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USP22 regulates cell proliferation by deubiquitinating the transcriptional regulator FBP1

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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 10 January 2011

Thank you for the submission of your manuscript to EMBO reports. Please accept my apologies for the delay in getting back to you, which is due to the recent holiday season. I have now carefully read the referee reports on your manuscript that are copied below.

As you will see, while the referees agree that the study is potentially interesting and well-executed, they also raise a few concerns that need to be addressed before the manuscript can be considered for publication in EMBO reports.

Referee 3 points out that it should be demonstrated that USP22 interacts with and de-ubiquitinates also endogenous FBP1. It further needs to be clarified why the study reports a much longer half-life for FBP1 than the one reported previously and it should be discussed why less FBP1 may bind to chromatin after USP22 knock-down. Referee 1 further mentions that it should be investigated whether USP22 regulates p21 exclusively via FBP1 and whether p21 upregulation is responsible for the observed cell cycle arrest. Referee 2 also indicates that the method section needs to be expanded and we agree with the referee that at least a brief description of the most important methods for understanding the experiments described in the manuscript need to be included in the main part of the manuscript.

Given these evaluations and the constructive referee comments, I would like to give you the

opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In this article, B. Atanassov and S. Dent investigate the role of the de-ubiquitinating enzyme USP22 in cell proliferation. USP22 is required for appropriate progression through the cell cycle; however, its targets remain to be identified.
In a proteomic screen the author recovered the transcriptional regulator FBP1 (Far Upstream Element Binding Protein 1) as a specific USP22 target. USP22 physically interacts with FBP1 and consistent with a role of USP22 in FBP1 deubiquitination, polyubiquinated forms of FBP1 specifically accumulate upon USP22 depletion. These polyubiquinated chains are linked via ubiquitin K63, which regulates non-proteolytic events. Consistently, USP22 has no impact of FBP1 stability; instead, USP22 regulates FBP1 activity. In particular, USP22 specifically alters the expression of several FBP1 target genes including p21. Using chromatin immunoprecipitation, the authors further show that USP22 depletion specifically impairs the loading of FBP1 and the FIR repressor on the promoter region of target genes, thereby inducing target gene expression. Since p21 is a target of USP22 and USP22-depleted arrest in the G1 phase of the cell cycle, the authors conclude that USP22 regulates cell cycle progression by controlling p21 expression levels.
The study is very interesting and the data are well presented. Overall the conclusions are well supported by the experiments. However, a few points should be addressed before the manuscript can be accepted for publication:

Specific points:
1- USP22 co-immunoprecipitates with FBP1 in the presence of ethidium bromide, therefore the authors conclude that DNA is not mediating the interaction. To further confirm this observation, they should repeat the experiment after DNAse treatment.
2- The authors compared the expression levels of FBP1 target genes in USP22 and FBP1-depleted cells. It would be important to determine the expression levels of these genes after depletion of both USP22 and FBP1 to determine whether FBP1 is the only target of USP22, in the regulation of p21 gene expression.
3- The authors propose that up-regulation of p21 is sufficient to explain the cell proliferation defect of USP22-depleted cells. They are probably right but they should demonstrate this. In other words, they should test whether p21 down-regulation suppresses the cell cycle arrest of USP22-depleted cells.
4- The model presented in Figure 5E is not clear. The authors should include arrows between the two states and better illustrate that USP22 binding to the SAGA complex promotes FBP1 de-ubiquitination and loading of the FBP1/FIR complex on the chromatin.

Referee #2:

This manuscript characterises the role of USP22 as a modulator of transcription - this deubiquitination enzyme (DUB) has previously been proposed to act as a histone H2A DUB thereby

acting as a transcriptional activator. In this manuscript, the authors very convincingly show that this enzyme interacts with and affects the ubiquitination status but not the stability of FBP1. Depletion of USP22 leads to an increase of a poly-ubiquitinated species of FBP1, which at least in part comprises K63-linked ubiquitin chains, and a concomitant decrease of promoter occupancy. This is a well-executed and well-presented case study which adds significantly to our growing appreciation of ubiquitination as a modification that can both positively and as is the case here - negatively regulate protein function.

I only have a few minor issues which I have listed below.

1. The cycloheximide timecourse (Figure 2C and E) as it stands does not give any information on the turnover of this protein - a set of longer timepoints would be required here, but since there is no indication that the stability of FBP1 is affected this figure could also be moved into supplementary, if space constraints are an issue.

2. In contrast, the Method section is too short - even taking into account the supplementary section - to offer enough guidance for anyone who would wish to repeat these experiments. For example, specific information for lysis conditions (e.g. in Figure 2B) are generally missing, as is the preparation/precipitation of His-tagged proteins for Mass spec analysis. I would strongly suggest ensuring that each methodology is properly described and at least briefly listed in the main part of the manuscript.

Referee #3:

The manuscript by Atanassov and Dent reports the de-ubiguination of an unusual transcription factor, FBP1, by USP22, a subunit of the mammalian SAGA complex, and they explore the regulatory consequences of this event. The authors first purify on nickel resin, his-tagged-ubiquitin (Ub) expressed in USP22 knockdown or control 293 cells, and then interrogate the eluate by mass spectrometry for differential ubiqutination. They discover that multiple transcription regulators are enriched, Transfections with tagged FBP and tagged Ub directly reveals an increase in FBP ubiquitination. Co-immunoprecipitations show that FBP1 only co-immunoprecipitates with catalytically active USP22 and that GCN5 is also present in the immunopreicpate which indicates that the SAGA complex binds FBP1, not just free USP22. Surprisingly, unlike the telomere component, and known USP22-target,TRF, USP22 knockdown did not change the levels or stability of FBP1. Transfection of mutant forms of tagged-Ub suggests that Usp22 knowdown led to a greater increase of K63 than K48 (the stability-related poly-ubiquitination site). Knockdown of USP22 influences the expression levels of two reported FBP1 targets, p21 and MMP, but barely influences the expression of its most studied target c-myc. Chromatin IP reveals that USP22 knockdown and FBP knockdown each decreases both FBP binding and binding of the repressor FIR, to the p21 promoter, suggesting that FBP plays a mainly negative role for this gene. While knockdown of USP22 and FBP1 did not clearly alter steady state myc levels in Hela, they seemingly did prevent the transient peak of myc that follows serum starvation and stimulation. The generality of this system was demonstrated by showing that knockdown of USP22 or FBP1 both decreased proliferation and increased p21 yielding the expected increase in the G0/G1 fraction of the cell cycle in MCF7 and H1299 cells as well as a decreased proliferation rate..

This would become an interesting, potentially important, and valuable contribution if some of the issues listed below were addressed. This will require the inclusion of some additional data or experiments.

Major points:

1. The major finding and starting point of this manuscript is that USP22 regulates FBP1 ubiquitination. This result is documented only after transient ectopic over-expression of FLAG-tagged FBP1 or myc-tagged FBP1. The authors need to provide some evidence that USP22 interacts with, changes the ubiquitin-state, and regulates endogenous FBP1.

2. The authors conclude that FBP is a long-lived protein and that USP22 does not influence its half-life or levels in Hela cells. This is at odds with a previous report by (Bazar L, Harris V, Sunitha I, Hartmann D, Avigan M.A, 1995, A transactivator of c-myc is coordinately regulated with the proto-oncogene during cellular growth. Oncogene, 10:2229-38.) that found a shorter half-life for FBP1 (1.5 hrs.) in HL60 cells. Considering that Hela cells express papilloma virus proteins that are known to modulate host protein levels, this finding should be confirmed in an additional cell line(s).

3. If FBP1 protein levels do not change, then the authors need to comment on why there is less FBP1 binding by ChIP following USP22 knockdown. If FBP levels don't change what happens upon K63 ubiquitination?

4. The effect of FBP1 on serum stimulated c-myc expression in Hela seems clear, yet Hela, as cancer cell-line, is sub-optimal for serum-starvation. The authors should either repeat this with a non-transformed cell-line or probe their extracts with other antibodies (western blots) to ensure that a normal cycle of serum stimulation occurred.

5. Considering that GCN5 is the main subunit of SAGA, it would seem relatively easy and important to probe FBP1 pulldowns with anti-acetyl lysine to test if FBP1 is acetylated and if USP22 might indirectly influence this.

6. In figure 2C, lane 14, since the USP22 was knocked down in the presence of MG132, we expect to see a band of ubiquitinated FBP-1, which is missing. It would be good if the authors can give an explanation of this result.

Minor point:

The middle panel of figure 2C revealed that p53 did not show any difference of stability between the control and USP22 knock down cells. It would be better to use a USP22 target protein here such as TRF instead p53 to show the efficacy of knockdown?

1st Revision - authors' response 14 May 2011

POINT-BY-POINT RESPONSE

Referee #1

Q1: USP22 co-immunoprecipitates with FBP1 in the presence of ethidium bromide, therefore the authors conclude that DNA is not mediating the interaction. To further confirm this observation, they should repeat the experiment after DNAse treatment.

A: We repeated the co-immuporecipitation after DNAseI treatment of the lysates and as shown on the new Supplementary Fig S1C DNAseI treatment does not alter the ability of FBP1 to precipitate endogenous USP22.

Q2: The authors compared the expression levels of FBP1 target genes in USP22 and FBP1-depleted cells. It would be important to determine the expression levels of these genes after depletion of both USP22 and FBP1 to determine whether FBP1 is the only target of USP22, in the regulation of p21 gene expression.

A: To address this question, we depleted USP22, FBP1 or both together in MCF7 cells. Total RNA from the depleted cells was purified and the expression levels of p21 and MMP1 were monitored with qRT-PCR. As sown on the new supplementary figure S5A, we were able to confirm that USP22 and FBP1 depletion leads to up regulation of p21 or MMP1. However, simultaneous depletion of both USP22 and FBP1 did not lead to a further increase in the expression levels of the monitored genes. Simultaneous depletion of USP22 and FBP1 led to lower p21 and MMP1 mRNA levels compared to their levels in single depleted cells, likely reflecting the fact that depletion of USP22 and FBP1 in the double shRNA treated cells is not as efficient as in cells infected with single shRNA vectors (supp. figure S5B). Even though these results suggest that simultaneous USP22 and

FBP1 depletion does not have an additive effect on p21 expression, we cannot rule out the possibility that USP22 might regulate other cell cycle related genes independently of FBP1.

Q3: The authors propose that up-regulation of p21 is sufficient to explain the cell proliferation defect of USP22-depleted cells. They are probably right but they should demonstrate this. In other words, they should test whether p21 down-regulation suppresses the cell cycle arrest of USP22-depleted cells.

A: We are thankful to the reviewer for this question.
Our new data presented in fig5E and F clearly demonstrates that depletion of p21 can rescue the growth retardation of USP22 or FBP1 depleted cells. p21 depletion completely rescues the growth retardation in USP22 depleted cells and restores the proliferation rate of FBP1 depleted cell up to ~80% of the proliferation rate of control cells. The incomplete rescue in FBP1 depleted cells is most likely due to the fact that the p21 depletion using the shRNA we have in hands is not 100% efficient (see fig5F, the anti-p21 blot), while this depletion efficiency is sufficient to rescue the USP22 effect.

Q4: The model presented in Figure 5E is not clear. The authors should include arrows between the two states and better illustrate that USP22 binding to the SAGA complex promotes FBP1 de-ubiquitination and loading of the FBP1/FIR complex on the chromatin.

A: Fig 5 is now revised, and the model was omitted due to space /figure limitations.

Referee #2

Q1: The cycloheximide time course (Figure 2C and E) as it stands does not give any information on the turnover of this protein - a set of longer timepoints would be required here, but since there is no indication that the stability of FBP1 is affected this figure could also be moved into supplementary, if space constraints are an issue

Q2: In contrast, the Method section is too short - even taking into account the supplementary section - to offer enough guidance for anyone who would wish to repeat these experiments. For example, specific information for lysis conditions (e.g. in Figure 2B) are generally missing, as is the preparation/precipitation of His-tagged proteins for Mass spec analysis. I would strongly suggest ensuring that each methodology is properly described and at least briefly listed in the main part of the manuscript.

A: We reformatted the new manuscript according to the reviewerís suggestions.

Referee#3

Q1: The major finding and starting point of this manuscript is that USP22 regulates FBP1 ubiquitination. This result is documented only after transient ectopic over-expression of FLAG-tagged FBP1 or myc-tagged FBP1. The authors need to provide some evidence that USP22 interacts with, changes the ubiquitin-state, and regulates endogenous FBP1.

A: In the current version of the manuscript, we provide evidence that USP22 interacts with endogenous FBP1 (Fig1E), regulates its ubiquitination levels (Fig1B) and deubiquitinates endogenous FBP1 (supplementary Fig S1B).

Q2: The authors conclude that FBP is a long-lived protein and that USP22 does not influence its half-life or levels in Hela cells. This is at odds with a previous report by (Bazar L, Harris V, Sunitha I, Hartmann D, Avigan M.A, 1995, A transactivator of c-myc is coordinately regulated with the proto-oncogene during cellular growth. Oncogene, 10:2229-38.) that found a shorter half-life for FBP1 (1.5 hrs.) in HL60 cells. Considering that Hela cells express papilloma virus proteins that are known to modulate host protein levels, this finding should be confirmed in an additional cell line(s).

A: We have repeated this experiment in MCF7 cells as well. As shown in supplementary figure S2D, the results are virtually identical to the results obtained using HeLa cells. A long half-life for FBP1 was also reported by another group: Ko HS, Kim SW, Sriram SR, Dawson VL, Dawson TM, Identification of far upstream element-binding protein-1 as an authentic Parkin substrate, J Biol

Chem. 2006 Jun 16;281(24):16193-6, where the authors show that FBP1 is stable up to 24 hours after the cycloheximide treatment in SH-SY5Y cells.

We also monitored FBP1 stability in HL60 cells after ceyclohexamide treatment (These data are not included). Indeed, FBP1 showed shorter half-life in these cells (~3 hours in our experimental conditions) but we also noted that HL60 cells are extremely sensitive to Cyclohexamide treatment and 5 hours after the treatment approximately 40% cells were dead (based on counting with trypan blue).
However, our main point made from this experiment is that while USP22 depletion increases FBP1 ubiquitination, it does not seem to alter its stability. We omitted the statement that FBP1 is a long-lived protein, because our studies did not address FBP1 stability per se.

Q3: If FBP1 protein levels do not change, then the authors need to comment on why there is less FBP1 binding by ChIP following USP22 knockdown. If FBP levels don't change what happens upon K63 ubiquitination?

A: This is in fact a very interesting question, and we are currently working to obtain more mechanistic details of how K63 ubiquitination may impact stable recruitment of FBP1 (or other chromatin associated proteins) to its target loci. These experiments, however, require development of additional experimental approaches and tools, and therefore we could not address this question in the time provided for the revision of the current report.

Q4: The effect of FBP1 on serum stimulated c-myc expression in Hela seems clear, yet Hela, as cancer cell-line, is sub-optimal for serum-starvation. The authors should either repeat this with a non-transformed cell-line or probe their extracts with other antibodies (western blots) to ensure that a normal cycle of serum stimulation occurred.

A. We repeated this experiment using Human Normal Fibroblasts. As sown on the new supplementary figure S3C the c-myc expression kinetic shows the same trend as the one obtained using in HeLa cells.

Q5: Considering that GCN5 is the main subunit of SAGA, it would seem relatively easy and important to probe FBP1 pulldowns with anti-acetyl lysine to test if FBP1 is acetylated and if USP22 might indirectly influence this.

A: We considered this possibility while we were working on the manuscript. In deed FBP1 was reported to be acetylated (Choudhary et al, Science 14 August 2009, Vol. 325 no. 5942 pp. 834-840) and we were able to detect acetylation using anti pan-acetyl antibody for blotting precipitated FBP1. However we did not see any impact of GCN5 or USP22 depletion on FBP1 acetylation levels detected with this particular antibody.

Q6: In figure 2C, lane 14, since the USP22 was knocked down in the presence of MG132, we expect to see a band of ubiquitinated FBP-1, which is missing. It would be good if the authors can give an explanation of this result.

A: On the original figure 2C is presented really lite exposure of the blot, so it can be used to judge the FBP1 steady levels in protein lysates. MG132 treatment does not have significant impact on either FBP1 steady levels or ubiquitination.

Minorpoint:

Q: The middle panel of figure 2C revealed that p53 did not show any difference of stability between the control and USP22 knock down cells. It would be better to use a USP22 target protein here such as TRF instead p53 to show the efficacy of knockdown?

A: The efficiency of USP22 depletion is now shown on separate panels adjacent to the FBP1 blot.

2nd Editorial Decision 07 June 2011

As I mentioned in my previous email, I have now heard back from the referees that were asked to assess your revised version, both of whom now support publication (see their reports below). Referee 1 only has a minor suggestion regarding a text change in page 5, line 15 that makes the text easier to follow and is worth considering.

I have also had time to go through your file in detail in preparation for the acceptance of your manuscript and a few minor things have come up:

- From the figure legends, it is unclear if the information regarding error bars, number of samples and P values in figure 4 pertains panels A, B and D or only D. Likewise, in figure 5, does the information pertain panels A, B, C and D? Please clarify this and provide information for all the panels in which error bars and/or statistical analysis is shown.

- Figure 5 E indicates that n=2, but it is incorrect to provide error bars when the sample number is less than 3. In such cases, it is more appropriate to plot the data points from both experiments and eliminate the error bars. Alternatively, it would be best to perform the experiment at least once more to achieve statistical power (for guidance, please refer to Cumming et al. JCB 2007).

- In addition, the statistical test performed needs to be indicated in all figures for which a p value was calculated (or alternatively, a brief description of the statistical analyses can be included in the Material & Methods section of the main text).

I look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

My sincere apologies for the delay in sending back my review. The manuscript is significantly improved and now suitable for publication in Embo reports. I personally think that determining the precise role of FBP1 ubiquitination is beyond the scope of this study.

I just have minor suggestions:
Page 5 line 8: "we over expressed and HA-tagged ubiquitin', replace by "we over expressed HA-tagged ubiquitin"
Page 5 line 15: "These findings suggest that ub-FBP-1 is targeted by USP22 for deubiquitination". I would suggest to replace this sentence by "These findings suggest that USP22 deubiquitinates FBP1".

Referee #3:

The authors have seriously addressed all major concerns. Admirably they have done this by experimentation rather than disputation. This manuscript makes a valuable contribution.

2nd Revision - authors' response 13 June 2011

We are now ready to resubmit our manuscript entitled "**USP22 regulates cell proliferation by deubiquitinating the transcriptional regulator FBP1**"

In the revised manuscript, we edited the title and the abstract according to your suggestions. The legends for all figures that contain error bars and p-values were updated so that they now clearly indicate to which panels in the figure the described parameters apply. The statistical method used in the analyses is also stated in the legends of the corresponding figures. A new Figure 5E was made according to your suggestion, which shows a plot of the average data points from two independent experiments. We also accepted all the suggestions about the manuscript body (i.e. page 5, lines 8 and 15) made by the referees.

While addressing your suggestions, we also found some typos and figure labeling errors. These are corrected in the current version of the manuscript, and the changes are listed below:
- On page 10 line17 "**completely**" was omitted from "**completely rescue**" and in the next sentence (line17-18):" **The incomplete restoration of the growth curve in shFBP1 treated cells**" was changed to "**The incomplete growth restoration in shFBP1 treated cells**" These changes were made to better describe the new format of Fig5E.
-On figure 1A "**Contrl**" was changed to "**Control**"
-On figure S3, C "**MCF7**" was changed to "**NHF**". This figure was mislabeled, but the experiments were correctly described in the text and the figure legend.
-On figure S5, A "**Fold Chanfe**" was changed to "**Fold Change**".

Thank you for your suggestions, and please thank the reviewers for us as well. All of the revisions have improved our paper, and we hope that the current version is now acceptable for publication.

3rd Editorial Decision 14 June 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

