JTV1 Co-activates FBP to induce USP29 transcription and stabilizes p53 in Response to Oxidative Stress

Juhong Liu, Hye-Jung Chung, Daniela Malide, Liusheng He, Miroslav Dundr, David Levens

Corresponding author: David Levens, National Cancer Institute Bethesda, US

Review timeline:

- Submission date: 17 March 2009
- Editorial Decision: 31 March 2009
- Resubmission: 10 August 2009
- Editorial Decision: 06 September 2010
- Revision received: 07 December 2010
- Editorial Decision: 22 December 2010
- Revision received: 08 January 2011
- Accepted: 11 January 2011

Transaction Report:

(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 March 2009

Thank you once more for submitting your research manuscript (EMBOJ-2009-70869) to our editorial office. I am very sorry for the really unusual delay of its editorial evaluation - we normally aim at reaching an initial decision within a few days or maximally one working week. However, in this case the situation was more complicated, with a substantial part of the delay being due to the fact that a related manuscript by Rabenhorst et al had not been cosubmitted for our consideration and only reached us early last week (just as I was about to leave on a business trip). I realized that this related manuscript was indeed rather peripheral to the evaluation of your present submission - however the way it was referred to in your study was somewhat unfortunate and confusing and lead to the misunderstanding of a similar or parallel mechanism being described there. This related story aside, I should say we were nevertheless hesitant given the precedent of a differentiation-inducing function of JTV1/p38 (by inducing FBP ubiquitination and thus blocking c-Myc transcription), and of JTV1/p38 directly binding p53 and thereby protecting it from Mdm2. I therefore decided at this stage to additionally consult with an expert member of our Editorial Advisory Board - which consequently delayed our decision somewhat further. The outcome of these editorial consultations was, I am afraid to say, was not a fully positive one. While the editorial board member appreciated the identification of a potential novel mechanism for p53 regulation by FBP1 and JTV1/p38, s/he remained at this stage unconvinced that the current level of analysis is sufficient to strongly support the conclusions on p53 regulation, and that the paper would therefore be unlikely to fare well under in-depth review by our (sometimes quite critical) referees (please find the pertinent part of his/her comments copied below). In light of this recommendation, I have therefore decided to return the manuscript to you with the message that we will not be able to consider it for
publication at this time; that said, I would however be happy to once more consider a new submission on this topic should future work allow you to strengthen the part in question. At this point, however, I am sorry to not be able to come to a more positive conclusion.

Yours sincerely,

Editor
The EMBO Journal

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Advisor's input:

On the other hand, it seems to me the validation of the proposed regulatory circuit using endogenous proteins, stability measurements for p53, relationship to known E3 ligases for p53, regulation of p53 downstream targets effectors is very low, for some elements of the story extremely low, so I would invite him to come back when he has a fully validated story.

Resubmission 10 August 2010

Enclosed you will find our manuscript "JTV1 and FBP Induce USP29 transcription to activate p53". In this manuscript we report that JTV1, a core component of a multi-tRNA aminoacyl synthetase complex, is released in response to oxidative stress and translocates to the nucleus where it serves as a transcriptional coactivator of the Far Upstream Element Binding Protein (FBP). A previously unstudied deubiquinitating enzyme, USP29 is up-regulated by the combined action of FBP and JTV1. USP29 in turn reverses mdm2 mediated p53 destabilization. The dramatic stabilization of p53 leads to expression of p53 targets and apoptosis.

This is an extensively reworked and greatly improved version of EMBOJ-2009-70869 submitted to you a bit more than a year ago. You returned the manuscript to us unreviewed after consulting an Editorial Advisory Board consultant who wrote, "...it seems to me the validation of the proposed regulatory circuit using endogenous proteins, stability measurements for p53, relationship to known E3 ligases for p53, regulation of p53 downstream targets effectors is very low, for some elements of the story extremely low, so I would invite him to come back when he has a fully validated story...". Indeed you suggested that you would "...be happy to once more consider a new submission on this topic should future work allow you to strengthen the part in question". We're back.

The new manuscript addresses all of the consultant’s concerns. Most importantly, whereas the previous version of the manuscript relied on the expression of transfected proteins to ascertain function, we have now demonstrated how the proposed scheme operates in response to oxidative stress using only endogenous proteins.

This work adds new dimensions to the functions of FBP and JTV1 and reveals a new and major player (USP29) that needs to be added to the p53 network. We believe that this manuscript will prove to be of broad interest and general importance to the readership of the EMBO Journal.

While we have complete confidence in whomever the Editors might select to review this manuscript, we respectfully request to exclude Dr. Sunghoon Kim whose model for JTV1 action is seriously challenged by the results reported here. We will be happy to provide any additional information or to address any concerns that you, the Editorial Advisory Board or the reviewers might have.

2nd Editorial Decision 06 September 2010

Thank you for submitting a new version of your previous manuscript EMBOJ-2009-70869 for our consideration. It has now been assessed in-depth by three expert reviewers, whose comments you
will find copied below. While these reviewers all consider your findings on a new regulatory link between JTV1, FBP, p53 and USP29 quite interesting and also potentially important, referees 1 and 3 however at the same time raise a number of substantive concerns that currently still preclude publication in The EMBO Journal. Should you be able to adequately address these issues, we should be able to consider a revised version further; but please note that for such a revision to be successful, it will be particularly important to provide more definitive evidence for the existence and relevance of the proposed new regulatory link under physiological conditions - including more careful choice of relevant cultured cell systems where appropriate, conclusive demonstration of interactions on the level of endogenous proteins, and supporting knock-down experiments. I realize that satisfactorily addressing these criticisms may require a significant amount of further time and effort, but I do agree with the referees that addressing these concerns is essential to make the study a strong candidate for publication in a broad general journal such as this one. Should you feel confident that you may be able to adequately address these critical points, then please prepare and submit a revised manuscript using the link below, keeping in mind that it is EMBO Journal policy to allow a single round of major revision only (making it important to diligently answer to all the various major and minor points raised at this stage). When preparing your revision, please also bear in mind that your letter of response will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html).

Finally, please also briefly specify the individual author contributions, either in the acknowledgements section or in an adjacent separate section, as we are attempting to adopt this as a common policy now. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding this revision.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREER REPORTS:

Referee #1 (Remarks to the Author):

The authors identify Jvt1 as a co-activator of Fbp1 and show that together they target Usp29, which de-ubiquitinates and thereby stabilizes p53. Jvt1 is normally a cytosolic protein, but translocates to PML bodies after oxidative stress. This is an interesting story: the strength is that it shows a novel and exciting signal transduction pathway. It contains many new observations and, as is the case with such stories, not all of them are documented in the current version in sufficient detail. In my view, the story deserves to be published provided a number of key issues are resolved.

1. The starting observation of the paper is that Jvt1 regulates expression of Usp29. This observation is documented after transient ectopic expression of Jvt1 in HeLa cells and sorting for transfected cells, indicating that massive overexpression may be necessary to observe this regulatory circuit. The authors need to provide evidence that Jvt1 is a physiological regulator of Usp29 expression, e.g. by depleting Jvt1 and by more moderate expression.
2. In Figure 1A, a better control would be to use HA antibodies on non-transfected cells, which would rule out that the a-HA antibody crossreacts with FBP.
3. It is not completely obvious whether the interaction of endogenous Jvt1 and Fbp has been documented and it would be good to see this in this paper.
4. Figure 3cff. would benefit from a quantification how many nuclear dots are seen per cell in unstressed and stressed situations.
5. Antibodies for Usp29 are commercially available and it is not clear why they were not used to demonstrate interaction of endogenous proteins. They could also be used to demonstrate induction of endogenous Usp29 expression in Figure 4c and after oxidative stress.
6. In Figures 4d and 6, the choice of HeLa cells is unfortunate since the status of p53 is unclear due to the presence of the papillomavirus E6 protein- most people would argue p53 is virtually inactive in these cells. Moreover, the key ligase degrading p53 in these cells is E6AP, nit Mdm2, so it is unclear how Usp29 would act here. These analyses have to be repeated in a better cell system.

7. Data showing that endogenous Jtv1 and Fbp1 are required for induction of Usp29 after oxidative stress appear to be missing unless I have missed them.

Referee #2 (Remarks to the Author):

In the manuscript by Liu et al., the authors report the intriguing transcriptional induction of USP29, a ubiquitin specific peptidase, by FBP and JTV1 resulting in stabilization of p53 and apoptosis in a model cell line. FBP is a protein known to positively regulate the MYC gene, while JTV1 is a Myc target that plays a central role in the structural integrity of the multi-aminocyl-tRNA synthetase complex. In this regard, Liu et al. sought to determine the function of JTV1 which localizes in the nucleus along with FBP. They found through gene expression profiling of cells ectopically expressing JTV1 or an alternatively spliced, constitutively nuclear JTV1-Alt (JA). USP29 is one of the induced target among 142 genes that were altered by JA expression. JTV1 was found to induce the expression of USP29 through FBP binding sites. Because of a previous implication of JTV1 in p53 stability, the authors sought and found that USP deubiquinates and stabilizes p53. To place this series of events in biological context, the authors document that oxidative stress (hydrogen peroxide treatment) induces JTV1 nuclear localization and p53 accumulation associated with cell death. Overall, the authors have uncovered an important role for JTV1 and FBP in stress response. The study is technically very well-executed and the data supports the conclusions drawn. In this regard, it is interesting to note that while FBP is upstream and JTV1 is downstream of MYC. Whether FBP and JTV1 may play a role in MYC oncogene stress response deserves some discussion. Is it possible on based on the authors' observations that over-expression of MYC, which is known to induce oxidative stress and JTV1, could utilize the same pathway to illicit a p53 response. Given the Levens' lab long standing interest in the regulation of MYC, this concept deserves some discussion particularly in the context of cells (primary) that have wild-type p53.

Referee #3 (Remarks to the Author):

In this manuscript by Liu et al., the authors have investigated changes in expression of genes upon overexpression of JTV1 or a splicing variant, JA. Both these JTV1 forms interact with FBP, a DNA binding protein that has been reported both to activate and repress transcription of target genes. The authors find among the genes changed in expression level upon JTV1/JA overexpression the USP29 gene. Indeed, the UPS29 gene appears to have an upstream FBP binding site, named uFUSE, and upon overexpression JTV1/JA can be found binding to that promoter element. In this respect it is surprising that JTV1/JA appear to bind to FBP with same affinity (figure 1A) and to bind similarly the uFUSE element as determined by ChIP (Figure 1F, and the activation of the endogenous USP29 promoter (Figure 2D) is also similar, while JA is found in the nucleus while JTV1 is (almost) exclusively in the cytoplasm (Figures 3A, 3B). Furthermore, with the use of a USP29 reporter construct, JA is more active than JTV1 (Figure 2C). How can the authors explain these apparent contradictions?

Can the authors explain why in figure 1A, 1B they use HeLa cells, in figure 1F MCF-7 cells, in figure 2A again HeLa, and in figure 2B, 2C H1299 cells. The choice of cells in figures 2D, 2E and 2F is not mentioned in text or Figure legends.

Is the basal level of USP29 higher in H1299 and MCF-7 cells compared to HeLa cells? The localization studies have been exclusively done in HeLa cells, while several of subsequent studies on USP29/p53 are done in MCF-7 cells. So, I would also like to see the localization of JTV1/JA in MCF-7 cells, at least.

In figure 2B the authors show that knocking down FBP reduces USP29 mRNA in H1299, as can be expected if there is a significant basal expression level. Also JTV1 knock-down reduces USP29 mRNA levels. This should indicate that JTV1 is expressed to some extent in the nucleus in these cells, but the authors state that 'under normal growth conditions, FBP and JTV1 were independently maintained as expected since FBP and JTV1 were segregated in separate subcellular compartments'. Can the authors explain?
As an introduction to the subcellular localization part, the authors state: Our data indicate that JA is a more potent activator of USP29 (and likely other FBP targets) than JTV1. This is, as mentioned above, not supported by the data shown in figures 1 and 2 regarding the endogenous USP29 gene.

In figure 3A the authors show that the subcellular localization of overexpressed JTV1 depends on the amount of plasmid transfected. However, they do not say how much is transfected relative to the number of cells in this experiment, which means that conditions cannot be compared to other transfection experiments. The authors should really provide this information, and discuss it in the light of the other experiments.

Is the amount of GFP-JTV1 transfected in figure 3A-c the same as in 3A-e? Would the authors not have expected that overexpressed FBP would by itself already recruit some JTV1 into the nucleus? Is the FBP/JTV1 interaction much weaker than the interactions between JTV1 and the ARS complex?

In figures 3C, 3D, 3E and 3F the authors show that treating cells with H2O2 (rather high doses, I think) does lead to some relocation of JTV1 into the nucleus, where it partly co-localizes with FBP, also with PML. For me, it is a bit unclear why the authors have chosen H2O2. Of course, it induces apoptosis and it does activate p53, but induction of apoptosis by H2O2 is only for a small part dependent on p53. Did the authors also try other, maybe more p53-specific, stresses like ionizing radiation or camptothecin? Or, is this effect on JTV1 very much stress specific? Did the authors try maybe a somewhat more physiologically relevant way to increase oxidative stress, like treating cells with menadione?

In figure 4 the authors show that overexpression of JTVB/JA increases p53 protein levels, in an USP29 dependent manner. Figure 4C suggests that knocking-down USP29 in MCF-7 does not alter basal p53 levels. So, USP29 is not necessary for maintaining basal p53 levels? Did the authors not test normal, not tumor cells, which might express higher levels of endogenous USP29?

In figure 5 the authors show that overexpressed USP29 can co-immunoprecipitate with p53, and that it leads to stabilization of p53 in MCF-7 cells. USP29 does not affect Hdm2 protein levels or Hdm2 stability directly. The experiment in figure 5B could suggest that USP29 overexpression reduces the ubiquitination of p53, but this experiment is not convincing. Why not transfec a tagged Ub construct in MCF-7 cells, perform IP or pull-down targeting the tag or anti-p53, and blot vice versa (with appropriate controls). That would be more convincing. Even such an experiment would not prove that USP29 is directly de-ubiquitinating p53. Therefore, in vitro experiment with purified proteins should be performed.

In figures 5F and 5G it is found that the overexpression of JTV1/JA or UPS29 leads to some induction of apoptosis, in a p53-dependent manner. This surprises me somewhat, especially in the HCT116 cells. One would expect that the observed induction of p53 levels is independent of, and not accompanied by post-translational modifications like phosphorylation. In that respect, it should work more-or-less the same as increasing p53 levels by Nutlin-3 treatment. Nutlin-3 has been shown to mainly induce cell cycle arrest and a senescence-like status in HCT116 cells.

So, is there a difference in p53 stabilization by USP29 and Nutlin-3? Did the authors check whether transfected cells (also) entered, maybe initially, a cell cycle arrest?

In relation to figures 5F and 5G the authors state: More apoptosis occurred 48hrs post transfection of JTV1 or JA than at 24hrs (Fig.5F), indicating that these factors must accumulate to trigger apoptosis in the absence of genotoxic stress. This statement must be removed. The difference between 5F and 5G is not only the time of analysis, but also a difference in cell line (HCT116 versus HeLa), so you never know why in HeLa the number of apoptotic cells is higher than in HCT116 cells.

In figure 6 it is investigated whether activation/relocalization of JTV1 into the nucleus upon H2O2 treatment leads to induction of UPS29 levels, and subsequent induction of p53 and apoptosis. Figures 6A and 6B are quite convincing: H2O2 treatment leads to increased USP29 mRNA levels and increased recruitment of JTV1 and FBP to the uFUSE element.

Figure 6C is not convincing. First, it is unclear what the indicated values mean. Is the increase in
percentage apoptotic cells compared to untreated cells? Does knock-down of JTV or USP29 affect the percentage of apoptotic cells in mock-treated conditions? The knock-down of JTV1 has a stronger effect than knocking-down USP29 or p53. So, there is no correlation between p53 levels and the induction of apoptosis. Therefore, my conclusion would be that knocking-down JTV1 partly inhibits H2O2-induced apoptosis, but this effect is largely p53-independent. It would have been much more informative if the authors had used HCT116 p53++ and p53-- cells. And, maybe not look at apoptosis but G2/M arrest since that is the main affect of H2O2 on HCT116 cells, although this seems to be mainly p53-independent. So, like mentioned before, a more clean p53-activating treatment that would also activate USP29 would be more informative. I feel that lysates for Western blots should also be made of the treated cells at the time of harvesting for FACS analysis.

In conclusion, the authors have shown clearly that JTV1/FBP can activate UPS29 transcription and that overexpressed USP29 can interact with p53 and can stabilize p53. Treatment with H2O2 also activates USP29 transcription and induces apoptosis in HeLa cells. At the same time H2O2 also increases p53 levels, but the contribution of increased p53 levels to the induction of apoptosis is not clear.

A potentially very interesting story, but some points have to be worked out. It is not proven that USP29 directly de-ubiquitinates p53, and a physiological relevant condition whereby USP29 (via JTV1/FBP or otherwise) regulates p53 activity has to be found.

Referee #1 (Remarks to the Author):

The authors identify Jvt1 as a co-activator of Fbp1 and show that together they target Usp29, which de-ubiquitinates and thereby stabilizes p53. Jvt1 is normally a cytosolic protein, but translocates to PML bodies after oxidative stress. This is an interesting story: the strength is that it shows a novel and exciting signal transduction pathway. It contains many new observations and, as is the case with such stories, not all of them are documented in the current version in sufficient detail. In my view, the story deserves to be published provided a number of key issues are resolved.

1. The starting observation of the paper is that Jvt1 regulates expression of Usp29. This observation is documented after transient ectopic expression of Jvt1 in HeLa cells and sorting for transfected cells, indicating that massive overexpression may be necessary to observe this regulatory circuit. The authors need to provide evidence that Jvt1 is a physiological regulator of Usp29 expression, e.g. by depleting Jvt1 and by more moderate expression.

We thank the reviewer for this clear directive. We have revised the title to better reflect our new understanding of the physiological role of JTV1 that developed from consideration of the reviewer’s helpful thoughts. We believe that we have now defined and confirmed a precise physiological for the JTV1 mediated induction of USP29. The data in the revised manuscript show that, in response to oxidative stress, endogenous JTV1 translocates to the nucleus, associates with the endogenous FBP and induces endogenous USP29 expression. USP29 then stabilizes p53 and elicits apoptosis. We show that this program occurs in several cell lines, including HCT116, a standard cell line for the analysis of p53 function and apoptosis. The results from experiments using knockdown of endogenous proteins were entirely consistent with the results and interpretations obtained from experiments relying upon transient transfection of the same proteins. Of note, USP29 has not been detected in cells under physiological growth conditions. Therefore, we believe USP29 contributes little, if any, to the stability of p53 under normal growth condition, but plays a predominant role in stabilizing p53 in response to oxidative stress.
2. In Figure 1A, a better control would be to use HA antibodies on non-transfected cells, which would rule out that the α-HA antibody crossreacts with FBP.

We in fact have performed other co-IP experiments where anti-HA antibody was used with lysates from HA empty vector transfected cells. The data indicate that this well-characterized, widely used anti-HA antibody does not cross-react with FBP. We have replaced Fig.1A with this set of experiments.

3. It is not completely obvious whether the interaction of endogenous Jtv1 and Fbp has been documented and it would be good to see this in this paper.

In the absence of signaling or stress, JTV1 and FBP are physically separated into different cellular compartments, with JTV1 being cytoplasmic and FBP being nuclear. Thus, FBP and JTV1 should not and do not interact with each other under physiological conditions. Our data indicate interaction between endogenous JTV1 and FBP was only observed after oxidative stress and not in untreated cells. This is shown in Figure 3F. This, together with other data, suggests that the FBP-JTV1 interaction is a stress response that regulates a pulse of transcription that in turn helps to determine cell fate.

4. Figure 3c ff. would benefit from a quantification how many nuclear dots are seen per cell in unstressed and stressed situations.

Since different nuclear foci are localized in different focal planes, an absolute count of nuclear foci would require quantitation using z-stacks. Although we have not collected enough z-stacks to obtain such an exact count, we nevertheless have reanalyzed the images collected to obtain the relative difference in the number of foci before and after oxidative stress. These data including the mean and standard deviation of this analysis are shown in the revised Fig. 3D. To confirm that oxidative stress induced JTV1 nuclear uptake is a common phenomenon, we have also examined MCF7 and HCT116 cells. The number of foci in MCF7 and HCT116 are also presented in the revised Figure 3D and in the confocal images included in Supplemental Figure 6. All these experiments showed a clear increase in both the number and size of the nuclear JTV1 foci after oxidative stress.

5. Antibodies for Usp29 are commercially available and it is not clear why they were not used to demonstrate interaction of endogenous proteins. They could also be used to demonstrate induction of endogenous Usp29 expression in Figure 4c and after oxidative stress.

We had meticulously tested all four commercially available anti-USP29 antibodies prior to the initial submission of the manuscript. Unfortunately none of the antibodies was able to detect endogenous USP29, even after oxidative stress. Only one of the antibodies detected as a faint band of the protein in the H1299 cell lysate after HA-USP29 expression vector transfection. Because the expression levels of USP29 in bacteria and in insect cells are also very low, we have been unable to generate a suitable polyclonal USP29 ourselves. Therefore we rely upon the detection of endogenous USP29 mRNA using qPCR.

6. In Figures 4d and 6, the choice of HeLa cells is unfortunate since the status of p53 is unclear due to the presence of the papillomavirus E6 protein- most people would argue p53 is virtually inactive in these cells. Moreover, the key ligase degrading p53 in these cells is E6AP, nit Mdm2, so it is unclear how Usp29 would act here. These analyses have to be repeated in a better cell system.

We appreciate this comment and fully agree with the referee’s concern. To confirm that JTV1’s role in p53 stabilization is not peculiar to HeLa, we have repeated the experiments shown in Figures 4d and 6 of the original manuscript in HCT116 cells. The localization of JTV in HCT116, MCF7 and HeLa cells were all similar. In HCT116, just as in HeLa, USP29 was induced in response to hydrogen peroxide. This induction is JTV1 dependent since JTV1 knockdown
compromised this response. In HCT116 cells, both JTV and JA induced pro-apoptotic Bax and PUMA (new Fig. 4F) and provoked apoptosis (new Figure 6C). As a result in HCT116, just as in HeLa, knockdown of JTV1 increased cellular resistance to H2O2 triggered apoptosis. The data are presented in the revised version as new Figure 7D, E and F. In addition, we also tested JTV1 and JA induced apoptosis in another commonly used p53+/+ and p53−/− cell pair, U2OS and SAOS2. The experiments consistently showed that cells lacking p53 better tolerate JTV1/JA over-expression.

Why did our experiments with HeLa work so well? Our data indicate UPS29 is very efficient and specific for the removal of poly-ubiquitin from p53. We detected no USP29-Hdm2 interaction, and in ubiquitination assays, USP29 had only a minimal effect on Hdm2 polyubiquitination at the highest levels of expression. Hdm2 is a very poor, low affinity USP29 substrate, if it is one at all. So the effect of USP29 on p53 is attributable to the direct deubiquitination and stabilization of the latter. This data is now presented in the revised Figure 5B and 5C. Thus USP29 is able to directly reverse p53 ubiquitination and so it may override destabilizing E3-ligases, whether Hdm2 or E6AP.

We include here a table that summarizes the additional cell systems employed. All of these cell lines generated equivalent results attesting to the generality of our findings.

Table 1: Experiments completed and data added to the revised manuscript:

<table>
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<tr>
<th>Cell Line</th>
<th>HeLa</th>
<th>HCT116, p53+/+</th>
<th>HCT116, p53−/−</th>
<th>MCF7</th>
<th>U2OS</th>
<th>SAOS2</th>
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<td>H2O2 sensitivity after siJTV1 or siUSP29</td>
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✓: Presented in the original manuscript; ✓: Performed and included in the revised manuscript.

7. Data showing that endogenous Jvt1 and Fbp1 are required for induction of Usp29 after oxidative stress appear to be missing unless I have missed them.

We thank the referee for his/her careful observation. We regret that such important evidence was omitted in the initial manuscript. During the revision, we have performed a series of experiments with HCT116 cells, and our data clearly show that knockdown of JTV1 attenuated the H2O2-induced activation of endogenous USP29. The data are now shown in Figure 7D.

Referee #2 (Remarks to the Author):
In the manuscript by Liu et al., the authors report the intriguing transcriptional induction of USP29, a ubiquitin specific peptidase, by FBP and JTV1 resulting in stabilization of p53 and apoptosis in a model cell line. FBP is a protein known to positively regulate the MYC gene, while JTV1 is a Myc target that plays a central role in the structural integrity of the multiaminoacyl-tRNA synthetase complex. In this regard, Liu et al. sought to determine the function of JTV1 which localizes in the nucleus along with FBP. They found through gene expression profiling of cells ectopically expressing JTV1 or an alternatively spliced, constitutively nuclear JTV1-Alt (JA). USP29 is one of the induced target among 142 genes that were altered by JA expression. JTV1 was found to induce the expression of USP29.
through FBP binding sites. Because of a previous implication of JTV1 in p53 stability, the authors sought and found that USP deubiquinates and stabilizes p53. To place this series of events in biological context, the authors document that oxidative stress (hydrogen peroxide treatment) induces JTV1 nuclear localization and p53 accumulation associated with cell death. Overall, the authors have uncovered an important role for JTV1 and FBP in stress response. The study is technically very well-executed and the data supports the conclusions drawn. In this regard, it is interesting to note that while FBP is upstream and JTV1 is downstream of MYC. Whether FBP and JTV1 may play a role in MYC oncogene stress response deserves some discussion. Is it possible on based on the authors' observations that over-expression of MYC, which is known to induce oxidative stress and JTV1, could utilize the same pathway to illicit a p53 response. Given the Levens' lab long standing interest in the regulation of MYC, this concept deserves some discussion particularly in the context of cells (primary) that have wild-type p53.

We thank the reviewer for his strong support. We appreciate his desire to have us interpret our current work within the framework of our MYC-FBP-FIR studies. Besides the published work showing that JTV1 is a MYC target, it turns out that we have unpublished data indicating that FBP1 is also a Myc target. The balance between FBP, JTV1, USP29 and p53 is likely to occur along a razor’s edge and will require systems approaches to sort out. While we definitely intend to pursue such investigations, we would prefer to refrain from making qualitative assessments concerning the nature of the JTV1 network because such a complex network may possess unexpected or emergent properties. We would prefer to collect more quantitative data concerning this issue so that we can contribute a predictive model rather than seat-of-the-pants speculation.

RESPONSE TO REVIEWER THREE
Referee #3 (Remarks to the Author):

In this manuscript by Liu et al., the authors have investigated changes in expression of genes upon overexpression of JTV1 or a splicing variant, JA. Both these JTV1 forms interact with FBP, a DNA binding protein that has been reported both to activate and repress transcription of target genes. The authors find among the genes changed in expression level upon JTV1/JA overexpression the USP29 gene. Indeed, the UPS29 gene appears to have an upstream FBP binding site, named uFUSE, and upon overexpression JTV1/JA can be found binding to that promoter element.

In this respect it is surprising that JTV1/JA appear to bind to FBP with same affinity (figure 1A) and to bind similarly the uFUSE element as determined by ChIP (Figure 1F, and the activation of the endogenous USP29 promoter (Figure 2D) is also similar, while JA is found in the nucleus while JTV1 is (almost) exclusively in the cytoplasm (Figures 3A, 3B). Furthermore, with the use of a USP29 reporter construct, JA is more active than JTV1 (Figure 2C). How can the authors explain these apparent contradictions?

Since JA and JTV1 bind FBP through a segment of protein that is common in both forms, it is highly likely that both bind to FBP with similar affinity. What distinguishes the two forms is, as the referee clearly noted, their localization. Under normal growth conditions, and without overexpression, JTV1 is contained within the ARS complex and is exclusively cytoplasmic and so is unlikely to have access to FBP. Transient transfection is sufficient in those cells to drive some of the overexpressed JTV1 into the nucleus, and those cells are expected to support reporter activity. In contrast, JA is always predominantly nuclear and is readily accessible to FBP. In reporter assays, JA indeed supports higher levels of reporter activity than does JTV1. At the endogenous USP29 gene, transfected JTV1 and JA appear to bind equivalently and support similar levels of expression. So for the USP29 promoter, as for many other promoters, transient transfection appears not to fully recapitulate the behavior of the endogenous gene. Indeed this is the situation with the c-myc FUSE where FBP was first identified. One possibility might be that the induced activity of the natural gene is delimited by a negative factor (for example FIR) or it doesn’t make a difference if you are driving a Smart Car or a Ferrari if the cruise control is set 50 km/hr. While the basal USP29 promoter is strong in transient transfection assays, endogenous USP29
expression, even after induction, is relatively weaker, suggesting that other parameters help constrain USP29 promoter strength in vivo. One such parameter may be superhelical density. It is difficult to recapitulate the proper level of supercoiling in reporter assays, and because the activity of FUSE element is tuned to the level of supercoiling in their embracing chromosomal domain, the expression of the reporter may not perfectly parallel that of the native gene. In the revised manuscript we now acknowledge this difference at the bottom of page 9.

Can the authors explain why in figure 1A, 1B they use HeLa cells, in figure 1F MCF-7 cells, in figure 2A again HeLa, and in figure 2B, 2C H1299 cells. The choice of cells in figures 2D, 2E and 2F is not mentioned in text or Figure legends.

The majority of initial experiments, including the microarray analysis and initial reporter assays were performed in HeLa cells before we found out JTV1 affected p53 level. After that, it appeared clearly to us that reporter assays had to be done in p53 null cells such as H1299 to avoid interference of JTV1-induced cell death on reporter activity; we now explicitly note this on page 7 of the revised manuscript. We also wanted to study the regulation of USP29 by JTV/FBP/USP29 in a cell line in which p53 is regulated by its natural E3 Hdm2, such as MCF7 and HCT116; Figure 1F shows such results using MCF7. We appreciate the referee’s concern, and so tested additional cell lines to check whether JTV1 stabilization of p53 is a general phenomena in cells where p53 levels are regulated by Hdm2 rather than E6AP. The subcellular localization of JTV1 as well as its role in the cellular response to H2O2 are now shown also in HCT116 cells in Figure 4F, Figure 6, Figure 7D-F and Supplemental Figure 6. The primary cell Hs68 behaved similarly, although the response was somewhat smaller. Our data reveal that the role of JTV1 in cellular response to oxidative stress is quite general. We have also revised the legend to clearly identify cell lines used in each experiment.

Is the basal level of USP29 higher in H1299 and MCF-7 cells compared to HeLa cells? The localization studies have been exclusively done in HeLa cells, while several of subsequent studies on USP29/p53 are done in MCF-7 cells. So, I would also like to see the localization of JTV1/JA in MCF-7 cells, at least.

USP29 appears to be a stress protein. Oxidative stress-induced (or transfected) USP29 expression appears to quickly stabilize p53 and induce apoptosis rapidly (Figure 6A). Under normal growth conditions, USP29 is barely expressed, if at all, in all of the cell lines we tested, and appears to have minimal influence on basal p53 activity. We appreciate the referee’s concern and repeated the subcellular localization and apoptosis assays in several other cell lines for the revised manuscript. These data are now shown in Supplemental Figure 6.

In figure 2B the authors show that knocking down FBP reduces USP29 mRNA in H1299, as can be expected if there is a significant basal expression level. Also JTV1 knock-down reduces USP29 mRNA levels. This should indicate that JTV1 is expressed to some extent in the nucleus in these cells, but the authors state that ‘under normal growth conditions, FBP and JTV1 were independently maintained as expected since FBP and JTV1 were segregated in separate subcellular compartments’. Can the authors explain?

The referee is astute. There are two non-exclusive possibilities now mentioned on page 9 of the revised manuscript. First, published work (Kim, et al, 2002, and Kim et al, 2003b) has shown that cytoplasmic JTV1 is essential to maintain the integrity of the ARS complex, and that the ARS supports more efficient translation than the dissociated tRNA synthetases. Second, we cannot exclude that a very low level of JTV1 leaks into the nucleus. As discussed above, the USP29 reporter may be more responsive to JTV1 than the endogenous gene.

As an introduction to the subcellular localization part, the authors state: Our data indicate that JA is a more potent activator of USP29 (and likely other FBP targets) than JTV1. This is, as mentioned above, not supported by the data shown in figures 1 and 2 regarding the endogenous USP29 gene.
We agree with the referee that our data indicated JA is a more potent activator for USP29 reporter constructs. We have re-phrased this statement accordingly.

In figure 3A the authors show that the subcellular localization of overexpressed JTV1 depends on the amount of plasmid transfected. However, they do not say how much is transfected relative to the number of cells in this experiment, which means that conditions cannot be compared to other transfection experiments. The authors should really provide this information, and discuss it in the light of the other experiments.

Is the amount of GFP-JTV1 transfected in figure 3A-c the same as in 3A-e?

In Figure 3A and all other panels in which transfected GFP-JTV1 or GFP-JA localization were directly compared (Figure 3A-b and -c; 3A-d and -e in the original manuscript; Fig. 3A-b, -c and -d, and 3A-e and -f in the revised version), the images came from the same slide. The difference of green fluorescence intensity is due to different levels of expression in individual cells within the same transfection. It is well known that even in the same transfection, the expression level of transfected protein in each individual cell could vary significantly. Our FACS analysis of transfected cells also revealed more than 50-fold variation in the green fluorescence intensity in cells from the same sample. This seems to be the nature of transient transfection.

We replaced some of the images with the higher quality confocal images, now shown in the revised Figure 3A. Again, all these images were collected from different fields of the same slide.

Would the authors not have expected that overexpressed FBP would by itself already recruit some JTV1 into the nucleus? Is the FBP/JTV1 interaction much weaker than the interactions between JTV1 and the ARS complex?

Although the interaction between JA/JTV1 and FBP is very strong (even stronger than the p53 and SV40 T-antigen interaction in yeast two-hybrid assays), unless stressed, cells look working very hard to keep JTV1 and FBP away from each other. We have never seen FBP in the cytoplasm, even in cells expressing very high levels of recombinant FBP. There is little or no free JTV1 in the cytoplasm, and even if there were, we also have preliminary evidence that JTV1 has nuclear exporting signals, one of which is located in the second exon, that drive it out of the nucleus until the cell is stressed. Furthermore, nuclear JTV1 is also quickly relocated to PML bodies. It seems that cells utilize multiple mechanisms to keep JTV1 out of the nucleus and away from FBP, possibly to prevent stabilization of p53. Interaction with FBP alone is unlikely to retain JTV1 in the nucleus. It is likely that stress or signaling induced post-translational modifications such as phosphorylation, ubiquitination or sumoylation of JTV1 is necessary for JTV1 nuclear uptake. We believe that the full elucidation of the cell biology and signaling mediating the phenomena that we report here, is beyond the scope of this manuscript.

In figures 3C, 3D, 3E and 3F the authors show that treating cells with H2O2 (rather high doses, I think) does lead to some colocalization of JTV1 into the nucleus, where it partly colocalizes with FBP, also with PML. For me, it is a bit unclear why the authors have chosen H2O2. Of course, it induces apoptosis and it does activate p53, but induction of apoptosis by H2O2 is only for a small part dependent on p53. Did the authors also try other, maybe more p53-specific, stresses like ionizing radiation or camptothecin? Or, is this effect on JTV1 very much stress specific? Did the authors try maybe a somewhat more physiologically relevant way to increase oxidative stress, like treating cells with menadione?

Oxidative stress by H2O2 was selected as a hunch based on previous JTV1 studies showing: (1) that JTV1 and FBP levels are increased in the brain regions that show pathology in Parkinson’s Disease whether due to a familial loss of Parkin, MPTP toxicity, or the sporadic disease, and that Parkinson’s disease is believed to be associated with oxidative stress (Ko, 2005; Cookson, 2005); (2) that over-expression of JTV1 resulted neuronal cell death in tissue culture of SH-SY5Y just as caused by H2O2 either due to direct application or generated by dopamine metabolism (Ko, 2005); (3) Parkin translocates to the nucleus in response to H2O2 (Kao, 2009). While we agree with the referee that oxidative stress is not one of the more conventionally employed DNA-damaging
agent for p53 studies, it is, nevertheless, recognized as a genotoxic stress. We have now tested HeLa and MCF7 cells, and found that treatment with either camptothecin (CPT) or UV elicits little or no JTV1 relocalization to the nucleus. Knockdown of either JTV1 or USP29 did not influence p53 stability following CPT treatment. The data are shown below. The referee is indeed correct that JTV1-induced USP29 transcription is an oxidative stress specific event. We thank the referee here and in the Acknowledgments for this insight.

With regard to selection of the H2O2 dose: at levels below 0.1 mM, H2O2 induces a short-lived cell cycle block, but not apoptosis. At levels higher than 0.2 mM, H2O2 induces dose-dependant apoptosis. The doses of H2O2 selected are within the physiological range of a typical inflammatory reaction where tissue concentrations are in the range of 0.2 ñ 0.8 mM (Grisham, 1984 and 1990; Suzuki, 1991).

In figure 4 the authors show that overexpression of JTVB/JA increases p53 protein levels, in an USP29 dependent manner. Figure 4C suggests that knocking-down USP29 in MCF-7 does not alter basal p53 levels. So, USP29 is not necessary for maintaining basal p53 levels? Did the authors not test normal, not tumor cells, which might express higher levels of endogenous USP29?

The referee is correct. Because USP29, when expressed, is very effective at stabilizing p53, and overrides p53 destabilization even by E6AP, under normal conditions, USP29 levels must be kept very low. In fact, in mouse, only the eyes and the brain have detectable USP29 mRNA; note that these neural cells are constantly subject to oxidative stress due to monoamine metabolism. We were unable to detect USP29 mRNA in cultured tumor cells. So USP29 must play a very limited, if any, role in maintaining basal p53 levels. In Figure 4C, the siUSP29 treatments were intended to eliminate the expression of the transfected USP29 expression vector, since the uninduced endogenous USP29 levels are inconsequential. We include here a table that summarizes the additional cell systems employed. All of these cell lines generated equivalent results attesting to the generality of our findings.
Table 1: Experiments completed and data added to the revised manuscript:

<table>
<thead>
<tr>
<th>Localization</th>
<th>HeLa</th>
<th>HCT116, p53+/+</th>
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<th>MCF7</th>
<th>U2OS</th>
<th>SAOS2</th>
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<tr>
<td>JTV1/USP29 induced apoptosis</td>
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<td>H₂O₂ sensitivity after siJTV1 or siUSP29</td>
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✓ : Presented in the original manuscript; ✓ : Performed and included in the revised manuscript.

In figure 5 the authors show that overexpressed USP29 can co-immunoprecipitate with p53, and that it leads to stabilization of p53 in MCF-7 cells. USP29 does not affect Hdm2 protein levels or Hdm2 stability directly. The experiment in figure 5B could suggest that USP29 overexpression reduces the ubiquitination of p53, but this experiment is not convincing. Why not transfect a tagged Ub construct in MCF-7 cells, perform IP or pull-down targeting the tag or anti-p53, and blot vice versa (with appropriate controls). That would be more convincing. Even such an experiment would not prove that USP29 is directly de-ubiquitinating p53. Therefore, in vitro experiment with purified proteins should be performed.

At the referee’s suggestion, we have co-transfected his-Ub, Hdm2 and p53 in the presence or absence of USP29. The results are now included in the revised manuscript as Figure 5B and 5C and convincingly show that USP29 is very efficient at removing poly-ubiquitin from p53. USP29 is relatively ineffective at removing poly-ubiquitin from, and stabilizing Hdm2. We have been unable to express USP29 in bacteria and could not perform insect cell expression within the time-frame allotted for revision by the editor. Further characterization of USP29’s enzymatic activity is beyond the scope of the current manuscript and will require a separate study.

In figures 5F and 5G it is found that the overexpression of JTV1/JA or UPS29 leads to some induction of apoptosis, in a p53-dependent manner. This surprises me somewhat, especially in the HCT116 cells. One would expect that the observed induction of p53 levels is independent of, and not accompanied by post-translational modifications like phosphorylation. In that respect, it should work more-or-less the same as increasing p53 levels by Nutlin-3 treatment. Nutlin-3 has been shown to mainly induce cell cycle arrest and a senescence-like status in HCT116 cells. So, is there a difference in p53 stabilization by USP29 and Nutlin-3? Did the authors check whether transfected cells (also) entered, maybe initially, a cell cycle arrest?

The reviewer raises a good point. These very reproducible results may indicate that USP29 and especially JTV1/JA not only deubiquitinate and stabilize, but also promote the modification/activation of p53. The identification and characterization of the putative pathway and relevant modifications could not be accomplished within the allotted timeframe, and in any case, would probably be best resolved and presented as part of another study.

In relation to figures 5F and 5G the authors state: More apoptosis occurred 48hrs post transfection of JTV1 or JA than at 24hrs (Fig.5F), indicating that these factors must
accumulate to trigger apoptosis in the absence of genotoxic stress. This statement must be removed. The difference between 5F and 5G is not only the time of analysis, but also a difference in cell line (HCT116 versus HeLa), so you never know why in HeLa the number of apoptotic cells is higher than in HCT116 cells.

We agree with the referee that the comparison in Figure 5F and 5G is inappropriate, and we have removed the offending sentence. We have performed apoptosis assay with HCT116 cells 48 hrs post transfection and the data are shown as Figure 6A and 6B of the revised manuscript. We have also expanded the assay to include the p53+/+ and p53/-/- pair, U2OS and SAOS2, and showed that JTV1, JA and USP29 induced apoptosis all require p53.

In figure 6 it is investigated whether activation/relocalization of JTV1 into the nucleus upon H2O2 treatment leads to induction of USP29 levels, and subsequent induction of p53 and apoptosis. Figures 6A and 6B are quite convincing: H2O2 treatment leads to increased USP29 mRNA levels and increased recruitment of JTV1 and FBP to the aFUSE element.

We thank the referee. We have also expanded the study that now includes HCT116 cells and shows that the JTV1-USP29 link is likely a general reaction to oxidative stress and is not limited to one certain cell line. This is now shown in Figure 7.

Figure 6C is not convincing. First, it is unclear what the indicated values mean. Is the increase in percentage apoptotic cells compared to untreated cells? Does knock-down of JTV or USP29 affect the percentage of apoptotic cells in mock-treated conditions?

We agree with the referee that the previous figure and legend were unclear and have revised them (new Figure 7, old Figure 6). The referee is correct that the percentage of apoptosis in siRNA transfected, but H2O2--untreated cells was subtracted from the percentage of apoptosis in the treated cells to reveal the excess cell death attributable to oxidative stress. This subtraction was necessary to clearly visualize apoptosis for the 0.2 mM and 0.4 mM H2O2 samples, since the induction of apoptosis was modest for these samples (in contrast, the 0.8mM results seem incontrovertible). Knockdown of JTV1, FBP or USP29 had no statistically significant effect on cell viability (now shown in Supplemental Figure 8).

The knock-down of JTV1 has a stronger effect than knocking-down USP29 or p53. So, there is no correlation between p53 levels and the induction of apoptosis. Therefore, my conclusion would be that knocking-down JTV1 partly inhibits H2O2-induced apoptosis, but this effect is largely p53-independent.

Our data in the new Figure 7C and 7F show that JTV1 drives apoptosis about equally through p53-dependent and p53-independent pathways. The data also indicate that USP29 may be the major DUB stabilizing p53 following oxidative stress, since cells without USP29 were just as resistant to oxidative stress as p53 knockdown cells. The data in these two panels, together with the ChIP data, show that JTV1 not only stabilized p53 via USP29, but may also affected other pathway(s) involved in oxidative stress-induced cell death.

It would have been much more informative if the authors had used HCT116 p53++ and p53-- cells. And, maybe not look at apoptosis but G2/M arrest since that is the main affect of H2O2 on HCT116 cells, although this seems to be mainly p53-independent. So, like mentioned before, a more clean p53-activating treatment that would also activate USP29 would be more informative. I feel that lysates for Western blots should also be made of the treated cells at the time of harvesting for FACS analysis.

As we gratefully acknowledge above, the referee is correct that "more clean" p53-activators fail to induce USP29, indicating that the response is specific for oxidative stress. With regard to the timing of the preparation of the cells lysates used for western blot analysis, we respectfully disagree with the reviewer on this point. It is well known that a pulse of p53 can
provoking subsequent apoptosis. Because it may take many hours to play out the apoptotic program following a genotoxic insult, the p53 levels at the time of FACS are irrelevant to the levels immediately following the death-inducing stimulus.

In conclusion, the authors have shown clearly that JTV1/FBP can activate USP29 transcription and that overexpressed USP29 can interact with p53 and can stabilize p53. Treatment with H2O2 also activates USP29 transcription and induces apoptosis in HeLa cells. At the same time H2O2 also increases p53 levels, but the contribution of increased p53 levels to the induction of apoptosis is not clear.

A potentially very interesting story, but some points have to be worked out. It is not proven that USP29 directly de-ubiquitinates p53, and a physiological relevant condition whereby USP29 (via JTV1/FBP or otherwise) regulates p53 activity has to be found.

We appreciate the referee’s comment and we believe our data as presented in the revised manuscript indicated that JTV1-mediated USP29 activation is a general cellular response to oxidative stress. The induction of USP29 stabilizes p53 that contributes to initiation of apoptosis of cells after oxidative stress.

Additional Reference:

2nd Editorial Decision 22 December 2010

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by two of the original reviewers, who both consider the manuscript significantly improved in response to the initial comments and thus in principle suited for The EMBO Journal. Nevertheless referee 3 retains a number of concerns that would still need to be addressed before acceptance, either through additional experimental clarification or more careful discussion and tempering of certain interpretations.

I am therefore returning the study to you once more for a final round of revision, kindly inviting you to modify the manuscript in response to the referee comments detailed below. When sending us the final version, please make sure to also include the following, in order to avoid any unnecessary further delays with the processing of the manuscript:
- a 'Conflict of Interest' statement in the text, following the 'material and methods' section
- an 'Author Contribution' statement in the text, to be included adjacent to the 'acknowledgements' section
- better quality versions of all figures, preferably not as PDF but maybe in TIF or EPS formats: in the current versions, most of the blot images are very difficult to assess, with bands/lanes often appearing somehow separate from the background staining (this could well be a PDF conversion artifact but needs to be clarified)
- a better quality version of the supplementary information PDF: again many figures there appear very blurry/pixelated, and I am hoping you may be able to improve this by altered PDF conversion procedures

Once we will have received your re-revised manuscript, we should then hopefully be able to proceed with its formal acceptance and publication.
Best regards and a happy holiday season,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The authors have substantively and scholarly addressed all major concerns.

Referee #3 (Remarks to the Author):

This revised version of the manuscript by Liu et al., "JTV1 Co-activates FBP to induce USP29 transcription and stabilizes p53 in Response to Oxidative Stress", has strongly improved in quality. Indeed, most of my comments have been adequately addressed. It is still too bad that endogenous USP29 cannot be monitored, and that a direct de-ubiquitination of p53 by USP29 has not been 100% proven. Still, and I still feel this to be a major criticism, it is known that the overexpression of USP's can lead to aspecific de-ubiquitination of targets. Authors will argue that USP29 only 'marginally' de-ubiquitinates Mdm2, but I do not fully agree with that. The authors have not quantified figures 5B and 5C, but I would say that the reduction in the Mdm2 Ub is also very significant. The fact that the total level of Mdm2 is not strongly increased in this experiment can be explained by the fact that cells have been incubated with MG132 for 8 hrs, so the authors cannot use that argument. A better argument is the observation that the half-life of Mdm2 appears not too be affected in H1299 cells upon USP29 overexpression. However, it is not possible to compare figures 5D and 5E. In 5D MCF-7 cells have been used to look at p53 half-life. Maybe these cells transfec much better than H1299 cells, so a higher percentage of cells is expressing USP29? Why not also investigated the Mdm2 half-life from these MCF-7 transfecants? The increased p53 in these cells should lead to increased basal levels of Mdm2, but should not affect the half-life of Mdm2. Regarding the H1299 cells: maybe try to select a polyclonal, stable USP29 overexpressing population, and then repeat the T1/2 experiment.

Another way to show that USP29 affects the endogenous p53 Ub could be the following experiment, which is not very difficult. The assumption is that the treatment with H2O2 leads to decreased Ub of p53, but increased USP29 expression, and, therefore, stabilization of p53. Ubiquitination of endogenous p53 can be easily monitored by transfection of e.g. His6-Ub, like the authors have performed in their co-transfection ubiquitination assay. Such transfected cells can be transfected with control or si-USP29, and subsequently mock-treated or H2O2-treated, and subsequently investigated for p53-ubiquitination.

So, either the authors should perform at least part of mentioned experiments, or should via text make very clear that the direct USP29 effect on p53 stability is not proven, and that an effect on Mdm2 levels cannot be fully excluded.

One other major remark: it has been reported that H2O2 or other oxidative stress mainly leads to G2/M cell cycle arrest or senescence in HCT116 cells. So, I find the percentage of increase in apoptosis upon H2O2 treatment (which is very low) not necessarily the best read-out. The authors really should comment on that or, better, perform FACS analyses to determine cell cycle distribution of the cells and not only perform Annexin-V staining.

Minor remark: Figure shown in rebuttal letter showing that CPT or UV-C treatment does not lead to nuclear entry of JTV1, and that CPT treatment does not need JTV1 or USP29 for p53 stabilization, should be included as Supple figure. It is as such mentioned in the text as Suppl. figure 8, but that figure shows other data.
Referee #2 (Remarks to the Author):
The authors have substantively and scholarly addressed all major concerns.
We thank the referee for his/her kind comment.

Referee #3 (Remarks to the Author):
This revised version of the manuscript by Liu et al., "JTV1 Co-activates FBP to induce USP29 transcription and stabilizes p53 in Response to Oxidative Stress", has strongly improved in quality. Indeed, most of my comments have been adequately addressed. It is still too bad that endogenous USP29 cannot be monitored, and that a direct deubiquitination of p53 by USP29 has not been 100% proven. Still, and I still feel this to be a major criticism, it is known that the overexpression of USP's can lead to aspecific deubiquitination of targets. Authors will argue that USP29 only 'marginally' deubiquitinates Mdm2, but I do not fully agree with that. The authors have not quantified figures 3B and 5C, but I would say that the reduction in the Mdm2 Ub is also very significant. The fact that the total level of Mdm2 is not strongly increased in this experiment can be explained by the fact that cells have been incubated with MG132 for 8 hrs, so the authors cannot use that argument. A better argument is the observation that the half-life of Mdm2 appears not too be affected in H1299 cells upon USP29 overexpression. However, it is not possible to compare figures 5D and 5E. In 5D MCF-7 cells have been used to look at p53 half-life. Maybe these cells transfect much better than H1299 cells, so a higher percentage of cells is expressing USP29? Why not also investigated the Mdm2 half-life from these MCF-7 transfectants? The increased p53 in these cells should lead to increased basal levels of Mdm2, but should not affect the halflife of Mdm2.

Regarding the H1299 cells: maybe try to select a polyclonal, stable USP29 overexpressing population, and then repeat the T1/2 experiment. Another way to show that USP29 affects the endogenous p53 Ub could be the following experiment, which is not very difficult. The assumption is that the treatment with H2O2 leads to decreased Ub of p53, but increased USP29 expression, and, therefore, stabilization of p53. Ubiquitination of endogenous p53 can be easily monitored by transfection of e.g. His6-Ub, like the authors have performed in their co-transfection ubiquitination assay. Such transfected cells can be transfected with control or si-USP29, and subsequently mock-treated or H2O2-treated, and subsequently investigated for p53- ubiquitination. So, either the authors should perform at least part of mentioned experiments, or should via text make very clear that the direct USP29 effect on p53 stability is not proven, and that an effect on Mdm2 levels cannot be fully excluded.

We thank the referee for kind suggestions for experiment to further improve the quality of the manuscript and to give us the option to revise the text. We have revised the text to clearly indicate that, since we were unsuccessful to express USP29 in either bacterial or insect cell-based system, we were unable to obtain the reagents to develop an in vitro enzyme assay to provide direct proof that p53 is the immediate substrate of USP29. Therefore, we could not fully exclude the possibility that the stabilization of p53 by USP29 as observed might be an indirect effect of USP29 over-expression. This stipulation is now included in page 16 of the revised manuscript.

One other major remark: it has been reported that H2O2 or other oxidative stress mainly leads to G2/M cell cycle arrest or senescence in HCT116 cells. So, I find the percentage of increase in apoptosis upon H2O2 treatment (which is very low) not necessarily the best read-out. The authors really should comment on that or, better, perform FACS analyses to determine cell cycle distribution of the cells and not only perform Annexin-V staining.

We have analyzed cell cycle distribution of HCT116 cells prior to and after treatment at
various $\text{H}_2\text{O}_2$ concentrations. Our data are in agreement with the referee’s point that, at concentrations lower than 0.1mM, $\text{H}_2\text{O}_2$ stress mainly led to G2/M cell cycle arrest. Above this concentration, increasing levels of $\text{H}_2\text{O}_2$ progressively augmented apoptotic cell population. At 0.4mM and 0.8mM, the concentrations used in the experiments shown in figure 7F, apoptosis became much more apparent. It is conceivable that after the initial G2/M arrest, cells that sustain damage beyond repair would eventually become apoptotic. The data is now included as supplemental figure 8 and the result is briefly discussed on page 18 of the revised manuscript.

**Minor remark:**

*Figure shown in rebuttal letter showing that CPT or UV-C treatment does not lead to nuclear entry of JTV1, and that CPT treatment does not need JTV1 or USP29 for p53 stabilization, should be included as Supple figure. It is as such mentioned in the text as Suppl. figure 8, but that figure shows other data.*

We have included these data as supplemental figure 9 and also revised the text on page 19 to correctly identify the figure. We regret the mix-up during the rush to meet the submission deadline for the initial revision, and thank the referee for pointing out our mistake.