Regulation of p53 stability and function by the deubiquitinating enzyme USP42

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 31 July 2011

Thank you for submitting your manuscript on USP42 as a new p53-stabilizing DUB for consideration by The EMBO Journal. It has now been assessed by three referees, who acknowledge the potential importance of these findings and would in principle also support eventual publication. Nevertheless, it is apparent from their comments that all of them feel that a deeper analysis of the USP42-p53 interplay and the underlying mechanisms will be required for EMBO J publication, in addition to clarifying a number of more specific technical points. In this respect, a key question is why USP42 selectively stabilizes p53 in response to stress signals, and how the interaction is modulated during the stress response. Another important issue is the clarification of how USP42 impinges on the p53/Mdm2 axis - which would require p53 ubiquitination status analyses and comprehensive pulse-chase half-life measurements for not only p53 but also Mdm2 and p21, as detailed by the referees; in this respect, the question of why USP42 would be needed in Nutlin-3-dependent p53 activation also becomes relevant. On the other hand, some issues we would consider less crucial within the scope of this study are the requests for USP42 isoform dissection or for assessing USP42 effects on other p53 pathway DUBs.

Should you be willing and able to extend the study with regard to these mechanistic aspects detailed in the referee reports, we could consider a revised version further for publication in The EMBO Journal. Please be reminded however that it is our policy to allow only a single round of major revision, and that it would therefore be crucial to satisfactorily address all the pertinent major and minor points within this revision round for the paper to be ultimately accepted. Alternatively, should you consider such mechanistic extension beyond the scope of the present manuscript and would rather be interested in publishing the basic observation as quickly as possible, we could envision publication of a revised version only addressing the key experimental issues pertaining directly to the present data as a shorter-format paper in our sister journal, EMBO reports. In either situation, we...
would be happy to discuss further proceedings on the basis of a tentative point-by-point response letter should you wish so. At any rate, please do not hesitate to contact me with any questions you may have regarding this revision.

Thank you for the opportunity to consider this work for publication. We look forward to your revision.

With best regards,
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript by Hock et al., 'Regulation of p53 stability and function by the deubiquitinating enzyme USP42', the authors have identified USP42 as another DUB that is able to de-ubiquitinate p53 and under certain settings appears to be needed for efficient p53 stabilization. It is interesting that USP42 expression appears only to affect p53 stabilization at relatively early time-points after applying a stress to the cells.

Is is furthermore interesting to note that USP42 appears also to be important for the stability of a mutant p53 in C33A cells. This observation might make USP42 a putative drug target for cells expression mutant p53.

However, in spite of these interesting observations the manuscript needs improvement. Most importantly, the authors give no mechanistic insights into the mechanism which makes USP42 necessary for the stress-induced stabilization of wt-p53 but is apparently not needed for maintaining basal levels of wt-p53. The latter is actually not discussed or mentioned at all in the manuscript, which is very surprising. Under non-stressed conditions the complex between p53 and USP42 can be found already, which would make it likely that USP42 is needed for maintaining basal wt-p53 levels. Therefore, I would urge the authors to perform CHX chases on siUSP42 cells with wt-p53.

What happens with the interaction under stress conditions? Is it enhanced? It would be interesting to determine the binding site of USP42 on p53.

Another very striking observation is that USP42 appears to be needed even for Nutlin-3 mediated stabilization of p53. Nutlin-3 disrupts very quickly the interaction between Mdm2 and p53, preventing ubiquitination of p53 by Mdm2. So, is USP42 then functioning by removing ubiquitins on p53 put there by other p53 ub-ligase(s)?

I also would like to see an experiment aimed at investigating the ubiquitination status of endogenous p53 under stressed and unstressed conditions, in control and USP42 knock-down cells.

All in all, I feel that more insight into the mechanism by which USP42 is regulating p53 stability is needed.

Other comments:

General:
1. It is known that regulation of levels of Mdm4 is also involved in the stabilization and activation of p53 upon stress. Have the authors investigated Mdm4 levels upon USP42 overexpression or knock-down?
2. All experiments have been performed in only one wt-p53 cell line, U2OS, which has really high levels of Mdm2. I feel that at least some key-experiments like shown if figures 4A, B and figure 6 need to be performed also in at least one, but better two other wt-p53 cell line; preferable one other tumor cell line, and in normal, untransformed cells.

Similarly, the effect of USP42 knock-down on mutant p53 is only investigated in C33A cells. More cell lines with mutant p53 (with varying mutations) have to be investigated to show the generality of the effect.
Specific
Figure 2. No decrease at all of p53 levels in si-Ctrl? If that is indeed the case one has to assume that the observation that exogenously expressed p53 is so stable is caused by the fact that de-ubiquitination is dominant over ubiquitination.

Figure 3A: Why is the catalytic site mutant of USP42 not shown here? Does it work as dominant-negative in cells?

Figures 4A, 4B: I find it very surprising that after 24 hrs of treatment no effect at all of USP42 knock-down is seen, while there is a very strong effect at 12 hrs. I really would like to see a longer time-course experiments, including 16, 20 and 24 hrs time-points. Some blots in figure 4B are not of high quality.

Figure 4D: Not very good figure. There might be some effect on p53 half-life, but needs shorter exposure. Quantification would also be very helpful.

Figure 4A, 4B: Are USP42 levels downregulated by Act.D treatment?

Referee #2 (Remarks to the Author):

The manuscript by Hock et al., describes the identification of USP42 as a de-ubiquitinating enzyme for the p53 tumour suppressor. The authors screened for p53 regulators by using a USP/DUB siRNA library where they found that USP42 knockdown compromised p53 transcriptional activity. They then analysed the biochemical and biological function of USP42, showing that it directly interacts to p53 but not to Mdm2 (the main E3-ligase for p53) and controls p53 ubiquitination, half-life and transcriptional activity. They also provide evidence that USP42 may play a role in the early stages of p53 activation during a stress response. Finally the authors show that USP42 is required for G1 arrest induced during stress, in a p53 dependent manner.

The study is well performed, the experiments well presented (except maybe Fig. 6, see below) and the findings are interesting. The role of USPs/DUBs in protein function regulation is an important area of research.

Specific comments:

The authors have carefully described the implications of the multiple isoforms for USP42, which is a common phenomenon for USPs/DUBs. So it will important to present the data in a way that we can assess, which potential isoforms interact with p53 (fig. 2). The presented western blots do not allow for such evaluation. Also, which is the full length USP42 in these blots? Molecular weight markers should also be included.

The authors show that USP42 does not interact with Mdm2, at least in these experimental conditions, suggesting that USP42 is targeting p53 and not Mdm2. However, USP42 knockdown has a profound effect on Mdm2 protein levels upon stress. The authors suggest that the defect in the increase of Mdm2 levels during stress upon USP42 knockdown is due to compromised p53 activity. This clearly is a possibility but based on the fact that the Mdm2 (and p21) mRNA levels are not dramatically affected upon SUP42 knockdown, the authors should determine the effect of USP42 on Mdm2 half-life, and whether is p53 dependent or not. The p53 mutant cell line used in fig 4D will also be a good system to use for this.

I am a bit confused with the experiment on fig. 6. I am not sure I received the correct figure but I think there is a problem with the labelling in fig 6A and I hope I worked out which is the ActD and nutlin treated panels. The authors show that the effect of USP42 on cell cycle is p53 dependent by knocking down p53. These are valuable and well performed experiments but the authors should also use the USP42 mutants that are catalytically active but do not bind to p53 (fig. 2B). They should also include the full profile of cell cycle analysis.

There are a few USPs/DUBs that control the activity of p53 so the authors should also check the effect of USP42 knockdown on their levels.
The data in fig. 4D for the effect of UPS42 knockdown on mutant p53 are not terribly convincing. I acknowledge that more than 6 h treatment with CHX is a difficult experiment to interpret, so a pulse chase experiment may be more appropriate.

For figure 6B, would it be better if the data +/- ActD are presented on the same panel? In this way it will be easier for the readers of the article to assess the effect of USP42 knockdown.

Referee #3 (Remarks to the Author):

The investigators show that p53 is a binding partner and substrate of the deubiquitinating enzyme USP42. They present evidence that USP42 participates in the accumulation of p53 following the treatment of cells with several p53 activating agents. Evidence is also presented of a requirement for USP42 for full transcriptional activation of p53 and full p53-dependent cell cycle arrest.

This is the first demonstration of a functional interaction between p53 and USP42 and the first study indicating that USP42 contributes to activation of p53. There is a great deal of general interest in the specific roles of deubiquitinating enzymes and in the mechanisms of p53 activation. The manuscript is well written.

Major Points:

1) A pool of 3 siRNA was used to investigate the role of USP42 in upregulation of p53 by p53 activating agents. To control for off target effects multiple individual siRNA should be used. One option would be to look at the effect of multiple individual USP42 siRNA (e.g. exon 13 targeting siRNA, supplementary Figure 1B) on p53 accumulation and activity at a single time point of treatment with the drugs (e.g. 12 hours). An alternative would be to perform rescue experiments using a single siRNA and ectopically expressed USP42.

2) There is no direct evidence that USP42 knockdown interferes with the stabilisation of p53 following treatment with p53 activating agents. In addition, the effect of USP42 knockdown on p21 and Mdm2 protein levels appears to be greater than that on their mRNA levels (Figure 4 compared to 5). The effect of USP42 suppression on the half life of endogenous p53, Mdm2 and p21 should be determined in cells treated with a selected drug or drugs.

Other Points:

1) The investigators should make some comment regarding whether the p53 activating compounds used regulate USP42 or its interaction with p53. This is particularly intriguing because these agents activate p53 by causing different "stresses".

2) Real-time PCR was not used to determine mRNA levels of p53 in Figure 1B. How quantitative was the method used? It may be more appropriate to determine mRNA levels by real-time PCR as was done in the remainder of the manuscript.

3) It would be informative to show Mdm2 protein levels in the westerns in figures 1B and C and 3C.

4) Figure 3c is overexposed for the WT USP42 lanes making it difficult to judge whether stabilisation of p53 is responsible for the clear increase in p53 expression. It would be useful to show an additional lower exposure and also to quantify the westerns (as in Figure 1D).

5) Transfection with the catalytically inactive form of USP42 (C120A) appears to increase the level of p53 (Figure 3c) but this is not commented on.

6) The labelling or description of Figure 6A should be improved to clearly indicate which data is for actinomycin D and which is for nutlin.

7) The labelling of the Y axis in supplementary Figure 1B is incorrect.
Thank you for sending the referees’ comments on our paper on USP42 as a new p53-stabilizing DUB. We are grateful to the reviewers for their helpful comments, and we have revised the paper to take these into account. We now provide additional experimental data to address the mechanism of how USP42 impinges on the p53/Mdm2 axis, including:

- An analysis of the effect of stress on the USP42/p53 interaction (Figure 2)
- Inclusion of the catalytic mutant of USP42 in deubiquitination studies (Figure 3)
- Further evidence for the specificity of USP42 on p53 (Figure 3)
- Half-life studies of p53, p21 and MDM2 (Figure 4)
- The role of USP42 in deubiquitination of p53 in response to stress (Figure 4)
- An extension of the basic observations of that USP42 depletion impedes p53 stabilization in 3 further cell lines and the effects of USP42 depletion on p53-induced cell cycle in 2 further cell lines (Supplementary Figure S2 and Figure 6)
- Deconvolution and rescue of the USP42 siRNAs (Supplementary Figure S2)

However, as you suggested, we have not pursued the dissection of the isoforms or the effect of USP42 on other components of the p53 pathway. These are important and interesting questions, and we hope to address them more fully in future studies.

Referee 1
The referee points out that we cannot see a role for USP42 in the regulation of basal p53 levels. We agree that this is an interesting point, and we had interpreted this as showing that the full activity of MDM2 (and other E3s) that target p53 for degradation is not counteracted by USP42 under normal growth conditions. As the reviewer requested, we have carried out half-life studies in unstressed cells and as predicted - see no change in p53 half life following depletion of endogenous USP42 (new Figure 4A and 4B). Furthermore, as suggested by the referee we now include data to show that depletion of USP42 can affect p53 ubiquitination under conditions of stress (actinomycin D treatment) but that this is not seen under unstressed conditions (new Figure 4E). These results further support the observation that USP42 does not play a major role in determining the basal stability and levels of p53, but contributes to the efficiency of the initial phases of the stress response.

The referee suggested that the interaction between USP42 and p53 may be altered after stress and we agree that this is a very interesting possibility. We carried out a series of co-immunoprecipitations to examine this point. These experiments are quite challenging, since it is difficult to detect a stable interaction between DUBs and their targets. Nevertheless, as we now show in new Figure 2E, we can detect a complex between p53 and USP42 by immunoprecipitation of either partner. Although p53 levels start to increase after stress, we did not see a clear enhancement of the USP42/p53 interaction under these conditions. While USP42 activity may be regulated in other ways following stress (such as post-translational modification), we also consider the possibility that the activity of USP42 is constitutive, but only becomes important as the efficiency of MDM2-mediated ubiquitination of p53 declines in response to stress.

We agree with the referee that it would be interesting to examine the role of USP42 in removing ubiquitin resulting from E3s other than MDM2, but would like to investigate this in further studies.

Referee requested evidence that USP42 affects the ubiquitination status of p53 under stressed and unstressed conditions, and we have now added these data in new Figure 4E.

General Comments
1. We agree that it would be very interesting to look at the interaction of USP42 activity and other regulators of p53 stability, but as we mention above would like to reserve these studies for future investigations.
2. We have now added further key experiments in HCT116, RKO and RPE cells, showing the same effects as seen in U2OS (Supplementary Figure 2C and Figure 6D and E).
3. We have checked various other mutant p53 expressing cell lines, but have been unable to see a strong effect of USP42 knock down on the stability of mutant p53 in these lines. We therefore
would prefer to remove this section of the paper, while we investigate the effect of USP42 on mutant p53 more fully.

Specific
1. It is true that transfected overexpressed p53 has a much longer half-life than endogenous p53 (we now mention this on page 6), and we assume that this is a reflection of inadequate levels of endogenous MDM2 to efficiently target degradation. However, consistent with our suggestion that USP42 activity become evident only when MDM2 efficiency is decreased, we do see an effect of USP42 knock down under these conditions. This system is limited, however, since overexpressed p53 is not regulated in exactly the same way as the endogenous protein, and we would prefer not to draw too many conclusions from these studies. Figure 1 was provided only to describe how we initially identified USP42, all the other studies have been carried out looking at endogenous p53.

2. We have repeated Figure 3A (now Figure 3B) and included the catalytic site mutant. We have occasionally seen evidence that this mutant may act as a dominant negative, but this is so far not entirely reproducible and we would prefer not to claim this to be the case.

3. We have now included the missing time points (16, 20 and 24 hours) in Supplementary Figure 2A. These data confirm that the effect of USP42 is limited to a relatively narrow time frame within the initial phases of the stress response.

4. Figure 4D has been removed.

5. We have not seen a consistent down-regulation of USP42 in response to actinomycin D treatment, although a slight decrease is seen in some experiments. We are currently investigating further how the activity of USP42 may be regulated.

Referee 2
Specific Comments
1. We agree with the reviewer that an investigation as to the role of the various USP42 isoforms will be very interesting, but we hope that this will become part of a future study. We used siRNAs that should target all the isoforms and so believe our present study addresses the general activity of USP42. We have included molecular weight markers in Figures 2D and E.

2. We have examined the half-life of p21 and MDM2 after USP42 knock down, as suggested by the reviewer (Figure 4A and 4B). These results show that USP42 depletion does not clearly alter p21 or MDM2 stability.

3. We apologize for the mistake in labeling in Figure 6, which we have now corrected. We also provide the quantification for the full cell cycle analysis in Figure 6A.

4. All of the experiments in Figure 6 examine the effect of modulating endogenous USP42 levels, not overexpression of USP42. Once again, we are sorry for the confusion arising from our mislabeling of the Figure.

5. The referee makes the interesting suggestion that USP42 may regulate the stability of other DUBs that regulate the p53 pathway. We will pursue this suggestion but hope to make this part of a future study. We certainly agree that USP42 is likely to have additional functions that may be p53-independent and we now mention this possibility in the discussion.

6. Figure 4D has been removed.

7. We remade Figure 6B (new Figure 6F) to show all the data on the same Figure, but felt that this made things look more, rather than less confusing. We have been careful to keep the axes of the 4 graphs the same so that they can be easily and directly compared. We hope the reviewer will be satisfied with this approach.

Referee 3
Major points
1. As suggested, we have examined the effects of each individual siRNA on the expression levels of USP42 and p53 (Supplementary Figure 2D) and show all three can lower USP42 levels and
impede p53 stabilization. To further provide evidence of specificity of the siRNA effect, we have also carried out the requested rescue experiment, where we show that the reduction in p53 levels seen following depletion of endogenous USP42 can be rescued by overexpression of ectopic USP42 (Supplementary Figure 2E).

2. We have included results showing the effect of USP42 knock down on the half-life of p53, p21 and MDM2 under unstressed conditions (Figure 4A and 4B). Unfortunately, we were unable to obtain convincing half-life data under conditions of stress, probably because it is difficult to catch the window during which USP42 knock down is effective to measure time points for the half-life study. However, we have now included data to show that knock down of US42 leads to enhanced levels of p53 ubiquitination in response to stress (Figure 4E), consistent with a reduction in stability and overall protein levels.

Other points
1. As we discuss above, we have included data to show that stress does not appear to affect the interaction between USP42 and p53. While it is clearly possible that stress signals modulate the activity of USP42 in other ways, it is also possible that our data reflects a constitutive activity of USP42 that becomes important only during the initial phases of the stress response.
2. Figure 1 was provided to show the screen in which we initially identified USP42 and was not intended to reflect a detailed study of the effect of USP42 on p53. We have carried out the requested real time PCR to measure mRNA levels in all subsequent experiments, where we examine the effect of USP42 on endogenous p53.
3. We have repeated the results in Figure 3C by infecting cells to achieve a lower level of USP42 expression in most of the cells. The results show clearly that wild type USP42 increases the half life of endogenous p53, but not another rapidly degraded protein, Myc. Quantification of these data is shown in Figure 3D.
4. As mentioned above, we have occasionally seen evidence that the mutant USP42 can function as a dominant negative but this result is not yet completely reproducible.
5. We apologize for the mislabeling of Figure 6, which we have now corrected.
6. The labeling of the Y-axis of supplementary Figure 1B has been corrected ñ thank you for pointing this out.

We thank the reviewers for their time and helpful comments, which have allowed us to add further experimental data and significantly improve our study. I hope that you will now find the paper suitable for publication in the EMBO Journal.

2nd Editorial Decision 13 October 2011

Thank you for submitting your revised manuscript on p53 regulation by USP42 for our consideration. We have now received the feedback from two of the original referees, which you will find copied below. In light of their opinions and recommendations, I am please to inform you that we consider the manuscript now suited for The EMBO Journal without further experimental requirements. There are however several presentational and editorial aspects that will still need to be addressed in a final round of minor revision:

1) Please respond to the minor issues raised in the reviewers' comments by corrections or explanations in the text where appropriate. Further experiments addressing Mdm2 or p21 ubiquitination (as asked by referee 2) will in my view not be necessary at this point, but should you already happen to have such data it may be helpful to include them.

2) Please revise the reference list to conform to the citation style of The EMBO Journal (as specified in our Guide to Authors). Please also make sure to amend citation volumes for references that still lack them, such as Yuan et al 2010

3) We will require an Author Contribution statement in the manuscript text (next to the Acknowledgement section)
4) The first sentence of the abstract appears grammatically not correct (I suspect 'activated' should read 'activating'). Furthermore, I wonder whether the abstract could be revised to put more emphasis on the novel findings and conclusions rather than on general background; e.g. noteworthy results like the differential role of USP42 in cell cycle arrest vs apoptotic p53 functions are not really stressed.

5) Image quality or resolution is insufficient for production/publication in several of the blot or gel panels (e.g. Fig 3A, several panels in Fig 4, Fig S2), and there are also cases where contrast/brightness settings have been too extensively adjusted thus obscuring all background bands and noise (e.g. Fig S2D and S2E). Therefore please carefully review all primary and supplementary figures and incorporate higher resolution and/or quality source images into the panels where appropriate.

6) We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to ask you if you would be willing to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission selecting "Figure Source Data" as object type, and would be published online with the article as a supplementary "Source Data" file.

I am therefore returning the manuscript to you once more for a final round of modification, and hope that you will be able to get an accordingly revised final version back to us as soon as possible. Should you have any further questions in this regard, please do not hesitate to contact me.

Sincerely,
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This revised version of the manuscript by Hock et al. has strongly improved in quality. It is a pity that the authors have not been able to provide an explanation for the observation that USP42 is only needed at early stages upon stress but not at later stages, and is also not needed for basal levels of p53. However, the authors have clearly shown now a function for USP42 in the regulation of the p53 response upon various forms of stress.

Referee #2 (Remarks to the Author):

The revised manuscript has been improved with the addition of new data.

Specific points:
Fig. 4E: Could the authors look for ubiquitination levels of Mdm2 and/or p21? This experiment may help the authors to decipher the role of USP42 on Mdm2/p21 stability upon stress
Fig. 5: I am not sure what the labelling "mRNA variation rate (%)" really means? Also not clear in the reference Vigneron et al. 2010
Suppl. 3B: "vexpressed" instead of expressed
Thank you for sending the referees’ comments on our paper on USP42 as a new p53-stabilizing DUB. We are very happy that you now find the paper suitable for publication.

1. We have attended to the comments from reviewer 2 in the text, explaining mRNA variation rate in the legend to Figure 5 and correcting the typo in Supplementary Figure 3B. We have not examined the ubiquitination of MDM2 or p21, but would not anticipate these to be affected, since the half-lives of these proteins remain the same regardless of USP42 expression.

2. We have revised and corrected the reference list to conform to the EMBO Journal style.

3. The author contribution statement is included.

4. The first sentence of the abstract is corrected, and the abstract amended to more fully explain the novel findings of our study.

5. The image resolution and contrast of the Figures has been improved.

6. We have included the primary data for the blots. In many cases, however, we have cut membranes to be able to blot the same gel with several antibodies. This approach allows a direct comparison of the expression of numerous proteins in the same experiment, but limits the size of the original blot. In general, we find that the antibodies used for these studies are clean and do not give many background bands - I think this is clear from the source data that we have provided.