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# α-Arrestin ARRDC3 tumor suppressor function is linked to GPCR-induced TAZ activation and breast cancer metastasis

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**Editor**: John Heath

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Original submission: 24 September 2020
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**Original submission**

First decision letter

MS ID#: JOCES/2020/254888

MS TITLE: α-arrestin ARRDC3 tumor suppressor function is linked to GPCR-induced TAZ activation and breast cancer metastasis

AUTHORS: Aleena Arakaki, Wen-An Pan, Helen Wedegaertner, Ivette Roca-Mercado, Logan Chinn, and JoAnn Trejo
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 examines the role of ARRDC3 in YAP/TAZ signaling in breast cancer models indicating a link between GPCR induced TAZ activity specifically and role of arrestin.
This is a valuable interesting study to tease out TAZ functions alongside ARRDC( a tumor suppressor) and are of interest to both the YAP/TAZ community and the GPCR community as it defines a novel relationship between a arrestin in GPCR induced TAZ activity. The role in breast cancer specifically is however somewhat underdeveloped and superficial in nature.

*Comments for the author*

Key overall issues: The effects on breast cancer metastasis are underdeveloped as validation of the mechanism in the tumor tissues has not been done and the significance to TNBC is also not clear. TAZ levels and localization should be assessed in the tumors if the authors wish to establish a clear link.

Second key overall issues is some aspects of the hippo signaling mechanism which can be better detailed ( see specific comments below). Quality of some figures (Figure 3 and most microscopy images) is very low making it difficult to evaluate the findings.

Specific comments:
The effect of cell density on YAP localization and activity is well documented in the literature. In figure 1: PI has low density pictures where hippo is likely low as YAP is widely distributed that then translocated to the nucleus in response to thrombin, however in the westerns, PI suggests hippo is high: fundamental to these studies is knowing the cell density in the localization versus the signaling/western studies to be able to faithfully interpret these experiments. As such this needs to be addressed in all figures showing localization and signaling studies.

Figure 6: ARRDC induction seems to downregulate TAZ activation in the absence of thrombin, thus the effect of thrombin itself is marginal, can authors comment on this?

Nuclear localization studies with TAZ and ARRDC3 are not clear and fractionation is required in this case.

In vivo experiments as stated in the " overall issues" require additional examination of tumors to show the relative changes in TAZ and YAP to clearly pinpoint the mechanism.

Minor clarification: Does thrombin induce TAZ much more significantly only in the absence of YAP?- some of the westerns suggest this- perhaps a discussion if this is a case is adequate here.

Statistical tests: In multiple comparisons, why was ANOVA not used everywhere by only in select analysis- please clarify?

Title comment: The link to TAZ and metastasis is not very clear, and title may need to be amended

Reviewer 2

*Advance summary and potential significance to field*

The manuscript of Ararkaki et al. studies the connection of the tumorsuppressor ARRDC3 with the hippo downstream effector and oncogene TAZ in triple-negative breast carcinoma (TNBC). They

describe a novel mechanism, in which Par1 and GPCR signaling activates TAZ/YAP. Downstream, TAZ but not YAP stimulates invasion and migration by increased expression of CTGF and ANKRD1. Beside its direct effects on Par1, ARRDC3 also sequesters TAZ keeping it at check. For this cytoplasmic retention, the WW domain of TAZ and a PPXY motif in ARRDC3 interact. So, ARRDC3 has two modes in controlling the activity of TAZ.

This is an interesting and novel story presenting a novel mechanism that specifically affects TAZ but not YAP. This contributes to solve the fundamental question of distinct and overlapping functions of YAP and TAZ. In addition, it delivers one possible explanation for a dysregulation of hippo downstream signaling through GPCR signaling. Therefore, I feel this is an important topic and important research for the hippo (and the breast cancer) community.

*Comments for the author*

1. The quality or, more accurate, the resolution of Fig.3 (and of some IBs throughout the paper) is really low. Most likely a problem of the pdf conversion? I can't really comment on any blot or cell picture here.

2. Some of the western blots presented raise the question, of how exactly they have been performed. This should be mentioned in the methods section. Have membranes been re-stained? Have they been "stripped" to remove antibodies? Are there "reruns" of samples on different gels? In addition, I would always prefer to see the full blots as supplements. This makes things much easier for everyone involved, specifically for the reader… . This of course, is not my decision.

3. The authors show very nicely the effect of thrombin on two depicted YAP/TAZ target genes. Doing this with siRNA against YAP and TAZ (Fig 2) they conclude that primarily TAZ is transmitting GPCR signaling. It would be very interesting to see, how additional YAP/TAZ target genes react and whether they follow the same scheme. To this end, qPCR data for a larger set of bonafide targets would be helpful especially since both YAP and TAZ end up in the nucleus and loose their phosphorylation upon thrombin treatment (Fig 1). This should also be done for additional data points in the study. Relying on only 1-2 target genes (ANKRD1/CTGF) is critical and might not be sufficient, since those are regulated by many other inputs.

Minor points:
1. How exactly were the cell lysates generated? Are these "whole cell lysates" that contain nuclear proteins? Please add this information to the methods.
2. The authors should exclude that 48 hrs of doxycycline alone does not affect YAP/TAZ activity by using controls in Fig 4.

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**First revision**

Author response to reviewers' comments

Dear Dr. Heath,

Thank you for your email on 10/26/20 regarding the decision on our manuscript (JOCES/2020/254888). We have carefully considered the comments and suggestions from both reviewers and have addressed their concerns as outlined below.

We have performed new experiments particularly to address the link between ARRDC3 and the Hippo pathway in our *in vivo* studies and more thoroughly explained, quantified and displayed the data from our initial studies. We have also made minor modifications to the text in terms of methods and discussion points.

We believe that these changes have strengthened the manuscript significantly and we hope that you will now consider the paper for publication in *The Journal of Cell Science*.

We thank you for your time and consideration. Sincerely, JoAnn Trejo

**Reviewer #1 Comments**
Reviewer #1 stated "*This is a valuable interesting study to tease out TAZ functions alongside ARRDC (a tumor suppressor) and are of interest to both the YAP/TAZ community and the GPCR community as it defines a novel relationship between ▫-arrestin in GPCR induced TAZ activity*". However, the reviewer also raised some concerns and stated "*The role in breast cancer specifically is however somewhat underdeveloped and superficial in nature*.", which is related to key overall issue #1 as noted below. Several specific comments related to key issue #2 regarding aspects of Hippo signaling were also noted and addressed below.

**Key overall issues:**
**1. The effects on breast cancer metastasis are underdeveloped as validation of the mechanism in the tumor tissues has not been done and the significance to TNBC is also not clear. TAZ levels and localization should be assessed in the tumors if the authors wish to establish a clear link.**
We agree with the reviewer's suggestion that assessing Hippo pathway activation in tumor cells re-expressing ARRDC3 is important to link the two pathways in our *in vivo* assessment of breast cancer metastasis.
We show using tail vein model of breast cancer metastasis results in micro-metastatic lesions in the lungs of NSG mice (Fig. 8C and D). However, due to the ongoing COVID-19 pandemic, we were unable to access the histology core at the UCSD Moores Cancer Center to fix and embed the lungs from these tail vein experiments for assessment of TAZ expression.
As an alternative approach, we examined TAZ expression and nuclear localization in tissue sections derived from a mammary fat pad xenograft model study completed prior to the onset of the COVID-19 pandemic. In these studies, ARRDC3 re-expression in MDA-MB-231 cells decreased tumor growth (Fig. 8A). Moreover, in tumors from implanted MDA-MB-231 cells lacking ARRDC3 expression we observe nuclear TAZ localization by immunohistochemistry, whereas TAZ nuclear localization was diminished in tumors from MDA-MB-231 cells re- expressing ARRDC3. This information has been added to the text on page 11 and the data are now included **Figure 8**.

**2. Some aspects of the hippo signaling mechanism which can be better detailed (see specific comments below). Quality of some figures (Figure 3 and most microscopy images) is very low making it difficult to evaluate the findings.**
We agree with the reviewer that some aspects of Hippo signaling can be improved and are addressed below under Specific Comments. We apologize for the low quality of some figures, this was caused by the conversion to a pdf file and reduction to meet size limits. We have corrected this issue by the inclusion of higher resolution and quality images.

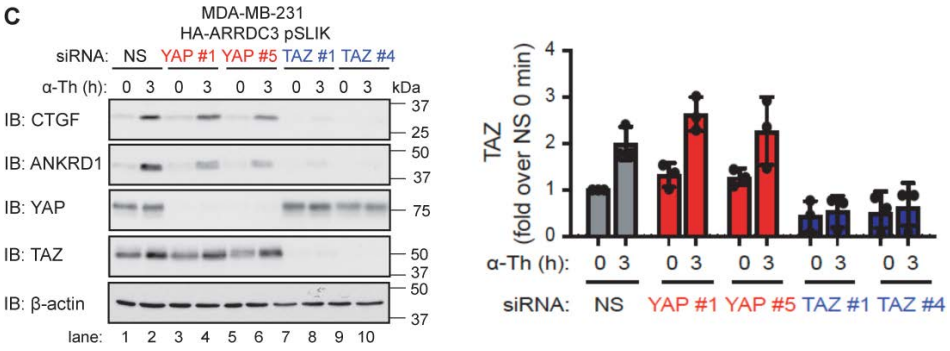
**Specific comments:**
1. **The effect of cell density on YAP localization and activity is well documented in the literature. In figure 1: PI has low density pictures where hippo is likely low as YAP is widely distributed that then translocated to the nucleus in response to thrombin, however in the westerns, PI suggests hippo is high: fundamental to these studies is knowing the cell density in the localization versus the signaling/western studies to be able to faithfully interpret these experiments. As such this needs to be addressed in all figures showing localization and signaling studies.**
We agree with the reviewer's concern. Despite loss of contact inhibition cancer cells including MDA-MB-231 cells display some sensitivity to cell density. To control for potential effects of cell density on YAP and TAZ analysis in different assays, breast cancer cells were seeded at specific densities to yield ~70-80% confluence at the time of the experiments for all assays (see Materials and Methods). The images shown in **Figure 1, A and D**, are representative images of 6 fields of cells from 3 independent experiments. Quantitative analysis of the images indicates that a greater amount of YAP and TAZ are present in cytoplasm versus the nucleus under basal (0 min) conditions. However, after thrombin stimulation an increase in nuclear versus cytoplasmic localization of YAP and TAZ was detected (**Figure 1, C and F**). The microscopy data are consistent with the basal level of YAP and TAZ phosphorylation detected in the immunoblot and correlate with the changes in phosphorylation (**Figure 1 G**). The same seeding and densities were used for all experiments conducted throughout the study. We have provided additional information in the text on page 18 in

the Materials and Methods section regarding cell seeding and density.

2. **Figure 6: ARRDC induction seems to downregulate TAZ activation in the absence of thrombin, thus the effect of thrombin itself is marginal, can authors comment on this?**
This is an interesting point raised by the reviewer. To address the potential effect of ARRDC3 induction on TAZ expression, we quantified total TAZ expression under basal conditions from MDA-MB-231 cells with and without ARRDC3 expression from experiments shown in Figure 6 and found no significant difference. This information has been added to the text on page 9 and the data have been added to **Supplemental Figure 2**.

3. **Nuclear localization studies with TAZ and ARRDC3 are not clear and fractionation is required in this case.**
The reviewer raised concerns regarding the lack of clarity of images showing the effect of ARRDC3 on nuclear translocation of TAZ in **Figure 6**. As stated above, we apologize for the low quality images shown in the original submission that was caused by the pdf conversion and size reduction. We have now corrected this issue and included higher resolution images that clearly show thrombin-induced TAZ nuclear translocation and the effect of ARRDC3 WT and AAXA mutant quantified from 10 fields of cells for each experiment, from 3 independent experiments using ImageJ Intensity Ratio Nuclei Cytoplasm Tool. We are hopeful that the reviewer will find the improved images and quantification now show more clearly the effect of ARRDC3 on thrombin-induced TAZ nuclear translocation acceptable as microscopy is the standard method for examining YAP/TAZ nuclear localization. Moreover, conducting new sets of experiments would be very difficult due to imposed restrictions on research activity at UC San Diego caused by COVID-19 and the pandemic.

**Minor points:**
**1. Does thrombin induce TAZ much more significantly only in the absence of YAP?- some of the westerns suggest this- perhaps a discussion if this is a case is adequate here.**
This is an interesting point raised by the reviewer. To determine if loss of YAP expression by siRNA knockdown results in increased TAZ expression after agonist treatment, we quantified total TAZ expression shown in Figure 2C from three independent experiments. While the data indicate a modest increase in TAZ expression in YAP depleted cells, the results were not significant based on one-way ANOVA analysis therefore not included in the manuscript.




**2. Statistical tests: In multiple comparisons, why was ANOVA not used everywhere by only in select analysis- please clarify?**
We thank the reviewer for the careful analysis of our data. We have reviewed all the data and data analysis and clearly state the method for statistical analysis. ANOVA was used for all multiple comparisons. Changes are indicated in red font.

**3. Title comment: The link to TAZ and metastasis is not very clear, and title may need to be amended.**
Now that we have shown a link between ARRDC3 re-expression and TAZ activity in our *in vivo* breast cancer model as well as in our *in vitro* breast cancer cell migration and invasion assays, we believe this study does address the role of TAZ in metastasis.

**Reviewer #2 Comments**
The manuscript of Arakaki et al. studies the connection of the tumor suppressor ARRDC3 with the hippo downstream effector and oncogene TAZ in triple-negative breast carcinoma (TNBC). They

describe a novel mechanism, in which Par1 and GPCR signaling activates TAZ/YAP. Downstream, TAZ but not YAP stimulates invasion and migration by increased expression of CTGF and ANKRD1. Beside its direct effects on Par1, ARRDC3 also sequesters TAZ keeping it at check. For this cytoplasmic retention, the WW domain of TAZ and a PPXY motif in ARRDC3 interact. So, ARRDC3 has two modes in controlling the activity of TAZ. This is an interesting and novel story presenting a novel mechanism that specifically affects TAZ but not YAP. This contributes to solve the fundamental question of distinct and overlapping functions of YAP and TAZ. In addition, it delivers one possible explanation for a dysregulation of hippo downstream signaling through GPCR signaling. Therefore, I feel this is an important topic and important research for the hippo (and the breast cancer) community.

**Key points:**

**1. The quality or, more accurate, the resolution of Fig.3 (and of some IBs throughout the paper) is really low. Most likely a problem of the pdf conversion? I can't really comment on any blot or cell picture here.**

We apologize for the low quality resolution of immunoblots and data in Fig 3 and other Figures. As the reviewer stated this was an issue associated with the conversion to pdf and file reduction due to size limitation. We have now corrected this issue and included higher resolution images.

**2. Some of the western blots presented raise the question, of how exactly they have been performed. This should be mentioned in the methods section. Have membranes been re-stained? Have they been "stripped" to remove antibodies? Are there "reruns" of samples on different gels? In addition, I would always prefer to see the full blots as supplements. This makes things much easier for everyone involved, specifically for the reader… . This of course, is not my decision.**

All western blot analysis was performed by running samples on separate gels especially when detecting phosphorylated and non-phosphorylated proteins to ensure remaining antibodies did not interfere with signal. In some cases, membranes were cut and probed with different antibodies only if the size difference was permitting. The only membranes that were stripped and reprobed were for the loading controls beta-actin and GAPDH. This information has been added to the methods section on page 18.

**3. The authors show very nicely the effect of thrombin on two depicted YAP/TAZ target genes. Doing this with siRNA against YAP and TAZ (Fig 2) they conclude that primarily TAZ is transmitting GPCR signaling. It would be very interesting to see, how additional YAP/TAZ target genes react and whether they follow the same scheme. To this end, qPCR data for a larger set of bonafide targets would be helpful especially since both YAP and TAZ end up in the nucleus and loose their phosphorylation upon thrombin treatment (Fig 1). This should also be done for additional data points in the study. Relying on only 1-2 target genes (ANKRD1/CTGF) is critical and might not be sufficient, since those are regulated by many other inputs.**

We agree that analysis of a larger set of Hippo pathway targets would allow for a more in-depth understanding of the distinct roles of YAP and TAZ in the context of GPCR signaling in breast carcinoma. Indeed, in future work (now delayed due to COVID-19), we are planning to examine global changes in gene expression by RNA-seq following GPCR activation in cells with either YAP or TAZ siRNA knockdown. This work is quite labor intensive and requires computational analysis and will yield other interesting TAZ-specific targets that would be the foundation of a separate study, and is beyond the scope of the present work.

**Minor points:**

**1. How exactly were the cell lysates generated? Are these "whole cell lysates" that contain nuclear proteins? Please add this information to the methods.**

Whole cell lysates were collected that contain nuclear proteins from non-stimulated and stimulated cells and used for biochemical analysis. This information has been added to the methods section on page 18.

**2. The authors should exclude that 48 hrs of doxycycline alone does not affect YAP/TAZ activity by using controls in Fig 4.**

We agree with the reviewer. This point was also raised by reviewer #1 and was addressed as follows. To address the potential effect of ARRDC3 induction on YAP and TAZ expression, we

quantified total YAP and TAZ expression under basal conditions from MDA-MB-231cells treated with or without doxycycline for 48 h (with or without ARRDC3 expression) from experiments shown in Figure 6 and found no significant difference. This information has been added to the text on page 9 and the data have been added to **Supplemental Figure 2**.

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Second decision letter

MS ID#: JOCES/2020/254888

MS TITLE: α-arrestin ARRDC3 tumor suppressor function is linked to GPCR-induced TAZ activation and breast cancer metastasis

AUTHORS: Aleena Arakaki, Wen-An Pan, Helen Wedegaertner, Ivette Roca-Mercado, Logan Chinn, Taranjit S Gujral, and JoAnn Trejo
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

*Advance summary and potential significance to field*

This is a revised manuscript wherein the authors have done their best to address issues raised by reviewers that improve the impact and rigor of the studies. No additional issues remain

*Comments for the author*

No additional revisions required.

Reviewer 2

*Advance summary and potential significance to field*

Please see the previous review.

*Comments for the author*

All my concerns have been addressed. I still feel that data on additional bona fide targets of TAZ/YAP would be helpful, but I understand that the pandemic is limiting possibilities. Therefore, I do not have further concerns.