Cooperative actions of p21/WAF1 and p53 induce Slug protein degradation and suppress cell invasion

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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.)

Editor: Barbara Pauly

1st Editorial Decision 13 March 2014

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although the referees find the topic of interest, especially referees 1 and 2 they all raise numerous serious concerns and question the conclusiveness and physiological relevance of your data.

As the reports are below, I will not detail them here. However, there are numerous technical concerns that need to be addressed, as well as new experiments to bolster your claims. In addition, referees 1 and 3 request an extensive rewriting of the manuscript and clarification of many of the procedures used. In essence, all referee concerns should be addressed except major points 4 and 8 of referee 1, and points 2 and 3 of referee 2, which would be beyond the scope of a short report. Please try to address point 4 of referee 1, as this is a valid point that would strengthen the study, but we would not make this a precondition for acceptance if technical problems preclude your from obtaining a clear answer. In addition, please note that the second issue raised by referee 2 is his/her point 3, regarding the interactions between p53, p21, Slug and Mdm2 should ideally be experimentally determined.
All other points of all referees would need to be fully addressed for the study to be considered for publication in EMBO reports. It is our policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Do not hesitate to get in touch with us if we can be of any further assistance.

REFEREE REPORTS:

Referee #1:

In this manuscript the authors found, that p21 is a new regulator of Slug protein stability. In particular p21 binds to p53 and Slug fostering Mdm2-dependent Slug degradation. This p21-p53 cooperation suppress invasion of cancer cells both in-vitro and in-vivo. Yet the authors dissected the p21-p53-Slug molecular interaction and proved that efficient binding of p21 to p53 and Slug is essential for its antimetastatic activity.

The finding, that p21 is a new regulator of Slug protein activity is interesting and unveils an additional mechanism to the oncosuppressor activities of p21.

Despite the impact of the p53-MDM2 complex on Slug degradation has already been shown by several groups (including previous paper of Jungdoo), undisputed novelties of this paper are: i) the connection of p21 to the complex involved in the Slug protein degradation (p53-Slug-Mdm2) ii) elucidation about mechanism of binding between the critical components of this complex and iii) the role of p21 in regulating Slug-mediated biological activities (EMT).

In general, the experiments presented in the manuscript have been well planned and executed step by step, explaining the layers of complexity of the p21-p53-Mdm2 axis in regulating the stability of Slug protein.

However, considering the EMBO Reports format, the manuscript is clearly laconically written within the limits of words and figures. The experiments despite being well preformed are not well explained in the manuscript due to the poor quality of English. Moreover this work suffers of several experimental weaknesses and requires of additional and important experiments. In the present form it is still preliminary for publication in EMBO Reports.

Major comments:

The materials and methods section of the manuscript requires an elaboration. The techniques are poorly described and the essential informations are not present.

1) The authors show that concomitant introduction of p53 and overexpression of p21 lead to the degradation of Slug protein and invasion decrease. It should be examined what impact will the introducing of the mutant p53 (ex. R280H) have on the Slug protein levels and invasion rate of H1299 cells. It is established, that introduction of mutant p53 to p53-/- cells leads to the upregulation of Slug protein and increased invasion. Will the overexpression of p21 have the effect on Slug protein level in the mutant p53 presence?

2) Moreover introduction of the mutant p53 should impact the ubiquitination rate of Slug. The authors should perform an experiment in H1299 mutant-p53 cells where they would overexpress the p21 and examine the ubiquitination rate of Slug.

3) The authors confirm, that Slug RNA level, on the contrary to the Slug protein, are not significantly altered by p53 or p21 knockdown in H460 cells (fig. S3). The authors should perform real-time PCR experiments in H460 and in H1299 and H1299/P53 to verify the stability of the Slug mRNA levels.

4) The authors should focus more on the specific p21 point mutants in the Serine 153 and Serine 160 C-terminal residues. It has been shown that the Serine 153 residue is a phosphorylation site; on the
other hand phosphorylation status of the residue Serine 160 is not known. The authors should determine the phosphorylation status of the Serine 160 residue; the phosphorylation might have the crucial impact on the binding of p21 and Slug in the complex.

5) The C-terminal domain of p53, which deletion caused the loss of binding to p21 is not the NLS domain of p53 as the authors claim in fig S5. There are two NLS domains in the structure of p53; one localized at 305-322 and the other at 370-384 (Liang and Clarke, 1999b). The figure needs to be corrected.

6) In the Fig.4 B the authors show, that the number of circulating tumor GFP expressing cells in mice, that bore the H460 xenograft tumors, increased after the knockdown of the p53 and decreased after the overexpression of p21. The immunofluorescence, that the authors present in Fig.4B to demonstrate their hypothesis is not convincing. The authors say: "to investigate whether p21 regulates cancer cell intravestation in vivo we established xenograft tumors in mice using GFP expressing H460 cells, and then monitored the appearance of the tumor cells in mouse blood (Fig 4B)". It is not clear what did the authors try to explain; is it an example of the principal of the technique? Moreover, the authors are not showing the specific immunofluorescence images for each of the p21 mutant isoforms shown on the right panel of the figure. Can the authors provide the pictures of the GFP circulating tumor cells for each of the variants? Yet, as we mentioned before, the authors should provide more specific explanation of the experimental procedures. Authors are not showing some crucially important data like: in which part of the body of the nude mice the GFP expressing H460 cancer cells were injected, how many cells have been injected, what was the xenograft tumor size (after injection and before starting of the counting of the circulating cells). The authors should show the pictures of the grown tumors and they should also provide the results of the measurement of tumor size. The size of the tumor might be the crucially important factor, that can directly impact on the number of the circulating cells.

7) There is no formal demonstration, over the paper, that the anti-invasiveness properties of p21 are mediated by Slug. To aim this the authors should silence p53 and p21 and demonstrate that concomitant silencing of Slug rescue the effect on migration.

8) If the effect of the Slug accumulation is p53 dependent as the authors hypothesize, any other p53 inducing treatment (that also leads to Mdm2 and p21 increase) should show the effect on Slug protein accumulation. For example the authors could activate the p53 with the treatment of the doxyrubicin (DNA damage inducing drug).

Minor comments:

1) The authors demonstrated that Slug degradation is also regulated by extra-cellular stimuli. As it has been reported before the gamma-irradiation of H460 cells caused the accumulation of p53, p21 and Mdm2. The Slug protein levels decreased after the irradiation, this effect was abolished after silencing of p53 and p21. The authors claim, that the silencing of Mdm2 has the same effect on the Slug protein levels as the silencing of p53 and p21. Looking at the results of the Western Blot it is not clear. The silencing of the Mdm2 does not down-regulate the Mdm2 protein level in the sufficient degree to claim, that the silencing of Mdm2 is the cause of the Slug protein accumulation. Moreover the effect of the silencing of Mdm2 on the Slug protein is not visible as the authors claim. Another problem is the general quality of the Western Blot (cut between silencing of Mdm2 and its control is unacceptable).

2) Silencing experiments appear done only with one siRNA. At least key observations need two indepent siRNA.

Referee #2:

Synopsis:
In this study by Kim et al., the authors present data that implicate the cooperative effect of p21<sup>WAF1</sup> and p53 on MDM2-dependent Slug degradation. The authors found that Slug degradation required both the presence of p21 and p53, and these three molecules could form a complex in vivo. They further mapped the interaction domains on p21 for Slug binding and p53 binding, which involved in the de-phosphorylation of two serine residues, S153 and S160, respectively. The final result of this cooperative actin of p21 and p53 is to reduce cell invasiveness in vitro and intravasation in vivo. Overall, this is a well-written report, concisely summing up the discoveries the authors have found. Nonetheless, the novelty of this finding is dimmed by the already-known p53-Mdm2-Slug axis. There remains to be certain questions left unaddressed, which are listed below:

Major criticisms:
1. Firstly, the authors carefully analyzed the interaction domain of p21 with Slug and p53, respectively, and they found that the phosphorylation status of S153 and S160 on p21 could determine these interactions. However, the authors did not address the reason why they suspected phosphorylation may mediate these interactions. There is some literature discussing this. For example, Child and Mann (Cell Cycle, 2006) have reviewed the importance of p21 phosphorylation. They could discuss this matter in the discussion.
2. Secondly, if the phosphorylation of S153 and S160 is so important to interfere with the binding of Slug and p53, ultimately changing the invasive phenotype of these cell lines, the authors need to analyze which kinase(s) are involved in this. S153 has been known to be regulated by PKC and Mirk while S160 by PKC. Or is there any other kinases they could identify in their own cell system?
3. Third, they need to provide the physiological scenario in which these kinases regulate p21 phosphorylation, eventually leading to Slug degradation. Since p53-Mdm2-Slug has already been studied, this would be paramount information for the readers that during gamma-irradiation, for example, a certain kinase is de-activated, and therefore facilitate Slug down-regulation. This shall provide more novelty if they can prove this correlation. Also, the claim in the last paragraph that “p53 and p21 were found to form a complex with Slug and Mdm2, in which the four molecules appear to directly contact each other.” is somehow inappropriate since they did not demonstrate it in the in vitro or in vivo cell line system. Although others have demonstrated that there is direct interaction between p21 and Mdm2 and p53 and Mdm2, these are fragmental data which cannot support the conclusion of a four-molecule complex. The authors need to rephrase it.
4. Another question is the functional study of p21 mutation. In Figure 4A, when p21-S153D, a mutant that disrupts the p53-p21 interaction, is transfected in H460 cells, Slug is conspicuously up-regulated, but the invasion percentage of H460 cells is not increased significantly. The same goes with the experiment using p21-S160D mutant, where Slug is slightly upregulated (Figure 3D). If these manipulations can increase Slug but cannot enhance invasion, the observations in Figure 1A and 1B where invasion is up or down due to p53 or p21 may imply that the downstream effector may not be Slug. Or is it possible that these Slug proteins are sequestered somewhere, i.e. the cytoplasm, so that Slug could not exert its function?

Minor criticisms:
5. The quality of the Western blotting in general is fairly good. However, there is some contrast or exposure the authors need to re-conduct.
   a. In Figure 1B, the authors used siRNA to knock down the expression of p21, but in this blot, the endogenous level of p21 (lane 1) is barely detectable due to short exposure. It may confuse the readers at first why to carry out the siRNA experiment since there is no p21 expression. The expression pattern of p21 should be like that in Figure 1D.
   b. In Figure 2B, the authors demonstrate that the degradation of Slug by p21 is dependent on MDM2. However, Slug is not accumulated when MDM2 is knocked down (lane 3 to lane 1). The authors need to conduct the blotting again.
   c. In Figure 2D, the input level of Slug overexpression can be hardly seen in H1299-p53 cells. The blot should be redone.
   d. In Figure 4C, knockdown of Mdm2 is not quite as effective as that in Figure 2B (right panel, lane 2 v.s. 3). Nor is there an obvious elevation of Flag-Slug after this knockdown (lane 2 v.s. 3). A new blot is required.
6. The authors show that p53 or p21 knockdown does not interfere with Slug at the RNA level (Fig S3) by RT-PCR. They should carry out a more discreet analysis by real-time PCR.
7. Page 7: The punctuation of the sentence, "To test the possibility, raised by these findings, that p21..." is not smooth. The deletion of the fragment, "raised by these findings," is suggested. Page 9: the sentence, "suggesting that p21 suppresses cancer cell intravasation through binding to p53 and Slug, implying operation..." is not smooth, either. A new sentence, "These data imply that the operation of the p21-p53-Slug-Mdm2 system may be present in vivo." or else should be started and is suggested. Page 11: "Transfectants were injected nude mice and allowed to form..." It should be "injected into nude mice." Moreover, the number of injected cells should be stated as well.

Referee #3:

The authors report about a cooperation of p21, p53 and Mdm2 to promote Slug degradation and cell invasion. The authors show that the suppression of cell invasion by p53 depends on the presence of p21 and vice versa. This interdependence is mirrored in the requirement of these proteins for ubiquitination and degradation of the slug protein. Slug, p21 and p53 form a ternary complex that allows Mdm2-mediated degradation of slug and thus reduces cell invasion.

This is a very interesting manuscript. The cooperation of p53 with its target p21 is astonishing and adds a completely new flavor. The experiments are convincing and the manuscript is nicely written. Therefore, I have only few comments that should be addressed prior to publication.

1) The reviewer missed the figure legends for the supplementary figures.
2) Page 3, the authors write that "p21 overexpression did not significantly affect the invasiveness..." and refer to Figure 1A and 1B. However, in both figures, the presence of p21 reduced the invasiveness. It might not be statistically significant, but there is a clear tendency, which should be mentioned in the text.
3) Figure 1A and 1B: It is unclear for which conditions the significance has been calculated. The same problem applies to the other figures where statistical significance has been calculated.
4) Fig. S3: has this experiment been done by qRT-PCR? If not, it should since RT-PCR is not really quantitative.
5) Figure S4B cannot be understood. Please label the signals.
6) Figure 3C: The Delta N26 mutant binds to GST with the same efficiency as to GST-Slug. Is this seen in all experiments? If yes, then it cannot be said that the binding occurs only via the C-terminus.
7) Figure 4A, middle part: There is also some increase in invasion with the alanine mutant and some decrease with the aspartic acid mutant. This should be corrected in the text.
8) Figure 4C, blots for Mdm2: These blots should come from the same gel with extracts loaded next to each other and should not be assembled in the way it is shown in the figure.
9) Description of the methods is very poor. Usually, methods should be described in a way that an experienced scientist can repeat the experiments. This is not the case for this manuscript.

Minor:
1) Figure S5: Please check the labelling for p53. (The last 3 of 393 has moved to the next lane)
2) There are spelling and grammar mistakes in the Methods section.

Correspondence - authors

02 June 2014

The authors deeply thank you for giving us an opportunity to revise our manuscript (EMBOR-2014-38587V1). You gave us three months for revision, which was notified us on 03-13-2014. Therefore, we believe that the revision is due on 06-13-2014. However, if possible, I wish to ask you to extend the due date, and also discuss with you regarding one additional experiment you demanded from us.

1. We have received ~30 comments from three reviewers. Although we have worked on the comments very hard, we still need more time to
successfully complete all the experiments additionally requested. In my own judgment, we may need three more weeks to complete experiments, and one additional week for English correction. This latter work is essential, as none of the authors are native English speakers. We would greatly appreciate if you extend our due date 3-4 weeks more, hopefully 4 weeks.

2. We proposed the possibility that p53, p21, Slug, and Mdm2 may form a complex, which was based on the following observations from this and other laboratories. (i) A pull-down assay using cell lysates has revealed that the four molecules can co-precipitate (Fig 3A). (ii) We and others have shown that the interactions between any two of the molecules are possible, as discussed on page 10. (iii) It has been previously shown that p53, Slug, and Mdm2 can form a complex (Nat Cell Biol 11:694-704). (iv) We directly demonstrated the complex formation of p53, p21, and Slug using a two-step co-precipitation assay (Fig 3E). Taking together the data from these physical analyses and many other functional analyses we mentioned in the manuscript, it seems highly possible that the four molecules can form a complex. To directly prove this, we may perform a three-step co-precipitation assay. However, this is technically very difficult, because the assay requires too much proteins and antibodies.

Therefore, if possible, can we simply rephrase the discussion instead of directly proving the possibility? This is what the review 2 actually demanded us in his/her point 3. If not, do you think it will be helpful for us to perform a pull-down assay using recombinant proteins of p53, p21, Slug and Mdm2, and show their co-precipitation?

Correspondence - editor 03 June 2014

Many thanks for your email and please accept my apologies for not being able to get back to you sooner.

An extension of four weeks is certainly not a problem. With regard to your question about the experimental proof of the quaternary complex between p53, p21, Slug and Mdm2: if you are not able to show the existence of this complex using endogenous proteins and co-immunoprecipitation, then it would at least be good if you were able to show this in a pull-down assay using recombinant proteins. If this does not work, then you should rephrase the discussion accordingly.

I look forward to receiving your revised study as soon as it is ready.

1st Revision - authors' response 07 July 2014

Responses to Referee 1’s comments

In response to the referee’s suggestion, we now provide additional detailed information and/or references related to key experiments in Methods. However, information on materials, cell cultures, mutagenesis, and transfection has been provided in Supplementary Methods because of the limit set on the character count. We hope that this change is acceptable to the referee.

1. As requested by the referee, we investigated whether a p53 mutant can cooperate with p21 in regulating Slug levels and cellular invasiveness. We selected R273H, a naturally occurring mutation that abolishes the DNA-binding and thus the trans-activating activities of p53 (Olivier M et al. Cold Spring Harb Perspect Biol 2: a001008, 2010). Our data suggest that p53R273H can
promote Slug ubiquitination/degradation and thereby suppress cell invasion. Notably, these effects of \( p53^{R273H} \) were observed in the presence but not absence of p21. Therefore, we conclude that like \( p53^{wt} \), \( p53^{R273H} \) can cooperate with p21 in reducing Slug levels and cellular invasiveness. These new data are presented in Fig 4D and described on p10 of the revised manuscript.

The referee noted that a p53 mutant has been previously reported to upregulate Slug protein and increase cell invasion. We were unable find this study in which a p53 mutant was introduced into p53-null cells in order to determine its effects on Slug levels and cellular invasiveness; however, we identified a report in which shRNA-mediated knockdown of endogenous \( p53^{R273H} \) in SW620 colorectal cells was shown to cause an increase in Mdm2 levels in association with a decrease in Slug levels and cellular invasiveness (Wang SP et al. Nat Cell Biol 11:694-704, 2009. Figure S3D). Based on these results, the authors proposed that \( p53^{R273H} \) reduces Mdm2 levels (through an undefined mechanism), which leads to an elevation in Slug levels and cellular invasiveness. These results sharply contrast our data. We determined that \( p53^{R273H} \) expression did not markedly influence Mdm2 levels under our experimental conditions, using which we confirmed that \( p53^{wt} \) effectively increased Mdm2 levels (Fig 4D, left). Therefore, we conclude that \( p53^{R273H} \) regulates Slug levels and cell invasion not by regulating Mdm2 levels but by cooperating with p21 under our experimental conditions. The reason for the discrepancy between the previous results and our results remain unclear, but it may reflect a difference between experimental settings. We now briefly mention this issue on p10.

2. As mentioned above, we performed the suggested experiment and found that \( p53^{R273H} \) promotes Slug ubiquitination in cooperation with p21. The data are now shown in Fig 4D and have been described on p10.

3. As suggested by the referee, we performed real-time PCR experiments; the results are shown in Fig S4B and described on p6.

4. As mentioned by the referee, Ser^{153} of p21 has been shown to be phosphorylated (Rodriguez-Vilarrula A et al. Mol Cell Biol 25:7364-7374, 2005) and Ser^{160} of p21 has been reported to be phosphorylated by PKC (Scott MT et al. J Biol Chem 275:11529-11537, 2000; Child ES and Mann DJ. Cell Cycle 5:1313-1319, 2006). Therefore, instead of repeating the same experiments, we have now cited these previous reports and mentioned that both serine residues are phosphorylatable (p8). We hope that this is acceptable to the referee.

5. Thank you for the information. Fig S6 (previous Fig S5) has been corrected.

6. (i) Tumor metastasis is a complex process that sequentially involves the invasion of cancer cells into neighboring tissues, intravasation, migration through the bloodstream, extravasation, and the formation of a distal metastatic tumor. According to this theory, the invasive potential of cancer cells influences the rate of their arrival at blood vessels, and thus their subsequent entry into the bloodstream. Therefore, to investigate whether p21 also suppresses cancer-cell invasiveness in vivo, we performed intravasation assays by using an animal model. We clearly state this rationale on p9. (ii) As requested by the referee, we now provide (in Fig. 4B) typical examples of confocal images (DAPI, GFP, and their merge images) for each of the experimental sets. (iii) Because of the word limitation applied to a short report, we previously indicated the number of injected cells (5 x 10^5) and the injection site in mice (the flank) in the Supplementary Methods. However, in response to the referee’s comment, we have moved this information to Methods. (iv) In Fig 4B of the revised manuscript, we also show the pictures of grown xenograft tumors and the results of their size measurement. The results indicate that the introduction of the indicated constructs did not significantly alter the size of tumors in our experimental model (as shown by statistical analysis). Therefore, a disparity in tumor size does not appear to cause the difference in the number of circulating tumors.

7. We performed the suggested experiment and the results (Fig S3) indicate that p53 or p21 knockdown fails to increase cellular invasiveness when Slug is concomitantly knocked down. This supports the notion that p53 and p21 suppress cell invasion in a Slug-dependent manner (p6).

8. To determine whether Slug levels decreases in response to a DNA-damaging agent, we had previously performed g-irradiation experiments (Fig 4C). However, in line with the editor’s
suggestion, we did not repeat the same experiment using doxorubicin.

Minor comments:

1. To improve the quality of the results of g-irradiation experiment, we repeated the experiment. The new data shown in Fig 4C demonstrate the silencing effects of siRNAs and their effects on Slug protein levels considerably more clearly than the previous data did. This time, we loaded relevant samples in neighboring lanes, and thus the cuts between lanes have been removed.

2. As requested by the referee, we repeated some of the key experiments by using 2 sets of siRNAs; the results of these experiments are shown in Figs 1C, S2, and S4 of the revised manuscript.

Responses to Referee 2’s comments

First, the authors would like to thank the referee for his/her judgment that our manuscript was written well and concisely summed up our discoveries.

Major criticisms:

1. As the referee mentioned, previous studies have shown that Ser\textsuperscript{153} and Ser\textsuperscript{160} of p21 can be phosphorylated (Scott MT et al. J Biol Chem 275:11529-11537, 2000; Child ES and Mann DJ. Cell Cycle 5:1313-1319, 2006). We have now mentioned this point and cited the previous reports on p8.

2. We agree with the referee that it is critical to identify the kinases that are involved in phosphorylating Ser\textsuperscript{153} and Ser\textsuperscript{160} of p21 in our experimental system. However, we do not address this point because, as mentioned by the editor, that would be beyond the scope of a short report such as this one.

3. (i) We again agree with the editor that determining the physiological conditions under which these kinases regulate p21 phosphorylation and Slug degradation is beyond the scope of this study, which is presented as a short report. (ii) We proposed the possibility that p53, p21, Slug, and Mdm2 form a complex; this was based on the following observations made by our group and by others. a, We used cell lysates in a pull-down assay and have shown that the 4 molecules can co-precipitate (Fig 3A). b, We and others have shown that any two of the molecules can interact (discussed on p11). c, Previously, p53, Slug, and Mdm2 were shown to form a complex (Nat Cell Biol 11:694-704). d, Using a 2-step co-precipitation experiment, we directly demonstrated that p53, p21, and Slug can form a complex (Fig 3E). e. We performed an additional experiment and now show that the 4 recombinant proteins can be co-precipitated (Fig S5B). The data obtained from these physical-interaction analyses and those from several other functional analyses described in the manuscript collectively indicate that it is highly possible that the 4 molecules form a complex. To demonstrate this directly, we could perform a 3-step co-precipitation assay. However, this is technically extremely challenging: for such an assay, excessive amounts of proteins and antibodies are required. Therefore, as suggested by the referee, we have rephrased the statement regarding the complex (p11). Moreover, we now refer to the model shown in Fig 4E as a “hypothetical” model.

4. Based on our results previously shown in Fig 4A, the referee asked why p21\textsuperscript{S153D} and p21\textsuperscript{S160D}, which increased Slug levels, did not increase cell invasion. To address this question, we repeated the experiments and found that p21\textsuperscript{S153D} and p21\textsuperscript{S160D} significantly influences neither Slug levels nor cell invasion. The previous data have now been replaced by the new data in Fig 4A.

Minor criticisms:

5. (a) We re-analyzed the samples and have replaced the previous data with new data to show the constitutive expression of p21 (Fig 1B; Western blotting for p21). (b) The referee suggested that we should repeat the Western blotting of Slug shown in Fig 2B, because siRNA-mediated knockdown of Mdm2 did not induce Slug accumulation. However, Mdm2 induces the degradation of Slug protein but stabilizes the Slug mRNA (Jung CH et al. Cancer Lett. 335:270-
277, 2013). These two opposing regulations of Slug by Mdm2 could explain why the knockdown of Mdm2 did not markedly alter Slug protein levels. Therefore, we did not repeat the suggested experiment. (c) To show the expression of Flag-Slug in H1299-p53 cells more clearly than before (Fig 2D), the previous blot was replaced with a new one that was exposed comparatively longer. However, we could not lengthen the exposure any further because of the presence of non-specific bands immediately below Slug. (d) We repeated the experiment shown in Fig 4C. The new data demonstrate the silencing effects of Mdm2 siRNAs and their effects on Slug protein levels considerably more clearly than the previous data did.

6. As requested by the referee, we performed real-time PCR experiments. The results are now shown in Fig S4B and described on p6.

7. As suggested by this referee and by the editor and other referees, we have extensively rewritten the manuscript in an effort to increase the clarity with which our points are stated. Moreover, the manuscript was edited by a native English speaker in order to improve the quality of English.

Responses to Referee 3’s comments

The authors would like to thank the referee for his/her judgment that our manuscript was very interesting and nicely written.

1. We sincerely regret that the referee did not receive the legends of the supplementary figures. We had submitted the supplementary figure legends in a separate file. To avoid any such “accident,” we have now combined the supplementary figures and legends.

2. As suggested by the referee, we now state on p5 that “Although cell invasion decreased slightly after p21 overexpression, the difference was not statistically significant.”

3. In response to the referee’s comment, we have now listed P values directly in the figures to avoid any ambiguity regarding the conditions for which the statistical significance was calculated.

4. As suggested by this referee (and also other referees), we performed real-time PCR experiments. The results are shown in Fig S4B and described on p6 of the revised manuscript.

5. We apologize for this. The signals are now labeled in Fig S5C (previously Fig S4B).

6. We repeated the experiments shown in Fig 3C. The new data indicate that whereas p21wt and p21ΔN26 bind to GST-Slug, none of the tested constructs of p21 bind to GST. Therefore, the previous data appear to have been obtained as a result of technical or experimental errors. We have replaced the old data with the new data.

7. As suggested by this referee and other referees, we repeated the invasion assay, and the results are shown in the middle panel of Fig 4A (n was increased from 2 to 6). The new data show that cellular invasiveness was neither markedly increased when the alanine mutant was expressed nor decreased when the aspartic acid mutant was expressed; the differences appear to be slight and not statistically significant. Moreover, because of facing strict a word limit in this short report, we have decided not to mention these slight and perhaps negligible differences in the text, and we sincerely hope that this is acceptable to the referee.

8. As suggested by this referee and other referees, we repeated the experiment shown in Fig 4C. This time, we loaded the relevant samples in the neighboring lanes, and thus the blots derived from the same gels are now shown.

9. In response to the referee’s suggestion, we provide additional detailed information and/or references related to key experiments in Methods. However, information on materials, cell cultures, mutagenesis, and transfection is provided in Supplementary Methods because of the word-count limit imposed. We hope that this is acceptable to the referee.
Minor comments:

1. Thank you for checking this detail. We have corrected the labels in Fig S6 (previously Fig S5).

2. As suggested by this referee and by the editor and other referees, we have extensively rewritten the manuscript in an effort to increase the clarity with which our points are stated. Moreover, the manuscript was edited by a native English speaker in order to improve the quality of English.

2nd Editorial Decision 22 July 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the report below, the referee that was asked to assess the revised version now supports its publication in our journal.

Before the manuscript can be officially accepted, please attend to the remaining, formal issues:

1. In cases in which you have calculated error bars and standard deviations, please provide information on how many independent times each of the experiments have been performed (biological, not technical replicates).

2. We have also started encouraging authors to submit the raw data of biochemical and/or microscopical images to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the final version of your study.

3. Please provide a short, two-sentence summary of the manuscript, two to three bullet points highlighting the main findings of the study, and a schematic figure that can be used as part of a visual synopsis on our new website.

Once you have made these minor revisions, please submit the final version of your study through our website.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORT:

Referee #1:

The authors addressed all the concerns I have raised. I don’t have restrictions of any sort.
The authors wish to thank the editor and the referee for supporting the publication of our manuscript (EMBOR-2014-38587V2) in *EMBO Reports*. As requested by the editor, we have provided the following information in the revised submission:

1. We calculated error bars and standard deviations using biological replicates. This is now clearly stated on p15 and p19.
2. We provide our raw data of biochemical analyses as supplementary files. The raw data of microscopic images are presented in Fig 4B, and not in the supplementary files.
3. We describe a short summary and bullet points of the manuscript on p2 and also provide a schematic figure of a visual synopsis as a separate file.

We sincerely hope that our manuscript is now deemed acceptable for publication in *EMBO Reports*.

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.