# Loss of U11 small nuclear RNA in the developing mouse limb results in micromelia

Kyle D. Drake, Christopher Lemoine, Gabriela S. Aquino, Anna M. Vaeth and Rahul N. Kanadia
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**Original submission**

First decision letter

MS ID#: DEVELOP/2020/190967

MS TITLE: Minor spliceosome disruption causes limb growth defects without altering patterning

AUTHORS: Kyle D. Drake, Christopher Lemoine, Gabriela S. Aquino, Anna M. Vaeth, and Rahul N. Kanadia

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 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.

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.

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Reviewer 1

*Advance summary and potential significance to field*

The minor spliceosome, responsible for mediating splicing in a small percentage (less than 2%) of genes, is nonetheless important for viability of vertebrate embryos. Hypomorphic defects in this complex result in a syndrome of defects. Interestingly, many of these morphological alterations parallel changes seen in domesticated animals, and previous work by the author's lab has show positive selection in minor spliceosome genes during domestication. One of the features of domesticated animals is shortened limbs. In the current paper, the impact of decreased minor spliceosome activity during limb development is directly tested. Conditional removal of the U11 small RNA (a constituent of the minor spliceosome) from developing limb buds indeed results in smaller limbs with decreased number of skeletal elements. Mechanistically, the authors show that U11-deficient limb progenitors have defects in the cell cycle by virtue of intron-retention in minor spliceosome-dependent cell cycle genes. This results in apoptosis and consequently a smaller limb. The authors also suggest that proper limb patterning is retained through a compensatory up-regulation of critical patterning genes (a conclusion I strongly dispute, see below.) Nonetheless, it is an interesting story, sheds light on the mechanisms underlying congenital changes seen in both human malformation syndromes and animal domestication, and is of potential interest to the readership of Development. The data are also nicely presented and convincing. However, the interpretations do need to be revised in a couple of key respects, and some additional work done, prior to being ready for publication.

*Comments for the author*

This is an interesting story, sheds light on the mechanisms underlying congenital changes seen in both human malformation syndromes and animal domestication, and is of potential interest to the readership of Development. The data are also nicely presented and convincing. However, the interpretations do need to be revised in a couple of key respects, and some additional work done, prior to being ready for publication.

(1) The biggest problem with this paper is the idea that patterning genes are somehow up-regulated in the U11 mutant, and that this is (for some unexplained reason) important for maintaining pattering. Sadly, this interpretation is the core of how the authors frame the developmental aspect of their work. First of all, smaller limb buds require lower levels of key signals (like Shh, Fgf8, etc.) to achieve proper patterning, not higher levels. More importantly, the apparent up-regulation is an artifact of comparing equivalently aged WT vs mutant limb buds. The levels of expression in the mutant are exactly what one would expect of a slightly younger WT limb bud. Moreover, it is very clear from the literature that when you transiently arrest the cell cycle in the limb bud (with colchicine, X-irradiation, etc.), the resulting limb bud is smaller, displays a loss of skeletal elements, and is developmentally delayed, just as observed here. Finally, in addition to the pattern of gene expression reflecting developmental delay, it is also apparent at the level of differentiation as seen in Figure 1, where digit cartilage condensations are present at E13.5 in WT but only at E14.5 in the mutant. The entire discussion of patterning gene up-regulation has to be dropped, developmental delay should be discussed, and also provided with further documentation (ie histology, staining for various markers of differentiation of various cell types, etc.)

(2) Much more minor, there is a statement that there is an absence of AP patter at E13 in the mutant, since there are no digit condensations. Digits are not required for AP pattern. The zeugopod has two different bones, even the single bone of the stylopod (humerus or femur) are themselves AP asymmetric, other tissues such as muscle and tendon reflect AP pattern, etc.

(3) Equally minor, the authors suggest that the cell cycle-based defects they observe are greater distally because the distal limb proliferates at a faster rate. However, the distal cells are also the undifferentiated progenitors. The more proximal cells, being different (more differentiated) cell types might be less reliant on U11 for other reasons, having nothing to do with the speed of their cell division.

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Reviewer 2

*Advance summary and potential significance to field*

This is an interesting paper that describes the phenotypes produced following conditional deletion of a component of the minor spliceosome, RNU4ATAC in the developing limbs.
It has implications on how the size of structures fortmed during embryogenesis is controlled and can be varied across different species. It also has implications for how we understand some forms of dwarfism and diseases caused by disruption of the spliceosome.

*Comments for the author*

I have several questions regarding presentation and interpretation of the data.

Figure 1 I would not agree that presence of two digits 'indicates the presence of an anterior-posterior axis". IN contrast, this indicates that this axis is fundamentally disrupted.
In addition, I would not agree "the P0 mutant forelimb contained three distinct skeletal units". I think none of them are really distinct. Here a molecular (IHC) analysis would be helpful-Some potentially useful data is shown in fig 6 but not commented on in this earlier section relating to Figure 1 data

Figure 4
I have difficulty in understanding how this data is presented and interpreted in light of what is shown in Fig1C, which maps the outline of the mutant buds from E11.5 to E13. Since Fig1C suggest the phenotype is apparent by E11.5 in both forelimb and hindlimb, the distal increase would seem to provide a minor effect at E12.5 . IN the hindlimb, you might expect to see a difference in E12.5 and E13 limb size but actually the size change is quite small. For me this would indicate much less of an impact from cell death and the major impact be from cell cycle length changes.
The statement "Despite these defects basic P-D and complete patterning were observed in P0 mutant forelimb and hindlimb, respectively" is not true in my opinion. This should be removed or significantly modified.

Transcriptomic analysis.
This is quite extensive in the current form of the manuscript but I was not convinced of its value overall.
I take issue with statements such as "the mutant transcriptome was attempting to maintain basic patterning." "..the mutant forelimb suggested that it was transcriptionally responding to cell cycle defects and apoptosis.."
This is imbuing properties on a transcriptome or a limb bud that they could never have. This should be removed.
Little of the transcriptional changes are independently verified and the significance and whether a primary or secondary effect) are not discussed at any length.

Discussion section:
The results are discussed in the context of domestication syndrome and minor spliceosome-related disease.
One alternative interpretation is that the data show that if you disrupt this complex during the establishment and timeframe when 'patterning genes'are having their most profound effects then you do disrupt A-P patterning (the forelimb phenotype). While, as the hindlimb phenotype shows, of you disrupt later when patterning is already well established you get ok patterning. The only consistent feature being cell cycle length effects and hence the earlier deletion has the most profound outgrowth defect.. One might therefore expect the DS and disease effects Perhaps bith on cell cycle length AND apoptosis) to be acting on events at later stages of limb development.

Partly as a result of the above, I did not find discussion of 'developmental bias' helpful.
The only differences describe relate to A-P and P-D. There is not data on D-V so this cannot be discussed.
The A-P defects (that are never really described in any detail) can possibly be explain by a loss of ZPA cells due to increased posterior apoptosis. This would be the most parsimonious explanation in

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light of what is already known about limb patterning genes. I do not feel inclusion of 'developmental bias' useful.

Since at several points the potential mechanism of a 'rheostat for tissue size control" is mentioned, I was surprised that no attempt or not mention is made of producing a heterozygous mutant or some type of hypomorph to test this idea.

Minor
Abstract: I don't think "resulting in" and "consequently" are appropriate. The final sentence could be expanded to explain more fully

Is it really helpful to express length of cell cycle/s=phase to 2 decimal places? I think this suggests a level of resolution that this experimental approach does not provide.

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**First revision**

Author response to reviewers' comments

We would like to thank the reviewers for their insightful comments and suggestions. Based on our reading of the reviewers' comments, we have attempted to perform additional experiments within the guidelines of COVID-19 restrictions. For the most part, the reviewers were positive about the data originally presented. However, they asked that we revise our interpretation of the original data. To that end, we have now made the appropriate changes to accommodate the reviewers' concerns. As you will see, the idea of developmental delay is not fully supported by the results of the additional data interpretation and experimentation aimed to address suggestions made by Reviewer 1. However, we cannot, with certainty, exclude the possibility of developmental delay. Therefore, in the revised discussion section of the manuscript, we describe evidence that supports and disputes developmental delay. We have addressed each comment by the reviewers in detail below (please note that all line numbers are correct under the manuscript version with tracked changes):

Reviewer 1 Comments for the Author:
This is an interesting story, sheds light on the mechanisms underlying congenital changes seen in both human malformation syndromes and animal domestication, and is of potential interest to the readership of Development. The data are also nicely presented and convincing. However, the interpretations do need to be revised in a couple of key respects, and some additional work done, prior to being ready for publication.

(1) The biggest problem with this paper is the idea that patterning genes are somehow up-regulated in the U11 mutant, and that this is (for some unexplained reason) important for maintaining pattering. Sadly, this interpretation is the core of how the authors frame the developmental aspect of their work. First of all, smaller limb buds require lower levels of key signals (like Shh, Fgf8, etc.) to achieve proper patterning, not higher levels. More importantly, the apparent up-regulation is an artifact of comparing equivalently aged WT vs mutant limb buds. The levels of expression in the mutant are exactly what one would expect of a slightly younger WT limb bud. Moreover, it is very clear from the literature that when you transiently arrest the cell cycle in the limb bud (with colchicine, X-irradiation, etc.), the resulting limb bud is smaller, displays a loss of skeletal elements, and is developmentally delayed, just as observed here. Finally, in addition to the pattern of gene expression reflecting developmental delay, it is also apparent at the level of differentiation as seen in Figure 1, where digit cartilage condensations are present at E13.5 in WT but only at E14.5 in the mutant. The entire discussion of patterning gene up-regulation has to be dropped, developmental delay should be discussed, and also provided with further documentation (ie histology, staining for various markers of differentiation of various cell types, etc.)

Response:
Here we understand the reviewer's contention to be that our transcriptome analysis showing

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upregulation of genes, such as *Shh* and *Fgf8*, in the E11.5 mutant forelimb bud is not the reason why the patterning is maintained. We agree that upregulation of these genes does not necessarily mean that they are actively maintaining limb patterning. However, the RNAseq analysis is evidence that these genes are indeed upregulated in the E11.5 mutant limb bud and morphological analysis shows that limb patterning in the mutant is also maintained. We had previously argued that perhaps these were linked by stating that this upregulation allowed for patterning to be maintained. In considering the reviewer's comments, we have now removed the idea that the upregulation of patterning genes allows patterning to be achieved (lines 60-63) and instead we state that, although the expression of these genes is changed, their spatial domains are not.

The reviewer states, "More importantly, the apparent up-regulation is an artifact of comparing equivalently aged WT vs mutant limb buds. The levels of expression in the mutant are exactly what one would expect of a slightly younger WT limb bud." In lines 236-257 we address this issue and found that it was only partially true. We show that one group of genes, representing about 1/3 of the upregulated genes in the E11.5 mutant forelimb, have expression levels highly similar to the E10.5 WT forelimb. However, a separate set of genes, representing about 2/3 of the upregulated genes in the E11.5 mutant forelimb, are aberrantly upregulated and are highly dissimilar to the E10.5 WT or mutant. Therefore, only the first set of genes is under developmental delay, whereas the majority of upregulated genes are aberrantly upregulated.

Furthermore, the reviewer states that developmental delay previously published in colchicine and x- irradiation experiments are in line with our findings. Therefore, developmental delay underlies the phenotype we observe in the U11-null forelimb. In lines 94-97 we address the issue of digit emergence in the mutant forelimb at E14.5 but not E13.5. Through H&E, we found that chondrogenesis was appropriately occurring at E12.5 and therefore the lack of obvious digits at E13.5 through SEM is not indicative of their absence. As such, in lines 396-407 we discuss evidence supporting and disputing the idea that the U11- null forelimb is under developmental delay.

(2) Much more minor, there is a statement that there is an absence of AP patter at E13 in the mutant, since there are no digit condensations. Digits are not required for AP pattern. The zeugopod has two different bones, even the single bone of the stylopod (humerus or femur) are themselves AP asymmetric, other tissues such as muscle and tendon reflect AP pattern, etc.

Response:
The reviewer is correct that absence of digits does not inform or dispute the presence of an AP axis. Therefore, we have removed any commentary on whether there is an AP axis in the mutant forelimb. These changes can be found in lines 90-92.

(3) Equally minor, the authors suggest that the cell cycle-based defects they observe are greater distally because the distal limb proliferates at a faster rate. However, the distal cells are also the undifferentiated progenitors. The more proximal cells, being different (more differentiated) cell types might be less reliant on U11 for other reasons, having nothing to do with the speed of their cell division.

Response:
The reviewer is correct that other features, besides cell cycle speed, may be the underlying cause for differential susceptibility for distal limb progenitor cells to the consequences of U11 loss. We have included commentary about this idea in lines 384-387.

Reviewer 2 Comments for the Author:

I have several questions regarding presentation and interpretation of the data.

Figure 1

- I would not agree that presence of two digits 'indicates the presence of an anterior-posterior axis". IN contrast, this indicates that this axis is fundamentally disrupted. In addition, I would not agree "the P0 mutant forelimb contained three distinct skeletal units". I think none of them are really distinct. Here a molecular (IHC) analysis would be helpful-Some potentially useful data is shown in fig 6 but not commented on in this earlier section relating to Figure 1 data

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Response:
Although we believe that two digits does indicate the presence (albeit disrupted, as the reviewer correctly points out) of an AP axis, we have removed any description of this feature in regard to the U11-null forelimb. These changes can be found in lines 90-92. Moreover, we maintain our stance that the U11-null forelimb does, indeed, contain three distinct skeletal units along the proximo-distal axis (as can be observed initially in Fig. 1G). However, we have changed our language to state that the forelimb is "appropriately segmented" which can be found in lines 102-103 and 336. We performed WISH for *Meis1* to show its expression domain in the mutant forelimb is comparable to the WT at E11.5 (lines 305 and 309; Fig. 6A,B). With this, we show that *Meis1*, *Hoxa11*, and *Hoxa13* have appropriate signaling domains, and therefore state that the skeletal units observed at P0 (Fig. 1G) are distinct (lines 335-339). Furthermore, what we describe here as segmentation is in line with previous publications (example: Harfe et al., PNAS, 2005).

Figure 4
- I have difficulty in understanding how this data is presented and interpreted in light of what is shown in Fig1C, which maps the outline of the mutant buds from E11.5 to E13. Since Fig1C suggest the phenotype is apparent by E11.5 in both forelimb and hindlimb, the distal increase would seem to provide a minor effect at E12.5. IN the hindlimb, you might expect to see a difference in E12.5 and E13 limb size but actually the size change is quite small. For me this would indicate much less of an impact from cell death and the major impact be from cell cycle length changes. The statement "Despite these defects basic P-D and complete patterning were observed in P0 mutant forelimb and hindlimb, respectively" is not true in my opinion. This should be removed or significantly modified.

Response:
The reviewer is correct in stating that the primary cause for limb size reduction- cell cycle defects or cell death- cannot be determined. We have gone through our manuscript and confirmed that we never make claim as to whether one of these is more substantially leading to defects in limb outgrowth. Moreover, we recognize the reviewer's concern with the statement "Despite these defects, basic proximo-distal and complete patterning were observed in the P0 mutant forelimb and hindlimb, respectively" and have removed it entirely (lines 191-193).

Transcriptomic analysis.
- This is quite extensive in the current form of the manuscript but I was not convinced of its value overall.
I take issue with statements such as "the mutant transcriptome was attempting to maintain basic patterning." "..the mutant forelimb suggested that it was transcriptionally responding to cell cycle defects and apoptosis.." This is imbuing properties on a transcriptome or a limb bud that they could ever have. This should be removed. Little of the transcriptional changes are independently verified and the significance and whether a primary or secondary effect) are not discussed at any length.

Response:
We recognize that the transcriptomic analysis was lengthy in the previous version. Therefore, we have consolidated the information presented and believe it communicates our findings more succinctly. These changes can be found in lines 217-286. In addition, we have removed all anthropomorphic language. Moreover, as a proof of principle, we validated the upregulation for four genes in the E11.5 mutant forelimb through qRT-PCR (lines 208-209; Fig. 5B). In response to the reviewer's comment regarding primary/secondary effects, we would like to share that we have purposefully made no mention of primary or secondary transcriptomic effects as we believe this is not possible given the large number of differentially expressed genes.
Discussion section:
- The results are discussed in the context of domestication syndrome and minor spliceosome-related disease.
One alternative interpretation is that the data show that if you disrupt this complex during the establishment and timeframe when 'patterning genes'are having their most profound effects then you do disrupt A-P patterning (the forelimb phenotype). While, as the hindlimb phenotype shows, of you disrupt later when patterning is already well established you get ok patterning. The only consistent feature being cell cycle length effects and hence the earlier deletion has the most profound outgrowth defect.. One might therefore expect the DS and disease effects Perhaps bith on cell cycle length AND apoptosis) to be acting on events at later stages of limb development.

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Response:
We agree with the reviewer and have included this discussion point in lines 344-349. However, in case of disease and domestication, the genetic changes are inherited and therefore would be acting at the onset and throughout the entirety of limb development. Therefore, we do not present this idea in discussion.

Partly as a result of the above, I did not find discussion of 'developmental bias' helpful. The only differences describe relate to A-P and P-D. There is not data on D-V so this cannot be discussed. The A-P defects (that are never really described in any detail) can possibly be explain by a loss of ZPA cells due to increased posterior apoptosis. This would be the most parsimonious explanation in light of what is already known about limb patterning genes. I do not feel inclusion of 'developmental bias' useful.

Response:
We have removed all commentary on developmental bias (see lines 408-418). The reviewer poses a possible argument that loss of AP bifurcation in the U11-null forelimb is due to loss of ZPA cells. However, we show that *Shh* is upregulated at E11.5 (Fig. 5D) and has a comparable expression domain through WISH (Fig. 6A,B) and therefore depletion of ZPA cells is most likely not occurring in the E11.5 mutant forelimb.

Since at several points the potential mechanism of a 'rheostat for tissue size control" is mentioned, I was surprised that no attempt or not mention is made of producing a heterozygous mutant or some type of hypomorph to test this idea.

Response:
The reviewer is correct to bring up this point. We have made mention that heterozygotes (*Rnu11*$^{WT/Flx}$ ::*Prrx1*-Cre$^{+}$) mice are aphenotypic (lines 536-537). Although not discussed in the manuscript, this is most likely due to the fact that U11 is produced five-fold higher than its binding partner U12 (Tarn, Yario, and Steitz, RNA, 1995), and therefore 50% loss is most likely insufficient to cause minor splicing defects.

Minor
Abstract: I don't think "resulting in" and "consequently" are appropriate. The final sentence could be expanded to explain more fully. Is it really helpful to express length of cell cycle/s=phase to 2 decimal places? I think this suggests a level of resolution that this experimental approach does not provide.

Response:
The word choice in the abstract has been adjusted and the last line has been expanded (lines 14-16). However, in our efforts to accommodate the reviewers comment, the abstract has exceeded the 150 word limit, which we will amend if the editor requires. The cell cycle speed values have been changed to one decimal place (lines 173-174).

In all, we believe we have addressed the reviewers' concerns and are confident that you will agree that the manuscript is suitable for publication.

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Second decision letter

MS ID#: DEVELOP/2020/190967

MS TITLE: Minor spliceosome disruption causes limb growth defects without altering patterning

AUTHORS: Kyle D. Drake, Christopher Lemoine, Gabriela S. Aquino, Anna M. Vaeth, and Rahul N. Kanadia

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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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. However as you will see Reviewer 2 still has some outstanding concerns that I think are entirely valid, especially with regard to the title and conclusion of the paper that indicates that patterning of the mutant limbs are not affected - this does not appear to be the case. They also express concerns about the degree to which the transcriptome analysis sheds mechanistic insights into the phenotype. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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 have adequately addressed my concerns. I am happy to support publication of this revised manuscript.

*Comments for the author*

The authors have adequately addressed my concerns. I am happy to support publication of this revised manuscript.

Reviewer 2

*Advance summary and potential significance to field*

as stated previously

*Comments for the author*

The authors have made extensive text changes throughout.

The central message (and title) remain the same however. Taken on face value the title "…causes limb outgrowth defects without altering patterning' (and in the Introduction "..maintain patterning") would suggest the skeletal phenotype to be a limb that is largely normal in regards to the number of skeletal elements that form but that all elements are reduced in size (ie. A dwarfism-like phenotype, consistent with the clinical links that are made)
This is not, however, the skeletal phenotype reported. Fig 1 shows profound patterning defects and not a reduction defect that can be explained as a simple growth defect. Patterning is clearly NOT maintained in the forelimb to a large extent.

The different temporal deletion in the FL vs HL illustrate a clear temporal component to the penetrance of defect-The significant difference in phenotype between FL and HL could be attributed to the early vs later deletion of U11 indicating that deletion of U11 at early limb bud stages has a more profound impact on limb patterning.
SO, despite the lack of dramatic changes seen in the domains of expression in the markers chosen (Fig 6) it would appear that there are effects at play from KO of U11 at stages E10 onwards that disrupt later skeletal pattern.

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I therefore still have concerns regarding the interpretation of the phenotype. The transcriptome analysis does not provide a significant level of clarity to the origins of the limb defects.

More Minor
The Meis 1 staining mentioned in the authors response is really not visible in either control or mutant limbs

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**Second revision**

Author response to reviewers' comments

I would like to thank reviewer 2 for their additional comments on our revised manuscript. We have now changed the title to address the reviewer's concern to: Loss of U11 small nuclear RNA in the developing mouse limb results in micromelia. We specifically use the word micromelia as it encompasses the phenotype for both the U11-null forelimb and hindlimb. In addition, in line with the suggestion of reviewer 2 (and your agreement), we have also removed the idea that the U11-null forelimb is patterned. Instead, we merely describe the phenotype as is, i.e. proximo-distally segmented. Furthermore, since the removal of our temporal transcriptome analysis (previous figure 5) did not fundamentally alter the claims made in the manuscript, we have decided to take out this analysis as per the reviewer's suggestion. I hope that these changes will be sufficient to warrant publication of the manuscript in its current form.

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Third decision letter

MS ID#: DEVELOP/2020/190967

MS TITLE: Loss of U11 small nuclear RNA in the developing mouse limb results in micromelia

AUTHORS: Kyle D Drake, Christopher Lemoine, Gabriela S Aquino, Anna M Vaeth, and Rahul N Kanadia
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.

Reviewer 1

*Advance summary and potential significance to field*

Loss of U11 small nuclear RNA in the developing mouse limb results in micromelia

*Comments for the author*

I was satisfied with the revisions made in the previous submission of this manuscript. The current round of changes were in response to additional comments from the other referee. I have nothing to add.

Reviewer 2

*Advance summary and potential significance to field*

As stated previously

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*Comments for the author*

I think the authors have made positive changes to the manuscript

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