TMEM8C-mediated fusion is regionalized and regulated by NOTCH signalling during foetal myogenesis

Joana Esteves de Lima, Cédrine Blavet, Marie-Ange Bonnin, Estelle Hirsinger, Emmanuelle Havis, Frédéric Relaix and Delphine Duprez
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MS TITLE: Regionalised expression and transcriptional regulation by NOTCH of TMEM8C fusion gene during foetal myogenesis

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript the authors further analyse a single cell sequencing dataset recently published from their laboratory. They performed scRNAseq of chick limb buds at E6 and E10. Here they focus on single cells marked by the expression of classic myogenic genes such as Pax7, Myod, Myog, MyHC. The analysis suggests progression of cells through the differentiation time course. They used TMEM8C (Myomaker) as a readout for myoblast fusion and found that it is coexpressed with MyoD and Myogenin, a differentiation marker, but is not overlapping with Pax7 positive progenitor cells. Thus MyoD/Myogenin/TMEM8c expressing cells are likely fusion competent.
To determine the location of TMEM8C fusion competent cells in vivo, they perform colorimetric in situ hybridisations on foetal limb serial sections. They analyse localisation of TMEM8C and myogenic and tendon markers. This shows that TMEM8C and Myogenin transcripts both regionalise to the central domains of foetal limb muscle mass in the FCU (Flexor Carpi Ulnaris). Immunostain for Myogenin with markers of the tips of muscle fibres (collagen, pSmad1/5/9) confirms its regionalisation to central domains.
They examine the possible role of Notch signalling, which is already known to regulate myoblast proliferation and differentiation. They show that expression of the ligand Jag2 is regionalised to the central domains of muscle fibres. Interference with Notch signalling results in increased differentiation and fusion, as was shown before by the group. In addition they show here that increased fusion correlates with TMEM8C becoming expressed more widely.
The authors use an in vitro culture system with the aim to uncouple myoblast differentiation and fusion. As they showed before Notch inhibition decreased markers for proliferative progenitors, but increased expression of differentiation markers, including TMEM8C, as well as fusion index. They next use a two step protocol, which leads to Myogenin expression in the majority (but not all) of myoblasts cultured at low density, thus the cells don't fuse.
They equate Myogenin positivity with differentiated cell. The cells are then replated at high density and with DAPT- mediated NOTCH inhibition they observe a higher fusion index. Based on this they conclude that Notch inhibition promotes fusion independently from differentiation in vitro. Finally, they show that the Notch target HEYL, a transcriptional repressor, binds to the TMEM8C promoter.

Overall this work advances our understanding as to how muscle fusion process is controlled and indicates that Notch regulates fusion by negatively regulating TMEM8c expression via HESL transcriptional repressor.

Comments for the author

While this finding is of potential interest to the field, the manuscript has gaps and is incomplete in my opinion. Additional experiments are required to test their hypothesis. The following are essential revisions needed in order to better support the conclusions.
1. The UMAP plots indicate the progression of cells through the differentiation program. This should be supported by using RNAvelocity to look at RNA splicing. Furthermore, additional markers used in this study should be identified in the scRNA data, such as Jag2 and HEYL.
2. Figures 2,3,4 and 5 are quite descriptive, looking at regionalised expression of various markers in foetal chick limb muscle. This is done in serial sections. There is a disconnect with the single cell data, as they now look at multi-nucleated muscle fibres. A limitation is that co-localisation of the different markers is not shown at single cell resolution. To address this, they should use fluorescent in situ and multiplexing probes. Expression of HEYL should also be shown.
In Fig. 3J,K it is difficult to see TMEM8C inside and outside of muscle fibres.
3. There are no rescue experiment shown to validate the mechanism they suggest. For example, can the phenotype obtained after DMB mediated immobilisation (Fig. 6), which is consistent with Notch LOF, be restored by Notch activation - using their RCAS-Delta1 virus in vivo. It is not clear where the section was taken (panels D, H) and in the control TMEM8c does not seem to be

regionalised centrally. Better describe the method to determine the distribution of myonuclei per fibre, not clear if was this done on serial transverse sections to capture the length of the fibre.
4. In Notch LOF (Fig. 6) they should also show what happens to expression and localisation of Jag2 and HEYL. Similarly, in the culture system (Fig. 7D) those markers should be added.
5. The two-step protocol described in the second part of Fig. 7 needs to uncouple differentiation and fusion better. At the moment there is a mixed population of cells including some Pax7 +ve progenitors. Can the authors obtain a pure population of Myogenin-only +ve cells by FACS. I am also not convinced that Myogenin positivity alone can be equated with 'differentiated'. Therefore, at the moment the statement that Notch inhibition in differentiated myoblasts promotes fusion independently from differentiation in vitro (line 453) is poorly supported.
As above (3.) rescue experiments are essential. It should be tested whether RCAS-Delta1 reverts the phenotype shown in Fig. 7J,K,L and what the expression levels are of TMEM8c, Jag2 and HEYL.
6. Panel 7A is not very informative, what are the genomic coordinates of these sequences. It is not clear what is plotted in Fig. 7B, C. The percentage of input (Y-axis) is very low, especially in panel C. The relevance of HESL binding to these sites for TMEM8c expression should be probed both in vitro (reporter assays wt/mutated site) and in vivo. Crispr mediated deletion of the elements should lead to premature expression of TMEM8c. This could be challenging, however the expression of an electroporated reporter construct could be assessed in the limb.
Minor typos:
Fig 7 legend says DMB when it should be DAPT (lines 915/916)

Reviewer 2

Advance summary and potential significance to field

See comments from initial review.

Comments for the author

In the revised version the authors attempted to deal with many of my concerns.
While the paper is somewhat improved and I think the data are solid, I'm worried that the system is not robust enough or there are technical limitations that give me pause when considering whether the the two main points - that fusion is regionalized and that NOTCH directly regulates fusion through a release of HeyL repression on TMEM8C - are likely to be true.
-The first point that fusion is regionalized - I think data say that fusion does not occur at the tips. Other than that, I don't think it's regionalized because the MyoG+ TMEM8C+ cells seem to be located across entire length of fibers. The fact that these cells are excluded from tips is still an important finding.
-The idea that NOTCH directly regulates fusion is very difficult to interpret.
They mention this in the text but there are still numerous instances where the authors clearly side with the interpretation that NOTCH directly regulates fusion.
-The experiments with DELTA1-DN and DMB (Figure 6) maybe do not strongly support the conclusions. First, there look to be fewer Tom+ cells in the DELTA1-DN sample (E,F compared to A,B). Second, the effect on TMEM8C is quite modest and it is questionable if that level of increase at the mRNA level would have any effect on fusion.
-The title does not accurately represent the findings. There is only correlational data showing that any potential NOTCH - TMEM8C pathway is regional. A direct experiment showing NOTCH activation in TMEM8C cells in vivo would be needed.

Reviewer 3

Advance summary and potential significance to field

The manuscript of Joana Esteves de Lima et al is a very elegant study concerning the crosstalk between Notch signaling and the fusion process of myogenic cells during embryonic development. Experiments of single cell RNA-seq on embryonic limbs at two developmental stages identify the transcriptomic programs characterizing the different fates of myogenic cells, from their cycling progenitor state to the post-cycling state when cells differentiate and fuse to form multinucleated

myofibers. Characterization of the rate of gene expression during the fusion competent state is associated with decreased NOTCH activity. With complementary in vivo and ex vivo culture experiments the authors show that myogenic progenitors may fuse with growing myofibers mainly in the center of the myofibers and not at their extremities, as inferred from the literature. NOTCH activity directly controls the expression of the HeyL transcription inhibitor. The authors demonstrate that HeyL binds the regulatory sequences of Myomaker (TMEM8C) when NOTCH activity is high in cycling progenitors and prevents expression of TMEM8C. Nevertheless the expression of Jagged 2 in the central part of the myofibers in vivo might activate NOTCH signaling in the surrounding myogenic progenitors and inhibit TMEM8C expression, while the authors show higher expression of TMEM8C expression and suggest that fusion is restricted to those central regions.
Altogether the experiments presented are of high quality, the manuscript is well written, but improvements must be performed to clarify the apparent contradictory explanations.

Comments for the author

Major concerns.
1- It is a regrettable that immunohistochemistry experiments with antibodies against TMEM8C were not performed. If the antibodies exist immunohistochemistry should be performed, it would greatly increase the quality of the characterization of the in vivo fusion processes of growing myofibers with the progenitor cells.
2- In figure 2 it is not clear whether TMEM8C is expressed in the myofibers themselves or/and in some myogenic progenitors, zoom of the figures with antibodies/dyes recognizing the cell membranes would help to determine whether this is the case. It is suspected that TMEM8C is expressed in both compartments (the progenitor and the myofiber). Whether the specific fusion of progenitors in the center of the myofiber (exclusion from the tips) is due to a specific expression of TMEM8C in specific myonuclei of the myofiber is unclear. Alternatively progenitors may express TMEM8C only in the center of the myofibers, precluding their fusion at the tips. In situ hybridization does not clearly establish if TMEM8C is detected in both the myofibers and the mononucleated progenitors, and this is crucial to understand the mechanisms. Is TMEM8C expressed in the same area than Jagged2 inside the myofibers? The authors clearly show a crosstalk between Notch and TMEM8C gene expression in myogenic progenitors, but how this crosstalk explains the regionalized fusion process must be better discussed. From the expression pattern of Jagged2, it would be expected that fusion would be increased at the tips of the myofibers where the NOTCH signaling crosstalk between myofibers and associated myogenic progenitors is lower.
3- The authors must explain why Jagged2 is expressed in the central myofibers/myonuclei where fusion takes place. Its expression should activate Notch signaling in surrounding myogenic progenitors, where HeyL should be high and suppress TMEM8C in those central regions of the myofibers. On the contrary, in this central region TMEM8C seems to be expressed in myogenic progenitors and fusion seems to occur. This is very confusing. The authors show that Notch signaling must be decreased to allow TMEM8C expression in myogenic progenitors, but in vivo Notch activity seems higher in the center of the myofibers where Jagged 2 is expressed.

First revision

Author response to reviewers' comments

Point-by-point response to the 3 reviewers
The original comments of the 3 reviewers are in *Italics* and highlighted in grey The revisions involving experiments are highlighted in **bold**.

Reviewer 1
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In this manuscript the authors further analyse a single cell sequencing dataset recently published from their laboratory. They performed scRNAseq of chick limb buds at E6 and E10. Here they focus on single cells marked by the expression of classic myogenic genes such as Pax7, Myod, Myog, MyHC. The analysis suggests progression of cells through the differentiation time course. They

used TMEM8C (Myomaker) as a readout for myoblast fusion and found that it is coexpressed with MyoD and Myogenin, a differentiation marker, but is not overlapping with Pax7 positive progenitor cells. Thus MyoD/Myogenin/TMEM8c expressing cells are likely fusion competent.
To determine the location of TMEM8C fusion competent cells in vivo, they perform colorimetric in situ hybridisations on foetal limb serial sections. They analyse localisation of TMEM8C and myogenic and tendon markers. This shows that TMEM8C and Myogenin transcripts both regionalise to the central domains of foetal limb muscle mass in the FCU (Flexor Carpi Ulnaris). Immunostain for Myogenin with markers of the tips of muscle fibres (collagen, pSmad1/5/9) confirms its regionalisation to central domains. They examine the possible role of Notch signalling, which is already known to regulate myoblast proliferation and differentiation. They show that expression of the ligand Jag2 is regionalised to the central domains of muscle fibres. Interference with Notch signalling results in increased differentiation and fusion, as was shown before by the group. In addition they show here that increased fusion correlates with TMEM8C becoming expressed more widely.
The authors use an in vitro culture system with the aim to uncouple myoblast differentiation and fusion. As they showed before Notch inhibition decreased markers for proliferative progenitors, but increased expression of differentiation markers, including TMEM8C, as well as fusion index. They next use a two step protocol, which leads to Myogenin expression in the majority (but not all) of myoblasts cultured at low density, thus the cells don't fuse. They equate Myogenin positivity with differentiated cell. The cells are then replated at high density and with DAPT-mediated NOTCH inhibition they observe a higher fusion index. Based on this they conclude that Notch inhibition promotes fusion independently from differentiation in vitro. Finally, they show that the Notch target HEYL, a transcriptional repressor, binds to the TMEM8C promoter.
Overall this work advances our understanding as to how muscle fusion process is controlled and indicates that Notch regulates fusion by negatively regulating TMEM8c expression via HESL transcriptional repressor.

Comments for the Author:
While this finding is of potential interest to the field, the manuscript has gaps and is incomplete in my opinion. Additional experiments are required to test their hypothesis. The following are essential revisions needed in order to better support the conclusions.

1. *The UMAP plots indicate the progression of cells through the differentiation program. This should be supported by using RNAvelocity to look at RNA splicing.*
Indeed, the UMAP plots show the expected progression of the muscle differentiation program. We applied the RNA velocity algorithm scvelo on our muscle single-cell RNAseq (scRNAseq) datasets to generate short-term trajectories (Bergen, V., Lange, M., Peidli, S. *et al*. Generalizing RNA velocity to transient cell states through dynamical modeling. *Nat Biotechnol* **38,** 1408– 1414 (2020). https://doi.org/10.1038/s41587-020-0591-3). However, the analysis of the phase *portraits and discussions with the algorithm authors led us to conclude that our datasets were not suitable for velocity analysis.*
Furthermore, additional markers used in this study should be identified in the scRNA data, such as Jag2 and HEYL.
We now include the *JAG2* and *HEYL* markers in the analysis of scRNAseq datasets (New Figure 5A,B, I,J). *JAG2* is mainly associated with differentiated muscle cells, while *HEYL* is associated with PAX7 progenitors at E6 and E10 (New Figure 5A,B). Nevertheless, there is a low proportion of *HEYL*+ cells that express MYOD and/or MYOG at E6 and E10 (New Figure 5I,J, New Fig. S6D,E). Among this population *HEYL* is significantly expressed in *TMEM8C*neg cells versus *TMEM8C*+ cells at E10 (New Figure 5I,J, New Fig. S6), suggesting that the downregulation of HEYL in *TMEM8C*+ cells allows fusion to occur.

2. *Figures 2,3,4 and 5 are quite descriptive, looking at regionalised expression of various markers in foetal chick limb muscle. This is done in serial sections. There is a disconnect with the single cell data, as they now look at multi-nucleated muscle fibres. A limitation is that co- localisation of the different markers is not shown at single cell resolution. To address this, they should use fluorescent in situ and multiplexing probes. Expression of HEYL should also be shown. In Fig. 3J,K it is difficult to see TMEM8C inside and outside of muscle fibres.*
Single cell data from limb cells (Figure 1) and immunolabelling/in situ hybridization to limb sections (Figures 2-5) are complementary experiments that do not address the same questions. Single-cell data analysis (Figure 1) provide us with gene expression at a single cell level within

muscles, while immunolabelling/in situ hybridization experiments provide us with spatial information at the muscle tissue level. Figures 2-5 show in situ hybridisation and immunolabelling experiments to transverse or longitudinal limb sections to show the regionalisation of gene expression at the level of muscle tissue. They show a preferential central location for *TMEM8C* and *MYOG*/MYOG compared to tips markers such as *FGF4* and PSMAD1/5/8. Single-cell data analysis tells us that *TMEM8C* is co-expressed with *MYOD* and *MYOG* in myoblasts and not in muscle progenitors (Figure 1). Following the reviewer concern and **to validate the scRNAseq datasets, we performed fluorescent in situ hybridization and looked at the co-expression of *TMEM8C* with *PAX7*, *MYOD* and *MYOG* at a single cell level. Consistent with the single-cell data analysis, *TMEM8C* is not expressed in PAX7+ cells, while being expressed in *MYOD*+ and *MYOG*+ cells (New Figure S4A-F'').**
To better visualise *TMEM8C* transcripts inside and outside muscle fibres, we analysed *TMEM8C* expression with fluorescent in situ hybridization and myosin immunolocalization to limb sections (New Figure S4G-H'). We found that *TMEM8C* transcripts rarely co-localized with myosins indicating a preferential location outside muscle fibres.

3. *There are no rescue experiment shown to validate the mechanism they suggest. For example, can the phenotype obtained after DMB mediated immobilisation (Fig. 6), which is consistent with Notch LOF, be restored by Notch activation - using their RCAS-Delta1 virus in vivo.*
Rescue experiments with DELTA1/RCAS overexpression after DMB treatments have been already performed in our eLife 2016 publication (PMID: 27554485, Figure 4). DELTA1/RCAS overexpression rescues the NOTCH LOF muscle phenotype observed in limbs of DMB-treated embryos (PMID: 27554485, Figure 4). DMB treatment induced a shift towards differentiation similar to NOTCH LOF (PMID: 27554485, Figures 1,2). DELTA1/RCAS rescue experiment showed that the increase in myosin expression observed after DMB treatment (PMID: 27554485, Figure 4C) is lost with DELTA1/RCAS overexpression (PMID: 27554485, Figure 4D). **Nevertheless, to address specifically the fusion step, we performed rescue experiments and analysed the fusion index in the DELTA1/RCAS grafted limbs treated with DMB and compared it with the contralateral limbs (DMB-treated only). As expected, we found that DELTA1/RCAS prevents the increase of fusion observed in limb muscles of DMB treated embryos (New Figure S7I-N).**

It is not clear where the section was taken (panels D, H) and in the control TMEM8c does not seem to be regionalised centrally.
TMEM8c is regionalised in control muscle, the central location depends on the section angle of the muscle.

Better describe the method to determine the distribution of myonuclei per fibre, not clear if was this done on serial transverse sections to capture the length of the fibre.
The method to determine the number of myonuclei per fibre was indeed performed on transverse sections. We better described it the methods.

4. *In Notch LOF (Fig. 6) they should also show what happens to expression and localisation of Jag2 and HEYL. Similarly, in the culture system (Fig. 7D) those markers should be added.*
Figure 6: The expression of *JAG2* and NOTCH target genes (*HEYL* and *HES5*) has been already analysed in limb muscles of control and DMB-treated embryos by in situ hybridization and qPCR in our eLife 2016 publication (PMID: 27554485, Figure 2A-C, G). These experiments show a decrease of J*AG2* and *HEYL* expression in DMB-treated limbs, consistent with a drop of NOTCH activity in limbs of DMB embryos. We prefer to cite the paper rather than duplicate the experiments.
Figure7: **We analysed the expression of the NOTCH target genes, *HEYL*, by RT-q-PCR in myoblast cultures following DAPT exposure, and found the expected down-regulation of *HEYL* upon DAPT treatment. (New panel D of Figure 7).**

5. *The two-step protocol described in the second part of Fig. 7 needs to uncouple differentiation and fusion better. At the moment there is a mixed population of cells including some Pax7 +ve progenitors. Can the authors obtain a pure population of Myogenin-only +ve cells by FACS.*
The two-step protocol is a recognised culture system to assess myoblast fusion *in vitro* (Nat Commun 2021, PMID: 33531476, PMID: 33531466). After 24h in differentiation (in low serum and with cells at low density), the cultures are composed of a majority of PAX7neg-MYOG+ differentiating myoblasts and of a residual double positive PAX7+MYOG+ myoblast population (Figure 7G,I). PAX7+MYOG+ cells do not constitute a progenitor population since the expression of the late

differentiation marker MYOG (Buckingham and Rigby 2014, PMID: 24525185) qualifies them as differentiated myoblasts. Moreover, it is recognized that this residual double population (PAX7+MYOG+) is virtually inexistent *in vivo* but can appear artificially *in vitro* (Buckingham and Rigby, 2014, PMID: 24525185). Consequently, we consider that the two-step protocol allows for uncoupling differentiation and fusion and that an additional step involving FACS sorting is unnecessary.

I am also not convinced that Myogenin positivity alone can be equated with 'differentiated'.
We do not understand the point to ask for a pure MYOG population (*see comment above with the request of a FACS-sorting step to obtain a pure population of Myogenin+ cells*) if the referee is not convinced that that the MYOG population represents a differentiated cell population.
We want to point out that MYOG is recognized to be expressed in differentiating and differentiated muscle cells. MYOG is the latest MRF to be expressed and is required for myoblast terminal differentiation. *Myog*-null mice is the only MRF single mutant that dies at birth due to muscle defects. The muscles of these mice are severely reduced due to impaired myoblast differentiation and lack of proper fiber formation (Hasty et al., Nature 1993, PMID**:** 393145; Nabeshima et al. Nature 1993, PMID: 8393146**)**. Therefore, we think that MYOG is a right marker for differentiated myoblasts (myocytes) according to the literature.

Therefore, at the moment the statement that Notch inhibition in differentiated myoblasts promotes fusion independently from differentiation in vitro (line 453) is poorly supported.
Because the two-step protocol is a recognized protocol to separate differentiation from fusion and MYOG a marker of differentiated myoblasts, we believe that NOTCH inhibition in this two-step myoblast cultures supports the conclusion.

*As above (**3**.) rescue experiments are essential. It should be tested whether RCAS-Delta1 reverts the phenotype shown in Fig. 7J,K,L*
Since the effect of the DAPT is downstream of DELTA1, the addition of DELTA1-RCAS in DAPT-treated myoblast cultures will not rescue the phenotype. Indeed, DAPT (an inhibitor of the gamma-secretase complex) prevents the proteolytic cleavage of the NOTCH intracellular domain (NICD) induced by DELTA1-NOTCH binding and required for NOTCH signalling activity.
and what the expression levels are of TMEM8c, Jag2 and HEYL.
We analysed the expression of *TMEM8C* and *HEYL* in the two-step myoblast cultures. We found that *TMEM8C* is increased upon DAPT exposure in this experimental design (new Figure S9C).
This strengthens the data about the effect of NOTCH inhibition on the expression of the fusion gene *TMEM8C*.

6. *Panel 7A is not very informative, what are the genomic coordinates of these sequences. It is not clear what is plotted in Fig. 7B, C. The percentage of input (Y-axis) is very low, especially in panel C.*
We suppose the referee means panel 8A and not panel 7A. The coordinates of the 3 regions containing E-box sequences are indicated from the transcription start site of *TMEM8C* gene and the genomic coordinates are detailed in the manuscript. We added them to Figure 8A for clarity. The percentage of input is low in limbs because we are dealing with tissues and not cell cultures. ChIP experiments from tissues are challenging compared to those made from cultured cells. ChIP experiments using nuclei from myoblast cultures show high percentage of input (panel B) since we are working with a myoblast pure population. However, ChIP experiments using nuclei from the entire limbs display lower percentage of input (panel C) compared to ChIP experiments using nuclei from myoblast cultures, since nuclei where HEYL binds to *TMEM8C* regulatory regions are diluted among all limb nuclei. Nevertheless, both DAPT-treated cultures and of DMB-treated limbs show a decrease of HEYL binding to *TMEM8C* regulatory regions.

The relevance of HESL binding to these sites for TMEM8c expression should be probed both in vitro (reporter assays, wt/mutated site) and in vivo. Crispr mediated deletion of the elements should lead to premature expression of TMEM8c. This could be challenging, however the expression of an electroporated reporter construct could be assessed in the limb.
This requested experiment is indeed very challenging in the chicken system. Moreover, given the mosaic expression after somite electroporation (PMID: 26518454), we believe that this experiment is unlikely to provide any clear answer.

Minor typos:
Fig 7 legend says DMB when it should be DAPT (lines 915/916)
DMB has been replaced with DAPT.

Reviewer 2
Advance Summary and Potential Significance to Field:
See comments from initial review.

Reviewer 2 Comments for the Author:
In the revised version the authors attempted to deal with many of my concerns. While the paper is somewhat improved and I think the data are solid, I'm worried that the system is not robust enough or there are technical limitations that give me pause when considering whether the the two main points - that fusion is regionalized and that NOTCH directly regulates fusion through a release of HeyL repression on TMEM8C - are likely to be true.

-The first point that fusion is regionalized - I think data say that fusion does not occur at the tips. Other than that, I don't think it's regionalized because the MyoG+ TMEM8C+ cells seem to be located across entire length of fibers. The fact that these cells are excluded from tips is still an important finding.
We do not claim that fusion does not occur at muscle tips. EdU/BrdU experiments tell us that there is cell fusion at muscle tips (PMID: 8589440, PMID: 26777211). In apparent contradiction, we observed that *TMEM8C* and *MYOG* transcripts are preferentially located at the middle versus the tips of fœtal muscles (Figures 2,3). Moreover, there are twice more MYOG+ cells in the middle versus tips of muscles (Figure 4). This regionalisation of fusing cells indicates that the fusion of MYOG+ cells dependent on myomaker (TMEM8C) preferentially occurs in the middle of fœtal muscles. We have better explained this important point in the manuscript.

-The idea that NOTCH directly regulates fusion is very difficult to interpret. They mention this in the text but there are still numerous instances where the authors clearly side with the interpretation that NOTCH directly regulates fusion.
We did not claim that "*NOTCH directly regulates fusion*", we say that our results indicate that NOTCH inhibition allows for fusion to happen by releasing the binding of the HEYL repressor to *TMEM8C* promoter.

-The experiments with DELTA1-DN and DMB (Figure 6) maybe do not strongly support the conclusions. First, there look to be fewer Tom+ cells in the DELTA1-DN sample (E,F compared to A,B). Second, the effect on TMEM8C is quite modest and it is questionable if that level of increase at the mRNA level would have any effect on fusion.
We agree that the DELTA1-DN phenotype is weaker than the DMB phenotype. In contrast to DMB, which is delivered *in ovo* and will target all muscle cells, the DELTA1-DN phenotype relies on the electroporation efficiency. Only localized cells close to electroporated tomato+fibers will be affected by NOTCH loss-of-function, explaining the modest effect. Taken together the DELTA1-DN and the DMB in vivo experiments combined with the *in vitro* myoblast cultures, we believe that NOTCH inhibition has an effect on myoblast fusion.

-The title does not accurately represent the findings. There is only correlational data showing that any potential NOTCH - TMEM8C pathway is regional.
We partially agree with the reviewer and have updated the title.
A direct experiment showing NOTCH activation in TMEM8C cells in vivo would be needed.
We do not see any activation of NOTCH in *TMEM8C*+ cells, we see a downregulation of NOTCH activity in *TMEM8C*+ cells, independently of the differentiation statut.

HEYL is a readout of NOTCH activity. **We have now analyzed the expression of *HEYL* in the myogenic population (New Fig. 5A,B,I,J; New Fig. S6). *HEYL* is mainly associated with PAX7 progenitors at E6 and E10 (New Figure 5A,B). Nevertheless, there is a low proportion of *HEYL*+ cells that express MYOD and/or MYOG at E6 and E10 (New Fig. 5I,J, New Fig. S6D,E). Among this differentiating myoblast population, *HEYL* is significantly expressed in *TMEM8C*neg cells versus *TMEM8C*+ cells at E10 (New Fig. 5I,J, New Fig. S6). This suggests that the downregulation of NOTCH in *TMEM8C*+ cells allows fusion to occur independently of the differentiation state.**

Reviewer 3

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The manuscript of Joana Esteves de Lima et al is a very elegant study concerning the crosstalk between Notch signaling and the fusion process of myogenic cells during embryonic development. Experiments of single cell RNA-seq on embryonic limbs at two developmental stages identify the transcriptomic programs characterizing the different fates of myogenic cells, from their cycling progenitor state to the post-cycling state when cells differentiate and fuse to form multinucleated myofibers. Characterization of the rate of gene expression during the fusion competent state is associated with decreased NOTCH activity. With complementary in vivo and ex vivo culture experiments the authors show that myogenic progenitors may fuse with growing myofibers mainly in the center of the myofibers and not at their extremities, as inferred from the literature. NOTCH activity directly controls the expression of the HeyL transcription inhibitor. The authors demonstrate that HeyL binds the regulatory sequences of Myomaker (TMEM8C) when NOTCH activity is high in cycling progenitors and prevents expression of TMEM8C. Nevertheless the expression of Jagged 2 in the central part of the myofibers in vivo might activate NOTCH signaling in the surrounding myogenic progenitors and inhibit TMEM8C expression, while the authors show higher expression of TMEM8C expression and suggest that fusion is restricted to those central regions. Altogether the experiments presented are of high quality, the manuscript is well written, but improvements must be performed to clarify the apparent contradictory explanations.

Reviewer 3 Comments for the Author:

Major concerns.

1- *It is a regrettable that immunohistochemistry experiments with antibodies against TMEM8C were not performed. If the antibodies exist immunohistochemistry should be performed, it would greatly increase the quality of the characterization of the in vivo fusion processes of growing myofibers with the progenitor cells.*

We could not find any good working antibody to label TMEM8C in chicken tissues.

2- *In figure 2 it is not clear whether TMEM8C is expressed in the myofibers themselves or/and in some myogenic progenitors, zoom of the figures with antibodies/dyes recognizing the cell membranes would help to determine whether this is the case. It is suspected that TMEM8C is expressed in both compartments (the progenitor and the myofiber). Whether the specific fusion of progenitors in the center of the myofiber (exclusion from the tips) is due to a specific expression of TMEM8C in specific myonuclei of the myofiber is unclear. Alternatively progenitors may express TMEM8C only in the center of the myofibers, precluding their fusion at the tips. In situ hybridization does not clearly establish if TMEM8C is detected in both the myofibers and the mononucleated progenitors, and this is crucial to understand the mechanisms.*

To better visualise *TMEM8C* inside and outside muscle fibres at a cellular resolution, we analysed *TMEM8C* location with fluorescent RNA in situ hybridization combined with myosin immunolocalisation. We found a preferential location of *TMEM8C* transcript outside muscle fibers (New Figure S4G-H'). This is consistent with the scRNAseq analysis that tells us that *TMEM8C* transcripts are located in mononucleated myogenic cells (there is no plurinucleated cell in the scRNAseq datasets) (Figure 1). *TMEM8C*+ myoblasts are preferentially located at the center of the muscle

Is TMEM8C expressed in the same area than Jagged2 inside the myofibers?

Because *TMEM8C* is mainly expressed outside myofiber, it is difficult to conclude on the location inside the myofiber. However, we state that *TMEM8C* and *JAG2* transcripts are regionalized in the centre of muscle.

The authors clearly show a crosstalk between Notch and TMEM8C gene expression in myogenic progenitors, but how this crosstalk explains the regionalized fusion process must be better discussed. From the expression pattern of Jagged2, it would be expected that fusion would be increased at the tips of the myofibers where the NOTCH signaling crosstalk between myofibers and associated myogenic progenitors is lower.

It is the inhibition of NOTCH activity, which promotes myoblast fusion. NOTCH is active in central muscles, based on the presence of NOTCH ligand *JAG2* in muscle fibres. Therefore, fusion occurs centrally in fœtal muscles, where the switch from NOTCH ON to NOTCH OFF is regulated and allows for fusion to happen by releasing the binding of the HEYL repressor to *TMEM8C* promoter. See below point 3 for detailed explanation.

3-*The authors must explain why Jagged2 is expressed in the central myofibers/myonuclei where fusion takes place. Its expression should activate Notch signaling in surrounding myogenic progenitors, where HeyL should be high and suppress TMEM8C in those central regions of the myofibers. On the contrary, in this central region TMEM8C seems to be expressed in myogenic progenitors and fusion seems to occur. This is very confusing. The authors show that Notch signaling must be decreased to allow TMEM8C expression in myogenic progenitors, but in vivo Notch activity seems higher in the center of the myofibers where Jagged 2 is expressed.*

We better explained this important point by **the analysis of NOTCH components with respect to *TMEM8C* and Myogenic markers (*PAX7*, *MYOD* and *MYOG*) in the scRNAseq datasets of muscle cells (New Figure S6). This analysis strengthens our conclusion that is the down- regulation of NOTCH signalling in differentiated myogenic cells may be required for fusion to occur.**

Indeed, *JAG2* expression in muscle fibers maintains a muscle PAX7+ progenitor pool by maintaining a NOTCH ON (HEYL+/HES5+) state in these progenitors (eLife 2016, PMID: 27554485). PAX7+ progenitors (known to be in a NOTCH ON state) do not express *TMEM8C* (scRNAseq Figure 1, **FISH New Figure S4**). Committed (MYOD+ and/or MYOG+) myoblasts in a NOTCH OFF state are *TMEM8C*+ (scRNAseq Figure 1, **FISH New Figure S4**). PAX7+ progenitors (NOTCH ON state) and MYOD+/MYOG+ myoblasts (NOTCH OFF state) co-exist in the muscle central regions (see Figure 4, **New Figure S4**). In committed myoblasts, MYOG positively regulates *TMEM8C* expression (PMID: 33001028, PMID: 26540045, PMID: 25085416).

We think that JAG2 signal via NOTCH to maintain the pool of adjacent muscle progenitors and that NOTCH inhibition in MYOD+ and/or MYOG+ cells allows for fusion to happen by releasing the binding of the HEYL repressor to *TMEM8C* promoter. We speculate in the discussion that there could be a competition between HEYL and MYOG to regulate *TMEM8C* expression, competition that could tune fusion events.

Second decision letter

MS ID#: DEVELOP/2021/199928

MS TITLE: TMEM8C-mediated fusion is regionalized and regulated by NOTCH signalling during foetal myogenesis

AUTHORS: Joana Esteves de Lima, Cedrine Blavet, Marie-Ange Bonnin, Estelle Hirsinger, Emmanuelle Havis, Frederic Relaix, and Delphine Duprez

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. However as you will see Reviewer 3 has identified an issue that they feel you might easily address in the Discussion section. If you would kindly respond to this comment and indicate how you have chosen to address this concern in the final version of your manuscript I would be very grateful. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. Your paper will not require any further review, rather I will look it over prior to acceptance.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

The authors examine the role of Notch signalling in myoblast fusion and provide some evidence that a downstream notch effector, HEYL, regulates TMEM8C, a marker for fusion. They also show that TMEM8C-positive fusion competent cells are located in central regions of foetal limb muscles, most likely outside of myofibers. The roles of Notch signalling in muscle are complex and the authors introduce and discuss these appropriately. Some explanation/ interpretation, such as the expression of the notch-ligand, Jagged, in the central muscle mass, remains a bit speculative. However, this is signposted as such.

Comments for the author

The authors have answered my previous queries.

Reviewer 2

Advance summary and potential significance to field

See previous reviews

Comments for the author

I think the manuscript is improved and clearer. I support publication.

Reviewer 3

Advance summary and potential significance to field

The manuscript of Joana Esteves de Lima et al is a very elegant study concerning the crosstalk between Notch signaling and the fusion process of myogenic cells during embryonic development. Experiments of single cell RNA-seq on embryonic limbs at two developmental stages identify the transcriptomic programs characterizing the different fates of myogenic cells, from their cycling progenitor state to the post-cycling state when cells differentiate and fuse to form multinucleated myofibers. Characterization of the rate of gene expression during the fusion competent state is associated with decreased NOTCH activity. With complementary in vivo and ex vivo culture experiments the authors show that myogenic progenitors may fuse with growing myofibers mainly in the center of the myofibers and not at their extremities, as inferred from the literature. NOTCH activity directly controls the expression of the HeyL transcription inhibitor. The authors demonstrate that HeyL binds the regulatory sequences of Myomaker (TMEM8C) when NOTCH activity is high in cycling progenitors and prevents expression of TMEM8C.

Comments for the author

The authors have answered most of my criticisms. However, the absence of TMEM8C mRNA in the myofiber itself (point 2 of my previous review) at the sites where TMEM8C is detected in cells expressing Myogenin suggests a mechanism of new fiber creation by fusion between Myog+TMEM8C+ cells rather than a fusion between these Myog+TMEM8C+ cells and the existing myofiber.
This hypothesis is not mentioned by the authors, but their experiments do not allow to revoke it in the absence of lineage tracing, or follow-up of BrdU+ nuclei in the growing fiber after injection of this agent. The authors should discuss/refute this hypothesis as they do not show at the stages studied on longitudinal sections expression of TMEM8C in the myofiber, and it is known that both fusing cells must express this protein for fusion to occur.
It is therefore conceivable to imagine from the experiments presented that the fusion studied in vivo takes place mainly in the center of the muscle between TMEM8C +Myog+ cells to allow the creation of new muscle fibers and allow muscle growth, and that existing fetal myofibers grow by accretion of new nuclei at their extremities, as discussed.

The authors should also recall in the discussion the rules that concern fusion between cells that must express the Myomaker and Myomixer proteins.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
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Reviewer 1 Comments for the Author:
The authors have answered my previous queries.
We thank the reviewer for the positive decision.

Reviewer 2 Advance Summary and Potential Significance to Field:
See previous reviews

Reviewer 2 Comments for the Author:
I think the manuscript is improved and clearer. I support publication.
We thank the reviewer for the positive decision.

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The manuscript of Joana Esteves de Lima et al is a very elegant study concerning the crosstalk between Notch signaling and the fusion process of myogenic cells during embryonic development. Experiments of single cell RNA-seq on embryonic limbs at two developmental stages identify the transcriptomic programs characterizing the different fates of myogenic cells, from their cycling progenitor state to the post-cycling state when cells differentiate and fuse to form multinucleated myofibers. Characterization of the rate of gene expression during the fusion competent state is associated with decreased NOTCH activity. With complementary in vivo and ex vivo culture experiments the authors show that myogenic progenitors may fuse with growing myofibers mainly in the center of the myofibers and not at their extremities, as inferred from the literature. NOTCH activity directly controls the expression of the HeyL transcription inhibitor. The authors demonstrate that HeyL binds the regulatory sequences of Myomaker (TMEM8C) when NOTCH activity is high in cycling progenitors and prevents expression of TMEM8C.

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The authors should also recall in the discussion the rules that concern fusion between cells that

must express the Myomaker and Myomixer proteins.

We thank the reviewer for this comment and for highlighting this point. Indeed, this hypothesis is valid and we have referred to it in the discussion of the new version of the manuscript. We also stressed out the myomaker requirements for fusion.

Third decision letter

MS ID#: DEVELOP/2021/199928

MS TITLE: TMEM8C-mediated fusion is regionalized and regulated by NOTCH signalling during foetal myogenesis

AUTHORS: Joana Esteves de Lima, Cedrine Blavet, Marie-Ange Bonnin, Estelle Hirsinger, Emmanuelle Havis, Frederic Relaix, and Delphine Duprez
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.