the introduction (Page 2):

"For example, Evx1as was found to promote the transcription of its neighboring TF Evx1 and regulate mesendodermal differentiation of mouse ES cells (Luo et al, 2016)."

1st Editorial Decision 24th Aug 2020

Dear Igor,

Thank you for the submission of your revised manuscript. We have now received the comments from all referees, which are pasted below. I am happy to say that all referees support the publication of your study now.

However, in order to proceed with manuscript acceptance, a few more changes will be required:

- Please address the last comments by referee 2.

- Please add up to 5 keywords to the manuscript file.

- Please add the Author Contributions to the manuscript.

- Please add a Conflict of Interest statement to the manuscript file.

- Please upload a completed authors checklist, that can be found here: <https://www.embopress.org/page/journal/14693178/authorguide> The completed checklist will also be part of our transparent peer-review process file, so will be openly accessible upon publication of your paper.

- Please upload all figures as separate files.

- The manuscript contains 9 supplementary figures. 5 of these can be added as EV Figures that expand when clicked in the online version of the manuscript. All EV Figures need to be uploaded as separate files as well. The remaining, or all of the supplementary figures, can be included in an Appendix file that needs a table of content with page numbers and needs to be uploaded as a single pdf file. You can find more information on our file types in our guide to authors online. Please also use the correct callouts for the EV figures and Appendix figures in the manuscript text, i.e. EV Figure 1, etc, Appendix Figure S1, etc.

- The 2 Data Sets that are uploaded need to be split into separate table files. It seems that one Data Set refers to the methods, these tables could be uploaded as separate EV table files (you can upload as many EV tables as you want), or some tables could be regular tables in the method section. The other Data Set seems to contain data that should be uploaded as separate EV Datasets (as many as necessary). All tables need a title or legend in the first excel tab. It is currently unclear what the tables show. Please remember to update the nomenclature accordingly (EV Table 1, etc, Dataset EV1, etc).

- The statement "Data not shown" on page 23 needs to be removed per journal policy, or the data need to be shown.

- Please add the funding info to the manuscript file.

- Please add a DATA AVAILABILITY SECTION to the end of the methods section, which lists data deposited in public databases. If no data were deposited please add a sentence to the section that explains that.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript file.

I look forward to seeing a final version of your manuscript as soon as possible.

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have done a thorough job addressing any previous concerns. The identification of functions in neurogenesis for conserved lncRNAs is important and I believe that this publication is now acceptable for publication in EMBO Reports.

Referee #2:

Overall, the authors have done a great job in preparing the revision and have strengthened the manuscript by new results and interpretations. A few questions outlined below can be addressed by reorganizing results and by further revising explanations/discussion in the larger context of the findings.

1) What is the relationship between Reno and Bahcc1 is still not clear. As they showed in figure 4B, knock-down of Reno did not regulate Bahcc1 expression, suggesting that they are not functionally related. Additionally, overexpression of Reno did not have a significant effect on Bahcc1 expression level in Reno depleted cells (Figure S6H).

2) Commonly regulated genes between Bahcc1 and Reno are not large, why? Raising the possibility again that these two genes could be functionally related by indirect and different mechanisms.

Referee #3:

The reviewers have addressed and or clarified all of my concerns. I really appreciate the extra effort in the suggest RNA Fish experiments and feel they strengthen the manuscript and better guides future studies. Moreover, the authors were incredibly diligent to the other reviews as well. The revised manuscript will be of interest to the general readership of EMBO reports.

1) What is the relationship between Reno and Bahcc1 is still not clear. As they showed in figure 4B, knock-down of Reno did not regulate Bahcc1 expression, suggesting that they are not functionally related. Additionally, overexpression of Reno did not have a significant effect on Bahcc1 expression level in Reno depleted cells (Figure S6H).

We added a comment in the discussion about the potential relationship between Reno and Bahcc1 (page 13):
*We speculate that Reno1 may bind the BAHCC1 protein, potentially affecting its participation in the Sin3a complex. If such activity, that can potentiate the complex activity at H3K4me3-decorated loci, occurs near the Reno1/Bahcc1 site of transcription, it can explain the preferential effect of Reno1 and Bahcc1 depletion on chromatin accessibility in the 1Mb region flanking the locus (**Figure 6A**).*

2) Commonly regulated genes between Bahcc1 and Reno are not large, why? Raising the possibility again that these two genes could be functionally related by indirect and different mechanisms.

We added a comment in the results section addressing this point, emphasizing that most genes affected by Reno1 depletion are also affected by Bahcc1 depletion (page 8):
Notably >80% of the genes significantly affected by Reno1 depletion were also affected by Bahcc1 depletion, suggesting that Reno1 mainly functions in the Bahcc1 pathway, whereas Bahcc1 may have additional, Reno1-independent functions.

1st Revision - Editorial Decision 1st Sep 2020

Dr. Igor Ulitsky
Weizmann Institute of Science
Biological Regulation
Hertzl St.
Rehovot 76100
Israel

Dear Igor,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to

our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-51264V2 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

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: Igor Ulitsky

Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-51264V1

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.

USEFUL LINKS FOR COMPLETING THIS FORM

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

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical calculations were performed to estimate sample size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data exclusion was performed
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.	NA
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	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used a-parametric tests (Spearman and Wilcoxon) in most cases, except for qRT-PCR experiments, where t-test statistics are the standard
Is there an estimate of variation within each group of data?	Yes

Is the variance similar between the groups that are being statistically compared?	Variance similarity has not been tested, standard tests for qRT-PCR were performed.

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).	Antibodies used: rabbit anti-Oct4 (Santa Cruz sc-9081), mouse anti-Nestin (Abcam ab6142), rabbit anti-beta III Tubulin (Tuj1, Abcam ab18207), Donkey anti Mouse Alexa 594 (Molecular Probes A21203), Goat anti Rabbit Alexa 594 (Abcam ab150080)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	R1 cells were obtained from… N2a cells were obtained from ATCC, catalogue number: CCL-131. All cell lines were tested negative for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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

F- Data Accessibility

18: Provide 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'. 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	All the sequencing data have been deposited to GEO, accession GSE124517
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).	All the sequencing data have been deposited to GEO, accession GSE124517
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. 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

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.	NA