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TAZ exhibits phase separation properties and interacts with Smad7 and ß-catenin to repress skeletal myogenesis

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MS TITLE: TAZ exhibits phase separation properties and interacts with Smad7 and ß-catenin to repress skeletal myogenesis

AUTHORS: Soma Tripathi, Tetsuaki Miyake, and John C. McDermott
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes 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 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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Tripathi et al. studies mechanisms whereby the transcriptional regulator TAZ regulates skeletal myoblast differentiation. TAZ co-immunoprecipitates with Smad7 and inhibits Smad7's enhancing effects on expression of a muscle-specific reporter construct in cultured myoblasts; siRNA against TAZ has the opposite effect. TAZ has complex effects: it activates expression of a TEAD-driven reporter but represses beta-catenin-, MyoD (256/-2.5)-, and myogenin enhancer-driven reporters. TAZ is shown to display properties consistent with liquid-liquid phase separation.

While the work presented in this paper appears to be carefully performed overall, it suffers from two problems: 1) many of the observations and conclusions have been published previously; and 2) there is an overreliance on artificial constructs. It is my opinion, therefore, that the paper is not well-suited for a journal like JCS.

Comments for the author

1. The physical interaction between TAZ and Smad7 is interesting (although co-IP between the two endogenous proteins was not demonstrated). However, the specific role of this interaction is not clear. All the work showing TAZ function is with reporter constructs, and no investigation of endogenous genes in the myogenic program that might be targeted by this interaction is included. Yet the strengths of a reporter construct system are not exploited – there is no analysis of the specific domains in TAZ and Smad7 that interact and/or are required for the effects on the various reporters was undertaken.
2. The expression pattern of YAP, TAZ, and their phosphorylation during myoblast differentiation, and the general effect of TAZ on myogenesis in vitro, were performed in greater detail than in this paper and reported in 2017 (Sun et al. Stem Cells, 35:1958-1972, 2017). Oddly, this paper was not cited.
3. The liquid-liquid phase separation properties of TAZ were also previously reported, again in much greater detail (Lu et al., 2020 in the references). This paper does not add significant new information to this phenomenon.

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors report on the functional interactions between Smad7 and TAZ in cultured skeletal muscle cells to repress the expression of muscle specific genes (e.g. ckm, myogenin and MyoD). They also show that Smad7-TAZ interferes with ß-catenin activity and that TAZ expression appears confined in nuclear speckles in myoblasts, while during differentiation, Ser 89 phosophorylation promotes TAZ cytoplasmic sequestration. Finally, the authors provide evidence that TAZ exhibits properties of liquid-liquid phase separation (LLPS).
In general, the data fairly support the author's conclusion that TAZ mediates Hippo signaling to repress myogenic differentiation. The cross-talk between TGF-ß, Hippo signaling and Wnt/ß-catenin in regulating myogenic differentiation is interesting and, to a certain extent, novel. However, there are a number of issues that need to be addressed in order to convincingly substantiate the author's conclusions.

Comments for the author

Major Points
1) Figure 1A and Fig. 4A-D – the immunofluorescence analysis does not fully support the author's conclusion that nuclear TAZ is significantly reduced during differentiation (Pearson R Value suggests slight differences). It seems more like the nuclear amount of TAZ is the same in myoblasts and myotubes, with the cytoplasm fraction of TAZ accumulating in myotubes, as also suggested by the increase in TAZ levels during differentiation (detected by WB in Fig. 4A). Only the phosphorylated form appears to accumulate in the cytoplasm of myotubes. A functional dissections

between phosphorylated vs non-phosphorylated form of TAZ in myotubes is required to fully elucidate the role of Hippo signaling to TAZ in skeletal muscle cells.
2) Most of the experiments are performed by over-expression or knock-down of effectors of individual pathways (SMAD7, TAZ, ß-catenin). While this approach provides a valid proof, in principle, of physical and functional interactions, it is not very informative on the physiological functions of these interactions. The authors should use specific ligands/culture conditions and analyze interactions between endogenous proteins (by IP) and function (by qPCR) as reliable outcomes, to support their conclusions
3) A preliminary validation of the data shown on TAZ levels localization and response to Hippo signaling should be performed in muscle satellite cells (minor point)

Reviewer 3

Advance summary and potential significance to field

In this report by Tripathi, Miyake, and McDermott, the researchers discovered an interesting phenomenon that TAZ forms phase-separated condensates to sequester other transcription factors to repress skeletal myogenesis. The paper is novel since it is the first to show that phase separation of transcription related factors is implicated in skeletal myogenesis. Different from most of the previous papers, condensates formed by TAZ is repressive in nature, which also indicates that condensates may serve diverse roles inside the cell.

Comments for the author

The manuscript will benefit from the following points of revision before acceptance by the journal:
1. The authors claimed that in Fig. 3A, ectopic expression of TAZ repressed beta-catenin activity shown by TOP FLASH assay. But it is not obvious how the authors arrived at such conclusion. Which two data points should we look at? In addition it is not clear from figure legends how they did statistical analysis and what "****" indicates: what is significantly different from what?

2. The authors also started using TAZ S89A instead of WT TAZ in the later experiments without giving an explanation. Why did they not use WT TAZ consistently? Is WT TAZ not giving a strong phenotype?

3. In Fig. 3F, it will be great that the authors can give quantification, instead of showing a couple of isolated cells. Can they also stain for myogenic conversion markers to show definitely that multinucleated cells are myogenic?

4. It is obvious in Fig. 3F that the transfection efficiency is low for the two plasmids. Is the luciferase assay in Fig. 3D done with similar setup? If so, you will wonder how the authors can see a big difference in luciferase activity.

5. The endogenous staining of TAZ in Fig. 4E is interesting. Will the authors see a colocalization of TAZ foci with SMAD or beta-catenin?

6. In Fig 4A, p-TAZ is decreased at 96h, while in Fig 4D, you see a dramatic increase of p-TAZ at Day 4, which is also 96h. How would the authors reconcile the difference in these two experiments?

7. In Fig. 5, the authors overexpressed EGFP-TAZ, which showed a hollow structure.
We see these structure when the TAZ is VERY overexpressed. It will be great if the authors can tune down their overexpression to make is similar to endogenous TAZ expression level, and see if the condensates sequester beta-catenin.

8. Are the TAZ condensates similarly sequestering SMAD?

First revision

Author response to reviewers' comments

Response to reviewers:

Reviewer 1 Advance summary and potential significance to field
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While the work presented in this paper appears to be carefully performed overall, it suffers from two problems: 1) many of the observations and conclusions have been published previously; and 2) there is an overreliance on artificial constructs. It is my opinion, therefore, that the paper is not well-suited for a journal like JCS.

Response: We appreciate the reviewers comment that our work has been carried out carefully. We believe that care and reproducibility are a hallmark of all of our studies. In terms of the reviewer's point that the observations and conclusions have been previously published. We agree that some aspects of the manuscript have been reported previously in the sense that the role of TAZ in satellite cells (myogenic progenitors in adult muscle) was addressed in one study and also the property of TAZ to form biomolecular condensates was published in another recent study using a different cell system (this study was published while the current manuscript was in preparation). However, we would contend that our manuscript is the first to combine and confirm these observations in a myogenic context. I would also respectfully suggest that it's not strictly negative to have some confirmation of previous studies embedded in a manuscript in view of the widespread issues concerning reproducibility plaguing modern science at present. In addition, I would add that the main focus of our study concerns the effects of TAZ as a repressor of the myogenic transcription complex. We believe this to be a completely novel aspect of our study in terms of the molecular details we have discovered in dissecting the transcription factor targets of TAZ in the myogenic transcription complex. In using reporter gene assays, while we agree that they are artificial constructs, we would like to point out that they do report on the "endogenous" activity of the transcriptional regulators that target those promoter/enhancer regions (in terms of reporter genes we also use highly specific 'synthetic' transcription factor based cis elements such as TOP flash, Hip/Hop and 4x MEF2-luc as well as natural promoter/enhancers for genes such as ckm, myog, and myod). Moreover, we also extensively utilize siRNA technology to observe the effects of depletion of the 'endogenous' transcriptional regulators on these well characterized target promoters. Again, since siRNA technology targets depletion of the endogenous mRNAs and their respective protein products we also see this as assessing the function of the endogenous genes in a targeted and specific manner. In our view this is the most precise way to unambiguously address the research questions at hand. In terms of describing the formation of biomolecular condensates by TAZ- we believe that, at least in a cellular context, using fluorescently labeled fusion proteins (eg. EYFP-TAZ) coupled with confocal microscopy and live cell imaging is the clearest and currently most state of the art approach to document this phenomenon. However, we do also acknowledge the reviewers point as the goal should always be to try to ultimately determine the physiological relevance on endogenous genes and we have now made some inroads to do this by including some new data documenting the effect of TAZ manipulation on the endogenous protein level expression of the muscle creatine kinase and myogenin genes (see Figs 2E and 3F). While these new data only begin to address the role of TAZ on endogenous gene expression in muscle cells, these data do support the main theme of the manuscript.

Reviewer 1 Comments for the author
1. The physical interaction between TAZ and Smad7 is interesting (although co-IP between the two endogenous proteins was not demonstrated). However, the specific role of this interaction is not clear. All the work showing TAZ function is with reporter constructs, and no investigation of endogenous genes in the myogenic program that might be targeted by this interaction is included.

Yet the strengths of a reporter construct system are not exploited – there is no analysis of the specific domains in TAZ and Smad7 that interact and/or are required for the effects on the various reporters was undertaken.

Response: We acknowledge that the reviewer makes an important point here. As mentioned above, we have now included data on the effects of TAZ on endogenous expression of MCK and Myogenin protein levels which are probably the most relevant in the context of this study. We believe that the reporter gene systems we have used are appropriate for the questions we framed. However, we do also see that mapping the domains of interaction between the two molecules would be of interest. This is definitely an approach that we will undertake. Secondly, I would point out that the absence of good commercial antibodies for IP of endogenous Smad7 has been a substantial hindrance in the Smad/TGF β-field in general and one that we have not been able to properly solve (even after trying 5 commercially available antibodies and also making our own rabbit polyclonal antibody in house). In view of this, we agree that co-IP of the endogenous proteins is an important test but so far we have not achieved this due to antibody issues. We have, however, provided both biochemical and functional evidence supporting the interactions to date.

2. The expression pattern of YAP, TAZ, and their phosphorylation during myoblast differentiation, and the general effect of TAZ on myogenesis in vitro, were performed in greater detail than in this paper and reported in 2017 (Sun et al. Stem Cells, 35:1958-1972, 2017). Oddly, this paper was not cited.

Response: Thanks to the reviewer for pointing this out. We apologize for this oversight- inexplicably we did not retrieve this manuscript in our original literature search and we have now integrated reference to the manuscript (in both the Introduction and Discussion sections of the manuscript) by Sun et al and have also cited it appropriately.

3. The liquid-liquid phase separation properties of TAZ were also previously reported, again in much greater detail (Lu et al., 2020 in the references). This paper does not add significant new information to this phenomenon.

Response: As stated above, the manuscript referred to, that we have cited and discussed in our study, came out while our manuscript was in preparation. While our data does confirm and reproduce the data in the Lu et al manuscript, our data also raises the possibility, as pointed out by one of the other reviewers, of TAZ condensates being important in skeletal myogenic cells and also documents the integration of ß-catenin into the TAZ condensates. So, we hope the reviewers, to some extent, see value in this confirmation and extension of knowledge concerning TAZ condensates.

Reviewer 2 Advance summary and potential significance to field
In this manuscript the authors report on the functional interactions between Smad7 and TAZ in cultured skeletal muscle cells to repress the expression of muscle specific genes (e.g. ckm, myogenin and MyoD). They also show that Smad7-TAZ interferes with ß-catenin activity and that TAZ expression appears confined in nuclear speckles in myoblasts, while during differentiation, Ser 89 phosophorylation promotes TAZ cytoplasmic sequestration. Finally, the authors provide evidence that TAZ exhibits properties of liquid-liquid phase separation (LLPS).
In general, the data fairly support the author's conclusion that TAZ mediates Hippo signaling to repress myogenic differentiation. The cross-talk between TGF-ß, Hippo signaling and Wnt/ß-catenin in regulating myogenic differentiation is interesting and, to a certain extent, novel. However, there are a number of issues that need to be addressed in order to convincingly substantiate the author's conclusions.

Response: We thank the reviewer for these positive general comments regarding our manuscript.

Reviewer 2 Comments for the author
Major Points

1)Figure 1A and Fig. 4A-D – the immunofluorescence analysis does not fully support the author's conclusion that nuclear TAZ is significantly reduced during differentiation (Pearson R Value suggests

slight differences). It seems more like the nuclear amount of TAZ is the same in myoblasts and myotubes, with the cytoplasm fraction of TAZ accumulating in myotubes, as also suggested by the increase in TAZ levels during differentiation (detected by WB in Fig. 4A). Only the phosphorylated form appears to accumulate in the cytoplasm of myotubes. A functional dissections between phosphorylated vs non-phosphorylated form of TAZ in myotubes is required to fully elucidate the role of Hippo signaling to TAZ in skeletal muscle cells.

Response: We appreciate the reviewers comment and have now quantitated the levels of phosphor-YAP/TAZ (Ser 89 phosphorylation) in Fig 4B based on the western analysis of phosphorylated and total YAP/TAZ which, unlike immunofluorescence data, is at least semi-quantitative. This analysis indicates that the phosphorylated forms of YAP and TAZ (expressed relative to the total YAP or TAZ protein) increase with the onset of cellular differentiation and that increase correlates with the induction of Myogenin protein (widely accepted to be the critical “no return” determinant of the commitment to the differentiation program).

2)Most of the experiments are performed by over-expression or knock-down of effectors of individual pathways (SMAD7, TAZ, ß-catenin). While this approach provides a valid proof, in principle, of physical and functional interactions, it is not very informative on the physiological functions of these interactions. The authors should use specific ligands/culture conditions and analyze interactions between endogenous proteins (by IP) and function (by qPCR) as reliable outcomes, to support their conclusions.

Response: As stated above, we have now documented the effects of TAZ manipulation on the endogenous protein levels of MCK and Myogenin and these data support the conclusions of our manuscript. We thought that, while a bit more difficult, assessing the protein levels has more relevance for physiological function than qPCR of mRNAs. We therefore hope that we have gone some way to addressing the reviewer’s comment. However, we acknowledge that there is much more to be done in terms of understanding the upstream activators of Hippo signaling and how it affects the physiological expression of muscle genes.

3)A preliminary validation of the data shown on TAZ levels localization and response to Hippo signaling should be performed in muscle satellite cells (minor point).

Response: We agree that studying Hippo signaling in satellite cells is highly relevant since C2C12 cells that we have used in this study were originally derived from mouse satellite cells. This approach may be of some interest in terms of muscle regeneration and we will eventually pursue it. During the recent course of these studies we have had no access to animals or FACS analysis to capably isolate satellite cells and therefore hope that the reviewer can consider this.

Reviewer 3 Advance summary and potential significance to field
In this report by Tripathi, Miyake, and McDermott, the researchers discovered an interesting phenomenon that TAZ forms phase-separated condensates to sequester other transcription factors to repress skeletal myogenesis. The paper is novel since it is the first to show that phase separation of transcription related factors is implicated in skeletal myogenesis. Different from most of the previous papers, condensates formed by TAZ is repressive in nature, which also indicates that condensates may serve diverse roles inside the cell.

Response: We appreciate the reviewer highlighting these aspects of our study.

Reviewer 3 Comments for the author
The manuscript will benefit from the following points of revision before acceptance by the journal:
1. The authors claimed that in Fig. 3A, ectopic expression of TAZ repressed beta-catenin activity shown by TOP FLASH assay. But it is not obvious how the authors arrived at such conclusion. Which two data points should we look at? In addition, it is not clear from figure legends how they did statistical analysis and what "****" indicates: what is significantly different from what?

Response: In looking at Fig 3A, the appropriate comparison related to the point above is between lane 2 which indicates the activation by β-catenin alone and lane 5 which indicates the level of TOP flash when TAZ is co-expressed with β-catenin (pcDNA is used to equalize the DNA concentrations in

all transfections). The **** indicate the adjusted p-value ****<0.0001 between those two treatments and this has now been clarified in the corresponding Fig legend.

2. The authors also started using TAZ S89A instead of WT TAZ in the later experiments without giving an explanation. Why did they not use WT TAZ consistently? Is WT TAZ not giving a strong phenotype?

Response: We do explain to some extent why we used TAZS89A in a number of experiments in the text.
The main reason is that our principal interest concerns the nuclear function of TAZ with myogenic regulators that are co-localized in the nucleus. Since S89A escapes the cytoplasmic sequestration of TAZ by 14-3-3 proteins we contend that using S89A gives us a clearer, less ambiguous picture of the nuclear function of TAZ when it accumulates there. This is particularly important when considering TAZ and β-catenin because a previous study has shown that under some conditions TAZ can be sequestered into the cytoplasmic APC degradation complex with β-catenin. In our studies we wanted to obviate this possibility which is difficult to control by using S89A to focus on the nuclear functions. In most cases we have documented that wt TAZ exhibits the same effects (since we did use the wt form in a number of experiments) but that we considered the effects seen with TAZ S89A are more interpretable and indicative of the nuclear function of TAZ.

3. In Fig. 3F, it will be great that the authors can give quantification, instead of showing a couple of isolated cells. Can they also stain for myogenic conversion markers to show definitely that multinucleated cells are myogenic?

Response: We agree with the reviewer. What we have now done is quantitated the length (since myotubes are elongated/ multinucleated cells) to compare the effects of TAZ in the MyoD induced 10T1/2 conversion assay (see Fig 3G). We believe this is a reasonable way to assess the myogenic conversion in MyoD positive cells in the presence or absence of TAZ. The data show that elongated cells (>110um's) occur less in MyoD positive, TAZ expressing cells (29.4%) whereas there are many in the MyoD positive, TAZ negative controls (89.7%). The issue with doing biochemistry to assess myogenic markers in this system (since both Red and Green channels are taken up by MyoD and TAZ detection in this assay) is that the frequency of conversion is extremely low (this was originally published in the classic paper on the cloning of MyoD from 10T1/2 converted by azacytidine treatment published in Cell by Weintraub's group). Thus, doing Western blots for myogenic markers when so many cells remain unconverted is not feasible.

4. It is obvious in Fig. 3F that the transfection efficiency is low for the two plasmids. Is the luciferase assay in Fig. 3D done with similar setup? If so, you will wonder how the authors can see a big difference in luciferase activity.

Response: The major advantage of doing luciferase assays in transient transfections is that the reporter genes are taken up along with the exogenous constructs so that one is only assaying the transfected cells. In this system, as long as the transfection efficiency is reasonable >10 % (efficiency is controlled for by the Renilla plasmid) there is no concern at all since all of the luciferase values are coming from the transfected cells. The untransfected cells do not factor in this particular assay system. Of course, if one is assaying endogenous genes then transfection efficiency of exogenous constructs is an important issue but that is not the case in these particular assays.

5. The endogenous staining of TAZ in Fig. 4E is interesting. Will the authors see a colocalization of TAZ foci with SMAD or beta-catenin?

Response: We have put considerable effort into this question, particularly with TAZ and β-catenin, and we have now included these data (Fig 5E) in the manuscript. The data depicts that some (but not all) β-catenin co-localizes with TAZ in the condensates.

6. In Fig 4A, p-TAZ is decreased at 96h, while in Fig 4D, you see a dramatic increase of p-TAZ at Day 4, which is also 96h. How would the authors reconcile the difference in these two experiments?

Response: As pointed out above, we would certainly favour the western data in terms of semi-quantitative evidence in the time course data. The IF data are definitely comparable to the other IF data points in terms of expression levels but they may not be absolutely synchronized with other data from the Western analysis. We try to normalize the differentiation time course as much as possible but there are some factors that cause variance such as exact confluency and growth conditions.

7. In Fig. 5, the authors overexpressed EGFP-TAZ, which showed a hollow structure. We see these structure when the TAZ is VERY overexpressed. It will be great if the authors can tune down their overexpression to make is similar to endogenous TAZ expression level, and see if the condensates sequester beta-catenin.

Response: We appreciate the reviewers comment. We took a look at this point and can report that we do see TAZ condensates that do not contain that "hollowed out"/ TAZ negative centre in some cells at a lower frequency, even when the expression level is high and equivalent to some cells that have the negative central region. We have now included a figure 5C that depicts both morphologies. At this point we are not sure whether the hollowed out structures reflect the maturation of the condensates (since they seem to occur after the condensates fuse together) or whether it is simply, as pointed out, a function of the expression level. It is possible that as the condensates increase in size/maturity that they sequester other components which occupy the central zone of the condensate (DNA/other proteins?). This is a very interesting question that we are looking into but don't have anything further to report at this stage. We have now alluded to these possibilities in the text.

8. Are the TAZ condensates similarly sequestering SMAD?
Response: We have not been able to adequately visualize Smad7 in these assays so far. We have confirmed that ß-catenin is co-localized in the condensates. However, our main point, as of now, is to highlight the formation of nuclear TAZ condensates in myogenic cells which requires further investigation.

Second decision letter

MS ID#: JOCES/2021/259097

MS TITLE: TAZ exhibits phase separation properties and interacts with Smad7 and ß-catenin to repress skeletal myogenesis

AUTHORS: Soma Tripathi, Tetsuaki Miyake, Jonathan Kelebeev, and John C. McDermott
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

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Comments for the author

The authors have addressed my comments in a satisfactory manner.

Reviewer 2

Advance summary and potential significance to field

The authors have satisfactorily addressed most of the reviewer concerns and the manuscript is in my opinion suitable for publication in JOCES

Comments for the author

none

Reviewer 3

Advance summary and potential significance to field

The paper made significant improvements after revision.

Comments for the author

There seems to be some confusions regarding reviewer comment #3:
3. In Fig. 3F, it will be great that the authors can give quantification, instead of showing a couple of isolated cells. Can they also stain for myogenic conversion markers to show definitely that multinucleated cells are myogenic?
I am not suggesting to use western blots to show that multinucleated cells are myogenic. I am suggesting to use immunofluorescence to show multinucleated cells are myogenic. This will be able to show in individual cells the myogenic conversion happens.