THE EMBO JOURNAL

A multifunctional locus controls motor neuron differentiation through short and long non coding RNAs

Andrea Carvelli, Adriano Setti, Fabio Desideri, Silvia Galfrè, Silvia Biscarini, Tiziana Santini, Alessio Colantoni, Giovanna Peruzzi, Matteo Marzi, Davide Capauto, Silvia Di Angelantonio, Monica Ballarino, Francesco Nicassio, Pietro Laneve, and Irene Bozzoni
DOI: 10.15252/embj.2021108918

Corresponding author(s): Irene Bozzoni (irene.bozzoni@uniroma1.it) , Pietro Laneve (pietro.laneve@cnr.it)

Review Timeline:

Submission Date:	8th Jun 21
Editorial Decision:	13th Jul 21
Revision Received:	7th Oct 21
Editorial Decision:	10th Nov 21
Revision Received:	26th Jan 22
Editorial Decision:	24th Feb 22
Revision Received:	13th Apr 22
Editorial Decision:	28th Apr 22
Revision Received:	29th Apr 22
Accepted:	2nd May 22

Editor: Stefanie Boehm

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. 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.)

Thank you for submitting your manuscript further characterizing the MN2 locus to The EMBO Journal. We have now received three referee reports on your study, which are included below for your information. In light of the referees' comments, we would like to invite you to prepare and submit a revised manuscript.

As you will see, the reviewers express an interest in the reported findings and appreciate that the study adds further insight into the locus' function(s). However, they also raise several major concerns that will need to be resolved before the manuscript can be considered further for publication here. Specifically, both referee #1 and #2 find certain aspects of the study more preliminary and that additional support for the proposed model and certain conclusions is needed. This must be experimentally addressed, in particular by adding further experiments to characterize lncMN2's proposed roles as a miRNA sponge and miRNA source (ref #1- point 1, (3), 4 ,5, 6, (7), 9; ref #3- point 2, 3). In addition, the functional role of the locus in motor neurons should be further analyzed to address referee #2's points 1, 2 and referee #3's points 4, 5. Please also carefully consider all other referee comments and revise the manuscript and figures as needed, as well as providing a detailed response to each comment.

Please note that it is our policy to allow only a single round of major revision. Acceptance depends on a positive outcome of a second round of review and therefore on the completeness of your responses included in the next, final version of the manuscript. We realize that lab work worldwide may currently still be affected by the COVID-19/SARS-CoV-2 pandemic and that an experimental revision can be delayed. We can extend the revision time when needed, and we have extended our 'scooping protection policy' to cover the period required for a full revision. However, it is nonetheless important to clarify any questions and concerns at this stage. We realize that fully resolving all referee concerns experimentally will likely require extensive work and time. However, to consider the study further here, it is critical that you adequately address the issues discussed above and provide further support for the conclusions. Therefore, I would encourage you to review the referees' comments and to contact me to discuss a preliminary revision plan to ensure beforehand that the critical points will most likely be addressed during the revision.

Referee #1:

The authors previously found that lncMN2 expression is enriched in terminally differentiated motor neurons using both mouse and human in vitro differentiation systems. The authors present a follow-up study here. They identified multiple lncRNA transcripts and embedded miRNAs expressed from the lncMN2 locus during MN differentiation and investigated their function using genome editing, in vitro differentiation, RNA biochemistry and single cell RNA-seq. This is an interesting study with potential high significance however I think it is too preliminary for publication in EMBO J. The data as presented do not fully justify the conclusions reached and more work is needed to improve scientific rigor. A summary of my concerns are shown below:

(1) The authors propose that lncMN2 acts as a sponge they should determine the copy number of lncMN2 and its proposed miRNA targets to investigate the feasibility of this hypothesis. This is a major weakness of the current study.

(2) PolyA and MAZ seq insertion is used to generate two independent knockout clones to investigate the function of this locus. This is a robust and well-designed strategy. KO clones showed a reduction in MN differentiation as measured by the number of Isl1 positive cells (Fig 1E). Why was this only carried out for one the KO cell lines. This should be done for both KO clones to show specificity.

(3) miRNA prediction algorithms generate many false positive results. Are the miRNA466 and 669 binding sites conserved in the mouse and human lncMN2? If so, this may provide additional evidence for the proposed important functional role of this interaction.

(4) Mutational analysis as opposed to a deletion strategy should be used to more accurately define the function of the predicted lncMN2-miRNA466 interaction in Fig 2. In Fig 2D, luciferase expression of the WT and deltaSP constructs is normalised to 1. Are there differences in luciferase expression between these constructs in the absence of miRNA knockdown? If so, this may affect the sensitivity of the assay and interpretation based on fold changes.

(5) To confirm the model that only the lncMN2-203 isoform interacts with the miRNA containing machinery RT-qPCR analysis should also be carried out to test for enrichment of 202 and 204 isoforms in the Ago CLIP experiment (even though they are more nuclear localised).

(6) The data in Fig 2F-I does not convince me that there is a specific interaction between lncMN2-203 and miRNA466. The fold enrichment in Fig 2G is not great. I believe that sense probes are better to use in pull down experiments compared to lacZ to illustrate specificity. How many times have these experiments been repeated? I can't find this info.

(7) In Fig 3 what size are the deletions that have been generated? If they are large they might also remove other functional

sequences (both RNA and DNA regulatory elements). Again, I think that mutation of the miRNA interaction site would be a better (more specific) approach or at least rescue experiments using lncRNA expressed from a transgene. In Fig 3D, the count of Islet positive cells again only uses one clone.

(8) I found the DGE analysis in Fig 5 confusing. I think this (along with Fig 4) are important for defining the differentiation defect. However this isn't really clear to me based on how the data is presented at the moment and should be better described. It would also be informative to describe the set of genes whose expression changes in both the KO and deltaSP mutant. This will help determine whether the lncMN2 functions mostly through miRNA binding. Also, the authors should define whether the shared GO categories are due to regulation of the same (or different genes). The number of genes in each cGI ategory should be stated. The x-axis in Fig 5A and B isn't labelled. I also think this figure and description could be made mor accessible and the conclusions clearer.

(9) For analysis of miRNA targets again predictions are not very reliable. The authors should perform RNA-seq of cells following transfection of LNA against miR-325 and mIR-384 to experimentally identify targets and then use this gene set to examine overlap with lncMN2 KO data. This dataset is not convincing.

(10) For Fig 6B, do Cdk6 and/or Cdc25b protein levels change?

Referee #2:

In this manuscript, Bozzoni and colleagues have investigated the function and molecular mechanism of a motor neuron-specific transcriptional unit that contains a lncRNA called lncMN2-203 and two miRNAs (miR-325-3p and miR-284-5p). There are three transcript isoforms from lncMN2, and a polyA-MAZ cassette was inserted into the beginning of the first exon common to all three isoforms. This KO abolishes production of all three isoforms as well as the two miRNAs. Cells with this KO are defective for motor neuron production. Deletion of sequences (deltaSP) of lncMN2 that bind miR-466i-5p also cause defective motor neuron production without affecting production of miR-325 and miR-284. Single cell RNA-seq shows that KO and deltaSP phenotypes differ, potentially related to differences in production of miR-325 and miR-466i. Transfection of LNAs against miR-466i-5p increase levels of Onecut2 and Ncam1, both of which are upstream regulators of motor neuron development.

Studies that demonstrate lncRNA function and molecular mechanism in development are of broad interest to the field, and the authors have tackled a lncRNA locus that appears to have multiple mechanisms. While the results point to lncMN2 having at least two functions (sponging of miR-466 and production of miR-325 and miR-284), this understanding is somewhat preliminary. Specific comments follow.

1. The MN2-deltaSP mutant (deleted for the region that can sponge miR466) is strongly defective for motor neuron production, and the authors claim that the miR466 sponging activity is a key aspect of the phenotype. Does expression of lncMN2-203 from a vector rescue motor neuron production in the MN2-deltaSP mutant? What about expression of just the MN2-deltaSP "fragment"? Similarly, does LNA against miR466 "rescue" the production of motor neurons in MN2-deltaSP cells?

2. The authors focus on miR466 regulation of Onecut2. Does manipulating Onecut2 rescue motor neuron production in either the MN2-KO or MN2-deltaSP mutants?

3. The lncMN2 transcriptional unit appears to produce a primary miRNA transcript (pri-miRNA) for miR-325 and miR-284. To more fully explore the MN2 locus (and more directly support the authors claim that "...additional pathways stand on miR325-p and miR384-5p activities..."), it would be very informative to delete the genomic regions that encode miR-325 and miR-384 and assess cellular/molecular phenotypes. Does deletion of these miRNAs affect proliferation but not motor neuron differentiation?

4. While perhaps beyond the scope of this manuscript, the authors might consider potential nuclear function of the MN2 locus. He et al (PNAS 2021) founds that lncRNA loci that host miRNAs are enriched for enhancer-like function, which is another potential mechanism that could be affected by the polyA-MAZ cassette insertion. Some of the MN2 transcripts seem to be nuclear. If not experimentally addressed, this could be discussed.

Referee #3:

In the current manuscript, Carvelli and colleagues investigate the role of a unit of different small- and long non-coding RNAs in the context of human motor neuron differentiation using different approaches, including iPSC-based modelling, CRISPR/Cas and scRNAseq. The locus examined harbors a lncRNA and two miRNAs, and interestingly the authors show that the lncRNA may act as a sponge for yet other miRNAs. Manipulation of the locus, either by inducing total knockout or deleting part of the lncRNA that binds miRNAs, the authors find defects in the differentiation of motor neurons in vitro. They attribute part of these cellular changes to miRNA-mediated effects exerted by the lncRNA locus (e.g. by the miRNAs produced or bound) and hint at Onecut2

as an ultimate downstream effector.

This is an interesting and well-performed study high-lighting a very intriguing locus of non-coding RNAs and unveiling its cellular effects. While this work underscores the complexity and importance of non-coding RNAs, I have a few questions.

- Do the authors have an explanation for the residual expression of the lncRNA splice forms and miRNAs in the KO lines (Fig. 1C)?
- In Fig. 2F the authors show that pull-down of lncMN2-203 leads to enrichment of miR-466i-5p. This association could of course still be indirect. If the miR-466i-5p binding sites in lncMN2-203 are mutated can this enrichment still be found?
- Did the authors consider that the large deletion (delta-SP) also effects the secondary RNA structure. Was this tested, e.g. in mfold?
- The effects of KO presented in the manuscript (e.g. Fig. 1D, E and 4B) seem subtle. How do the authors explain this observation? Are the locus and its RNAs only part of a larger pathway underlying MN differentiation or are they only only active in part of the motor neurons, etc? This should be discussed.
- In Fig. 6, the authors try to link the miRNAs studied to downstream pathways and indeed show that manipulation of the miRNAs leads to changes in targets such as Onecut2, which has a role in motor neuron differentiation. However, especially for miR-466i-5p these experiments do not formally link the lncRNA, miRNA, and downstream pathway. Is the described sponging function really important here? Is it possible to functionally link the lncRNA and miRNA with respect to Onecut2 expression and MN differentiation?

Point to point response to the reviewers' comments:

REFEREE #1

1) The authors propose that lncMN2 acts as a sponge they should determine the copy number of lncMN2 and its proposed miRNA targets to investigate the feasibility of this hypothesis. This is a major weakness of the current study.

We used single-cell RNA-seq expression data to assess the stoichiometry between lncMN2 and its targets Onecut2 and Ncam1 mRNAs in motoneuron progenitors (MNP). We counted deduplicated UMI belonging to cells clustered as MNP and assigned them to the genes of interest. We counted 108 UMIs for the lncMN2 locus in the WT1 (33% of which could be assigned to lncMN2-203 isoform) and 114 in WT2 (more than 50% assigned to lncMN2-203), 138 and 44 UMIs for Onecut2, and 658 and 677 UMIs for Ncam1. Moreover, lncMN2 displays 7 binding sites for miR-466i-5p, while Onecut2 has 5 and Ncam1 only 3 (see **Methods** for details).

Overall, besides the fact that specific compartmentalization inside the cell could increase the relative concentration of these factors, these figures are compatible with a sponging mechanism. These data have been included in the discussion and in the novel sheet of **Table S5**.

2) PolyA and MAZ seq insertion is used to generate two independent knockout clones to investigate the function of this locus. This is a robust and well-designed strategy. KO clones showed a reduction in MN differentiation as measured by the number of Isl1 positive cells (Fig 1E). Why was this only carried out for one the KO cell lines. This should be done for both KO clones to show specificity.

As suggested by the referee, we have separately presented the 2 sets of data. The results show that the two clones for either the KO or the ΔSP mutants behave similarly. These new data have been substituted to the previous ones in **Figure 1E** (KO) and **3D** (ΔSP).

3) miRNA prediction algorithms generate many false positive results. Are the miRNA466 and 669 binding sites conserved in the mouse and human lncMN2? If so, this may provide additional evidence for the proposed important functional role of this interaction.

Compared to the mouse, in humans a slightly different and interesting situation occurs. A syntenic locus exists (MIR325HG) which contains the miR-325 and miR-384 coding regions but lacks the miR466i-5p sponging region. Moreover, miR466i-5p is not conserved in humans, where Onecut2 and Ncam1 mRNAs are predicted to be regulated by other miRNAs (let7c and miR-18a). Intriguingly, we found that, similarly to the murine system, these miRNAs can be sponged by a long non coding RNA specifically expressed in MNs but transcribed from another region of the genome. This very interesting genomic diversification, despite a conserved regulatory mechanism, is currently being studied in the lab and it will be the subject of future work.

4) Mutational analysis as opposed to a deletion strategy should be used to more accurately define the function of the predicted lncMN2-miRNA466 interaction in Fig 2. In Fig 2D, luciferase expression of the WT and ΔSP constructs is normalised to 1. Are there differences in luciferase expression between these constructs in the absence of miRNA knockdown? If so, this may affect the sensitivity of the assay and interpretation based on fold changes.

As requested by the referee, to highlight the basal luciferase level from the two reporter constructs (WT and ΔSP) and to clearly show the specificity of the effect of LNAs against miR-466i-5p, we folded all the luciferase values over that of the luc-WT construct set as 1.

Compared to the WT reporter (luc-WT/LNA SCR), the ΔSP construct (luc-ΔSP/LNA SCR), which is unable to bind the endogenous miR-466i-5p, displayed a significant increase of the basal level of luciferase. Consistently, no effect was registered for the ΔSP construct in the presence of LNAs targeting miR-466i-5p (luc-ΔSP/LNA 466i), while a significant increase of luciferase was obtained when LNAs against miR-466i-5p were co-transfected with the WT construct (luc-WT/LNA 466i). Altogether, these data show that a specific interaction occurs between miR-466i-5p and lncMN2. These results are shown in the new panel **D** of **Figure 2**.

5) To confirm the model that only the lncMN2-203 isoform interacts with the miRNA containing machinery RT-qPCR analysis should also be carried out to test for enrichment of 202 and 204 isoforms in the Ago CLIP experiment (even though they are more nuclear localised).

To corroborate the hypothesis that lncMn2-203 isoform specifically interacts with the miRISC complex, we analyzed by qRT-PCR the enrichment of the lncMN2-202 and 204 isoforms in the CLIP samples. As expected, we didn't find any enrichment for these transcripts in the IP fraction. This result, along with the nuclear localization of the 202 and 204 isoforms, confirms that only lncMN2-203 is able to bind AGO2. This new data has been added in the new **Figure 2E**.

6) The data in Fig 2F-I does not convince me that there is a specific interaction between lncMN2-203 and miRNA466. The fold enrichment in Fig 2G is not great. I believe that sense probes are better to use in pull down experiments compared to lacZ to illustrate specificity. How many times have these experiments been repeated? I can't find this info.

We agree with the referee about the enrichment observed; the problem was also linked to the poor enrichment of lncMN2-203 in the pull-down. Therefore, we set up a different pull-down experiment making use of longer oligos (90 nt, such as the conditions used in RAP experiments [1]) and we changed the control used (U1 RNA, for which we already experienced successful pull-down [2]). Through this approach, we got higher levels of lncMN2-203 pull-down and we obtained a significant enrichment of miR-466i-5p. The results of the experiment, performed in triplicate, are shown in the new panels **F-I** of **Figure 2**.

[1] McHugh, CA, Guttman, M. RAP-MS: a method to identify proteins that interact directly with a specific RNA molecule in cells. Methods Mol. Biol.1649, 473–488 (2018).

[2] Desideri F, Cipriano A, Petrezselyova S, et al. Intronic Determinants Coordinate Charme lncRNA Nuclear Activity through the Interaction with MATR3 and PTBP1. Cell Rep. 2020 Dec 22;33(12):108548. doi: 10.1016/j.celrep.2020.108548.

7) In Fig 3 what size are the deletions that have been generated? If they are large they might also remove other functional sequences (both RNA and DNA regulatory elements). Again, I think that mutation of the miRNA interaction site would be a better (more specific) approach or at least rescue experiments using lncRNA expressed from a transgene. In Fig 3D, the count of Islet positive cells again only uses one clone.

Given the repetitive nature of the miR-466i-5p binding sites on lncMN2-203, the introduction of single point mutations to affect the binding ability of all of them would have been quite challenging. For this reason, we choose to generate a deletion (260 bp) across the entire sponging region. However, in order to address the point raised by the reviewer, we tested whether the ectopic expression of the sponging region (lncMN2-Ex5) would rescue Onecut2 and Ncam1 levels in NSC-34 cells where miR-466i-5p is endogenously expressed. The new data indicate that upon expression of the sponging region, a significant increase of the Onecut2 levels and an upregulation trend for Ncam1 is obtained (**Figure S6G**). These results, together with the complementary experiment with LNAs against miR-466i-5p shown in **Figure S6F,** where Onecut2 and Ncam1 mRNA levels increase, indicate the existence of a lncMN2-203→mir-466i-5p/→Onecut2-Ncam1 axis also in the NSC-34 cell line.

Importantly, in the revision of the paper we have added another interesting experiment which answers the concern raised by the reviewer. We treated ΔSP clones with LNAs against miR-466i-5p and found that in EBs the expression of both Onecut2 and Ncam1 increases compared to untreated cells. These data are added in **Figure 6G**.

As for the count of Islet positive cells, similarly to what reported in previous point #2, we have analyzed separately the 2 clones of each lncMN2 mutant (KO and ΔSP). These results are now presented in the new **Figures 1E** (KO) and **3D** (ΔSP).

8) I found the DGE analysis in Fig 5 confusing. I think this (along with Fig 4) are important for defining the differentiation defect. However, this isn't really clear to me based on how the data is presented at the moment and should be better described. It would also be informative to describe the set of genes whose expression changes in both the KO and ΔSP mutant. This will help determine whether the lncMN2 functions mostly through miRNA binding. Also, the authors should define whether the shared GO categories are due to regulation of the same (or different genes). The number of genes in each cGI ategory should be stated. The x-axis in Fig 5A and B isn't labelled. I also think this figure and description could be made mor accessible and the conclusions clearer.

According to the reviewer comments, we modified **Figure 5**. In order to better describe the relationship between the two mutants and the categories presented, we added aside pie plots to discern for each macro-category (Neuronal, Development and Proliferation) how many genes are in common or differentially affected. With regard to the down-regulated genes, the two mutants mainly share the Development and Neuronal categories. These data suggest that, at least for these categories, the downregulation effect is mainly due to the depletion of the sponging region. On the

other hand, for the up-regulated genes, the overall effect is more heterogeneous across mutants. The number of genes in each category has been added.

9) For analysis of miRNA targets again predictions are not very reliable. The authors should perform RNA-seq of cells following transfection of LNA against miR-325 and mIR-384 to experimentally identify targets and then use this gene set to examine overlap with lncMN2 KO data. This dataset is not convincing.

In order to further validate the putative targets of miR-325-3p and miR-384-5p we analyzed, besides CDK6 and CDC25B, additional eleven transcripts. Among the six that we found to be expressed in NSC-34 cells, we measured a significant increase of TBX20 and H2AFZ levels upon transfection of LNAs against miR-325-3p (new **Figure S6C**) and a significant increase of ALCAM and WSL by treatment with LNA against miR-384-5p (new **Figure S6D**)**.** Overall, we were able to validate six (CDK6, CDC25B, TBX20, H2AFZ, ALCAM and WSL) out of eight NSC-34 expressed putative targets for either one of the two miRNAs, confirming the computational predictions.

10) For Fig 6B, do Cdk6 and/or Cdc25b protein levels change?
Cdk6 and Cdc25b protein levels were analyzed in lysates collected from NSC-34 transfected with LNA against miR-325-3p or LNA SCR. The results are in agreement with the increase of mRNA levels and are now presented in **Figure S6E**.

REFEREE #2

1) The MN2-ΔSP mutant (deleted for the region that can sponge miR466) is strongly defective for motor neuron production, and the authors claim that the miR466 sponging activity is a key aspect of the phenotype. Does expression of lncMN2-203 from a vector rescue motor neuron production in the MN2-ΔSP mutant? What about expression of just the MN2-ΔSP "fragment"? Similarly, does LNA against miR466 "rescue" the production of motor neurons in MN2-ΔSP cells?
The rescue experiment with a lncMN2-203 expression vector is quite difficult to perform in differentiated mESCs. In fact, mESC-derived EBs are difficult to transfect and DNA plasmids, provided to undifferentiated mESCs, result in a very heterogeneous expression in differentiated MNs. Therefore, we performed such experiment in the MN-like NSC-34 cell line. Upon overexpression of lncMN2-203 exon 5 we observed an increase of the Onecut2 and Ncam1 levels, thus supporting the hypothesis that lncMN2-203 acts as a sponge in the miR-466i-5p/Onecut2-Ncam1 pathway (see new **Figure S6G**). As an additional approach in support of this model and in line with what requested by the reviewer, we made use of LNAs which can be efficiently transfected in mESCs and survive until the early EBs stage. The results show that upon treatment with LNAs against miR-466i-5p the Onecut2 and Ncam1 mRNA levels increase. These new results are shown in the new panel **G** of **Figure 6**.

2) The authors focus on miR466 regulation of Onecut2. Does manipulating Onecut2 rescue motor neuron production in either the MN2-KO or MN2-ΔSP mutants?
This is a very interesting question, though difficult to answer since: i) stable and homogeneous expression of exogenous DNA in mESCs induced to MN differentiation is difficult due to the long time of the differentiation process (more than 5 days) and ii) it is likely that Onecut2 works in combination with other miR-466i-5p targets, such as Ncam1, making it difficult to reconstitute the entire differentiation pathway.

The alteration of these targets, and in particular of Onecut2, is already well established in the literature to affect MN differentiation [1, 2, 3, 4].

[1] Roy A, Francius C, Rousso DL, et al.,. Onecut transcription factors act upstream of Isl1 to regulate spinal motoneuron diversification. Development. 2012;139:3109-19. doi: 10.1242/dev.078501.
[2] Francius C, Clotman F. Dynamic expression of the Onecut transcription factors HNF-6, OC-2 and OC-3 during spinal motor neuron development. Neuroscience. 2010; 165:116-29.
[3] Toch M, Harris A, Schakman O, et al. Onecut-dependent Nkx6.2 transcription factor expression is required for proper formation and activity of spinal locomotor circuits. Sci Rep. 2020;10(1):996.. doi:10.1038/s41598-020-57945-4
[4] Rhee HS, Closser M, Guo Y, et al.Expression of Terminal Effector Genes in Mammalian Neurons Is Maintained by a Dynamic Relay of Transient Enhancers. Neuron. 2016;92:1252-1265. doi:10.1016/j.neuron.2016.11.037

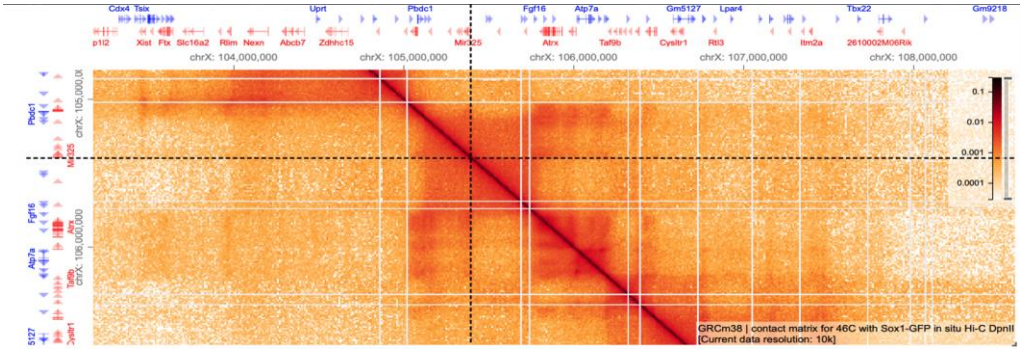
3) The lncMN2 transcriptional unit appears to produce a primary miRNA transcript (pri-miRNA) for miR-325 and miR-384. To more fully explore the MN2 locus (and more directly support the authors claim that "...additional pathways stand on miR325-p and miR384-5p activities..."), it would be very informative to delete the genomic regions that encode miR-325 and miR-384 and assess cellular/molecular phenotypes. Does deletion of these miRNAs affect proliferation but not motor neuron differentiation?

As requested by the reviewer, we produced through CRISPR/Cas9 two additional mESC mutants for miR-325 and miR-384. These two new clones were differentiated and GFP+ recording was carried out. The results (shown in the new **Figures S1K** and **S1N**) indicate that neither the depletion of miR-325 nor of miR-384 affects MN production.

4) While perhaps beyond the scope of this manuscript, the authors might consider potential nuclear function of the MN2 locus. He et al (PNAS 2021) founds that lncRNA loci that host miRNAs are enriched for enhancer-like function, which is another potential mechanism that could be affected by the polyA-MAZ cassette insertion. Some of the MN2 transcripts seem to be nuclear. If not experimentally addressed, this could be discussed.

This is an interesting question that we would like to answer in the future. However, in order to address this point, we did a specific analysis based on a recent finding that pri-miRNA regions embedded in lncRNA loci could control neighboring genes (He et al., 2021).

In order to inspect a putative miRNA independent function of lncMN2 pri-miRNA transcripts, we investigated whether genes related to the MN2 TADs were affected in KO mutant conditions where the pri-miRNAs are depleted. We used Hi-C data published by the Cavalli group (Multiscale 3D Genome Rewiring during Mouse Neural Development) in mESC differentiated to cortical neurons and specifically we selected the widest TAD linked to MN2 in the dataset (chrX:105000000-106215000, ~350 Kb upstream of MN2 and ~825 Kb downstream, observed in mESC differentiated to Neuronal Progenitors and sorted by Sox1-GFP). We identified 27 genes associated to this region and we intersected this set with the genes deregulated in KO mutant compared to WT condition. We found no overlapping genes in both MNP or MN differentially expressed genes. Even if we cannot exclude a nuclear effect on genes outside the specified TAD, these results suggest that pri-miRNA MN2 transcripts are not involved in the regulations of proximal genes.



Legend. Contact matrix of MN2 locus associated genomic regions in mESC Hi-C data published by the Cavalli group (Multiscale 3D Genome Rewiring during Mouse Neural Development). The contact matrix were retrived by 4DN Data Portal

(https://data.4dnucleome.org/browse/?type=ExperimentSetReplicate&experimentset_type=replicate&dataset_label=Hi-C+on+mESC+during+neural+differentiation).

REFEREE #3

1) Do the authors have an explanation for the residual expression of the lncRNA splice forms and miRNAs in the KO lines (Fig. 1C)?

The depletion of lncMN2 has been obtained by insertion of a poly-A signal. The residual expression in the KO clones might be due to the efficiency of termination which is not 100%.

2) In Fig. 2F the authors show that pull-down of lncMN2-203 leads to enrichment of miR-466i-5p. This association could of course still be indirect. If the miR-466i-5p binding sites in lncMN2-203 are mutated can this enrichment still be found?

We understand the concern; however, it is difficult to properly answer this question. The mutation we have in the ΔSP is still quite large due to the repetition of the miR-466i-5p binding sites, therefore it is quite difficult to specifically address this point. We believe that the most convincing experiment is the luciferase assay which shows that while the ΔSP construct does not show any significant alteration upon miR-466i-5p LNA administration, the luciferase activity from the WT construct is significantly increased. These data are presented in **Figure 2D**.

3) Did the authors consider that the large deletion (ΔSP) also effects the secondary RNA structure. Was this tested, e.g. in mfold?

We predicted the structures of lncMN2-203 WT and ΔSP transcripts with the RNALfold algorithm [1], which allows the assembly of the whole sequence RNA secondary structure by local predictions. In order to compare the predicted structures, we used the BEAGLE alignment algorithm [2] obtaining a structure identity score of 96.63% with a p-value of 0.0001. These results support the hypothesis that the ΔSP deletion does not substantially alter the lncMN2-203 secondary structure.

[1] Lorenz R, Bernhart SH, Höner Zu Siederdissen C, et al. ViennaRNA Package 2.0. Algorithms Mol Biol. 2011;6:26. Published 2011 Nov 24. doi:10.1186/1748-7188-6-26

[2] Eugenio Mattei, Marco Pietrosanto, Fabrizio Ferrè, Manuela Helmer-Citterich, Web-Beagle: a web server for the alignment of RNA secondary structures, Nucleic Acids Research, Volume 43, Issue W1, 1 July 2015, Pages W493–W497, https://doi.org/10.1093/nar/gkv489

4) The effects of KO presented in the manuscript (e.g. Fig. 1D, E and 4B) seem subtle. How do the authors explain this observation? Are the locus and its RNAs only part of a larger pathway underlying MN differentiation or are they only active in part of the motor neurons, etc? This should be discussed.

We thank the reviewer for the request of clarification on this important point since we were not clear enough in describing the timing differences between the distinct experiments. Those shown in **Figures 1D** and **4B** (Hb9-GFP quantification and SC-RNA seq) have been carried out on day 6 of differentiation (EB6). At this time point, the decrease in the number of MNs is of approximately 30%. Instead, the Islet quantification of **Figure 1E** has been performed at 14 days (DIV14) after EB6 dissociation, when MNs have undergone functional and morphological maturation. In this case, the effect is much more drastic and the number of MNs decreases by almost 80%. This strong decrease supports the claim that the MN2 locus plays an important role in MN differentiation and maturation. We have better specified these differences in the text.

5) In Fig. 6, the authors try to link the miRNAs studied to downstream pathways and indeed show that manipulation of the miRNAs leads to changes in targets such as Onecut2, which has a role in motor neuron differentiation. However, especially for miR-466i-5p these experiments do not formally link the lncRNA, miRNA, and downstream pathway. Is the described sponging

function really important here? Is it possible to functionally link the lncRNA and miRNA with respect to Onecut2 expression and MN differentiation?

In this revised version we included a new experiment which we hope answers the reviewer concern. We treated ΔSP clones with LNAs against miR-466i-5p and found that at EB stage 3 the expression of both Onecut2 and Ncam1 increases compared to untreated cells. These data are shown in panel **G** of **Figure 6**.

To further reinforce this point, we also performed the overexpression of the fifth exon of lncMN2-203 (which contains the sponge region for miR-466i-5p) in NSC-34 cells and found upregulation of Onecut2 and Ncam1 mRNA levels (new **Figure S6G**). This experiment, together with the one shown in Fi**gure S6F**, where the same effect is obtained with LNAs against miR-466i-5p, consolidates the direct link between lncMN2-203/miR466i-5p and the Onecut2 and Ncam1 targets.

1st Revision - Editorial Decision **10th Nov 2021**

Thank you again for submitting your revised manuscript. We have now received comments from the three initial referees (please see below), and I am pleased to say that they overall find that their concerns have been satisfactorily addressed. Referee #2 has two final questions, which could likely be addressed by textual revision. In addition to revising the manuscript as appropriate, please also briefly respond to these comments in a short point-by-point response. In addition, in this final version, I would also like to ask you to address a number of editorial issues that are listed in detail below.

Referee #1:

The authors have performed a substantial set of new experiments and these have satisfactorily addressed all my specific comments. I think it is very hard to conclusively prove the miRNA sponge mode of action but the data presented is now much more convincing. I recommend publication of the revised manuscript.

Referee #2:

Many apologies for the delay in my response. In this revised manuscript from Prof. Bozzoni and colleagues, the authors have performed several new experiments that address my earlier comments. While they were unable to perform rescue experiments of lncMN2-203 in mESCs, they did analyze the effect of lncRNA overexpression in a motor neuron-like neural stem cell line, producing results consistent with their overall model. Similarly, LNA against miR466 also increased the expression of Onecut2 and Ncam1 levels.

The authors have suggested that miR325 and miR384 have roles in regulating cell proliferation but not motor neuron production. For this revision, they generated deletions of these miRNAs, and as predicted by their model, motor neuron production was not affected. Did they analyzed effects on cell proliferation?

In the letter to reviewers, the authors present data to suggest that lncMN2 doesn't affect local gene expression, and this analysis is reasonable. Could the authors discuss these findings in the manuscript?

Referee #3:

The authors have addressed my major concerns and I thank them for performing the requested additional experiments.

While checking the R code used for the analysis of single-cell RNA sequencing data included in our manuscript, we realized that we made a mistake in the type of analysis performed.
Specifically, for the differential gene expression analysis we used the integrated RNA matrix instead of the original RNA matrix. While the integrated matrix, which relies on batch corrected gene expression values, was appropriate for clustering and visualizing the different samples (WT, ΔSP, KO) in order to remove possible technical biases across samples processed in different moments, it might have affected the specific identification of genes when used for differential gene expression analysis.
Therefore, once identified the problem, we have repeated the analyses using the recommended original RNA matrix (Stuart et al., 2019, Cell 177, 1888–1902).

We resubmit now the amended version of the article and summarize here below the new findings and differences with respect to the previous data.

We acknowledge that the new analysis has led to a better distinction of the different motor neuron (MN) subpopulations: in addition to MN progenitors (MNPs), we were able to specifically distinguish early and late MN (EMNs and LMNs). Interestingly, the latter resulted not only the most affected in both KO and ΔSP clones but also their decrease was stronger than in previous analyses, with almost a 50% reduction (new **Figure 4C**). Therefore, this analysis has not only confirmed but has even emphasized, at the single-cell level, the MN reduction phenotype shown by cytofluorimetry and immunofluorescence assays shown in **Figures 1E** and **3D**.

The novel differential gene expression analysis, performed on the different MN subpopulations (new **Figure 5**), has confirmed that the miR-466i-5p sponging activity of lncMN2-203 impinges on MN differentiation, while miR-325-3p and miR-384-5p control cell cycle-related genes.
Indeed, a conspicuous enrichment of neuronal development related factors was found among the downregulated genes, common to KO and ΔSP mutants (**Figure 5F)** and preferentially enriched for miR-466i-5p targets **(Figure 6E)**. Moreover, the category of cell cycle-related genes was more specifically linked to the KO clones; in fact, this category resulted much more abundant in the upregulated genes KO mutants compared to ΔSP (**Figure 5C and 5D)**.

However, according to this new analysis Onecut2 and Ncam1 were not found among the significantly downregulated genes. Their differential expression remained under the threshold of significance. It is also possible that their downregulation occurs at a specific differentiation phase that is absent in the conditions used for the single cell differentiation experiment, such as mature MN, where lncMN2 is expressed at high levels. Therefore, we have removed these two factors from the proposed regulatory circuitry.
The reduction of Onecut2 and Isl1, detected by bulk RNA analysis (previous **Figure 6F**), can now be explained by the observed drastic decrease of LMN in ΔSP and KO clones. We have included these data in **Appendix Figure S2D** and appropriately discussed in the text.

Nonetheless, the new analysis revealed other interesting miR-466i-5p targets, which are strongly and significantly downregulated in ΔSP and KO clones and which have a well testified role in neuronal development and function; the most relevant one being *Scrt1*, a transcription factor with

an important role in neuronal fate commitment and in neuron generation and maintenance (Matsuda T, et al. Neuron. 2019).
The new candidates have been validated as part of the miR-466i-5p ceRNA network in LNA transfection experiments in NSC-34 (**Figure 6G**) as well as in ΔSP mESCs induced to differentiate to EB (**Figure 6H**). In conclusion, even if we couldn't maintain the emphasis on the Onecut2 and Ncam1 factors, we can still justify the decrease in MNs through the miR-466i-5p ceRNA network and the involvement of important regulatory factors of neuronal differentiation and function.

Finally, with regard to miR-325-3p and miR-384-5p, we have confirmed their ability to regulate a pool of cell cycle-related genes, specifically upregulated in KO MNPs (**Figure 6B**). Several significantly upregulated targets were found and also in this case they were validated in LNA experiments performed in NSC-34 cells.

In conclusion, the corrected computational analysis and the consequent results strengthen our data and reinforce the manuscript conclusions. The major findings of the study as well as the underlying molecular mechanisms remain the same: by modulating the activity of miR-466i-5p, lncMN2-203 controls genes involved in neural development to regulate MN differentiation. In parallel, the co-expressed miR-325-3p and miR-384-5p mainly impact on the regulation of MNP proliferation by regulating cell-cycle related genes.

Thank you again for alerting us to the technical issues you noticed in your analyses and for submitting a revised manuscript to resolve these. As discussed, given the extent of changes to the main figures, we sent this version back to the initial referees. We have now received their comments, which are included below for your information. Overall, in our view the referees are still supportive of publication, but referee #1 and #2 do raise some concerns regarding the new experimental data and the conclusions. In this exceptional case, where the data has changed over the course of the revision, we would ask you to revise the manuscript to address the new issues. Please add the additional knockdown control referee #1 is asking for (related to Fig. 6G). To address referee #2's concerns, please incorporate any further available data to support a role for Scrt1 in motor neuron generation and carefully review and/or revise the manuscript to ensure all conclusions on lncMN2-203- mediated motor neuron generation are fully supported by experimental data. Please also remember to revise the synopsis text and bullet points to reflect the altered data.

Referee #1:

My understanding is that the new manuscript corrects a finding in the previous version regarding the set of lncMN2 target genes that were identified. I think that in general the altered results do not change the overall conclusion of the study. Instead, the authors now show a different set of genes that are regulated in the lncMN2 mutants. The new results are mostly consistent with a model in which lncMN2-203 controls genes involved in neural development via a mechanism involving miR-466i-5p. However, I am not convinced by the RT-qPCR experiments in Fig 6G to validate Scrt1, Ncan, Nrxn1 and Snap25 as miR-466i-5p targets. Only a single LNA is used to knockdown miR-466i-5p and best practice is to use more than one LNA against each specific target to control for off-target effects. I think this should be corrected before publication, especially as the expression changes in Scrt1, Ncan, Nrxn1 and Snap25 are in general very small.

Referee #2:

During the preparation of this revised manuscript about lncMN2-203, the authors discovered an error in the code used for single cell RNA-seq analysis. After correcting the analysis, Onecut2 was no longer found to be downregulated in the lncMN2-203 mutant cell lines. Instead, the authors now describe a different set of genes (Scrt1, Ncam, Nrxn1, and Snap25) as being downregulated in the mutants. The authors describe Scrt1 as the most relevant to their story, since Scrt1 has roles in neuronal differentiation and cell migration.

I truly appreciate the diligence that the authors have shown in these late stages of review. However, I find that overall impact of the revised story is diminished by the change in focus from Onecut2 (and other genes highly relevant to motor neuron differentiation) to those that have less defined roles in this particular model of neurogenesis. In the first round of reviews, I had asked the authors to try to experimentally test whether Onecut2 was indeed an important downstream regulator of lncMN2-203 in this model of motor neuron production (e.g., can Onecut2 rescue lncMN2-203 mutants). In response, the authors felt that Onecut2 was so clearly established as being critical to motor neuron production that they could simply cite the publications. In this new revision, given that the "molecular circuitry" related to motor neuron production now revolves around Scrt1 (and a few other genes involved in axon guidance and synapse function), this argument is now very weak. There is little data supporting a role for Scrt1 in motor neuron generation. Thus, while I think it is interesting that the authors show that lncMN2-203 can have a role as a miRNA sponge as well as a miRNA-producing gene, the knowledge of how lncMN2-203 regulates motor neuron generation is now much weaker.

Referee #3:

I thank the reviewers for sharing their issues with data analysis and for presenting a new manuscript with additional analyses and (in part) conclusions. I feel that in general the main conclusions of the study remain intact and that the authors have made sure that new conclusions can be drawn with alternative downstream targets. I therefore support publication of the present manuscript.

Referee #1:

My understanding is that the new manuscript corrects a finding in the previous version regarding the set of lncMN2 target genes that were identified. I think that in general the altered results do not change the overall conclusion of the study. Instead, the authors now show a different set of genes that are regulated in the lncMN2 mutants. The new results are mostly consistent with a model in which lncMN2-203 controls genes involved in neural development via a mechanism involving miR-466i-5p. However, I am not convinced by the RT-qPCR experiments in Fig 6G to validate Scrt1, Ncan, Nrxn1 and Snap25 as miR-466i-5p targets. Only a single LNA is used to knockdown miR-466i-5p and best practice is to use more than one LNA against each specific target to control for off-target effects. I think this should be corrected before publication, especially as the expression changes in Scrt1, Ncan, Nrxn1 and Snap25 are in general very small.

*We understand the referee's concern. However, in this case, since LNAs are directed toward microRNAs, we couldn't design more than one targeting molecule, due to sequence constraints. To address the point raised by the reviewer, we instead performed an experiment complementary to LNA-based LOF, by using Mimics RNAs to overexpress miR-466i-5p in NSC-34. By analyzing the expression of miR-466i-5p mRNA targets in cells overexpressing the miRNA, we observed a significant reduction of three out of the four identified miR-466-5p targets, namely Scrt1, Ncan and Nrxn1 (**new Appendix Figure S5D**). This demonstrates, through an alternative chemistry, the specificity of miR-466-5p/target interaction. As an additional control, we performed the LNA experiments using a molecule targeting miR-669a-5p, which is unable to interact with lnc-MN2-203 (**Figure 2H**) even though its sequence largely overlaps with that of miR-466i-5p (**Figure 2A**). We didn't observe any variation of the target mRNAs expression levels upon miR-669a-5p LNA administration (**new Appendix Figure S5C**). We believe that these experiments provide an adequate answer to the concern raised by the reviewer.*

Referee #2:

During the preparation of this revised manuscript about lncMN2-203, the authors discovered an error in the code used for single cell RNA-seq analysis. After correcting the analysis, Onecut2 was no longer found to be downregulated in the lncMN2-203 mutant cell lines. Instead, the authors now describe a different set of genes (Scrt1, Ncam, Nrxn1, and Snap25) as being downregulated in the mutants. The authors describe Scrt1 as the most

relevant to their story, since Scrt1 has roles in neuronal differentiation and cell migration.

I truly appreciate the diligence that the authors have shown in these late stages of review. However, I find that overall impact of the revised story is diminished by the change in focus from Onecut2 (and other genes highly relevant to motor neuron differentiation) to those that have less defined roles in this particular model of neurogenesis. In the first round of reviews, I had asked the authors to try to experimentally test whether Onecut2 was indeed an important downstream regulator of lncMN2-203 in this model of motor neuron production (e.g., can Onecut2 rescue lncMN2-203 mutants). In response, the authors felt that Onecut2 was so clearly established as being critical to motor neuron production that they could simply cite the publications. In this new revision, given that the "molecular circuitry" related to motor neuron production now revolves around Scrt1 (and a few other genes involved in axon guidance and synapse function), this argument is now very weak. There is little data supporting a role for Scrt1 in motor neuron generation. Thus, while I think it is interesting that the authors show that lncMN2-203 can have a role as a miRNA sponge as well as a miRNA-producing gene, the knowledge of how lncMN2-203 regulates motor neuron generation is now much weaker.

Among the factors implicated in lncMN2-203 ceRNA network, Scrt1 appeared as the most plausible player in motoneuron differentiation, due to its activity as a transcription factor in the brain and in the spinal cord (Nakakura et al., PNAS 2001).
Therefore, to address the issue raised by the reviewer in a reasonable timeframe, we set-up RNAi experiments to downregulate Scrt1 in mouse ESC-derived EBs (day 3). Efficient Scrt1 silencing (approximately 90%) caused a strong downregulation (more than 75%) of Onecut2 and Islet1, which are both crucially associated with MN differentiation and function. Even if this effect could be due to the decrease of the cell population expressing these markers, it anyway confirms a specific role of Scrt1 on MN differentiation.
*These results, included in the novel **Appendix Figure S5F**, highlight the activity of Scrt1 in MN generation and provide additional evidence about the relevant regulatory function of lncMN2-203 in MN development.*

Referee #3:

I thank the reviewers for sharing their issues with data analysis and for presenting a new manuscript with additional analyses and (in part) conclusions. I feel that in general the main conclusions of the study remain intact and that the authors have made sure that new conclusions can be drawn with alternative downstream targets. I therefore support publication of the present manuscript.

3rd Revision - Editorial Decision **28th Apr 2022**

Thank you again for submitting the revised manuscript. Please excuse the delay, I was away from the office for a few days and since this is a somewhat special case the procedure was a bit different. The revised figures and data have now been through the image integrity and data editor checks (no issues found). However, there are a few remaining editorial issues listed below, which I would like to ask you to resolve in a final revised version.

The authors have made all requested editorial changes.

Accepted **2nd May 2022**

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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: Irene Bozzoni, Pietro Laneve
Journal Submitted to: EMBO Journal
Manuscript Number: 2021-108918

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to determine sample size. At least three independent replicates were performed in all experiments. For single cell RNA-Seq all mESC clones available were analyzed (two for each condition).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	no animals were used in this study
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis. For cell counts analysis of each subtype in WT and mutant clones we used same batch samples (see methods and legends for more details).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	no animals were used in this study
For animal studies, include a statement about randomization even if no randomization was used.	no animals were used in this study
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 animals were used in this study
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.	in the case of single cell RNA-Seq, published state of the art software (e.g. Seurat, Monocle and COTAN) were used. These tools were specifically developed for single cell RNA-Seq data analysis. For the other experiments we assumed that all samples followed a normal distribution.
Is there an estimate of variation within each group of data?	in all experiments, we show data variability as mean and error bars that represent SD or Standard Error of the Mean (SEM). See legends or methods for details.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes

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).	See Table S2 for a complete list of antibodies information
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells used are already described in published studies: Capauto et al., 2018 and Biscarini et al., 2018. Cells were tested for micoplasma contamination before use.

* 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	The RNA-Seq data generated in this study have been deposited in the GEO database (GSE174671). A data availability section is included in the "Methods".
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. 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