Srsf3 mediates alternative RNA splicing downstream of PDGFRα signaling in the facial mesenchyme

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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 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 (see also Editor's note), 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.

Editor's note:
Points for clarification: (i) mechanistic attribute of the differential splicing of Srsf3 dependent genes in relation to the manifestation of mutant phenotype, and timing of sampling of tissues for transcriptome analysis, (ii) discrepancy of the results between analytics approaches of splicing events, and (iii) further evidence of EMT process has been disrupted.

Reviewer 1

Advance summary and potential significance to field

Alternative splicing and elaboration of cell type specific isoforms are important for morphogenesis. Detailed analysis of specific isoforms is an understudied area. This work contributes analysis of specific isoforms of key pathway (Pdgf) in craniofacial development.

Comments for the author

This study builds on the finding from previous phosphoproteomic screen and focus on one specific candidate Srsf3. The authors first investigated the differentially alternative-ly spliced genes in palatal shelves mesenchyme RNA between PdgfraPI3K/PI3K and control, and found that Srsf3 motifs were enriched in the significant exon skipped genes. Among these genes, 22 are associated to craniofacial malformation and 8 are associated to cleft secondary palate. They further showed that the expression of Srsf3 is enriched in the facial processes during development and the phosphorylation of Srsf3 in response to Pdgf ligand led to its nuclear translocation. The authors then tried to examine the func-tion of Srsf3 in neural crest cells using conditionally disruption in Wnt-1 lineage and found that severe facial cleft resulted, which could be due to delayed EMT. They then follow up by doing RNA-seq on E11.5 MxP mesenchyme RNA between conditional KO vs control and found out that alternative spliced genes are enriched in protein ser-ine/threonie kinase. Specifically, the authors found increased phosphorylation of Vasp (substrate of Prkd2, a serine/threonie kinase) in the conditional Srsf3 KO, thereby con-cluding that Srsf3 negatively regulate Pdgfra signaling through alternative splicing of protein serine/threonie kinase.

1. Major comments

i. L130 -131, “confirming the trend” observed in the rMATS analysis”. However, in Fig.1C, the PSI of Chrd and Smad7 between wild-type and mutants is very simi-lar. Reporting the value, 95% Confidence interval and the p-value would be rec-ommended to make a clearer claim to reader. Moreover, the PSI differences for these 2 genes are less than 5%, can the authors clarify why these 2 genes would be picked up by rMATS in the differentially alternative spliced events?

ii. According to Vandernbroucke et al (Nucleic Acids Research, 2001), the method of using RT-qPCR to detect exon skipping would usually take the approaches of “detection by a boundary spanning probe” and “detection by a boundary span-ning primer.” But the authors are using a different way of using “exon spanning probe.” Can the authors clarify the benefits of using this approach?

iii. L133-134. There is significant discrepancy between the analysis results between rMATS and and DESeq2 in terms of differentially alternatively spliced genes in both E13.5 PS RNA and cKO E11.5 MxP mesenchyme RNA. Could the authors ad-dress this difference?

iv. Figure 3B. For the 2nd row, can the authors also present the nuclear fraction of WB Srsf3? L166-167, the claim “increased band intensities over baseline levels in response to PDGF-AA ligand treatment” does not really match what the data shows. Can the authors provide a full image of the WB experiment? Besides, cy-toplasmic fraction with addition of both PDGF-AA and LY294002 has a much dimmer ß-globulin band (control) but the darkest band in Srsf3. Does that mean addition of both chemicals would lead to further increase in phosphorylation of Srsf3?

v. L336, Melk doesn’t seem to be differentially alternative spliced in Fig.7C, but again it is picked up by rMATS. This trend does not seem to be confirmed. Can the authors explain this discrepancy?

vi. L361. Can the authors clarify whether the increased phosphorylation of Vasp is due to more functional Prkd2 isoform in cKO?

2. Minor comments
No minor comments.

Reviewer 2

Advance summary and potential significance to field

In this manuscript Dennison et al reports that PI3K/AKT signalling downstream of PDGFRα results in phosphorylation of the splicing factor SRSF3. This increased phosphorylation is associated with enrichment of the splicing factor in nuclear extract, suggesting that PI3K/AKT regulates translocation of SRSF3 into the nucleus. The authors performed RNAseq analysis and reported a small number of differentially expressed genes in palate of E13.5 PdgfrαPI3k/PI3k mutant embryos, but a significant number of transcripts that were differentially spliced. They further showed that regions flanking skipped exons were enriched for SRSF3 binding sites. These observations lead the authors to examine expression and requirement for Srsf3 in the developing face. The authors showed enriched expression of Srsf3 mRNA and protein in the developing head. They also showed that craniofacial development was severely disrupted when Srsf3 was mutated in neural crest cells and that these abnormalities were associated with a large number of differentially expressed genes and abnormal splicing. Differentially expressed genes were enriched in terms associated with protein kinase activity in Gene Ontology. In addition, Prkd2 and its target VASP - previously identified target of PDGFRα signalling - was differentially spliced and have increased phosphorylation, respectively in Srsf3 neural crest cell mutant embryos.

This work make novel contributions to the field. The authors have identified an alternative splicing program that is regulated by PDGFR, and a role for Srsf3 as a downstream effector of PDGFRα signalling. It is an interesting paper, with major strengths including the careful and detailed characterization of the Srsf3 mouse models and the extensive RNAseq analysis.

Comments for the author

Some of the data included in the manuscript does not fully support the authors conclusion. For example, the authors concluded that pre-incubation with LY294002 resulted in reduced phosphorylation of SRSF3 (Fig 3B). However, this is not clear in the blot. In figure 2 it appears as if SRSF3 is exclusively nuclear, is there in vivo evidence that this protein needs to be translocated to function? What is the evidence that this antibody is specific to SRSF3? No controls were provided and the authors did not cite any previous paper indicating that this antibody was validated.

Along the same line, the evidence for delayed cranial neural crest cell epithelial to mesenchymal transition in cKO embryos was also insufficient. SOX10 is a transcription factor that is normally localized in the nucleus. The immunofluorescence image provided in Figure 5C and 5D shows extensive cytoplasmic signal. This data was not very convincing, maybe separating the fluorescent and DAPI signal would allow for the nuclear signal to be made obvious? In addition, the sections do not appear to be in the same plane.

Finally, additional EMT markers would also need to be analyzed to support the conclusion of a delay in EMT.

The authors should discuss why they found increased proliferation and apoptosis? How do those two things contribute to the phenotype? The authors cannot rule out that an earlier increase in apoptosis contribute to the reduced number of neural crest cells seen at E9.5?

The authors examined splicing of Fgfr2 but do not explain why they selected this particular transcript. They also did not explain why E11.5 was chosen for the RNAseq analysis since the mutant phenotype was severe by this stage of development. The authors also did not show any of

the gels for their splicing analysis. This is an important piece of data that must be included alongside the quantification, especially as they did not indicate which exons were spliced in the transcripts picked for validation.

The data regarding alternative splicing of Prkd2 and phosphorylation of VASP needs to be improved. The alternative splicing event found in the Vasp transcript in PdgfrαPI3k/PI3k mutant needs to be shown and validated. For example, which exon was spliced, was this an exon skipping event? was it significant? Is VASP phosphorylation reduced in PdgfrαPI3k/PI3k mutants?
Finally, for this reviewer, there were several key tables referred to in the text that were not included, in the manuscript. Especially Table S2 and S8, which had important data for understanding the work.

Some minor comments:
Mating described in Table S5 is mislabelled, Srsf3 heterozygous flox embryos would not be observed if homozygous mutant flox mice were mated to each other.

Reviewer 3

Advance summary and potential significance to field

Craniofacial abnormalities are among the most common of all birth defects. Neural crest (NC) contribution to the craniofacial tissues is well demonstrated. Compared to signaling pathways and transcription factors, functions of RNA binding proteins including splicing factors are now extensively studied in Neural crest cells (NCCs). In this manuscript, the authors attempted to demonstrate that NCCs specific AS events are regulated by PDGFRa-PI3K/Akt-Srsf3 axis. This is an interesting manuscript but there are several severe issues related to the experimental plans and conclusions made based on the data presented.

Comments for the author

Craniofacial abnormalities are among the most common of all birth defects. Neural crest (NC) contribution to the craniofacial tissues is well demonstrated. Compared to signaling pathways and transcription factors, functions of RNA binding proteins including splicing factors are now extensively studied in Neural crest cells (NCCs). In this manuscript, the authors attempted to demonstrate that NCCs specific AS events are regulated by PDGFRa-PI3K/Akt-Srsf3 axis. This is an interesting manuscript but there are several severe issues related to the experimental plans and conclusions made based on the data presented. Overall, the conclusions are not fully supported by the data presented. Some of the major issues are described below.

1. In this work, authors claim that they have demonstrated that PI3K/Akt-mediated PDGFRa signaling regulates the expression of genes involved in palatal shelf morphogenesis through AS, in part through the phosphorylation and subsequent nuclear translocation of Srsf3. This conclusion is not supported by the data presented here. For example, RNAseq analysis on PS mesenchyme showed 523 significant AS events between Pdgfra+/+ versus PdgfraPI3K/PI3K embryos. However, only 13 genes were differentially expressed. None of the differentially expressed genes were alternatively spliced. This does not make sense when authors are suggesting that PDGFRa signaling-dependent AS is mediated through Srsf3. The authors should have compared PDGFRa signaling-dependent and Srsf3-dependent AS to identify a common target to support their conclusions.

2. Similarly, the conclusion derived from Figure 1 “Collectively, these findings demonstrate that AS is an important mechanism of gene expression regulation downstream of PI3K/Akt-mediated PDGFRa signaling in the mid-gestation PS” is not supported when there are no common genes that are differentially spliced and differentially expressed.

3. In Figure 1C, any of the changes observed in terms of percent spliced in between both genotypes are significant? Which exons are being analyzed here? Better to show gel images to compare which isoform is affected? If altered significantly, how do they affect the phenotype in PdgfraPI3K/PI3K embryos? As AS can also affect protein stability, modification, and functions, it is important to check their protein expression levels?

4. In Figure 2, as the focus of the study is Neural crest-derived craniofacial structures, it is important to show Srsf3 expression in Neural crest cells using an established marker by double immunostaining.

5. In Figure 3B, WB for Srsf3 in the first two panels are not convincing. Why all nuclear fractions are not analyzed similarly to cytoplasmic fractions?
6. In Figure 5C-D, the Authors conclude that Srsf3fl/fl;Wnt1-Cre+/Tg cKO littermates had very few Sox10-positive NCCs in the mesenchyme of the cranial neural folds, with extensive Sox10 staining in the overlying ectoderm. This is not clear from the images presented. Looks like more Sox10 positive cells in the Srsf3 KO. Similar sections need to be analyzed at this stage as there is no obvious morphological differences observed. Quantification needed.

7. In Figure 5 (A-B and E-H), as authors are using mTmG reporter, it's better to show Merged images with both RFP and GFP for all the panels so the migration of GFP cells can be analyzed in the context of RFP background in the same embryo.

8. In Figure 5 (I-N), it is not clear what cells are being analyzed for proliferation and apoptosis? Lineage tracing analysis suggests that there are no GFP+ neural crest-derived cells in the MxP and MdP of the Srsf3 KO, then what cells are being quantified in the KO? Authors should perform GFP and Ki67 double immunostaining to show proliferation defects in the neural crest-derived craniofacial structures. Better to analyze both at E9.5 and E10.5 timepoint. Similarly, apoptosis should be analyzed in with double immunostaining for GFP and apoptotic markers (TUNEL or caspases, etc.). TUNEL quantification shown that apoptosis is not affected in MxP and increased in MdP. This increase is driven by one sample in the KO. Include more samples to make the changes robust.

9. It is not clear how robust increase in cell proliferation and a modest increase in apoptosis will lead to this phenotype where most of the craniofacial structures are severely hypoplastic?

10. Defective EMT claimed by authors should be analyzed properly. EMT markers such as E-cadherin/N-cadherin or others need to be analyzed by immunostaining/in situ hybridization and qPCR.

11. To determine the alternatively spliced transcript regulated by Srsf3, authors harvested and sequenced MxP mesenchyme RNA from three biological replicates of E11.5 control versus cKO embryos. It is not clear what cells are being analyzed in this experiment. Lineage tracing data presented in Figure 5H-H' shows that even at an earlier time point (E10.5) there are no NC-derived GFP+ cells in the MxP so what is being analyzed here? Most of the transcriptional changes observed from RNAseq data are likely to secondary and not primary. As NC-contribution is relatively preserved in the MxP and MdP of Srsf3KO at E9.5 (Figure 5E-F'), authors could have sorted GFP+ cells from E9.5 control and Srsf3KO heads (or dissected just MxP and MdP) to determine the primary changes.

12. No molecular mechanism downstream of Srsf is provided to explain the molecular changes associated with the craniofacial phenotype. Due to the point made above the results presented in Figures 6 and 7 are due to secondary change in the hypoplastic MxP and not primary changes due to Srsf deletion in NCCs. In addition, Volcano plot analysis using RNAseq data from MxP mesenchyme shows downregulation of Zic5 and Pou3f4 (Figure 6A), however qRT-PCR analysis on MxP mesenchyme shows upregulation of these genes (Figure 6C). These two independent experiments show opposite results. Am I missing something here?

First revision

Author response to reviewers' comments

Response to Reviewers

Reviewer #1

1. L130 -131, "confirming the trend" observed in the rMATS analysis". However, in Fig.1C, the PSI of Chrd and Smad7 between wild-type and mutants is very similar. Reporting the value, 95% Confidence interval and the p-value would be recommended to make a clearer claim to reader. Moreover, the PSI differences for these 2 genes are less than 5%, can the authors clarify why these 2 genes would be picked up by rMATS in the differentially alternative spliced events?

For Figure 1C, we have added the ΔPSI values (with p-values) for the differentially alternatively-spliced transcripts as assessed by both rMATS analysis of RNA-seq data and qPCR analysis. We have additionally included the 95% confidence interval from the qPCR analysis. Further, we have added representative qPCR gels for each transcript. These additions demonstrate that *Chrd*, *Cask* and *Smad7* have ΔPSI values as assessed by rMATS analysis of 14.3, 11.3 and 9.5, respectively, representing a greater than 5% change in the alternative splicing of each event between genotypes. Though the ΔPSI values as assessed by qPCR analysis for *Chrd* and *Smad7* are less than 5%, the trends (i.e. positive ΔPSI values) are consistent between rMATS and qPCR analyses. We thank the Reviewer for this suggestion, as we agree that these additions present the rMATS and qPCR results more clearly to the reader.

2. According to Vandernbroucke et al (Nucleic Acids Research, 2001), the method of using RT-qPCR to detect exon skipping would usually take the approaches of "detection by a boundary spanning probe" and "detection by a boundary spanning primer." But the authors are using a different way of using "exon spanning probe." Can the authors clarify the benefits of using this approach?

In Vandenbroucke *et al.*, 2001, the authors compared three methods – detection by a boundary-spanning probe, detection by a boundary-spanning primer and quantification by subtraction - and ultimately endorsed use of a boundary-spanning primer. This approach requires two primer pairs that include one common primer and two additional primers, each specific to a particular splice variant. As discussed in Londoño and Philipp, "A reliable method for quantification of splice variants using RT-qPCR" (*BMC Molecular Biology,* 2016), drawbacks of this approach include "false positives and reduced fidelity because of the high degree of similarity between the exon-exon junction sequences of differentially spliced mRNAs". Londoño and Philipp demonstrate that the use of primers that flank the splice site and anneal to both transcript variants (as employed in our manuscript) allows for simultaneous, accurate detection of both splice isoforms. Further, as this method uses two common primers, it eliminates misleading results due to differing amplification efficiencies of primers that recognize only one of two splice variants. The method used in our manuscript was also employed to detect differential alternative splicing events between control and mutant mouse tissue in the first paper to demonstrate a role for an RBP during craniofacial development, Bebee *et al.* (*eLife*, 2015), the authors of which include an expert in alternative RNA splicing during mouse development (Dr.Russ Carstens) and the developer of the rMATS model and program (Dr. Yi Xing). To avoid confusion, we have changed all instances of the phrase "by qPCR using primers spanning constitutively-expressed exons flanking the alternatively-spliced exon" to "by qPCR using primers within constitutively-expressed exons flanking the alternatively-spliced exon" (lines 133- 134, 356-357).

3. L133-134. There is significant discrepancy between the analysis results between rMATS and DESeq2 in terms of differentially alternatively spliced genes in both E13.5 PS RNA and cKO E11.5 MxP mesenchyme RNA. Could the authors address this difference?

Multiple studies have demonstrated that networks of tissue-specific transcripts which are differentially alternatively spliced do not have significant overlap with genes that are differentially expressed in the same tissue. For example, in their analysis of the RBPs Esrp1 and Esrp2 in the murine embryonic epidermis, Bebee *et al.*, (*eLife*, 2015) found that only 12 genes (representing 5.7% of all skipped exon rMATS events) were detected in both their differential splicing and differential gene expression analyses. The findings in that paper are similar to the low percentage of genes that were detected in this manuscript in both rMATS (skipped exon) and DESeq2 analyses in the *Pdgfra*$^{PI3K/PI3K}$ (0%) and *Srsf3* cKO (2.7%) RNA-seq experiments.

We have significantly revised our analysis of these findings in the Discussion as follows: "…there was little to no overlap between the genes detected in the rMATS and DESeq2 analyses within

either RNA-seq experiment, similar to previous analyses of differential AS and differential gene expression upon ablation of the RBPs Esrp1 and Esrp2 in the embryonic murine epidermis (Bebee et al., 2015). The majority of genes identified in the control versus *Srsf3* cKO MxP mesenchyme DESeq2 analysis encode transcripts that are likely regulated by Srsf3 through mechanisms other than AS, such as transcription, export, translation and degradation (Howard and Sanford, 2015). Notably, however, the rMATS and DESeq2 results from the control versus *Srsf3* cKO MxP mesenchyme RNA-seq analysis were commonly enriched for genes involved in focal adhesion, PI3K/Akt signaling and MAPK/Erk signaling, similar to results from a xenograft glioblastoma model in the presence or absence of SRSF3 (Fuentes-Fayos et al., 2020). These findings indicate that Srsf3 activity may influence each of these processes through multiple mechanisms of gene expression regulation. Relatedly, we found that phospho-Erk1/2 levels were significantly reduced in *Srsf3* cKO embryos [see Fig. 7E]. As the MAPK/Erk pathway has demonstrated roles in murine facial midline development and has been shown to function downstream from PDGFRα activation in this setting (Parada et al., 2015; Vasudevan et al., 2015), we propose that dysregulation of this signaling axis in *Pdgfra* and *Srsf3* mutant mouse models may underlie, at least in part, the common facial clefting phenotypes" (lines 478-496).

4. Figure 3B. For the 2nd row, can the authors also present the nuclear fraction of WB Srsf3? L166-167, the claim "increased band intensities over baseline levels in response to PDGF-AA ligand treatment" does not really match what the data shows. Can the authors provide a full image of the WB experiment? Besides, cytoplasmic fraction with addition of both PDGF-AA and LY294002 has a much dimmer ß-globulin band (control) but the darkest band in Srsf3. Does that mean addition of both chemicals would lead to further increase in phosphorylation of Srsf3?

We have repeated the experiments in Figure 3B and have included two panels of the same blot (immunoprecipitation with the anti-Akt phosphosubstrate antibody followed by western blotting with the anti-Srsf3 antibody) imaged at different exposures to reveal the expression of phospho-Srsf3 in the cytoplasmic (top) and nuclear (bottom) fractions. We have also quantified the relative phospho-Srsf3 levels in both the nucleus and cytoplasm in untreated, PDGF-AA-treated, and PDGF-AA- and LY294002-treated cells across four experiments and represented this data in a scatter dot plot in a new panel, Figure 3C. Here, relative phospho- Srsf3 levels were determined by normalizing to Lamin B1 and ß-tubulin for the nuclear and cytoplasmic fractions, respectively. In the representative experiment shown, the ß-tubulin levels are essentially equal across the three cytoplasmic fraction samples. We have revised the following sentences in the Results section to summarize these findings: "This analysis revealed increased band intensities over baseline levels in response to PDGF-AA ligand treatment in the cytoplasmic fractions (1.851 ± 0.3222-fold induction over unstimulated levels), indicative of increased phospho-Srsf3 levels, and a return to band intensities near baseline levels upon treatment with LY294002 (1.136 ± 0.2930-fold induction over unstimulated levels) (Fig. 3B,C)" (lines 181-185). We thank the Reviewer for this suggestion, as we believe that it enhances our biochemical findings.

5. L336, Melk doesn't seem to be differentially alternative spliced in Fig.7C, but again it is picked up by rMATS. This trend does not seem to be confirmed. Can the authors explain this discrepancy?

We have removed the analysis of *Melk* alternative splicing as assessed by qPCR from Figure 7C, as we do not feel that it adds substantially to our conclusions. For Figure 7C, we have added the ΔPSI values (with p-values) for the differentially alternatively-spliced transcripts as assessed by both rMATS analysis of RNA-seq data and qPCR analysis. We have additionally included the 95% confidence interval from the qPCR analysis. Further, we have added representative qPCR gels for each transcript. These additions demonstrate that *Limk2*, *Dmpk* and *Prkd2* have ΔPSI values as assessed by rMATS analysis of -11.5, -26.7 and -15.8, respectively, representing a greater than 5% change in the alternative splicing of each event between genotypes. Though the ΔPSI value as assessed by qPCR analysis for *Prkd2* is less than 5%, the trend (i.e. negative ΔPSI values) is consistent between rMATS and qPCR analyses. Importantly, the ΔPSI values for *Prkd2* as assessed by both rMATS and qPCR analyses are significant, rendering Prkd2 a suitable candidate for our subsequent follow-up studies with its phosphorylation target Vasp.

6. L361. Can the authors clarify whether the increased phosphorylation of Vasp is due to more functional Prkd2 isoform in cKO?

We have added the following sentences to the Results section to clarify that increased phosphorylation of Vasp is due to more Prkd2 protein containing the protein kinase domain in cKO samples: “SPLINTER predicted that AS of *Prkd2* would result in a truncated protein in control embryos affecting amino acids that fall within the protein kinase domain and include the proton acceptor. Alternatively, *Srsf3* cKO embryos were predicted to express more full-length Prkd2 protein containing the protein kinase domain. Consistent with these predictions, phosphorylation of the Prkd2 substrate Vasp was significantly increased in pooled *Srsf3* cKO E11.5 MxP mesenchyme lysates compared to those of control embryos ($p = 0.02$) (Fig. 7D)” (lines 372-379). Unfortunately, to our knowledge no anti-Prkd2 antibodies are available that specifically recognize the truncated amino acid residues that would allow us to test the relative presence of these amino acid residues between genotypes. However, we feel that the significant changes in the alternative splicing of *Prkd2* exon 15 between control and *Srsf3* cKO genotypes (Fig. 7C) together with the changes in phosphorylation of the Prkd2 phosphorylation target Vasp between these same genotypes (Fig. 7D) provide compelling evidence that increased phosphorylation of Vasp in *Srsf3* cKO embryos is due to more Prkd2 protein containing the protein kinase domain in this setting.

Reviewer #2

1. Some of the data included in the manuscript does not fully support the authors conclusion. For example, the authors concluded that pre-incubation with LY294002 resulted in reduced phosphorylation of SRSF3 (Fig 3B). However, this is not clear in the blot. In figure 2 it appears as if SRSF3 is exclusively nuclear, is there in vivo evidence that this protein needs to be translocated to function? What is the evidence that this antibody is specific to SRSF3? No controls were provided and the authors did not cite any previous paper indicating that this antibody was validated.

We have repeated the experiments in Figure 3B, quantified the relative phospho-Srsf3 levels in both the nucleus and cytoplasm in untreated, PDGF-AA-treated, and PDGF-AA- and LY294002-treated cells across four experiments and represented this data in a scatter dot plot in a new panel, Figure 3C. Here, relative phospho-Srsf3 levels were determined by normalizing to Lamin B1 and ß-tubulin for the nuclear and cytoplasmic fractions, respectively. As represented in the graph, there is a significant difference in relative phospho-Srsf3 levels in the cytoplasm between PDGF-AA-treated and PDGF-AA- and LY294002-treated cells ($p = 0.0147$). We have revised the following sentences in the Results section to summarize these findings: “This analysis revealed increased band intensities over baseline levels in response to PDGF-AA ligand treatment in the cytoplasmic fractions (1.851 ± 0.3222-fold induction over unstimulated levels), indicative of increased phospho-Srsf3 levels, and a return to band intensities near baseline levels upon treatment with LY294002 (1.136 ± 0.2930-fold induction over unstimulated levels) (Fig. 3B,C)” (lines 181-185). We thank the Reviewer for this suggestion, as we believe that it enhances our biochemical findings.

We agree with the reviewer that the Srsf3 protein expression in the mouse embryonic sections in Figure 2 appears to be predominantly nuclear, consistent with previous observations in cultured HEK293 cells by Long *et al*. (*J Biol Chem*, 2019). As we state in the Introduction, “Phosphorylation of Akt consensus sites within the C-terminal arginine/serine-rich (RS) domain of Srsf3 has been shown to drive its translocation to the nucleus (Bavelloni et al., 2014; Long et al., 2019)”. This is consistent with our results in Figure 3D,E in which phosphorylation of Srsf3 at Akt consensus sites in response to PDGF ligand stimulation leads to nuclear translocation. As alternative RNA splicing takes place in the nucleus, these collective findings suggest that the ability of Srsf3 to regulate alternative RNA splicing is dependent on its ability to translocate to the nucleus. To clarify this point to readers, we have revised the following sentence in the Results section: “These results suggest that phosphorylation of Srsf3 at Akt consensus sites downstream of PDGFRα signaling drives translocation of phosphorylated Srsf3 into the nucleus, where alternative RNA splicing takes place” (lines 198-200). To our knowledge, there is not *in vivo* evidence that Srsf3 needs to be translocated to function. Though well outside the scope of the current manuscript, we plan to directly test the hypothesis that phosphorylation of Srsf3 is required for its alternative RNA splicing function through the generation of a novel mouse model.

We have added representative western blots of E11.5 control versus *Srsf3* cKO maxillary process mesenchyme lysates with the anti-Srsf3 antibody and an anti-vinculin antibody as a loading control in a new panel, Figure S2F, revealing an absence of Srsf3 protein expression in the *Srsf3* cKO sample. The following sentence has been revised in the Results section: “Quantitative PCR,

whole mount *in situ* hybridization and western blotting confirmed deletion of *Srsf3* exons 2 and 3 in the facial processes and loss of Srsf3 protein expression in MxP mesenchyme lysates from cKO embryos (Fig. S2A-F)" (lines 205-208). The Srsf3 antibody used in the present study (catalog #ab73891; Abcam plc) has previously been shown to be specific to Srsf3 in the publication Sen *et al.*, "Splicing factor SRSF3 is crucial for hepatocyte differentiation and metabolic function" (*Nat Commun*, 2013). In this publication, the same $Srsf3^{fl}$ allele used in the present study was used to ablate *Srsf3* in hepatocytes. In Supplementary Figure S1d of that publication, Srsf3 protein expression was assessed in hepatocytes derived from wild-type versus *Srsf3* cKO mice using the antibody referenced above, revealing a dramatic reduction or complete loss of Srsf3 protein expression across multiple cKO samples. The authors of that paper concluded from these results that "…primary hepatocytes [derived from *Srsf3* cKO mice] do not express SRSF3 protein". We have added the phrase "previously validated in (Sen et al., 2013)" following Srsf3 antibody information in both the "Immunofluorescence analysis" and "Immunoprecipitations and western blotting" subsections of the Materials and Methods section (lines 656, 710).

2. Along the same line, the evidence for delayed cranial neural crest cell epithelial to mesenchymal transition in cKO embryos was also insufficient. SOX10 is a transcription factor that is normally localized in the nucleus. The immunofluorescence image provided in Figure 5C and 5D shows extensive cytoplasmic signal. This data was not very convincing, maybe separating the fluorescent and DAPI signal would allow for the nuclear signal to be made obvious? In addition, the sections do not appear to be in the same plane.
We have re-optimized our Sox10 immunofluorescence protocol, which has minimized non-nuclear background staining. We have quantified the number of Sox10-positive nuclei in the cranial neural folds and underlying mesenchyme of E8.0 control versus *Srsf3* cKO embryos at the same axial level, revealing no significant differences in the percentage of Sox10-positive NCCs between genotypes at either location. We have revised our interpretation of the Sox10 immunofluorescence data in the Results section: "Consistently, sections through embryos at this same timepoint [E8.0] revealed that the percentage of Sox10-positive NCCs was not significantly different in the cranial neural folds nor underlying mesenchyme between genotypes (Fig. 5C-E), suggesting that cKO embryos do not have defects in NCC specification nor epithelial-to-mesenchymal transition" (lines 262-266). We thank the Reviewer for requesting this revision, as it has led us to re-examine our data and come to a new conclusion regarding the *Srsf3* cKO phenotype.

3. Finally, additional EMT markers would also need to be analyzed to support the conclusion of a delay in EMT.
Given our re-analysis of Sox10-positive nuclei described above, we no longer believe that *Srsf3* cKO embryos exhibit defective EMT and have removed such comments from the manuscript.

4. The authors should discuss why they found increased proliferation and apoptosis? How do those two things contribute to the phenotype? The authors cannot rule out that an earlier increase in apoptosis contribute to the reduced number of neural crest cells seen at E9.5?
We have performed both Ki67 and TUNEL staining at E8.0 and E9.5 (in addition to our previous staining at E10.5). We have added to the following sentences to the Results section to describe our new findings: "We next assessed both cell proliferation and cell death via Ki67 immunofluorescence analysis and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), respectively, in the mesenchyme underlying the cranial neural folds at E8.0 and the mesenchyme of PA 1 at E9.5 and E10.5 (including the maxillary and mandibular processes) in $Srsf3^{fl/fl}$;$Wnt1\text{-}Cre^{+/+}$ control versus $Srsf3^{fl/fl}$;$Wnt1\text{-}Cre^{+/Tg}$ cKO embryos. Consistent with the above findings, cKO embryos had an approximately three-fold reduction in the percentage of Ki67-positive cells ($p = 0.0002$) and a modest decrease in TUNEL-positive cells ($p = 0.03$) compared to control embryos at E8.0 (Fig. 6A-D,M,N). At E9.5, cKO embryos had a slight reduction in proliferation ($p = 0.03$) and a greater than 30-fold increase in cell death ($p = 0.01$) relative to control embryos (Fig. 6E-H,M,N). By E10.5, cKO embryos had approximately four-fold increases in the percentage of Ki67-positive ($p < 0.0001$) and TUNEL-positive cells ($p = 0.007$) compared to control embryos (Fig. 6I-N). Together, these results reveal an early requirement for Srsf3 in promoting proliferation of cNCCs that have recently undergone an epithelial-to-mesenchymal transition and a later requirement for Srsf3 in promoting survival of the NCC-derived craniofacial mesenchyme" (lines 274-289). We have further revised our

discussion of these results in the Discussion section: “Upon ablation of *Srsf3* in the NCC lineage, cKO cells underlying the cranial neural folds exhibit a proliferation defect. These cranial NCCs are able to migrate into the facial processes, though fewer of these cells reach their target site. By E9.5, cKO embryos exhibit significantly increased cell death in the facial process mesenchyme, leading to hypoplastic facial processes. However, this cell death is partially offset by increased cell proliferation at E10.5, thereby allowing derivatives of these processes to form in embryos that survive past mid-gestation” (lines 449-455). We thank the Reviewer for requesting this revision, as it has led to a more complete understanding of the cellular processes underlying the *Srsf3* cKO phenotype.

5. The authors examined splicing of Fgfr2 but do not explain why they selected this particular transcript. They also did not explain why E11.5 was chosen for the RNAseq analysis since the mutant phenotype was severe by this stage of development. The authors also did not show any of the gels for their splicing analysis. This is an important piece of data that must be included alongside the quantification, especially as they did not indicate which exons were spliced in the transcripts picked for validation.

We have added to following sentences to the Results section to explain our choice of selecting *Fgfr2* to assess whether alternative splicing remained intact in *Srsf3* cKO embryos: “*Fgfr2* is subject to AS that produces tissue-specific isoforms depending on the inclusion of exon 8 (epithelial “b” isoforms) or exon 9 (mesenchymal “c” isoforms). Both isoforms are required for proper craniofacial development (De Moerlooze et al., 2000; Eswarakumar et al., 2002)” (lines 294-297).

The goal of this study was to identify the mechanisms by which gene expression changes occur downstream of PI3K-mediated PDGFRα signaling. Palatal clefting is the main craniofacial phenotype in embryos in which this signaling pathway is disrupted. As such, we conducted our previous phosphoproteomic screen in primary mouse embryonic palatal mesenchyme (MEPM) cells derived from E13.5 embryos (Fantauzzo and Soriano, 2014) and the first RNA-seq experiment in this study on palatal shelf mesenchyme derived from E13.5 wild-type versus $Pdgfra^{PI3K/PI3K}$ embryos in which PDGFRα is unable to bind PI3K (Figure 1). Our subsequent biochemical analyses were similarly performed in immortalized MEPM cells derived from E13.5 embryos (Figure 3). We were initially hoping to perform the second RNA- seq experiment in this study on palatal shelf mesenchyme derived from E13.5 control versus *Srsf3* cKO embryos to better compare the two RNA-seq experiments. However, our analyses revealed that the majority of *Srsf3* cKO embryos die just past mid-gestation (Table S5), with E11.5 being the latest timepoint that we were able to recover *Srsf3* cKO embryos at Mendelian frequencies. Our gross morphological analyses demonstrated that *Srsf3* cKO embryos did not have significant morphological defects until E10.5 (Figures 4 and 5). Interestingly, these embryos developed palatal shelves by E12.5, which were hypoplastic compared to those of control embryos (Figure S3). We therefore chose to perform the second RNA-seq experiment on E11.5 embryos for multiple reasons: 1) to profile AS and gene expression changes between control and *Srsf3* cKO embryos in the maxillary processes that will give rise to the palatal shelves just before the onset of palatal shelf development; and 2) to harvest tissue from control and *Srsf3* cKO embryos as close to the E13.5 timepoint of the first RNA-seq experiment as possible to better compare the results from the two sequencing experiments. We have added the following sentence to the Results section to explain our choice of timepoint: “We chose this timepoint in order to profile AS and gene expression changes between control and cKO embryos just before the onset of PS development and to harvest tissue close to the E13.5 timepoint of the RNA-seq experiment above to better compare the results from the two sequencing experiments” (lines 303-307). We thank the Reviewer for this suggestion, as we believe that this explanation will provide clarity to the reader.

We have added representative qPCR gels for all transcripts assayed in Figure 1C, Figure 7C and Figure S6B, as well as depictions of the differentially alternatively-spliced exon and upstream and downstream sequences that were assessed by qPCR in each case.

6. The data regarding alternative splicing of Prkd2 and phosphorylation of VASP needs to be improved. The alternative splicing event found in the Vasp transcript in PdgfrαPI3k/PI3k mutant needs to be shown and validated. For example, which exon was spliced, was this an exon skipping event? was it significant? Is VASP phosphorylation reduced in PdgfrαPI3k/PI3k mutants?

We have added qPCR validation of *Vasp* differential AS between E13.5 wild-type and *Pdgfra*$^{PI3K/PI3K}$ palatal shelf mesenchyme samples to Figure S6B. The following sentences have been added to the Results section: "*Vasp* was also found to be differentially alternatively spliced in our E13.5 PS mesenchyme rMATS analysis (Table S2), a finding which we confirmed by qPCR (Fig. S6B), resulting in skipping of exon 9 and loss of amino acids containing the Akt consensus motif and the final Prkd2 consensus motif more often in *Pdgfra*$^{PI3K/PI3K}$ samples, indicating an additional mechanism of *Vasp* regulation downstream of PI3K/Akt-mediated PDGFRα signaling" (lines 367-372).
We have added quantification of western blot data demonstrating decreased phosphorylation of Vasp in *Pdgfra*$^{PI3K/PI3K}$ E13.5 palatal shelf mesenchyme lysates to Figure S6A. The following sentence has been added to the Results section: "Of interest, we detected increased phosphorylation of the protein serine/threonine kinase Prkd2 and its substrate Vasp upon PDGF-AA ligand treatment of primary MEPM cells in our previous phosphoproteomic screen (Fantauzzo and Soriano, 2014), and have confirmed that phospho-Vasp levels are decreased in *Pdgfra*$^{PI3K/PI3K}$ E13.5 PS mesenchyme lysates (Fig. S6A)" (lines 362-367).

7. Finally, for this reviewer, there were several key tables referred to in the text that were not included, in the manuscript. Especially Table S2 and S8, which had important data for understanding the work.
Tables S2, S4, S6 and S8 are too large to have been included in the Supplemental Material .pdf file. As instructed by the journal, these files were instead submitted as separate .xlsx Microsoft Excel files.

Some minor comments:
8. Mating described in Table S5 is mislabelled, Srsf3 heterozygous flox embryos would not be observed if homozygous mutant flox mice were mated to each other.
We thank the Reviewer for pointing out this error. We have revised the title of Table S5 to read "Progeny from *Srsf3*$^{fl/fl}$ x *Srsf3*$^{+/fl}$;*Wnt1-Cre*$^{+/Tg}$ crosses."

Reviewer #3
1. In this work, authors claim that they have demonstrated that PI3K/Akt-mediated PDGFRa signaling regulates the expression of genes involved in palatal shelf morphogenesis through AS, in part through the phosphorylation and subsequent nuclear translocation of Srsf3. This conclusion is not supported by the data presented here. For example, RNAseq analysis on PS mesenchyme showed 523 significant AS events between Pdgfra+/+ versus PdgfraPI3K/PI3K embryos. However, only 13 genes were differentially expressed. None of the differentially expressed genes were alternatively spliced. This does not make sense when authors are suggesting that PDGFRa signaling- dependent AS is mediated through Srsf3. The authors should have compared PDGFRa signaling-dependent and Srsf3-dependent AS to identify a common target to support their conclusions.
Multiple studies have demonstrated that networks of tissue-specific transcripts which are differentially alternatively spliced do not have significant overlap with genes that are differentially expressed in the same tissue. For example, in their analysis of the RBPs Esrp1 and Esrp2 in the murine embryonic epidermis, Bebee *et al.*, (*eLife*, 2015) found that only 12 genes (representing 5.7% of all skipped exon rMATS events) were detected in both their differential splicing and differential gene expression analyses. The findings in that paper are similar to the low percentage of genes that were detected in this manuscript in both rMATS (skipped exon) and DESeq2 analyses in the *Pdgfra*$^{PI3K/PI3K}$ (0%) and *Srsf3* cKO (2.7%) RNA-seq experiments.

We have performed a new GO analysis of the genes represented by the 38 alternative splicing events commonly detected between the two rMATS analyses and significantly revised our analysis of these findings in the Discussion as follows: "Comparing the two RNA-seq experiments performed here, there were 38 AS events commonly detected in both rMATS analyses. Among these, only 12 (32%) were differentially alternatively spliced in the same direction (included or excluded) in *Pdgfra*$^{PI3K/PI3K}$ and *Srsf3* cKO embryos compared to their respective control embryos. A GO analysis of the genes represented by the 38 common AS events using the WikiPathways 2019 Mouse and KEGG 2019 Mouse libraries of Enrichr demonstrated that the top terms were focal adhesion-

PI3K-Akt-mTOR-signaling pathway (p = 0.006) and PI3K-Akt signaling pathway (p = 0.008), respectively. For the DESeq2 analyses, only two genes were commonly detected, *Aldh1a2* and *Rragd*. The relatively low extent of overlap between identified genes is not surprising given that these RNA-seq experiments were performed in different, but related, tissues across a 48-hour timeframe. In fact, AS changes in the developing murine face from E10.5-E12.5 are more significant across age than across facial prominence location (Hooper et al., 2020). Further, there was little to no overlap between the genes detected in the rMATS and DESeq2 analyses within either RNA-seq experiment, similar to previous analyses of differential AS and differential gene expression upon ablation of the RBPs Esrp1 and Esrp2 in the embryonic murine epidermis (Bebee et al., 2015). The majority of genes identified in the control versus *Srsf3* cKO MxP mesenchyme DESeq2 analysis encode transcripts that are likely regulated by Srsf3 through mechanisms other than AS, such as transcription, export, translation and degradation (Howard and Sanford, 2015). Notably, however, the rMATS and DESeq2 results from the control versus *Srsf3* cKO MxP mesenchyme RNA-seq analysis were commonly enriched for genes involved in focal adhesion, PI3K/Akt signaling and MAPK/Erk signaling, similar to results from a xenograft glioblastoma model in the presence or absence of SRSF3 (Fuentes-Fayos et al., 2020). These findings indicate that Srsf3 activity may influence each of these processes through multiple mechanisms of gene expression regulation. Relatedly, we found that phospho-Erk1/2 levels were significantly reduced in *Srsf3* cKO embryos. As the MAPK/Erk pathway has demonstrated roles in murine facial midline development and has been shown to function downstream from PDGFRα activation in this setting (Parada et al., 2015; Vasudevan et al., 2015), we propose that dysregulation of this signaling axis in *Pdgfra* and *Srsf3* mutant mouse models may underlie, at least in part, the common facial clefting phenotypes" (lines 465-496). Only one gene represented by the 38 AS events commonly detected in both rMATS analyses has a demonstrated role in craniofacial development, *Gng8*. *Gng8*-deficient mice exhibit abnormal vomeronasal sensory neuron morphology (Montani *et al.*, *J Physiol*, 2013), which is not particularly relevant to the midline facial clefting phenotypes of *Pdgfra* and *Srsf3* mutant mouse models described in this manuscript. As such, we chose not to pursue this particular target at this time.

2. Similarly, the conclusion derived from Figure 1 "Collectively, these findings demonstrate that AS is an important mechanism of gene expression regulation downstream of PI3K/Akt-mediated PDGFRa signaling in the mid-gestation PS" is not supported when there are no common genes that are differentially spliced and differentially expressed.
See the answer to point 1 above.

3. In Figure 1C, any of the changes observed in terms of percent spliced in between both genotypes are significant? Which exons are being analyzed here? Better to show gel images to compare which isoform is affected? If altered significantly, how do they affect the phenotype in PdgfraPI3K/PI3K embryos? As AS can also affect protein stability, modification, and functions, it is important to check their protein expression levels?
For Figure 1C, we have added the ΔPSI values (with p-values) for the differentially alternatively-spliced transcripts as assessed by both rMATS analysis of RNA-seq data and qPCR analysis. We have additionally included the 95% confidence interval from the qPCR analysis. We have added representative qPCR gels for all transcripts assayed in Figure 1C, as well as depictions of the differentially alternatively-spliced exon and upstream and downstream sequences that were assessed by qPCR in each case. These additions demonstrate that *Chrd*, *Cask* and *Smad7* have ΔPSI values as assessed by rMATS analysis of 14.3, 11.3 and 9.5, respectively, representing a greater than 5% change in the alternative splicing of each event between genotypes. Though the ΔPSI values as assessed by qPCR analysis for *Chrd* and *Smad7* are less than 5%, the trends (i.e. positive ΔPSI values) are consistent between rMATS and qPCR analyses. We have added a column to Table S3 indicating SPLINTER predictions for skipped exon events with one or more Srsf3 motifs and a corresponding mouse model with a craniofacial phenotype from the $Pdgfra^{+/+}$ versus $Pdgfra^{PI3K/PI3K}$ RNA-seq analysis. We have added the following sentences to the Results section to summarize these new SPLINTER findings: "We next employed SPLINTER (Low, 2020) to predict outcomes stemming from the AS of the 25 events in Table S3, revealing that 10 (40%) are predicted to result in a truncated protein, six (24%) in nonsense-mediated decay and potential downregulation of expression and two (8%) in an alternative protein (Table S3). All 10 truncated protein outcomes, including those for *Cask* and *Smad7*, and five of six nonsense-mediated decay

outcomes, including that for *Chrd*, are predicted to occur more often in *Pdgfra*$^{PI3K/PI3K}$ as opposed to wild-type samples.
Identifying how the differential AS of such transcripts ultimately affects protein function and contributes to the *Pdgfra*$^{PI3K/PI3K}$ palatal clefting phenotype will be the topic of future research studies" (lines 135-144). Such studies are beyond the scope of the current manuscript. For example, the *Cask* and *Smad7* events are predicted to result in a truncated protein. As the truncated amino acid residues do not fall within a known protein domain or include amino acid residues for which post-translational modification affects protein activity in either case, these changes have as yet unknown effects on protein stability and/or function.

4. In Figure 2, as the focus of the study is Neural crest-derived craniofacial structures, it is important to show Srsf3 expression in Neural crest cells using an established marker by double immunostaining.
We have performed Srsf3 co-immunofluorescence analysis with Sox10 at E8.5 and E9.5, revealing expression of both proteins in neural crest cells. We have added six new panels to Figure 2 (G-G'',I-I'') and added the following sentences to the Results section to summarize these findings: "Srsf3 was expressed in Sox10-positive NCCs migrating away from the cranial neural folds and in PAs 1 and 2 from E8.5-E9.5 (Fig. 2G-G'',I-I'')" (lines 163-165). We thank the Reviewer for this suggestion.

5. In Figure 3B, WB for Srsf3 in the first two panels are not convincing. Why all nuclear fractions are not analyzed similarly to cytoplasmic fractions?
We have repeated the experiments in Figure 3B and have included two panels of the same blot (immunoprecipitation with the anti-Akt phosphosubstrate antibody followed by western blotting with the anti-Srsf3 antibody) imaged at different exposures to reveal the expression of phospho-Srsf3 in the cytoplasmic (top) and nuclear (bottom) fractions. We have also quantified the relative phospho-Srsf3 levels in both the nucleus and cytoplasm in untreated, PDGF-AA-treated, and PDGF-AA- and LY294002-treated cells across four experiments and represented this data in a scatter dot plot in a new panel, Figure 3C. Here, relative phospho- Srsf3 levels were determined by normalizing to Lamin B1 and ß-tubulin for the nuclear and cytoplasmic fractions, respectively. We have revised the following sentences in the Results section to summarize these findings: "This analysis revealed increased band intensities over baseline levels in response to PDGF-AA ligand treatment in the cytoplasmic fractions (1.851 ± 0.3222-fold induction over unstimulated levels), indicative of increased phospho-Srsf3 levels, and a return to band intensities near baseline levels upon treatment with LY294002 (1.136 ± 0.2930-fold induction over unstimulated levels) (Fig. 3B,C)" (lines 181-185). We thank the Reviewer for this suggestion, as we believe that it enhances our biochemical findings.

6. In Figure 5C-D, the Authors conclude that Srsf3fl/fl;Wnt1-Cre+/Tg cKO littermates had very few Sox10-positive NCCs in the mesenchyme of the cranial neural folds, with extensive Sox10 staining in the overlying ectoderm. This is not clear from the images presented. Looks like more Sox10 positive cells in the Srsf3 KO. Similar sections need to be analyzed at this stage as there is no obvious morphological differences observed. Quantification needed.
We have re-optimized our Sox10 immunofluorescence protocol, which has minimized non-nuclear background staining. We have quantified the number of Sox10-positive nuclei in the cranial neural folds and underlying mesenchyme of E8.0 control versus *Srsf3* cKO embryos at the same axial level, revealing no significant differences in the percentage of Sox10-positive NCCs between genotypes at either location. We have revised our interpretation of the Sox10 immunofluorescence data in the Results section: "Consistently, sections through embryos at this same timepoint [E8.0] revealed that the percentage of Sox10-positive NCCs was not significantly different in the cranial neural folds nor underlying mesenchyme between genotypes (Fig. 5C-E), suggesting that cKO embryos do not have defects in NCC specification nor epithelial-to-mesenchymal transition" (lines 262-266). We thank the Reviewer for requesting this revision, as it has led us to re-examine our data and come to a new conclusion regarding the *Srsf3* cKO phenotype.

7. In Figure 5 (A-B and E-H), as authors are using mTmG reporter, it's better to show Merged images with both RFP and GFP for all the panels so the migration of GFP cells can be analyzed in the context of RFP background in the same embryo.

In our experience, the Tomato signal from the *ROSA26*^{mTmG} allele is weak, diffuse and does not provide the same resolution of morphological features as DAPI staining. We have revised the panels in Figure 5A',B',F',G',H' and I' to include merged images of the GFP and DAPI signals so that migration of GFP-positive neural crest cells can be visualized in the context of the entire embryo.

8. In Figure 5 (I-N), it is not clear what cells are being analyzed for proliferation and apoptosis? Lineage tracing analysis suggests that there are no GFP+ neural crest- derived cells in the MxP and MdP of the Srsf3 KO, then what cells are being quantified in the KO? Authors should perform GFP and Ki67 double immunostaining to show proliferation defects in the neural crest-derived craniofacial structures. Better to analyze both at E9.5 and E10.5 timepoint. Similarly, apoptosis should be analyzed in with double immunostaining for GFP and apoptotic markers (TUNEL or caspases, etc.). TUNEL quantification shown that apoptosis is not affected in MxP and increased in MdP. This increase is driven by one sample in the KO. Include more samples to make the changes robust.

When imaging embryos with the *ROSA26*^{mTmG} allele in Figure 5, we imaged both control and *Srsf3* cKO embryos using the same microscope settings (light source intensity, exposure, etc.) to be able to compare GFP expression in each case. While GFP-positive cells are present in E10.5 *Srsf3* cKO embryos, increasing the exposure enough to visualize these cells would have resulted in severe overexposure of the control embryos. We have added a new panel (Fig. 5I'') of an image of the same embryo in Fig. 5I' with increased GFP exposure which clearly demonstrates the presence of GFP-positive cells in the facial processes of an *Srsf3* cKO embryo at E10.5. These GFP-positive, neural crest-derived cells are being analyzed for proliferation and apoptosis. We have revised our description of these cells in the Results section as follows: "…cKO littermates had noticeably fewer and less intense GFP-positive cells in the facial processes and no obvious NCC streams entering the PAs (Fig. 5I-I'')" (lines 271-273). We thank the Reviewer for this suggestion, as we believe that it will provide clarity to the reader.
Unfortunately, technical restrictions did not allow us to perform co-immunofluorescence analysis with either Ki67 or TUNEL on embryos possessing the *ROSA26*^{mTmG} allele. However, we have performed both Ki67 and TUNEL staining at E8.0 and E9.5 (in addition to our previous staining at E10.5). We have added to the following sentences to the Results section to describe our new findings: "We next assessed both cell proliferation and cell death via Ki67 immunofluorescence analysis and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), respectively, in the mesenchyme underlying the cranial neural folds at E8.0 and the mesenchyme of PA 1 at E9.5 and E10.5 (including the maxillary and mandibular processes) in *Srsf3*$^{fl/fl}$*;Wnt1-Cre*$^{+/+}$ control versus *Srsf3*$^{fl/fl}$*;Wnt1-Cre*$^{+/Tg}$ cKO embryos. Consistent with the above findings, cKO embryos had an approximately three-fold reduction in the percentage of Ki67-positive cells ($p = 0.0002$) and a modest decrease in TUNEL-positive cells ($p = 0.03$) compared to control embryos at E8.0 (Fig. 6A-D,M,N). At E9.5, cKO embryos had a slight reduction in proliferation ($p = 0.03$) and a greater than 30-fold increase in cell death ($p = 0.01$) relative to control embryos (Fig. 6E-H,M,N). By E10.5, cKO embryos had approximately four-fold increases in the percentage of Ki67-positive ($p < 0.0001$) and TUNEL-positive cells ($p = 0.007$) compared to control embryos (Fig. 6I-N). Together, these results reveal an early requirement for Srsf3 in promoting proliferation of cNCCs that have recently undergone an epithelial-to-mesenchymal transition and a later requirement for Srsf3 in promoting survival of the NCC-derived craniofacial mesenchyme" (lines 274-289). Importantly, we only quantified NCC-derived mesenchymal cells for these analyses that fell within domains in which both Srsf3 and Sox10 are expressed, as determined by co-immunofluorescence analysis (Figure 2). To maintain consistency between timepoints, we analyzed the first pharyngeal arch mesenchyme at E9.5 and 10.5 (including both the maxillary and mandibular processes at the latter timepoint). These analyses were thorough, with up to five sections analyzed per embryo, across three embryos per genotype per timepoint. Our revised results demonstrate significant changes in both proliferation and cell death between genotypes at E8.0-E10.5. We thank the Reviewer for requesting this revision, as it has led to a more complete understanding of the cellular processes underlying the *Srsf3* cKO phenotype.

9. It is not clear how robust increase in cell proliferation and a modest increase in apoptosis will lead to this phenotype where most of the craniofacial structures are severely hypoplastic?

As mentioned in point 8 above, we have performed both Ki67 and TUNEL staining at E8.0 and E9.5 (in addition to our previous staining at E10.5). We have revised our discussion of these results in the Discussion section: “Upon ablation of *Srsf3* in the NCC lineage, cKO cells underlying the cranial neural folds exhibit a proliferation defect. These cranial NCCs are able to migrate into the facial processes, though fewer of these cells reach their target site. By E9.5, cKO embryos exhibit significantly increased cell death in the facial process mesenchyme, leading to hypoplastic facial processes. However, this cell death is partially offset by increased cell proliferation at E10.5, thereby allowing derivatives of these processes to form in embryos that survive past mid-gestation” (lines 449-455). We thank the Reviewer for requesting this revision, as it has led to a more complete understanding of the cellular processes underlying the *Srsf3* cKO phenotype.

10. Defective EMT claimed by authors should be analyzed properly. EMT markers such as E-cadherin/N-cadherin or others need to be analyzed by immunostaining/in situ hybridization and qPCR.
Given our re-analysis of Sox10-positive nuclei described above, we no longer believe that *Srsf3* cKO embryos exhibit defective EMT and have removed such comments from the manuscript.

11. To determine the alternatively spliced transcript regulated by Srsf3, authors harvested and sequenced MxP mesenchyme RNA from three biological replicates of E11.5 control versus cKO embryos. It is not clear what cells are being analyzed in this experiment. Lineage tracing data presented in Figure 5H-H’ shows that even at an earlier time point (E10.5) there are no NC-derived GFP+ cells in the MxP so what is being analyzed here? Most of the transcriptional changes observed from RNAseq data are likely to secondary and not primary. As NC-contribution is relatively preserved in the MxP and MdP of Srsf3KO at E9.5 (Figure 5E-F’), authors could have sorted GFP+ cells from E9.5 control and Srsf3KO heads (or dissected just MxP and MdP) to determine the primary changes.
As detailed in point 8 above, there are GFP-positive, neural crest-derived cells in the maxillary processes of *Srsf3* cKO embryos at E10.5. It is these neural crest-derived cells that are being analyzed in the E11.5 control versus *Srsf3* cKO RNA-seq experiment. The goal of this study was to identify the mechanisms by which gene expression changes occur downstream of PI3K-mediated PDGFRα signaling. Palatal clefting is the main craniofacial phenotype in embryos in which this signaling pathway is disrupted. As such, we conducted our previous phosphoproteomic screen in primary mouse embryonic palatal mesenchyme (MEPM) cells derived from E13.5 embryos (Fantauzzo and Soriano, 2014) and the first RNA-seq experiment in this study on palatal shelf mesenchyme derived from E13.5 wild-type versus $Pdgfra^{PI3K/PI3K}$ embryos in which PDGFRα is unable to bind PI3K (Figure 1). Our subsequent biochemical analyses were similarly performed in immortalized MEPM cells derived from E13.5 embryos (Figure 3). We were initially hoping to perform the second RNA-seq experiment in this study on palatal shelf mesenchyme derived from E13.5 control versus *Srsf3* cKO embryos to better compare the two RNA-seq experiments. However, our analyses revealed that the majority of *Srsf3* cKO embryos die just past mid-gestation (Table S5), with E11.5 being the latest timepoint that we were able to recover *Srsf3* cKO embryos at Mendelian frequencies. Our gross morphological analyses demonstrated that *Srsf3* cKO embryos did not have significant morphological defects until E10.5 (Figures 4 and 5). Interestingly, these embryos developed palatal shelves by E12.5, which were hypoplastic compared to those of control embryos (Figure S3). We therefore chose to perform the second RNA-seq experiment on E11.5 embryos for multiple reasons: 1) to profile AS and gene expression changes between control and *Srsf3* cKO embryos in the maxillary processes that will give rise to the palatal shelves just before the onset of palatal shelf development; and 2) to harvest tissue from control and *Srsf3* cKO embryos as close to the E13.5 timepoint of the first RNA-seq experiment as possible to better compare the results from the two sequencing experiments. We have added the following sentence to the Results section to explain our choice of timepoint: “We chose this timepoint in order to profile AS and gene expression changes between control and cKO embryos just before the onset of PS development and to harvest tissue close to the E13.5 timepoint of the RNA-seq experiment above to better compare the results from the two sequencing experiments” (lines 303-307). We do not believe that the majority of the AS and gene expression changes observed in the second RNA-seq experiment are secondary, especially given our goal to characterize both just before the onset of palatal shelf development. A third RNA-seq experiment at E9.5 is beyond the scope of the current manuscript and our current capabilities.

12. No molecular mechanism downstream of Srsf is provided to explain the molecular changes associated with the craniofacial phenotype. Due to the point made above the results presented in Figures 6 and 7 are due to secondary change in the hypoplastic MxP and not primary changes due to Srsf deletion in NCCs. In addition, Volcano plot analysis using RNAseq data from MxP mesenchyme shows downregulation of Zic5 and Pou3f4 (Figure 6A), however qRT-PCR analysis on MxP mesenchyme shows upregulation of these genes (Figure 6C). These two independent experiments show opposite results. Am I missing something here?
We have addressed this point in multiple ways. First, we have added a column to Table S9 indicating SPLINTER predictions for skipped exon events with one or more Srsf3 motifs and a corresponding mouse model with a craniofacial phenotype from the control versus *Srsf3* cKO RNA-seq analysis. We have added the following sentences to the Results section to summarize these SPLINTER findings: "We again employed SPLINTER to predict outcomes stemming from the AS of the SE events (Table S8). Of the 51 events in Table S9, 14 (26%) are predicted to result in nonsense-mediated decay and potential downregulation of expression, nine (17%) in a truncated protein, four (8%) in no termination codon and two (4%) in an alternative protein (Table S9). Half of the nonsense-mediated decay and alternative protein outcomes, and the majority of truncated protein and no termination codon outcomes, are predicted to occur more often in *Srsf3* cKO as opposed to control samples" (lines 342-349). Second, we performed a gene ontology analysis of the 21 genes encoding the differentially alternatively-spliced protein serine/threonine kinase transcripts, identifying an enrichment for MAPK signaling, which we corroborated by demonstrating that phospho-Erk1/2 levels are significantly decreased in *Srsf3* cKO E11.5 maxillary process lysates compared to those of control embryos (Fig. 7E). We have added the following sentences to the Results section to summarize these gene ontology and biochemical findings: "…a separate GO analysis of the 21 genes encoding the above protein serine/threonine kinases using the WikiPathways 2019 Mouse library of Enrichr revealed that the second and fourth most significant terms were MAPK signaling pathway ($p = 2.1 \times 10^{-5}$) and focal adhesion-PI3K-Akt-mTOR-signaling pathway ($p = 3.3 \times 10^{-4}$), respectively. In line with changes to MAPK signaling between genotypes, phospho-Erk1/2 levels were significantly decreased in pooled *Srsf3* cKO E11.5 MxP mesenchyme lysates compared to those of control embryos ($p = 0.003$) (Fig. 7E)" (lines 380-386). Finally, we have significantly revised our analysis of the commonalities between the rMATS and DESeq2 findings from the control versus *Srsf3* cKO RNA-seq analysis in the Discussion as follows: "…the rMATS and DESeq2 results from the control versus *Srsf3* cKO MxP mesenchyme RNA-seq analysis were commonly enriched for genes involved in focal adhesion, PI3K/Akt signaling and MAPK/Erk signaling, similar to results from a xenograft glioblastoma model in the presence or absence of SRSF3 (Fuentes-Fayos et al., 2020). These findings indicate that Srsf3 activity may influence each of these processes through multiple mechanisms of gene expression regulation. Relatedly, we found that phospho-Erk1/2 levels were significantly reduced in *Srsf3* cKO embryos. As the MAPK/Erk pathway has demonstrated roles in murine facial midline development and has been shown to function downstream from PDGFRα activation in this setting (Parada et al., 2015; Vasudevan et al., 2015), we propose that dysregulation of this signaling axis in *Pdgfra* and *Srsf3* mutant mouse models may underlie, at least in part, the common facial clefting phenotypes" (lines 485-496). We thank the Reviewer for this suggestion, as we feel that these changes tie together results from multiple experiments and significantly enhance the manuscript.
Zic5 and *Pou3f4* have a negative log2(fold change) value from the RNA-seq analysis, indicating that their expression is upregulated in *Srsf3* cKO embryos compared to control embryos. This trend is confirmed in the qRT-PCR results in Figure S5C. To avoid confusion, we have flipped the x-axis in the volcano plot in Figure S5A so that the trends of expression are consistent between Figure S5A and S5C. We thank the Reviewer for this suggestion, as we believe that it will provide clarity to the reader.

Second decision letter

MS ID#: DEVELOP/2021/199448

MS TITLE: Srsf3 mediates alternative RNA splicing downstream of PDGFRα signaling in the facial mesenchyme

AUTHORS: Brenna J.C. Dennison, Eric D. Larson, Rui Fu, Julia Mo, and Katherine A. Fantauzzo

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, provided that the critique of Reviewer #2 can be satisfactorily addressed. Please attend to comments on the data of alternative splicing and phosphorylation 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 carried out major revision of this work, responsive to this reviewer. The paper is improved with the additional clarifications and experiments.

Comments for the author

the authors have carried out major revision of this work, responsive to this reviewer. The paper is improved with the additional clarifications and experiments.

Reviewer 2

Advance summary and potential significance to field

In this revised manuscript Dennison et al reports that PI3K/AKT signalling downstream of PDGFRα results in phosphorylation of the splicing factor SRSF3. This increased phosphorylation is associated with enrichment of the splicing factor in nuclear extract, suggesting that PI3K/AKT regulates translocation of SRSF3 into the nucleus. The authors performed RNAseq analysis and reported a small number of differentially expressed genes in palate of E13.5 PdgfrαPI3k/PI3k mutant embryos, but a significant number of transcripts that were differentially spliced. They further showed that regions flanking skipped exons were enriched for SRSF3 binding sites. These observations lead the authors to examine expression and requirement for Srsf3 in the developing face. The authors showed enriched expression of Srsf3 mRNA and protein in the developing head.
They also showed that craniofacial development was severely disrupted when Srsf3 was mutated in neural crest cells and that these abnormalities were associated with a large number of differentially expressed genes and abnormal splicing. Differentially expressed genes were enriched in terms associated with protein kinase activity in Gene Ontology. Finding that the SRSF3 splicing factor is regulated downstream of PDGFR is a major advance in the field.

Comments for the author

In this reviewers' opinion, the major strength of this manuscript is the careful and detailed characterization of the Srsf3 mouse models and the extensive RNAseq analysis. The reviewer appreciates that the authors have improved their immunofluorescence experiment using the SOX10 antibody and included necessary references for the Srsf3 antibody that they used, thereby addressing some of the concerns initially flagged.

However, there are still some issues between the data being shown and conclusions made by the authors.
The major issue being the report of Srsf3-regulated alternative splicing of protein kinases and increased phosphorylation of VASP.
The scatterplot presented in fig 7C is not sufficient. There appears to be at least a 10-fold difference in the amount of product with exon2 in the first gel. Therefore, the authors should show gels with similar loading. In fact, there is consistently less product in the mutant samples. For the Prkd2 gel, there does not seem to be a clear band shown and this needs to be addressed. Also, it would be useful for the authors to describe how they calculated "percent spliced in" in the Materials and Methods section.
Similarly, the data regarding phosphorylation of VASP is insufficient. Could the authors show the gels used for quantification? Finally, the alternative splicing event reported for Vasp in PdgfrαPI3k/PI3k mutant was not visible on the gel attached, as there were no alternatively spliced products in either lane. Was the wrong gel included?

Reviewer 3

Advance summary and potential significance to field

Craniofacial abnormalities are among the most common of all birth defects. Neural crest (NC) contribution to the craniofacial tissues is well demonstrated. Compared to signaling pathways and transcription factors, functions of RNA binding proteins including splicing factors are now extensively studied in Neural crest cells (NCCs). In this manuscript, the authors attempted to demonstrate that NCCs specific AS events are regulated by PDGFRa-PI3K/Akt-Srsf3 axis.

Comments for the author

After revision, the manuscript has improved significantly and, my concerns have been addressed diligently. I do not have any further comments.

Second revision

Author response to reviewers' comments

Response to Reviewers

Reviewer #2
1. The scatterplot presented in fig 7C is not sufficient. There appears to be at least a 10- fold difference in the amount of product with exon2 in the first gel. Therefore, the authors should show gels with similar loading. In fact, there is consistently less product in the mutant samples.
We were very careful to set up each qPCR reaction with identical amounts of cDNA (1 µg, as indicated in the Materials and Methods section). The differential alternative splicing of *Limk2* was assessed by qPCR in three biological replicates of paired control and *Srsf3* cKO samples, each of which were analyzed independently and revealed the same trend of increased inclusion of exon 2 in *Srsf3* cKO samples compared to control samples. As we have now indicated in the Materials and Methods section (see point 3 below), the percent spliced in (PSI) was calculated independently for each sample as the percentage of the larger isoform divided by the total abundance of all isoforms within the given gel lane. As indicated in Tables S8 and S9, SPLINTER predicted that alternative splicing of *Limk2* would result in nonsense-mediated decay, which could explain why different overall amounts of *Limk2* amplicon were detected between genotypes. Confirming the qPCR results, *Limk2* was found to be expressed more highly in control versus *Srsf3* cKO samples in the DESeq2 analysis in Table S6 (log2(foldchange) = 0.35), though this differential gene expression between genotypes was not significant. As we note in the Discussion section, Srsf3 can regulate transcripts through mechanisms other than alternative splicing, such as transcription, export, translation and degradation. While it is possible that one or more of these mechanism(s)

contributed to the different overall amounts of *Limk2* amplicon detected between genotypes, exploring these mechanisms further is beyond the scope of the current manuscript, which is specifically focused on Srsf3-mediated alternative splicing.

2. For the Prkd2 gel, there does not seem to be a clear band shown and this needs to be addressed.
We have re-optimized our *Prkd2* qPCR protocol, which resulted in clear bands at both 508 and 409 bp. Importantly, quantification of these new qPCR products confirmed the trend observed in the rMATS analysis and our previous qPCR analysis in all three biological replicates.

3. Also, it would be useful for the authors to describe how they calculated "percent spliced in" in the Materials and Methods section.
The following sentence has been added to the Materials and Methods section: "The percent spliced in (PSI) was calculated independently for each sample as the percentage of the larger isoform divided by the total abundance of all isoforms within the given gel lane."

4. Similarly, the data regarding phosphorylation of VASP is insufficient. Could the authors show the gels used for quantification?
We have added representative phospho-Vasp western blot images which were used for quantification to Figures 7D and S6A. For consistency, we have also added a representative phospho-Erk1/2 western blot image to Figure 7E.

5. Finally, the alternative splicing event reported for Vasp in PdgfrαPI3k/PI3k mutant was not visible on the gel attached, as there were no alternatively spliced products in either lane. Was the wrong gel included?
We have re-optimized our *Vasp* qPCR protocol, which resulted in clear bands at both 198 and 164 bp. Importantly, quantification of these new qPCR products confirmed the trend observed in the rMATS analysis and our previous qPCR analysis in all three biological replicates.

Third decision letter

MS ID#: DEVELOP/2021/199448

MS TITLE: Srsf3 mediates alternative RNA splicing downstream of PDGFRα signaling in the facial mesenchyme

AUTHORS: Brenna J.C. Dennison, Eric D. Larson, Rui Fu, Julia Mo, and Katherine A. Fantauzzo
ARTICLE TYPE: Research Article

I am satisfied with your response to review and the revision. This manuscript has been accepted for publication in Development, pending our standard ethics checks.