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The role of USP7 in the Shoc2-ERK1/2 signaling axis and Noonan-like syndrome with loose anagen hair

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MS TITLE: USP7 regulates ERK1/2 signals in the Shoc2 axis and contributes to the pathogenicity of Noonan-like syndrome with loose anagen hair

AUTHORS: Patricia Wilson, Lina Abdelmoti, Rebecca G Norcross, Eun Ryoung Jang, Malathy Palayam, and Emila Galperin
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. The reviewers indicate that there are several inconsistencies and interpretation of the data, and in some instances parts of figures are not referenced appropriately. These concerns need to be fully addressed. 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

This manuscript contains a huge number of data, representing probably a very significant amount of work. The text is very well written and the presentation of the manuscript is tidy. The images shown appear to result from experiments performed in reliable conditions, and the interpretations of the results appear to be sound and convincing. In brief, the scientific quality of this work seems to be more than enough for publication (assuming that the topic and the significance of the question addressed fit with the the editorial line of the journal).

Comments for the author

In my opinion, this manuscript has only minor issues, as follows:
- In Supplemental Fig. 1C, the interpretation of the results may be somewhat unclear for the reader. Indeed, one may wonder how the expression of Cyclin D is related to Shoc-2 half-life.
- The country of origin (probably USA) is not indicated in the authors' affiliation
- Possible inappropriate spacing is sometimes present around hyphens (e.g., lines 175, 201, 287, 367, 598) or in "feedback" (line 217) or "immunoprecipitates" (line 224)
- From a mathematical point of view, one may wonder whether the use of Student's t tests or ANOVAs could be applied to data found in this study, as it is possible that these data do not fulfill all prerequisites for this type of test (notably in terms of Gaussian distribution).

Reviewer 2

Advance summary and potential significance to field

In this manuscript Wilson et al study the regulation of ERK1/2 pathway activation evoked by the interaction between Shoc2 with the ubiquitin-specific protease USP7. Such interaction subsequently controls the E3 ligase HUWE1 via its deubiquitination, which unleashes its activity, resulting in Shoc2 and RAF ubiquitination. This generates a negative feedback loop that quenches ERK activation. in this line, authors claim that disruption of such mechanisms underlie the role that Shoc2 mutations play in Noonan Syndrome and NSLAH. Even though the proposed model is quite interesting. Most of the tenets are not supported by the data provided and mostly rest on unproven conceptual inferences

Comments for the author

In general, the manuscript is far from being reader-friendly. It is confusing, sometimes convoluted. Hypotheses and claims are expressed as facts and data is not adequately described. Some experiments are difficult to follow as the technical approaches are not adequately explained, so the rationale is lost. All this does not contribute at all to convey the author's message. Added to this, the main flaw of this manuscript is that some of its central tenets are not adequately supported by the data provided ; and some of its conclusions are unfounded.

Specific points.
1) In their experiments, authors utilize a series of Shoc2 mutants these are sometimes referred to as, for example: Shoc2 E89D, Shoc2 QH269/270HY. On other occasions these mutants are named Shoc2 variants E89; GLN270 (HysTyr). This is rather confusing for the reader. It is recommended that the same nomenclature is maintained throughout the manuscript.

2) Figure 1. In cells expressing WT Shoc2, ERK activation shows an acute peak at 7 min (Fig 1B upper panel and support fig 1B) whereas on other occasions it is sustained up to 15 min (Fig 1B lower panel). What are these differences due to?. Obviously, in a study dealing with ERK activation this is no minor nuance and interpretations could vary enormously depending on this. For example,

authors mention that mutant L4731 does not fully rescue ERK activation. This would be so if compared to Shoc2 wt in Fig 1B lower panel, but it does rescue ERK activation if compared to Shoc2 wt in Fig 1B upper panel.

3) On several occasions authors perform experiments under "denaturing" conditions. The rationale of such procedure should be explained as it is far from evident to the general readership.

4) Supp Fig 2. Using a series of deletion mutants for USP7, to identify the domains responsible for Shoc2 interaction, authors conclude that "Shoc2 interacts equally well with the TRAF domain and the UBL regulatory domain". This conclusion is unfounded, since the deltaTRAF construct, which harbors an intact UBL domain, does NOT interact with Shoc2. How do authors explain this?

5) Figure 4A. Authors state "Huwei-induced Shoc2 ubiquitination peaks between 15 to 30 min following EGF treatment". In this experiment Huwei participation is not being tested at all. For reaching such conclusion, the same experiment should be performed with Huwei being down-regulated.

6) In the abstract, authors claim that "disruption of Shoc2-USP7 binding leads to an aberrant activation of Shoc2-bound RAF". Such a claim has never been put to test in the present study

7) Also in the abstract author claim that "our studies reveal a hitherto unknown role of USP7 in the pathogenic mechanisms underlying NSLAH". This is a rather far-fetched conclusion as nothing of this sort has been tested either.

8) In Figure 8D authors propose a model in which USP7 binding to Shock triggers the deubiquitination of Huwei, which would become activated and would subsequently ubiquitinate Shock2 and RAF. This model is hard to reconcile with the data presented in Fig 4A in which inhibition of USP7 enhances Shoc2 ubiquitination. According to the model proposed by the authors, if USP7 is inhibited it would not deubiquitinate/activate Huwei and Shoc2 should not be ubiquitinated.

Reviewer 3

Advance summary and potential significance to field

In the present paper, the authors establish that the USP7 deubiquitinating enzyme is involved in the Shoc2 complex that regulates the ERK pathway activation. USP7 modulates the E3 ligase HUWE1 auto-inhibition and control the Shoc2 ubiquitination levels by HUWE1 deubiquitination. Interestingly, Wilson et al. also identify new Shoc2 mutations associated with the NSLAH syndrome that display aberrant ubiquitination and altered USP7 binding properties.

This is an interesting and well-written manuscript. The data are convincing and presented in a logical way. I have few suggestions that could further improve this publication.

Comments for the author

1. Since no destabilization of the protein was observed, how the authors can explain the biological effect on ERK pathway?

2. In figure 1, there are some inconsistencies between Ub and phospho-ERK levels in E89D et L473I mutants. The E89D mutant displays no Ub alterations but an impaired ERK activation whereas the L473I mutant shows increased Ub levels but no defect in ERK activation. Could the authors comment?

3. Page 6 / lane 150 : the conclusion on the E89D mutant is not true since Ub and phosphor-ERK levels are non-significantly altered following treatment by EGF.
4. Is there a way to induce the activation of USP7 instead of inhibition by P22077 or si-RNA interference in order to observe deubiquinination?

5. In Figure 4A, a control should be added in the input part (total ERK or GAPDH).

6. In Figure 8, a comment should be added on the basal level of USP7 binding by Shoc2 mutants.

7. A clear correspondence between Subject Number in Table 1 and Decipher ID in the Supplemental Clinical Notes should be indicated.

8. Some figures are not used in the manuscript : Figures 2C, 5B, 5C, 8D, Supplemental Figures 1A, 3A, 3B, 3D, 4A.

9. Corrections :
- page 5 / lane 121 : “L437I” instead of “L473I”
- table 1 : replace “kb, kilobases” by “b, bases”
- table 1: replace “missence” by “missense”
- table 1 : indicate what “v” means

First revision

Author response to reviewers' comments

Response to critique

We thank the editor and reviewers for their positive and encouraging response to our paper reporting the discovery of a new molecular mechanism by which USP7 regulates signal transduction via the Shoc2 scaffold complex. We have carefully read the reviewer's concerns, and attempted to rigorously address them in the detailed responses below.

Reviewer #1

We are encouraged by the positive comments of this reviewer and his/her appreciation of the significance of our findings.

1) In Supplemental Fig. 1C, the interpretation of the results may be somewhat unclear for the reader. Indeed, one may wonder how the expression of Cyclin D is related to Shoc-2 half-life.

Cyclin D is a short half-life protein. Using Cyclin D in the experiments testing protein turn- over provides a necessary positive experimental control and often used is similar experiments. We have added this information in legends to Suppl. Fig. 1.

2) The country of origin (probably USA) is not indicated in the authors' affiliation

Corrected in text.

3) Possible inappropriate spacing is sometimes present around hyphens (e.g., lines 175, 201, 287, 367, 598) or in "feedback" (line 217) or "immunoprecipitates" (line 224).

Corrected in text.

4) From a mathematical point of view, one may wonder whether the use of Student's t tests or ANOVAs could be applied to data found in this study, as it is possible that these data do not fulfill all prerequisites for this type of test (notably in terms of Gaussian distribution).

We thank the reviewer for this comment. We have now used a mathematically more appropriate analysis (i.e. Kruskal-Wallis test that is used for non-parametric data with three or more groups or parametric data when unequal variances between groups are assumed). New statistical analysis did not affect our conclusion in terms of the effect of each of the Shoc2 variants on the amplitude of the ERK1/2 signals and is now incorporated into Figure 1C.

Reviewer #2

“In general, the manuscript is far from being reader-friendly.” **The manuscript was read for clarity to streamline the work and the message.**

1) In their experiments, authors utilize a series of Shoc2 mutants these are sometimes referred to

as, for example: Shoc2 E89D, Shoc2 QH269/270HY. On other occasions these mutants are named Shoc2 variants E89; GLN270 (HysTyr). This is rather confusing for the reader. It is recommended that the same nomenclature is maintained throughout the manuscript.

As suggested by the reviewer, we corrected the nomenclature to maintain it throughout the manuscript.

2) Figure 1. In cells expressing WT Shoc2, ERK activation shows an acute peak at 7 min (Fig 1B upper panel and support fig 1B) whereas on other occasions it is sustained up to 15 min (Fig 1B lower panel).
What are these differences due to?. Obviously, in a study dealing with ERK activation this is no minor nuance and interpretations could vary enormously depending on this. For example, authors mention that mutant L4731 does not fully rescue ERK activation. This would be so if compared to Shoc2 wt in Fig 1B lower panel, but it does rescue ERK activation if compared to Shoc2 wt in Fig 1B upper panel.

In the revised manuscript we show new blots (Figure 1B) that are more representative of our results. Data in Figure 1B suggest that Shoc2 mutant L473I cannot rescue ERK1/2 phosphorylation to the level of the WT Shoc2. Additionally, as ERK1/2 phosphorylation is known for being very sensitive to subtle experimental variations, we never compare pERK1/2 signals from different blots or experiments. Nevertheless, results of the several independent experiments convincingly show that when expressed in Shoc2 CRISPR/Cas9 knock-out cells, the Shoc2 L437I mutant cannot fully rescue ERK1/ phosphorylation. These findings are also supported by our new statistical analysis (see Rev 1 comments).

3) On several occasions authors perform experiments under "denaturing" conditions. The rationale of such procedure should be explained as it is far from evident to the general readership.

To assure that no ubiquitinated proteins other than the protein of interest is analyzed, experiments detecting ubiquitination of HUWE1, Shoc2 or RAF-1 are performed under denaturing conditions. All immunoblots of the "denatured" immunoprecipitates are tested to confirm that only the protein of interest is detected in the immunoprecipitates analyzing ubiquitination. We have clarified this point in the text.

4) Supp Fig 2. Using a series of deletion mutants for USP7, to identify the domains responsible for Shoc2 interaction, authors conclude that "Shoc2 interacts equally well with the TRAF domain and the UBL regulatory domain". This conclusion is unfounded, since the deltaTRAF construct, which harbors an intact UBL domain, does NOT interact with Shoc2. How do authors explain this?

We apologize for this oversight. We have repeated experiments using proteins purified from *E. coli* and now show that the ΔTRAF mutant of USP7 recognizes Shoc2. The expression levels of the ΔTRAF mutant of USP7 in 293 cells and *E. coli* were unusually low indicating that the ΔTRAF mutant is not stable. Hence, low detection of the interaction in our immunoprecipitation experiments. Corresponding changes are made in text.

5) Figure 4A. Authors state "Huwei-induced Shoc2 ubiquitination peaks between 15 to 30 min following EGF treatment". In this experiment Huwei participation is not being tested at all. For reaching such conclusion, the same experiment should be performed with Huwei being down-regulated.

Per the reviewer's suggestion, we analyzed Shoc2 ubiquitination in cells depleted of HUWE1 and treated with USP7 inhibitor P22077 (see Figure R1). Our new data show that Shoc2 ubiquitination in cells depleted of HUWE1 was not affected by P220077 treatment. Additionally, in Jang ER *et al., MCB*, 2014 we also demonstrated that *i)* overexpression of full-length HUWE1 and the HECT domain of HUWE1 enhances ubiquitination of endogenous Shoc2 and RAF-1 in cells, *ii)* siRNA depletion of HUWE1 lead to decreased levels of Shoc2 and RAF-1 ubiquitination and *iii)* the HECT domain of HUWE1 is sufficient to ubiquitinate Shoc2 *in vitro*. In this study we also reported that K6, K48 and K63-links are conjugated to Shoc2. Together these experiments validate that Shoc2 is ubiquitinated and that HUWE1 is the E3 ligase that modified Ub links on Shoc2.

Figure R1

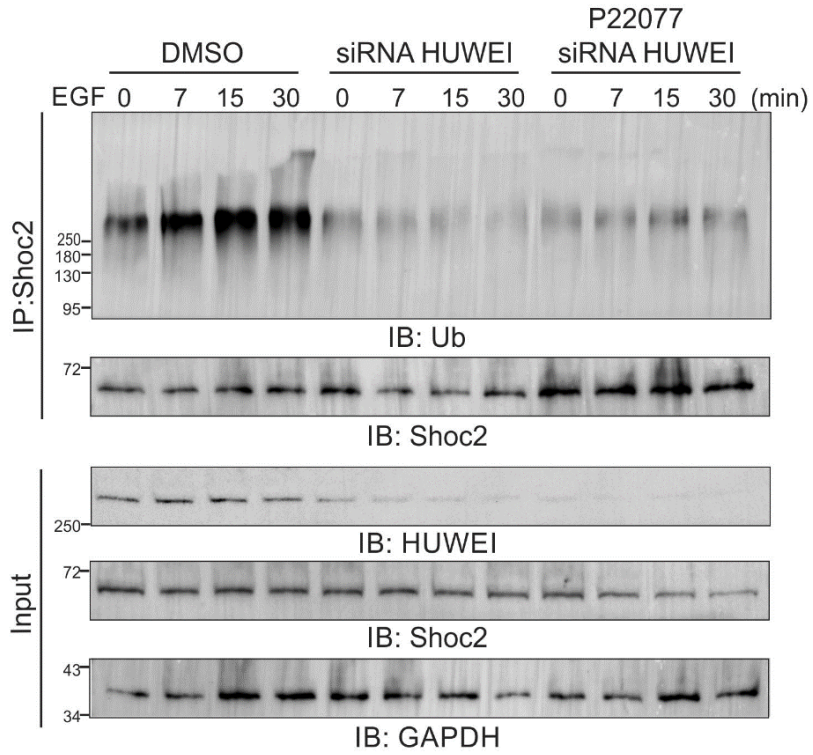

Figure R1. USP7 modulates HUWE1-dependent Shoc2 ubiquitination. Control 293FT cells and 293FT cells depleted of HUWE1 were serum starved for 16 h, treated with USP7 inhibitor P22077 (25μM) for 4 hours and then stimulated with EGF (0.2 ng/ml) for 7, 15- and 30-min. Endogenous Shoc2 was immunoprecipitated under denaturing conditions using Shoc2 antibodies. Shoc2 ubiquitination was detected by immunoblotting using anti- Ub antibody. The immunoprecipitates and cell lysates were analyzed by immunoblotting with anti-Ub, -Shoc2, - HUWE1 and -GAPDH antibodies.

6) In the abstract, authors claim that "disruption of Shoc2-USP7 binding leads to an aberrant activation of Shoc2-bound RAF". Such a claim has never been put to test in the present study.

We have made a correction in text.

7) Also in the abstract author claim that "our studies reveal a hitherto unknown role of USP7 in the pathogenic mechanisms underlying NSLAH". This is a rather far-fetched conclusion as nothing of this sort has been tested either.

We have made changes in the text.

8) In Figure 8D authors propose a model in which USP7 binding to Shock triggers the deubiquitination of Huwei, which would become activated and would subsequently ubiquitinate Shock2 and RAF. This model is hard to reconcile with the data presented in Fig 4A in which inhibition of USP7 enhances Shoc2 ubiquitination. According to the model proposed by the authors, if USP7 is inhibited it would not deubiquitinate/activate Huwei and Shoc2 should not be ubiquitinated.

We agree with Reviewer 2, our data does provide direct support to the model in which USP7 deubiquitinates HUWE1 to become activated. As written in the Discussion, we suggest that EGF-dependent activation of the ERK1/2 pathway induces binding of USP7 to the Shoc2 complex (step1), where USP7 controls the ability of HUWE1 to modify the noncatalytic scaffold Shoc2 (step 2). HUWE1-mediated ubiquitination of Shoc2 and RAF-1 allows for the dynamic range of RAF-1 activity to be fine-tuned, thereby actively monitoring transmission of ERK1/2 signals (step 3). The studies that address the question of in what way USP7 and HUWE1 controls the activity of HUWE1 bound to Shoc2 are outside the scope of this manuscript.

Reviewer #3

We are encouraged by the positive comments of this reviewer and his/her appreciation of the of our findings.

1) Since no destabilization of the protein was observed, how the authors can explain the biological effect on ERK pathway?

As was previously demonstrated in our studies, Shoc2 ubiquitination is a negative-feedback mechanism to fine-tune transmission of ERK1/2 signals. Shoc2 ubiquitination has very little effect on its turn-over. Thus, it was not surprising to find that aberrations in Shoc2 ubiquitination did not result in changes in protein turn-over, but rather had a profound effect on ERK1/2 phosphorylation.

2) In figure 1, there are some inconsistencies between Ub and phospho-ERK levels in E89D et L473I mutants. The E89D mutant displays no Ub alterations but an impaired ERK activation whereas the L473I mutant shows increased Ub levels but no defect in ERK activation. Could the authors comment?

The E89D variant is in the Ras-RAF-1 binding unstructured N-terminal domain of Shoc2. Thus, it is possible that impaired ability of this mutant is related to Shoc2's binding of Ras and RAF-1 signaling proteins and not to proteins of ubiquitin machinery that recognize LRR domain of Shoc2. However, further experiments that are beyond the scope of this manuscript will be needed to validate this hypothesis.

3) Page 6 / lane 150: the conclusion on the E89D mutant is not true since Ub and phosphor-ERK levels are non-significantly altered following treatment by EGF.

Corrected in test.

4) Is there a way to induce the activation of USP7 instead of inhibition by P22077 or si-RNA interference in order to observe deubiquinination?

We appreciate reviewer's comment. However, to the best of our knowledge no activating USP7 mutation has been reported, and the reasons for that would be the complexity of the structural changes that USP7 undergoes to reach its full activity (Kim, R.Q., et al., Kinetic analysis of multistep USP7 mechanism shows critical role for target protein in activity. *Nat Commun* 10, 231 (2019)).

5) In Figure 4A, a control should be added in the input part (total ERK or GAPDH).

We appreciate reviewer's comment. GAPDH control is included in multiple experiments in the manuscript, including Fig. C. We had not observed changes in Shoc2 or GAPDH protein levels in cells treated with P22077 inhibitor.

6) In Figure 8, a comment should be added on the basal level of USP7 binding by Shoc2 mutants.

In the revised manuscript we comment on the basal level of USP7 binding by Shoc2.

7) A clear correspondence between Subject Number in Table 1 and Decipher ID in the Supplemental Clinical Notes should be indicated.

We have included Decipher ID were included in Supplemental Table 1. Supplemental Clinical Notes are now presented as Supplemental Table 2.

8) Some figures are not used in the manuscript: Figures 2C, 5B, 5C, 8D, Supplemental Figures 1A, 3A, 3B, 3D, 4A.

We apologize for this oversight, this was corrected in text.

9) Corrections:
-page 5 / lane 121 : "L437I" instead of "L473I"
-table 1 : replace "kb, kilobases" by "b, bases"
-table 1: replace "missence" by "missense"
-table 1 : indicate what "v" means
Corrected

Second decision letter

MS ID#: JOCES/2021/258922

MS TITLE: The role of USP7 in the Shoc2 - ERK1/2 signaling axis and Noonan-like syndrome with loose anagen hair (NSLAH)

AUTHORS: Patricia Wilson, Lina Abdelmoti, Rebecca G Norcross, Eun Ryoung Jang, Malathy Palayam, and Emila Galperin
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

Please see my report regarding the first version of this manuscript

Comments for the author

In this version, the authors seemed to have appropriately addressed the remarks I made regarding the first version of this manuscript. Therefore I believe the manuscript is now suitable for publication.

Reviewer 3

Advance summary and potential significance to field

Authors answered to all the points. Only a minor modification in figures has been performed by reloading an experiment.

Comments for the author

Authors answered to all the points and performed text corrections.
In Figure 4A, a control should have been added in the input part (total ERK or GAPDH)by reloading the experiment or performing a new one even if the authors claimed that they never observed changes in Shoc2 or GAPDH protein.