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Deficiency in the nuclear long noncoding RNA *Charme* causes myogenic defects and heart remodeling in mice

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30th May 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, our three referees all highlight the quality and importance of the data and support publication, pending adequate revision. In addition to the reports here I conducted a round of referee cross-commenting in which referee #2 pointed out that the suggested rescue experiments would be very time-consuming without necessarily adding much new insight on the story.

Given the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:

-> ref #1 finds that additional mechanistic insight would strengthen the study but we see the value of study to lie in the in vivo data and will therefore not ask for more mechanism. However, I would ask you to comment on/clarify the minor points raised by this referee

-> ref #2 mainly asks for technical clarification that should be straightforward to include

-> ref #3 suggests a few more possible rescue experiments and additional clarification on the relevance to human cells. For the rescue experiments, this would be nice to include if it can be done in cell culture but we do not expect you to set up additional mouse lines to test this. I'd be happy to discuss the exact data that you would be able to include in response to this point.

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REFEREE REPORTS

Referee #1:

In this manuscript Ballarino et al. study the function of a nuclear lncRNA, which they call Charme, during myogenesis. Charme is mainly expressed in multinucleated myofibers and inhibition of Charme leads to differentiation and the downregulation of genes involved in muscle function and contraction. In a pull-down experiments the authors identified several loci to interact with Charme, among which nctc. This region contains a core muscle enhancer important for muscle differentiation through the regulation of several important myogenic genes, and inhibition of Charme leads to a reduction in the expression of these genes. Further analysis also showed that Charme is responsible for regulating the long-range interaction between the Charme locus and nctc.
Genetic deletion of Charme in vivo, at least partly phenocopies the effects observed in vitro by influencing the myogenic process in skeletal muscle and inducing a cardiac phenotype.

This story is a well taken care of manuscript that represents interesting data on yet another lncRNA regulating myogenic differentiation. The data look solid and show Charme to be functioning as a myogenic regulator through the regulation of chromatin interactions
While the effects of Charme appear striking, mechanistically it remains unclear how this lncRNA is exactly regulating chromatin interactions and gene expression.

- How many lncRNA by now have shown to be regulating myogenic differentiation and why would so many different lncRNAs be involved in the myogenic program?
- On Page 6 the authors mention that overexpression of pCharme fails to recover the effects induced by Charme inhibition. The authors suggest several explanations for this observations, but instead of suggesting these options they should be tested.
- Based on the effects in vitro one would expect a more dramatic phenotype in the global deletion of Charme. Can the authors comment on this?
- In is unclear what is meant by nctc region and why it is called this way
- How is Charme regulated transcriptionally and does overexpression result in an increased expression of the myogenic program?
- It would be good to show the proximity assay for Charme and nctc in skeletal muscle and in adult heart tissue.
- To get a more complete view on the function of Charme on skeletal muscle and cardiac muscle it would be good to perform RNA seq on these tissues too?
- Are any of the other myogenic lncRNAs regulated in response to Charme deletion?

Referee #2:

The study by Ballarino et al. demonstrates the in vivo function of a muscle-specific lncRNA that the authors called Charme. The most important finding of the study is that Charme regulates proper heart muscle development in mice. The loss of its expression results in morphological abnormalities in the embryonic heart that persist through the adulthood. As a consequence of this heart defect, Charme-/- mice have a significantly shorter life span. This is an important study reporting an in vivo phenotype for a lncRNA, sequence and potentially function of which is also conserved in human. Moreover, the study is carried out at the high technical level including generation of the Charme null allele in mice by an insertion of a premature polyA signal that, in contrast to commonly used big deletions of the lncRNA loci, is a state-of-the-art. The authors also show the role of Charme in myogenesis using its knock-down in the simplified murine and human cell line systems. In addition, the authors demonstrate that Charme interacts with chromatin to form a chromosomal domain that is required for normal expression of a set of myogenic genes.

Major comments:
As mentioned above, the findings of the study are of a high importance for the lncRNA field and the in vivo phenotype is convincing, however, the manuscript should be extensively re-worked. The data is presented in a way that the most interesting and important findings of the study are buried in

less important experiments and should be described in more detail. The authors should re-structure the manuscript: instead of being one coherent story, it consists of several parts that are partially repetitive (phenotypic consequences of Charme KD in C2C12 cells, mechanistic studies by ChIRP and microscopy in C2C12, phenotypic characterization of Charme-/- mice, mechanistic studies by microscopy in mice, Charme KD in human cell lines). My suggestion is to re-shuffle the parts of the manuscript: (1) describe the Charme depletion/phenotype in C2C12, then (2) show the in vivo mouse Charme-/- phenotype, (3) then move to the mechanistic studies by transcriptome analyses, ChIRP and FISH microscopy in both cell lines and in vivo and (4) only then talk about potential Charme's conservation in human.

Specific comments:
1. Summary:
The authors should avoid usage of generic sentences such as "a very strong cardiac phenotype" in the abstract and be more specific in their description, especially because it is one of the most important findings of the manuscript.
2. Introduction:
- "Charme ... highly conserved in human". What does it mean? The authors should be more precise: this lncRNA was found in mouse and shows sequence conservation to human. What about other species? Is there any sequence conservation deeper in the evolution?
3. Results:
3.1 "Charme depletion affects myogenesis" section
- "Upon knock-down, 50% decrease of the myosin creatin kinase (MCK) and the myosin heavy chain (MHC) mRNAs was found (Figure 1E and Table S1), indicating quite a clear effect on differentiation." The authors should elaborate more what are these genes for non-specialists (e.g. are markers of myogenesis).
- "...mCharme cDNA construct failed to recover.." I guess, the authors meant mature, spliced Charme transcript instead of cDNA? In the same sentence the authors speculate about why their rescue with the mature Charme transcript failed without having any data for it. While it is important to report also negative results, this paragraph appears out of place here and should be re-worked, at least, how it is phrased.
3.2 "Charme functional knock-out in mice affects the myogenic process" section
- The whole section reads like a material and methods section and should be reworked. I would suggest to add half a sentence at the beginning, stating why the authors decided to move forward with mouse genetics.
- The authors should elaborate more on the Charme-/- mice phenotype understandable to non-specialists. For example, what is a fiber caliber?
3.3 "Charme-/- mice exhibit an altered cardiac phenotype" section
- The description of the mouse phenotype is quite sparse. Instead of saying "Hematoxylin/eosin staining in both adult (Figure 5B) and neonatal (Figure S6B) mice indicated a strong alteration of heart morphology..." the authors should elaborate on the phenotype and what a non-heart specialist reader is looking at.
- The authors should indicate how penetrant is the observed Charme-/- heart phenotype.
3.4 "Identification of a functionally conserved human Charme transcript" section
- "...the hs-Charme upstream region contains many binding sites for MyoD..". The authors should precise how many MyoD binding sites are located in the human Charme promoter region.
4. Figure 5/Figure legend 5
- Indicate sample number for each experiment.
- Does Figure 5C show adult cardiac tissues or neonatal?
- Same for Figure 5D

Referee #3:

The authors of the study entitled "Deficiency in the nuclear long noncoding RNA Charme causes myogenic defects and heart remodeling in mice" follow up on their previous discovery of the lncRNA Charme to understand the physiological role on an organismal scale. They find my oligo

based depletion that MHC, MYH7 and other key skeletal/cardiac muscle genes are misrelated but cannot be rescued. Considering most key regulatory changes are in trans, the lack of rescue points to an insufficient cDNA rescue, or a dramatic cis regulatory role that has indirect effects on cardia/skeletal muscle genes. Consistent with this the authors see the Charme lncRNA localized to many of the misrelated genes by ChIRP. The authors proceed to generate a genetically modified mouse model with a PA terminator and MAZ sites to limit genetic alterations while depleting Charme. Interestingly the authors observe several clear anatomical and physiological defects in heart development and physiology. In a very succinct section the authors perform an LNA depletion in human myoblasts they find similar gene expression changes suggesting a conserved transcriptional regulatory role. Overall, this is a strong characterization of lncRNA that has a key physiological role in mouse models. As such, it will be of great interest to the general readership of EMBO. However, I have a few suggestions that may help increase this studies impact.

1) The analysis in human is very preliminary but makes an important conclusion: conserved gene regulation. This is a critical aspect considering the possible roles in muscle and cardiac disease (e.g Figure 6B). However, this section is very short and seems as a bit of an add on despite this important aspect. I suggest further characterization of hCharme. Specifically, the cloning and northern analyses used to characterize mouse Charme. As it stands this locus is identified by synteny and characterized by MYOD binding to the promoter. Yet it maybe more compelling if MyoD knockdown results in a concomitant decreases of Charme, as I believe was determined in mouse in a previous study. The knockdown is compelling but with out a real understanding of what the transcript in human cell is this remains somewhat elusive to make the strong conclusion of conserved regulation.

2) The transgene rescue study was uninterpretable, yet the authors give good explanations for why this may be the case. However, it would make the argument that there isn't a cis effect from the LNAs. It is recommended to try another cDNA or delivery construct to disentangle if the transcript needs to be localized in cis. It would also be prudent to see Charme over-expression in WT cells results in an increase or decrease in a reciprocal manner relative to knockdown. This would determine the genes that are regulated in an RNA specific manner. More so a transgenic mouse that could be investigated for reciprocal or other heart defects upon GOF and can be used for breeding to rescue the PA-MAZ depletion of Charme in vivo. These studies would conclusive distinguish between the two models proposed by the authors of why the cDNA did not rescue.

1st Revision - authors' response — 14th June 2018

Referee#1:

- How many lncRNA by now have shown to be regulating myogenic differentiation and why would so many different lncRNAs be involved in the myogenic program?

This is an interesting point since current literature is continuing reporting the identification of many different types of lncRNAs functionally correlated with a large number of different biological processes; however, only for a minority of them a mechanism of action has been discovered. Myogenesis, due to the availability of suitable *in vitro* cellular systems that faithfully reproduce the entire differentiation process, has represented one the biological systems in which the role of lncRNAs was studied first and so far there is quite a big collection of data indicating their participation in a large number of regulatory processes occurring both in the nucleus (i.e. histone modification, gene imprinting, chromatin dynamics) and in the cytoplasm (i.e. miRNA sponges, protein stabilizers, translational regulators). **In our case, *Charme* represents the first example of a lncRNA whose ablation produces a clear pathological phenotype *in vivo* in the absence of any stress condition.**

Here below some of the relevant references indicating the relevance of lncRNAs in the myogenic process. Most of them are included in the review by Ballarino *et al.* (JCI 2016). Nuclear lncRNAs mainly act as enhancer (***MUNC***, Mueller AC, *et al* Mol Cell Biol. 2015) or scaffold RNAs to guide epigenetic regulators onto specific chromosomal loci (***Bvht***, Klattenhoff CA, *et al* Cell. 2013; ***Fendrr***, Grote P, *et al* Dev Cell. 2013; ***Chaer***, Wang Z, *et al* Nat Med. 2016; ***Dum,*** Wang L, *et al* Cell Res. 2015; ***DBET***, Cabianca DS, *et al* Cell. 2012). Cytoplasmic lncRNAs include miRNA sponges (***Linc-MD1***, Cesana M, *et al* Cell. 2011; ***H19,*** Kallen AN, *et al* Mol Cell. 2013;

CHFR, Wang K, *et al* Circ Res. 2014; ***Malat1***, Han X, *et al* FASEB J. 2015) and translation regulators (***linc-31***, Dimartino et al., Cell Rep. 2018).
We have included in the Introduction the References that were missing in the previous version of the manuscript.

- On Page 6 the authors mention that overexpression of pCharme fails to recover the effects induced by Charme inhibition. The authors suggest several explanations for this observations, but instead of suggesting these options they should be tested.
In the paper we show that the overexpression of *Charme* (*mCharme*) through a cDNA expressing vector does not rescue the phenotype. We have now included in the new **Fig S4G** the results showing that this construct leads to the production of RNA species which are exclusively accumulated in the cytoplasm. This will remain a problem also with other cDNA constructs since they will all give rise to transcripts efficiently exported to the cytoplasm. Unfortunately, since the gene is quite large to be accommodated in state of the art vectors, it is quite a problem to conceive an appropriate construct able to express the primary transcript; indeed, one major point of the paper is the demonstration that the active *Charme* species is the one retained in the nucleus as an unspliced isoform at the sites of its own transcription (note for instance that ChIRP data demonstrate that only *pCharme* co-precipitates with the *nctc* region). Therefore, it is quite a problem for us to conceive a vector ensuring all these features. Finally, it seems generally accepted now in the field that for chromatin associated lncRNA species acting in *cis* and retained at the sites of their own transcription, rescue phenotypes are not expected to be obtained with exogenous gene overexpression (Goff and Rinn, Genome Res. 2015; Wang L, *et al*. Cell Res. 2015). Our future goal to clarify this issue will be to produce an edited *Charme* gene depleted of intron 1 in order to be able to unequivocally attribute to the intron retention the *Charme cis*-activity.
As a final point, we believe that the lack of rescue with the cDNA construct proves once more that the active species is the nuclear one.

- Based on the effects in vitro one would expect a more dramatic phenotype in the global deletion of Charme. Can the authors comment on this?
The phenotype is indeed deleterious since mice depleted of *Charme* do not survive the 1 year of age. We think, and we comment this in the paper, that *Charme* activity is to fine tune the expression of a large set of genes leading to conditions compatible to life; nonetheless, producing pathological conditions only in chronic states and becoming lethal only at later times after birth. This is not particularly in contrast with the *in vitro* data where we observed a partial decrease in the ability of myoblasts to fuse in mature myofibers and delaying the myogenic process. *In vivo* the effects become relevant with time producing a strong alteration mainly in the heart architecture and eventually leading to death.

- In is unclear what is meant by nctc region and why it is called this way
The target region was named ***nctc*** since the ChIRP peak (chr7:149746850-149747033) was identified close to the *nctc* gene locus (see also Table S2).

- How is Charme regulated transcriptionally and does overexpression result in an increased expression of the myogenic program?
In our previous paper, we reported an analysis performed on ChIP-seq datasets indicating that in differentiating C_2C_{12} cells MyoD binds to four E-box *consensus* sequences in the *Charme* promoter region (former lnc-405, Ballarino *et al*, Mol. Cell Biol. 2015), thus indicating a direct control of *Charme* by MyoD. This is also in line with the timing of *Charme* expression which follows that of MyoD. Interestingly, the dependence of *Charme* expression from MyoD is conserved in human, where three canonical E-boxes bound by MyoD (ChIP-seq data, MacQuarrie et al., 2013) are present in the region upstream to the *hs-Charme* TSS. These data have been added to the **new Fig S7B and C**.
At present it will be difficult, as previously discussed, to overexpress a functional *Charme* isoform. This is an interesting issue to answer when we will hopefully find a way to overcome the problems of OE.

- It would be good to show the proximity assay for Charme and nctc in skeletal muscle and in adult heart tissue.

We have performed the proximity assay also in adult hearts with results similar to the neonatal ones. The new data are added in the new **Fig S5E**. We hope this will be enough to respond to the referee's comment since C_2C_{12} are considered *bona fide* skeletal muscle cells.

- To get a more complete view on the function of Charme on skeletal muscle and cardiac muscle it would be good to perform RNAseq on these tissues too?
This is a very interesting part of the project and experiments on this issue are ongoing in the lab. This will certainly open very new avenues for future work. We thank the reviewer for this suggestion.

- Are any of the other myogenic lncRNAs regulated in response to Charme deletion?
As shown in the Supplemental Table S1 (*common targets*) and in the extracted list reported below, the Snhg6 transcript is the only annotated myogenic lncRNA down-regulated upon *Charme* depletion with both GAPmers utilized in our study (q-value<0.1, abslog2 Fold Change>0.5).

List of the myogenic lncRNAs as extracted from Supplemental Table S1.

Gene	Genomic position	Group1	Group2	log2	qvalue
H19	chr7:149761436-149764051	Scramble	Gap_Spliced	-0.816138	0.156731
Neat1	chr19:5842301-5845478	Scramble	Gap_Spliced	-0.452575	0.167022
Malat1	chr19:5795689-5802671	Scramble	Gap_Spliced	-0.0676754	0.953377
Dancr	chr5:74489107-74490361	Scramble	Gap_Spliced	0.858354	0.180798
Dnm3os	chr1:163917432-164408165	Scramble	Gap_Spliced	0.626217	0.0509583
Snhg1	chr19:8797976-8800816	Scramble	Gap_Spliced	0.297474	0.50458
Pvt1	chr15:61869541-62082530	Scramble	Gap_Spliced	-0.34538	0.501256
Dleu2	chr14:62217062-62301210	Scramble	Gap_Spliced	0.825386	0.824087
Snhg6	chr1:9932105-9934199	Scramble	Gap_Spliced	0.742601	0.080109
Snhg7	chr2:26492695-26495764	Scramble	Gap_Spliced	0.519407	0.388227
Airn	chr17:12875271-13061009	Scramble	Gap_Spliced	1.39946	0.858672
Igf2as	chr7:149836672-149856261	Scramble	Gap_Spliced	-1.5956	0.764825
2310015B20Rik	chr10:69667414-69682459	Scramble	Gap_1	-0.0332669	0.978149
Gene	**Genomic position**	**Group1**	**Group2**	**log2**	**qvalue**
H19	chr7:149761436-149764051	Scramble	Gap_1	-0.613328	0.358848
Neat1	chr19:5842301-5845478	Scramble	Gap_1	-0.526811	0.0797259
Malat1	chr19:5795689-5802671	Scramble	Gap_1	-0.0734751	0.948427
Dancr	chr5:74489107-74490361	Scramble	Gap_1	0.900215	0.145087
Dnm3os	chr1:163917432-164408165	Scramble	Gap_1	0.441525	0.254985
Snhg1	chr19:8797976-8800816	Scramble	Gap_1	0.567121	0.0586732
Pvt1	chr15:61869541-62082530	Scramble	Gap_1	0.416117	0.324065
Dleu2	chr14:62217062-62301210	Scramble	Gap_1	0.152771	0.982413
Snhg6	chr1:9932105-9934199	Scramble	Gap_1	0.999838	0.00585558
Snhg7	chr2:26492695-26495764	Scramble	Gap_1	0.412772	0.561036
Airn	chr17:12875271-	Scramble	Gap_1	0.705941	0.947855

	13061009				
Igf2as	chr7:149836672-149856261	Scramble	Gap_1	-1.05831	0.86833
2310015B20Rik	chr10:69667414-69682459	Scramble	Gap_1	-0.0332669	0.978149

Referee#2:

- Major comments:

As mentioned above, the findings of the study are of a high importance for the lncRNA field and the in vivo phenotype is convincing, however, the manuscript should be extensively re-worked. The data is presented in a way that the most interesting and important findings of the study are buried in less important experiments and should be described in more detail. The authors should re-structure the manuscript: instead of being one coherent story, it consists of several parts that are partially repetitive (phenotypic consequences of Charme KD in C2C12 cells, mechanistic studies by ChIRP and microscopy in C2C12, phenotypic characterization of Charme-/- mice, mechanistic studies by microscopy in mice, Charme KD in human cell lines). My suggestion is to re-shuffle the parts of the manuscript: (1) describe the Charme depletion/phenotype in C2C12, then (2) show the in vivo mouse Charme-/- phenotype, (3) then move to the mechanistic studies by transcriptome analyses, ChIRP and FISH microscopy in both cell lines and in vivo and (4) only then talk about potential Charme's conservation in human.

We thank the reviewer for this helpful comment. The rational behind the organization was to assemble the manuscript in two main parts with the first showing the experiments performed in the C_2C_{12} cell line (including all the mechanistic studies) and the second showing the experiments performed *in vivo*. We have tried to consider the suggested possibility (and originally we already thought of that), however we have found many difficulties. In particular, it is not easy to anticipate the *in vivo* experiments since most of the molecular data reported there require the *in vitro* RNAseq and ChIRP data. What we have been able to do, following the reviewer's suggestion, was to move the rescue experiment at the end of the third paragraph.

Specific comments:

1. Summary:

The authors should avoid usage of generic sentences such as "a very strong cardiac phenotype" in the abstract and be more specific in their description, especially because it is one of the most important findings of the manuscript.

The sentence "*resulted in a very strong cardiac phenotype*" was rephrased into:
"*resulted in a peculiar cardiac remodeling phenotype consisting in changes in size, structure and shape of the heart".*

2. Introduction:

- "Charme ... highly conserved in human". What does it mean? The authors should be more precise: this lncRNA was found in mouse and shows sequence conservation to human. What about other species? Is there any sequence conservation deeper in the evolution?

Sorry for the confusion, indeed the term ***highly*** is meaningless. By synteny and sequence comparison (40% of sequence identity, quite good for a noncoding RNA) we could define the conservation between mouse and human. A *Charme* transcript originating from a syntenic locus was also found in rat (chr1:101,544,378-101,556,038). What makes the story interesting is that human *Charme* is up-regulated upon differentiation and its depletion affects the same set of genes observed in mouse. We have better described and discusses this point.

3. Results:

3.1 "Charme depletion affects myogenesis" section

- "Upon knock-down, 50% decrease of the myosin creatin kinase (MCK) and the myosin heavy chain (MHC) mRNAs was found (Figure 1E and Table S1), indicating quite a clear effect on differentiation." The authors should elaborate more what are these genes for non-specialists (e.g. are markers of myogenesis).

We have appropriately rephrased the paragraph.

"...mCharme cDNA construct failed to recover.." I guess, the authors meant mature, spliced Charme transcript instead of cDNA?

Yes, sorry for the inaccuracy. We have corrected the sentence.

In the same sentence the authors speculate about why their rescue with the mature Charme transcript failed without having any data for it. While it is important to report also negative results, this paragraph appears out of place here and should be re-worked, at least, how it is phrased.
We agree with the reviewer and we have rephrased these data and moved them at the end of the third paragraph. Moreover, we have added in the new **Fig S4G**, the experiment showing the cytoplasmic localization of the overexpressed RNA.

3.2 "Charme functional knock-out in mice affects the myogenic process" section
- The whole section reads like a material and methods section and should be reworked. I would suggest to add half a sentence at the beginning, stating why the authors decided to move forward with mouse genetics.
According to this suggestion we have introduced the *in vivo* section with a starting sentence.

- ***The authors should elaborate more on the Charme-/- mice phenotype understandable to non-specialists. For example, what is a fiber caliber?***
We agree with the reviewer and we have specified the meaning of fiber caliber in the text. Moreover, we have checked that all the details are correctly provided in the Materials and Methods section.

3.3 "Charme-/- mice exhibit an altered cardiac phenotype" section
- The description of the mouse phenotype is quite sparse. Instead of saying "Hematoxylin/eosin staining in both adult (Figure 5B) and neonatal (Figure S6B) mice indicated a strong alteration of heart morphology..." the authors should elaborate on the phenotype and what a non-heart specialist reader is looking at.
Thanks again for this request that has allowed us to be more comprehensible. We have rephrased the sentence with more clearness.

- The authors should indicate how penetrant is the observed Charme$^{-/-}$ heart phenotype.
The remodeling of heart muscle was observed in 100% of the analysed *Charme* $^{-/-}$mice. We have introduced this data in the text.

3.4 "Identification of a functionally conserved human Charme transcript" section
The authors should precise how many MyoD binding sites are located in the human Charme promoter region.
Thank you for the clarification. We have specified that ChIP-seq analysis indicated the presence of one major binding region for MyoD in the *hs-Charme* upstream region which includes three canonical E-boxes (new **Fig S7C**).

4. Figure 5/Figure legend 5 - Indicate sample number for each experiment.
Thank you for noticing the mistake; the number is now better specified in the legend.

- Does Figure 5C show adult cardiac tissues or neonatal? - Same for Figure 5D
Thank you for noticing the omission; the information has been added in the figure and in the legend.

Referee #3:

1) The analysis in human is very preliminary but makes an important conclusion: conserved gene regulation. This is a critical aspect considering the possible roles in muscle and cardiac disease (e.g Figure 6B). However, this section is very short and seems as a bit of an add on despite this important aspect. I suggest further characterization of hCharme. Specifically, the cloning and northern analyses used to characterize mouse Charme. As it stands this locus is identified by synteny and characterized by MYOD binding to the promoter. Yet it maybe more compelling if MyoD knockdown results in a concomitant decreases of Charme, as I believe was determined in mouse in a previous study. The knockdown is compelling but with out a real understanding of what the transcript in human cell is this remains somewhat elusive to make the strong conclusion of conserved regulation.

The structure of the human *Charme* transcript was derived from our own RNAseq data (GSE70389, Legnini et al., 2017, see new **Fig S6B**) and the combined analysis of FANTOM5 (Phase 1 and 2) CAGE datasets across 1829 samples (see new **Fig S6C and D**). These analyses indicated a similar exon-intron structure with a 41,6% of sequence identity in the exons (see **Fig S6E** and new **Table S4**).
The dependence of *Charme* expression from MyoD is mainly suggested, in analogy with the murine counterpart, by the presence of three MyoD canonical E-boxes in the region upstream to the human *Charme* TSS as well as by MyoD ChIP-seq data (MacQuarrie *et al*., 2013) (new **Fig S7B and C**), which confirm its binding to these elements. Since MyoD, which is the major inducer of differentiation, is upstream to *Charme* expression (see new **Fig S7A, left panel**), we believe that the down-regulation of MyoD would provide a general block in differentiation not allowing to distinguish between direct and indirect effects on *Charme* transcription, as observed in Duchenne Muscular Dystrophy myoblasts (see new **Fig S7A, right panel**).
We thank the referee for this comment that has allowed us to improve the characterization of *hs-Charme* and to better present the data on human *Charme*. We believe that the additional data add more value to the paper.

2) The transgene rescue study was uninterpretable, yet the authors give good explanations for why this may be the case. However, it would make the argument that there isn't a cis effect from the LNAs. It is recommended to try another cDNA or delivery construct to disentangle if the transcript needs to be localized in cis. It would also be prudent to see Charme over-expression in WT cells results in an increase or decrease in a reciprocal manner relative to knockdown. This would determine the genes that are regulated in an RNA specific manner. More so a transgenic mouse that could be investigated for reciprocal or other heart defects upon GOF and can be used for breeding to rescue the PA-MAZ depletion of Charme in vivo. These studies would conclusive distinguish between the two models proposed by the authors of why the cDNA did not rescue.
In the paper we show that the overexpression of *Charme* (*mCharme*) through a cDNA expressing vector does not rescue the phenotype. We have now included in **Fig S4G** the results showing that this construct leads to the production of RNA species which are exclusively accumulated in the cytoplasm. This will remain a problem also with other cDNA constructs since they will give rise to transcripts efficiently exported to the cytoplasm. Indeed, one major point of the paper is the demonstration that the active *Charme* species is the one retained in the nucleus as an unspliced isoform at the sites of its own transcription (note, for instance, that ChIRP data demonstrate that only *pCharme* co-precipitates with the *nctc* region). Therefore, it is quite a problem for us to conceive a vector ensuring all these features. On the other hand, we believe that the lack of rescue with the cDNA construct proves once more that the active species is the nuclear one. Finally, it seems generally accepted now that for chromatin associated lncRNA species acting in *cis*-, rescue phenotypes are not expected to be obtained (Goff and Rinn, Genome Res. 2015; Wang L, *et al*. Cell Res. 2015). Our future goal to clarify this issue will be to produce an edited *Charme* gene depleted of intron 1 in order to be able to unequivocally attribute to the intron retention the *Charme cis*-activity.

2nd Editorial Decision 12th July 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by one of the original referees and this person's comments are shown below. As you will see the referee finds that all criticisms has been sufficiently addressed and recommends the manuscript for publication. However, before we can go on to officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in final revision.

Referee #1:

I this updated version of the manuscripts the authors were able with most of the comments that were raised by the reviewers.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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-repo
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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?	Due to the low experimental variability, the experiments performed in C2C12 cells were performed (at least) in triplicates. Due to the higher variability, a sample size of 3-9 independent observations was chosen for mouse studies to be able to detect statistical differences with a statistical power values of about 70%.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We selected a sample size of 3-9 independent observations in order to be able to detect statistical differences with a statistical power values of about 70%. This was assuming that we would like to detect differences of 25% of in the means of control and experimental groups and that taking into account that the SEM is around 10 to 20 % of mean values.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We only made sure that we used age-matched mice with appropriate genotypes for comparison. No other criteria were used to include or exclude mice. Littermates mice were prefered for survivor measurement analyses. In all the other experiments, indipendently chosen animals were selected
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.	Due to the clear differences between Charme+/+ and Charme -/- no particuular steps were taken to minimize the effects of subjective bias when allocating mice to treatments.
For animal studies, include a statement about randomization even if no randomization was used.	For animal studies, no randomization was used.
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.	Due to the clear differences between Charme+/+ and Charme -/- no particuular steps were taken to minimize the effects of subjective bias during animal group allocation.
4.b. For animal studies, include a statement about blinding even if no blinding was done	In experiments involving mice, no blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes, the statistical test used is indicated for each experiment in the figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	In the case of RNA-seq, Cuffdiff assumes that the read counts associated to transcripts follow a beta binomial negative distribution. It is well accepted that this is a quite effective way to model read counts. In order to test if our data fit to this distribution, we should have more than two replicates per condition. Furthermore, the usage of a non parametric test for differential expression would have required more replicates in order to correctly estimate p-values. 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 studies, we show the Standard Error of the Mean (SEM).
Is the variance similar between the groups that are being statistically compared?	Yes, since animals analysed belong to the same strain and the celsl used for the analyses belong to the same cell line

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).	The antibodies used in the study were: RNA Pol II (Millipore) cat. 17-620; anti-acetyl-HistoneH3 (Lys9) (Millipore) cat. 07-352; MHC (eBioscience) cat. 14-6503; MCK (Santacruz Biotechnology) cat. sc-15161. For more informations, please refer to details of the antibodies used in 'Materials and Methods"
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	C2C12 line was purchased from ATCC and tested for mycoplasma 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.	Both Charme +/+ and Charme -/- mice belong to C57BL/6 strain. Charme -/- genetic modified mouse is properly described in the paper. All the mice were housed in standard conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animals were treated in respect to housing, nutrition and care according to the guidelines of Good laboratory Practice (GLP).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance of ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
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.	Not applicable
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.	Not applicable

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 (GSE94498). A data availability section is included in the "Materials and 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).	Not applicable
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).	Not applicable
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.	Not applicable

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.	Not applicable