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miR-605 joins p53 network to form a p53:miR-605:Mdm2 positive feedback loop in response to stress

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1st Editorial Decision 12 April 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the slight delay in getting back to you with a decision on your manuscript: this was due to a delay in receiving the reports back from the reviewers because of the Easter break. However, I have now received comments from three referees, which are enclosed. As you will see, all three reviewers recognise the potential interest in your work, but all three raise very substantial concerns, at both a technical and a conceptual level, that would have to be addressed in a major revision of your manuscript before we could consider publication in the EMBO Journal.

Technically, all three referees highlight concerns as to the choice of cell lines used throughout the study, and also as to the methods used for manipulating p53 activity. For example, referee 1, in particular, argues that several aspects your data would be much more convincing if you could use isogenic cell lines differing only in their p53 status: I would encourage you to follow this suggestion where appropriate. At a more conceptual level, all three referees argue that further analysis of the physiological relevance of the identified positive feedback loop would be important: particularly in terms of analysing in greater detail the effects of altered miR-605 levels on p53-dependent responses. Again, from an editorial standpoint, we would view a significant extension of your data in this direction as critical for any eventual acceptance of your manuscript for publication. Referee 1 also states that some assessment of the pathological relevance of miR605 in carcinogenesis would be important. While we do agree with the referee that this would be valuable, we realise that it may lie beyond the scope of this study, and so would not deem such data as essential at this stage.

In the light of the referees' recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This is an interesting manuscript describing a microRNA that potentially regulates Mdm2 and p53. Unfortunately, technical shortcomings and incomplete data make it difficult to believe the major conclusions drawn by the authors. It is very difficult to accept that tumor cell lines containing wild type p53, such as MCF-7, should synthesize almost the highest detectable amounts of miR-605 in comparison to other cells and tissues, whereas miR-605 should act as an activator of p53 at the same time. Also, for publication on a high level, it would be important to show at least some clinical data that support the relevance of miR-605 for cancerogenesis. For instance, is miR-605 downregulated or deleted in some tumors, when compared to corresponding normal tissue? Perhaps in rhabdomyosarcoma (since PRKG1 and presumably miR-605 are mostly expressed in heart and skeletal muscle)? Do miR-605 levels inversely correlate with the presence of wild type p53 (as is the case for the p53 activator p14/arf)? Without some evidence for the relevance of miR-605 in cancer, the interpretation of the manuscript remains quite speculative.

Specific points:
1) In Fig. 1A, it is curious that miR-605 and the antagonist AMO-605 were each used in A549 cells, whereas miR-605 overexpression was not even tried in MCF7 cells. Transfection of synthetic microRNA should in any case exceed the levels of endogenous miR-605 by far, even if more endogenous miR-605 seems to be present in MCF7 cells. The same applies to Fig. 1B (right panel) and Fig. 1C (lower panel).
2) In Fig. 1B, it would be important to show p53 levels (do they go up in response to Mdm2 removal when miR-605 is overexpressed?). Showing a p53-responsive gene product, e. g. p21/CDKN1A, would help to assess p53 activity.
3) In Fig. 2A, the authors use a decoy p53 binding site to compete p53 off the putative PRKG1 promoter. In other assays, they use PFTalpha to inhibit p53. Both are rather unusual approaches, prone to artefacts. It would be much more helpful to overexpress p53 along with the reporter plasmid, in a p53 null cell line (e. g. H1299, Saos2), to see how much this induces the reporter. As a positive control, and for comparison, a bona fide p53-responsive promoter should be used (e. g. the 1st intron of the Mdm2 gene). Moreover, the putative p53 binding site within the PRKG1 promoter should be mutated at its core, to see whether this abolishes the induction of the promoter by p53. This is especially important since the putative p53 binding sites (Suppl. Fig. 2) are doubtful. First of all, there are only two such half sites visible (the TATA box does not constitute a

p53 binding site). Secondly, these two half sites would constitute only one full p53 binding site (the p53 tetramer can only bind if two sites of the sequence RRRCWWGYYY come in a row). However, to act as a full binding site, the two half sites are too far from each other (27 interspersed nucleotides, whereas the commonly accepted consensus tolerates only up to 13 such residues between the half sites). All this raises doubts about the validity of the putative p53 binding site. If the authors wanted to maintain their claim, they had to prove it by introducing mutations into these sites within their promoter construct.

4) In Fig. 2B, why don't the authors use Nutlin (rather than Doxorubicin) to induce p53 without all the other DNA-damage responsive transcription factors? If they insist on Doxorubicin, it would be by far preferable to use an isogenic system of cells that only differ in their p53 status, e. g. HCT116 cells with and without p53.

5) In Fig. 2C, the ChIP should be followed by quantitative real-time PCR. In such an experiment, the PRKG1 promoter should be compared to well-established p53-responsive promoters (e. g. p21, PIG3, PUMA).

6) In Fig. 2D, the EMSA experiment should be accompanied by a supershift using p53 antibodies (e. g. antibody mAb421 would also increase the efficiency of the p53-DNA-interaction). It should also be controlled with a bona fide p53 binding element (like the one used as a decoy). Finally, labelled vs. non-labelled oligonucleotides should be used in competitor studies. Such experiments are needed to rule out the typical artefacts associated with EMSA.

7) In Fig. 3, p53 target genes other than miR-34a should be quantified as to their expression. This would make it clear whether miR-605 generally induces p53-responsive genes, e. g. p21, PIG3, PUMA, and others.

8) In Fig. 4, the arrow from MiR-605 to Mdm2 should be replaced by an "inhibitory" error, e. g. "-----I", to distinguish inhibition from activation.

9) In Fig. 5, on top of the MTT assays, more specific assays for apoptosis would be needed to clarify the impact of miR-605 on cell survival. Also, rather than using two entirely different cell lines, it would be far better to use an isogenic system that only differs in the p53 status, such as HCT116 cells with or without p53. Flow cytometry should be performed to show the DNA content of the cells, since this would not only provide an estimate for the fraction of apoptotic cells, but also indicate whether cell cycle arrest occurred (another important activity of p53). Caspase cleavage and long term survival in clonogenic assays should also be assessed, in the presence or absence of DNA damage.

10) Suppl. Fig. 1 shows multiple putative binding sites for miR-605 in the Mdm2 3' UTR. How well are these conserved in other vertebrates, and how many of them match stringent criteria used by the various target-miRNA-matching algorithms? Visual inspection leaves one obvious candidate, nt 3493-3516. Would the authors consider mutating this sequence in their reporter construct, to see whether this at least decreases the responsiveness to miR-605?

11) Suppl. Fig. 5 suggests that transfection of synthetic miR-605 increases the PCRable levels of this microRNA only less than 4-fold, when compared to endogenous miR-605. This is almost impossible, given the high concentrations of RNA that are transfected by lipofection. A more typical increase would be more than 1000-fold. Unless this can be explained, it raises serious doubts about the quantification method, or about the transfection efficiency.

12) Text, p 13, last line. The authors did not correctly point out the core sequence of a consensus p53 binding site, which is (capital letters): rrrCWWGyyy where R is purine, W is A or T, and Y is pyrimidine.

Referee #2 (Remarks to the Author):

In this study, Xiao et al report the involvement of a new microRNA, miR-605, in the p53 pathway. More specifically, they show that miR-605 targets and downregulates Mdm2, leading to enhanced p53 activity. On the other hand, p53 upregulates the expression of miR-605 through p53 binding sites in the host gene of miR-605, the PRKG1 gene. Together, this defines a new amplification loop for p53 activity.

This is an interesting paper, which adds a new dimension to the study of p53 regulation and the Mdm2-p53 cross-regulation. However, some of the experimental data is too limited, and further data is required to make the conclusions sufficiently robust, as detailed below. There are also several errors and issues that need clarification and validation.

1. Page 4. It is unclear how the authors conclude that the PRKG1 gene has three p53 binding sites.

From Supplemental Fig. 2, it looks like the second and third sites are actually derived from the two complementary strands of the SAME sequence!
2. Figure 1B. It is very unusual to see Mdm2 migrating as 62kDa; it usually runs much higher than that. The authors need to confirm that this band is truly Mdm2, for instance by showing that it is knocked down by Mdm2 siRNA.
3. Figure 1C. The increase in Mdm2 mRNA after transfection of miR-605 is ascribed to p53 activation (page 5). This is supported by the PFTa data in Fig. 3. However, PFTa is not always affecting only p53. Therefore, it also needs to be shown that miR-605 does not increase Mdm2 mRNA in cells that do not have wild type p53. Moreover, in such cells it will be possible to tell if miR-605 can also destabilize Mdm2 mRNA and reduce Mdm2 mRNA levels in addition to blocking its translation.
4. Page 6 implies that Dox induces PRKG1 mRNA. However, this information is not shown in Fig. 2B, unlike what one might understand from the text. Data showing that Dox induces PRKG1 mRNA in a p53-dependent manner should be provided.
5. Figure 2C shows semi-quantitative ChIP-PCR analysis. The ChIP data should be quantified by qPCR and shown as % of input, as is now common practice in the field. Furthermore, qPCR should also be performed in parallel for a genomic region that does not contain a p53 binding site, to prove that the enrichment with p53 antibodies is specific. This, too, is common practice nowadays.
6. Figure 3: to demonstrate more convincingly that miR-605 increases the transcriptional activity of p53, it should be shown that there is an elevation not only of miR-34a but also of mRNA transcripts derived from classical p53 target genes (e.g. p21, Bax, Gadd45a etc).
7. Page 8. DAN-damaging written instead of DNA-damaging.

Referee #3 (Remarks to the Author):

In this manuscript, Jiening et al. describes a story about a new mir-605 involved in the p53-mdm2 feedback loop. MiR-605 is upregulated when p53 is activated, mir-605 then targets mdm2 by repressing mdm2 translation. They claim that mir-605 can create a positive feedback loop between p53 and mdm2. The study is potentially interesting, but there are a number of caveats in the manuscript as following:
1. In the whole paper, the legends are too complicated and hard to distinguish each group. The authors may want to make concise and clear versions of them.
2. The authors try to compare mir-605 with another well-studied miRNA mi34. However, sometime they used mir34a, sometime they used mir-34c. Even in the figure legend there is no a right match among the terms, such as, figure 1.
3. In figure 1B, are the bands around 62KD mdm2? However, endogenous mdm2 should be much bigger than 62KD. If mir-605 targets mdm2 in response to p53 activation, why mdm2 levels are elevated in response to most of stress signals? Thus, it is important to specify at what stage or time point after stress when mir-605 plays a role in downregulation of mdm2 expression. In other words, it is important to clarify the physiological outcome of mir-605 targeting of mdm2 in response to p53 activation.The only data showing reduction of mdm2 protein levels by this miRNA is figure 1B by overexpressing this miRNA in A549 cells. How about in other cells?
4. In page 5, the authors claim that mRNA of host gene PRKG1 is increased as shown in figure 2B, but the legend only mentioned mir-605 mRNA levels.
5. The manuscript needs to be polished by fixing up some grammar errors and sentences.
6. It is odd to put a model in between data sets. It is suggested to move the model to the final figure.

1st Revision - authors' response 26 July 2010

EMBOJ-2010-74108-R1

Response to Referee #1's Comments

"*This is an interesting manuscript describing a microRNA that potentially regulates Mdm2 and p53.*"

We thank the reviewer for his/her appreciation of and constructive suggestions on our work.

"*Unfortunately, technical shortcomings and incomplete data make it difficult to believe the major conclusions drawn by the authors. It is very difficult to accept that tumor cell lines containing wild type p53, such as MCF-7, should synthesize almost the highest detectable amounts of miR-605 in comparison to other cells and tissues, whereas miR-605 should act as an activator of p53 at the same time. Also, for publication on a high level, it would be important to show at least some clinical data that support the relevance of miR-605 for cancerogenesis. For instance, is miR-605 downregulated or deleted in some tumors, when compared to corresponding normal tissue? Perhaps in rhabdomyosarcoma (since PRKG1 and presumably miR-605 are mostly expressed in heart and skeletal muscle)? Do miR-605 levels inversely correlate with the presence of wild type p53 (as is the case for the p53 activator p14/arf)? Without some evidence for the relevance of miR-605 in cancer, the interpretation of the manuscript remains quite speculative*."

We are afraid that the reviewer misunderstood the point; our point is that in cells expressing both p53 and miR-605, miR-605 participates in the p53 network. We do not try to generalize that all tumor cell lines containing wild-type p53 have higher miR-605; like any other genes, expression of miR-605 must be regulated by multiple transcription factors and our study merely shows that p53 plays an important role in promoting the expression of miR-605.

Though we agree with the reviewer on that it is interesting to conduct a survey type of study to for miR-605 expression in various tumor tissues versus normal tissues, we feel this is out of the scope of the present study. The aim of our study is to elucidate the mechanisms for the participation of miR-605 in the p53 regulatory network in cells expressing this miRNA.

Specific points:

"1) In Fig. 1A, it is curious that miR-605 and the antagonist AMO-605 were each used in A549 cells, whereas miR-605 overexpression was not even tried in MCF7 cells. Transfection of synthetic microRNA should in any case exceed the levels of endogenous miR-605 by far, even if more endogenous miR-605 seems to be present in MCF7 cells. The same applies to Fig. 1B (right panel) and Fig. 1C (lower panel)."

We also performed the experiments with exogenous miR-605 and AMO-605 in MCF-7 cells. But we did not show the data because they do not provide any further information beyond what is seen in A549 cells and inclusion of these data in the main text could results in redundancy. We believed that it makes more sense to use MCF-7 cells for observing the effects of endogenous miR-605 by using AMO-605 alone as miR-605 likely imposes tonic repression on Mdm2 with a higher expression level in this cell line. To address this point, we have now included these data in Supplementary Figure 4S.

"2) In Fig. 1B, it would be important to show p53 levels (do they go up in response to Mdm2 removal when miR-605 is overexpressed?). Showing a p53-responsive gene product, e. g. p21/CDKN1A, would help to assess p53 activity."

The focus of Figure 1 is on the role of miR-605 in repressing Mdm2. The consequences of Mdm2 repression (changes of p53 activity and level) are presented in Figure 3 and in the text under the section subtitled "*miR-605* enhances transactivation activity of p53". Our results showed that repression of Mdm2 by miR-605 did not cause significant upregulation of p53 level though there is a tendency of increase. This is consistent with the common view that Mdm2 primarily inhibit the activation of p53 but not the expression of p53. We feel that addition of p53 data to Figure 1 would create an "early jump" and break the logical flow of the article. We therefore decided to place the p53 data in Supplementary Figure 6S.

As far as the p53 activity concerned, please note that miR-34 is also an established p53-responsive gene (this is clearly indicated in our manuscript with many references) and Figure 3 is focused on this point. We feel that adding another p53-responsive gene will not add any further information but will create redundancy and make the manuscript unnecessarily lengthy. However, to address the reviewer's concern, we performed additional experiments on p21/CDKN1A to confirm our findings with miR-34. The data are presented in Supplementary Figure 6S to avoid redundancy.

"3) In Fig. 2A, the authors use a decoy p53 binding site to compete p53 off the putative PRKG1 promoter. In other assays, they use PFTalpha to inhibit p53. Both are rather unusual approaches, prone to artefacts. It would be much more helpful to overexpress p53 along with the reporter plasmid, in a p53 null cell line (e. g. H1299, Saos2), to see how much this induces the reporter. As a

positive control, and for comparison, a bona fide p53-responsive promoter should be used (e. g. the 1st intron of the Mdm2 gene). Moreover, the putative p53 binding site within the PRKG1 promoter should be mutated at its core, to see whether this abolishes the induction of the promoter by p53."

Ever since its invention by Morishita *et al* in 1997 [Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A, Maeda K, Sawa Y, Kaneda Y, Higaki J, et al. (1997) In vivo transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. *Nat Med* 13: 894–899], decoy ODN technique has been widely and routinely used as an efficient tool for functional genomics studies involving transcriptional regulation of gene expression. One obvious advantage of the activator/inhibitor approach, over the overexpression method is that it answers the question whether endogenous p53 plays a role in the regulation, which better links to pathophysiological conditions. Based on our experience, overexpression of p53 can create far more severe artifacts compared with decoy ODN and p53 inhibitor approaches. Indeed, anything (non-specific off-target effects) could happen with overexpression, particularly when the level of overexpression is excessively high. However, to add an extra piece of evidence, we have conducted additional experiments with p53 overexpression in H1299 cells (Figure 2E; page 6, paragraph 2) and with the 1st intron of the Mdm2 gene as a positive control (Figure 2B; page 6, lines 11-13), as suggested by the reviewer.

"This is especially important since the putative p53 binding sites (Suppl. Fig. 2) are doubtful. First of all, there are only two such half sites visible (the TATA box does not constitute a p53 binding site). Secondly, these two half sites would constitute only one full p53 binding site (the p53 tetramer can only bind if two sites of the sequence RRRCWWGYYY come in a row). However, to act as a full binding site, the two half sites are too far from each other (27 interspersed nucleotides, whereas the commonly accepted consensus tolerates only up to 13 such residues between the half sites). All this raises doubts about the validity of the putative p53 binding site. If the authors wanted to maintain their claim, they had to prove it by introducing mutations into these sites within their promoter construct."

First, by labeling the TATA box, we do not try to say it constitutes a p53 binding site and in nowhere in our manuscript we declare so.

Second, we entirely agree with the reviewer on that there are only two half-sites in the promoter region of PRKG1/miR-605. However, it has been shown by numerous studies that p53 dependent-transactivation could be detected on a half-site, though the transactivity of p53 was found lower than a full p53 binding site (please see references below). According to the recent study by Jordan *et al* (see below for reference), a full-site with a spacer length between two decamers $\geqq$ 3 nts reduces transactivity of p53 to the level comparable to a decamer half-site. This would imply that for many of the established target genes of p53 with spaces $\geqq$ 3, the transactivities are expected to be equal to that with a half-site. Consistent with this notion, it was demonstrated that p53 dependent-transactivation was detected in association with a decamer half-site created by a single nucleotide polymorphism in the promoter of the FLT-1 gene (the vascular endothelial growth factor receptor-1 gene) (Menendez *et al* 2006). The p53 consensus sequence of p21 promoter can be activated by p53 only at 5' site one (GAACATGTCCcAACATGTTg), equivalent to a half-site. The 14-3-3s promoter (AGGCATGTgCcAcCATGCCC) can be activated by p53 only at 3' site but not at 5' site (Funk *et al* 1992); this also makes the full p53 binding site equivalent to a half-site. Furthermore, the p53 DNA binding consensus sequence is evolved to possess the degenerate nature to expand the universe of p53 regulated genes. For example, the p53 binding sites in Mdm2 promoter have great degeneracy even in the core region, but they work well for p53 (Zauberman *et al* 1995).

In our case, we have performed additional experiments to confirm the transactivity of p53 at the two half-sites in the PRKG1/miR-605 promoter. First, we observed substantial abrogation of p53 transactivity with mutations in the p53 binding sites using luciferase assay (Figure 2A). Second, we showed a loss of p53 binding to the half-sites when mutated in our EMSA experiments (Figure 3B). Third, we performed supershift with anti-p53 antibody for our EMSA (Figure 3B). And forth, we performed quantitative real-time PCR experiments following ChIP (Figure 3A).

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"4) In Fig. 2B, why don't the authors use Nutlin (rather than Doxorubicin) to induce p53 without all the other DNA-damage responsive transcription factors? If they insist on Doxorubicin, it would be by far preferable to use an isogenic system of cells that only differ in their p53 status, e. g. HCT116 cells with and without p53."

We have actually used nutlin-3 in our study (see Figures 3 and 5). To address the reviewer's concern, we have also added new nutlin-3 data to Figure 2A and 2B.

"5) In Fig. 2C, the ChIP should be followed by quantitative real-time PCR. In such an experiment, the PRKG1 promoter should be compared to well-established p53-responsive promoters (e. g. p21, PIG3, PUMA)."

We have done so as suggested (Figure 3A).

"6) In Fig. 2D, the EMSA experiment should be accompanied by a supershift using p53 antibodies (e. g. antibody mAb421 would also increase the efficiency of the p53-DNA-interaction). It should also be controlled with a bona fide p53 binding element (like the one used as a decoy). Finally, labelled vs. non-labelled oligonucleotides should be used in competitor studies. Such experiments are needed to rule out the typical artefacts associated with EMSA."

Supershift using the mAb421 p53 antibody, as suggested by the reviewer, was conducted (Figure 3B).

Positive control experiments for both luciferase and EMSA with a bona fide p53 binding element the same as the decoy used in our study were conducted, as suggested (Figure 2B & 3B).

Experiments with labelled vs. non-labelled probes were performed (Figure 3B), as suggested.

"7) In Fig. 3, p53 target genes other than miR-34a should be quantified as to their expression. This would make it clear whether miR-605 generally induces p53-responsive genes, e. g. p21, PIG3, PUMA, and others."

The data are shown in Figure 3. miR-34a (and also miR-34b and miR-34c) is an established target gene of p53 by numerous studies; this point is clearly stated and referenced in our manuscript. Please also see the references below. Though we do not totally agree on the requirement of another p53-responsive gene, we performed additional experiments looking at p21 mRNA. As expected, miR-605 increases p21 mRNA level and this increase is abrogated by its antisense and p53 inhibitor PFT-a. To avoid redundancy, we place these results in Supplementary Figure 6S.

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Kumamoto K, Spillare EA, Fujita K, Horikawa I, Yamashita T, Appella E, Nagashima M, Takenoshita S, Yokota J, Harris CC. Nutlin-3a activates p53 to both down-regulate inhibitor of growth 2 and up-regulate mir-34a, mir-34b, and mir-34c expression, and induce senescence. Cancer Res. 2008 May 1;68(9):3193-203.

"8) In Fig. 4, the arrow from MiR-605 to Mdm2 should be replaced by an "inhibitory" error, e. g. "-----I", to distinguish inhibition from activation."

The arrows have been replaced by "inhibitory" signs (Figure 6).

"9) In Fig. 5, on top of the MTT assays, more specific assays for apoptosis would be needed to clarify the impact of miR-605 on cell survival. Also, rather than using two entirely different cell lines, it would be far better to use an isogenic system that only differs in the p53 status, such as HCT116 cells with or without p53. Flow cytometry should be performed to show the DNA content of the cells, since this would not only provide an estimate for the fraction of apoptotic cells, but also indicate whether cell cycle arrest occurred (another important activity of p53). Caspase cleavage and long term survival in clonogenic assays should also be assessed, in the presence or absence of DNA damage."

We have performed additional experiments for apoptosis. First, we measured caspase 3 activity. The new data are presented in Figure 5D and are described in the text (page 9, lines 14-16). Second, we carried out TUNEL study to investigate chromosomal cleavage and the data are presented in Figure 5E. We did not performed measurement of DNA content because we have good expertise in other techniques and we have used these techniques in many of our previous studies. Though use of flow cytometry for DNA content can also yield information on cell cycle arrest, it is not immediately pertinent to the question we asked and will not provide any further insight with regard of our specific goal. We truly believe that with ELISA, caspase 3 and TUNEL, we can convincingly claim that the cell death observed in our study is largely apoptotic cell death.

We were unable to obtain HCT116 cells with different p53 status; they do not seem to be commercially available. But we performed additional experiments using human breast cancer cell line MDA-MB-436 that is known to not have p53 expression nor its responsive gene p21 because of the insertion mutation on p53 gene. We believe that comparison between MCF-7 and MDA-MB-436 is valid since they both are adenocarcinoma **derived from metastatic site** with pleural effusion: the former has functional p53 status whereas the latter does not. The data are added to Figure 5. In addition, MDA-MB-436 expresses much lower level of miR-605 (Supplementary Figure 3S).

"10) Suppl. Fig. 1 shows multiple putative binding sites for miR-605 in the Mdm2 3' UTR. How well are these conserved in other vertebrates, and how many of them match stringent criteria used by the various target-miRNA-matching algorithms? Visual inspection leaves one obvious candidate, nt 3493-3516. Would the authors consider mutating this sequence in their reporter construct, to see whether this at least decreases the responsiveness to miR-605?"

We used an earlier version of miRBase to predict putative binding sites for miR-605 in the Mdm2 3' UTR; this is stated in the last paragraph of Materials & Methods. The sites are conserved between humans and primates. We did not compare with other algorithms; they have different criteria for target prediction and no one is better than the others, which makes comparison among them difficult and hardly justified. Experimental verification is the ultimate judgment. Because there are too many candidate sites for miR-605 which may act cooperatively and independently which could well confound mutation study, we did not touch this complicated issue.

"11) Suppl. Fig. 5 suggests that transfection of synthetic miR-605 increases the PCRable levels of this microRNA only less than 4-fold, when compared to endogenous miR-605. This is almost impossible, given the high concentrations of RNA that are transfected by lipofection. A more typical increase would be more than 1000-fold. Unless this can be explained, it raises serious doubts about the quantification method, or about the transfection efficiency."

First, we need to clarify that the concentrations of miRNAs (10 nM) used for transfection in our study are generally much lower than in many other studies. Second, the quantity of miRNA to be transfected is controllable by manipulating the ratio of miRNA and lipofectamine and the duration of cell incubation. There is no doubt that we could obtain 1000-fold increase in miR-605 level, but this amount of increase is rather non-physiological and will undoubtedly produce non-specific effects. To limit the increase of miRNA to the range attainable under physiological or pathological conditions, we set up our protocols (miR-605/lipofectamine=1:0.5; normally, the ratio is 1:3) to achieve less than 10-fold increase in miR-605 level 48 hr after transfection. Third, miRNA concentration varies along the time after transfection. Under our experimental conditions, significant penetration of miRNA into the cells was observed 1 hr after transfection, peak entry occurs between 6-12 hr after transfection, miRNA level starts to decline from 30 hr after transfection, and around 40% miRNA remains in the cells 48 hr after transfection.

"12) Text, p 13, last line. The authors did not correctly point out the core sequence of a consensus p53 binding site, which is (capital letters): rrrCWWGyyy where R is purine, W is A or T, and Y is pyrimidine."

The mistakes have been corrected (Supplementary Figure 2S).

Response to Referee #2's Comments

"*This is an interesting paper, which adds a new dimension to the study of p53 regulation and the Mdm2-p53 cross-regulation.*"

We thank the reviewer for his/her very positive comments and constructive suggestions on our work.

"1. Page 4. It is unclear how the authors conclude that the PRKG1 gene has three p53 binding sites. From Supplemental Fig. 2, it looks like the second and third sites are actually derived from the two complementary strands of the SAME sequence!"

We thank the reviewer for pointing out the error. There are indeed three putative p53 binding half-sites within this region, but we mistakenly presented the results in the previous version. We have now corrected the mistakes (Supplementary Figure 2S).

"2. Figure 1B. It is very unusual to see Mdm2 migrating as 62kDa; it usually runs much higher than that. The authors need to confirm that this band is truly Mdm2, for instance by showing that it is knocked down by Mdm2 siRNA."

We thank the reviewer again for picking up the issue. We have checked our data and found that we wrongly labeled the Mdm2 molecular mass: it should be 92 kDa, instead of 62 kDa. We used an antigenic peptide for Mdm2 to verufy the specificity of the antibody used in our study (Figure 1B). We also used siRNA as a control to verify our results (Figure 1B), as suggested by the reviewer.

"*3. Figure 1C. The increase in Mdm2 mRNA after transfection of miR-605 is ascribed to p53 activation (page 5). This is supported by the PFTa data in Fig. 3. However, PFTa is not always affecting only p53. Therefore, it also needs to be shown that miR-605 does not increase Mdm2 mRNA in cells that do not have wild type p53. Moreover, in such cells it will be possible to tell if miR-605 can also destabilize Mdm2 mRNA and reduce Mdm2 mRNA levels in addition to blocking its translation.*"

Additional experiments were carried out in HCT-166 p53 negative cells to address the issue raised by the reviewer. Our results showed that miR-605 decreased Mdm2 mRNA level in this cell line (Figure 1C & Figure 3C) though the percent decrease in mRNA was smaller than that in protein.

"*4. Page 6 implies that Dox induces PRKG1 mRNA. However, this information is not shown in Fig. 2B, unlike what one might understand from the text. Data showing that Dox induces PRKG1 mRNA in a p53-dependent manner should be provided.*"

The data have now been added into Figure 2D.

"5. Figure 2C shows semi-quantitative ChIP-PCR analysis. The ChIP data should be quantified by qPCR and shown as % of input, as is now common practice in the field. Furthermore, qPCR should also be performed in parallel for a genomic region that does not contain a p53 binding site, to prove that the enrichment with p53 antibodies is specific. This, too, is common practice nowadays."

We have redone our ChIP experiments with quantitative real-time ChIP-PCR and a genomic region that does not contain a p53 binding site as a control, as suggested by the reviewer. The results are shown in Figure 3A and text (page 6, lines 22-23 and page 7, lines 1-2).

"*6. Figure 3: to demonstrate more convincingly that miR-605 increases the transcriptional activity of p53, it should be shown that there is an elevation not only of miR-34a but also of mRNA transcripts derived from classical p53 target genes (e.g. p21, Bax, Gadd45a etc).*"

miR-34a (and also miR-34b and miR-34c) is an established target gene of p53 by numerous studies; this point is clearly stated and referenced in our manuscript. Please also see the references below. However, we performed additional experiments looking at p21 mRNA, as suggested. As expected, miR-605 increases p21 mRNA level and this increase is abrogated by PFT-a. To avoid redundancy, we place these results in Supplementary Figure 6S.

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"7. Page 8. DAN-damaging written instead of DNA-damaging."
Error corrected (page 10, line 2)

Response to Referee #3's Comments

"*1. In the whole paper, the legends are too complicated and hard to distinguish each group. The authors may want to make concise and clear versions of them.*"
The legends have been changed to make concise and clear.

"*2. The authors try to compare mir-605 with another well-studied miRNA mi34. However, sometime they used mir34a, sometime they used mir-34c. Even in the figure legend there is no a right match among the terms, such as, figure 1.*"
We investigated all three members of the miR-34 family in this study. In the main text, we show only the data from miR-34a and the results for miR-34b and miR-34c are shown in Supplementary materials. The data in Figure 1 of the original version were from miR-34a, not miR-34c. This wrong labeling has been corrected. We thank the reviewer for picking up the mistake.

"*3. In figure 1B, are the bands around 62KD mdm2? However, endogenous mdm2 should be much bigger than 62KD.*
We thank the reviewer again for picking up the issue. We have checked our data and found that we wrongly labeled the Mdm2 molecular mass: it should be 92 kDa, instead of 62 kDa. We used an antigenic peptide for Mdm2 to verify the specificity of the antibody used in our study (Figure 1B). We also used siRNA as a control to verify our results (Figure 1B), as suggested by the reviewer.

"If mir-605 targets mdm2 in response to p53 activation, why mdm2 levels are elevated in response to most of stress signals? Thus, it is important to specify at what stage or time point after stress when mir-605 plays a role in downregulation of mdm2 expression. In other words, it is important to clarify the physiological outcome of mir-605 targeting of mdm2 in response to p53 activation."
Expression of Mdm2, like any other genes, is controlled by many factors and the expression level at any particular situation is a balance between the positive and the negative regulatory factors. miR-605 likely acts to fine-tune Mdm2 expression mainly at the protein level to limit the expression

level of Mdm2. It is expected that as long as miR-605 is present in a cell, it can play a role in repressing Mdm2 expression at any stage or time point after stress and contribute to decreasing Mdm2 expression as a negative regulator, though under these conditions Mdm2 expression may still be increased as a result of actions of positive regulators. This is an interesting but complicated issue requiring a specific study to resolve.

"The only data showing reduction of mdm2 protein levels by this miRNA is figure 1B by overexpressing this miRNA in A549 cells. How about in other cells?"

We must clarify that in addition to the data in Figure 1B with miR-605 overexpression in A549 cells, our data showing upregulation of Mdm2 protein with AMO-605 in MCF-7 cells also testify the repression of Mdm2 by miR-605. And probably this later result is more important because it indicates that endogenous miR-605 produces tonic repression of Mdm2. Moreover, repression of Mdm2 by miR-605 was also demonstrated in MCF-7 cells as depicted in Supplementary Figure 4S.

"*4. In page 5, the authors claim that mRNA of host gene PRKG1 is increased as shown in figure 2B, but the legend only mentioned mir-605 mRNA levels*."

The data have now been added into Figure 2D.

"*5. The manuscript needs to be polished by fixing up some grammar errors and sentences*."

Careful reading has been performed to correct the errors.

"*6. It is odd to put a model in between data sets. It is suggested to move the model to the final figure*."

It has been done, as suggested.

2nd Editorial Decision 26 August 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-74108R to the EMBO Journal. I'm sorry for the slight delay in getting back to you with a decision on this - all three reviewers wanted to see the paper again, and over the summer months, these things take a little longer than we would like. However, we do now have the comments of all three referees, which are enclosed below.

As you will see, all find the manuscript to be substantially improved, but while referee 2 is now happy for the manuscript to be accepted without further changes, referees 3 and particularly 1 still raise a few concerns that have not been adequately addressed during the revision. While we do normally limit papers to a single round of revision, I think their concerns should be addressable, and I would therefore like to offer you the exceptional possibility to undertake a second round of revision.

To spell out in detail what would be necessary for eventual acceptance of your manuscript:
- Referee 1 is still concerned that you have not adequately demonstrated that the effects of miR-605 on apoptosis are via its regulation of Mdm2. There are two facets here. Firstly he/she points out an inconsistency between the results of your Nutlin3 experiments and your proposed model: in situations where Mdm2 is already inhibited by Nutlin3, miR-605 activity would be irrelevant. Some explanation as to this would be important. Secondly, he/she still finds that using isogenic cell lines differing only in their p53 status would be critical. I do recognise that you had not been able to obtain these, but the referee points out that they should be readily available from the Vogelstein lab if nowhere else. I would therefore strongly encourage you to try to get hold of these cells and to conduct this experiment.
- Both referees 1 and 3 express their disappointment that you have not attempted to generate 3' UTR mutants that are non-responsive to the miRNA. I do appreciate that there are multiple potential target sites, but it should be possible to generate constructs that address this - by mutating first the highest scoring candidates, by mutating multiple sites at once, or by first narrowing down the likely relevant sites by truncation analysis before specific mutagenesis. Since the central claim of your study is that miR-605 directly regulates Mdm2, this is a central experiment that needs to be done.
- Referee 1 also raises a concern with the ChIP/qPCR data presented in Figure 3 - I guess that the

scale you show does not really represent percent of input, but please can you clarify this. It would also be beneficial to include a positive control here, as requested by the referee. Finally, from an editorial point of view, I agree with the referee that the experiment shown in Figure 3B is not adequately explained in either the text or the figure legend.

I hope that you should be able to address all these points; please let me know if you have any further questions or comments with this, or if you foresee difficulty with any of these experiments. I hope you agree that addressing these remaining concerns will significantly strengthen the conclusiveness of the study.

With best wishes,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

EMBOJ-2010-74108R
In their paper, the authors describe with improved data that a) miR-605 and its host gene PRKG1 are inducible by p53, that b) miR-605 is capable of negatively regulating Mdm2 levels, resulting in c) p53-activation by miR-605, perhaps generating a positive feedback loop.
For a high-ranking journal, it would have been desirable to show the physiological relevance of this regulation, e. g. with regard to its relevance to cancer. No such data are presented.
The study was improved in response to the reviewer comments. However, some points still remain questionable.

Specific points:
1) Does p53 really bind to the promoter of PRKG1? The presented data still do not unequivocally answer this question.
a. The authors say that they now performed the ChIP-PCR by real-time, quantitative PCR, as was demanded by two out of three reviewers. It is very unusual, though, that they precipitated 50% of the available promoter DNA with p53. This would be the most efficient ChIP ever seen for p53. Any ChIP result found by other groups for p53 was less than 1% of input. Moreover, the authors unfortunately ignored the advice from Reviewer 1 to include more established p53-responsive promoters as positive controls in this ChIP experiment. Such an experiment would simply consist in using the same DNA preparations in PCRs with additional primers. (Minor point: For negative control genes, the figure labeling indicates NFAT, but the text and legend say HERG).
b. The authors try to fortify the function of the claimed p53-responsive element in the PRGK1 promoter. In their rebuttal letter, they say that they have now included a mutated sequence in their EMSA experiments, and they talk about the right panel of Fig. 3B. Unfortunately, this experiment is not described at all in the figure legend. It is neither clear what the faint bands in these panels should tell the reader, nor is it clear what kind of mutation was introduced into the oligo used in this study.
2) Mir-605 appears to induce apoptosis when overexpressed. Fig. 4B also argues that antagonizing miR-605 reduces doxorubicin-induced apoptosis. But does miR-605-induced apoptosis work through p53 activation? It might, since p53-inducible miR-34 appears necessary for miR-605-induced apoptosis, and since pftalpha also impairs miR-605-induced apoptosis. On the other hand, it is difficult to understand why antagonizing miR-605 also reduces Nutlin-3-induced apoptosis. After all, Nutlin-3 abolishes the interaction between p53 and Mdm2, and thus should be rendering the effects of miR-605 on Mdm2 irrelevant. But this is not what is seen in Fig. 4B, raising the suspicion that miR-605 induces apoptosis also by p53-independent mechanisms. The use of an entirely different, p53-mutant cell line is not really helpful, since many properties (including transfection efficiency) are may differ between these cell lines on top of the p53 status. Isogenic HCT116 cells with or without p53 are available from public depositories or from B. Vogelstein (Johns Hopkins, Baltimore), against the statements of the authors in their rebuttal letter. Similarly, it is very curious that they declare the regulation of p53 levels by Mdm2 as irrelevant in their rebuttal letter, despite the overwhelming literature on Mdm2 as a ubiquitin ligase for p53. Their failure to detect changes in p53 levels in response to miR-605 should at least be discussed in the text.

3) How does miR-605 regulate Mdm2? The authors identified a number of putative binding sites for miR-605 within the 3' UTR of Mdm2. They decided not to test the relevance of any of them by mutational analysis, since they feel this is too much of a complicated issue (as said in their rebuttal letter). It is doubtful whether other high-ranking journals would accept such a statement. After all, even combining a limited number of candidate target sites would be very doable, and it would definitely improve the specificity and credibility of the model proposed by the authors.
4) Minor point: The model in the last figure should also have an "inhibitor"-like arrowhead (-----/) at the end of the arrow going from Mdm2 to p53.

Referee #2 (Remarks to the Author):

The paper has been strengthened substantially by new experimental data. It is now more convincing and better controlled than before.
Publication is recommended.

Referee #3 (Remarks to the Author):

The authors have addressed most of the previous questions verbally and experimentally. The manuscript has been improved significantly. However, it is necessary to make the mutants of miR-605 targeting site of Mdm2 3UTR, and to check the effect of miR-605 on these mutants, since the main finding of this study is that miR-605 targets mdm2 directly.

2nd Revision - authors' response 24 November 2010

EMBOJ-2010-74108-R2

Response to Referee #1's Comments

"In their paper, the authors describe with improved data that a) miR-605 and its host gene PRKG1 are inducible by p53, that b) miR-605 is capable of negatively regulating Mdm2 levels, resulting in c) p53-activation by miR-605, perhaps generating a positive feedback loop. For a high-ranking journal, it would have been desirable to show the physiological relevance of this regulation, e. g. with regard to its relevance to cancer. No such data are presented."
As stated clearly, the aim of our study is to elucidate the mechanisms for the participation of miR-605 in the p53 regulatory network at the molecular and cellular level. While the reviewerís suggestion is definitely a good one, it must be examined as a separate study involving various gain-of-function and loss-of-function of miR-605 in whole animal models of tumorigenesis, which is beyond the scope of the present study. Nonetheless, the present study lays the groundwork for further investigations into the potential roles of miR-605 relevant to cancer.

Specific points:
"1) Does p53 really bind to the promoter of PRKG1? The presented data still do not unequivocally answer this question.
a. The authors say that they now performed the ChIP-PCR by real-time, quantitative PCR, as was demanded by two out of three reviewers. It is very unusual, though, that they precipitated 50% of the available promoter DNA with p53. This would be the most efficient ChIP ever seen for p53. Any ChIP result found by other groups for p53 was less than 1% of input. Moreover, the authors unfortunately ignored the advice from Reviewer 1 to include more established p53-responsive promoters as positive controls in this ChIP experiment. Such an experiment would simply consist in using the same DNA preparations in PCRs with additional primers. (Minor point: For negative control genes, the figure labeling indicates NFAT, but the text and legend say HERG)."
We apologize for the lack of clarity of description of our data. We actually calculated the fold differences between the PRGK1 band as a signal and the laminA band as a control, following the procedures published in Nat Protocols [Mukhopadhyay A, Deplancke B, Walhout AJ, Tissenbaum HA. Chromatin immunoprecipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in Caenorhabditis elegans. Nat Protoc 2008;3:698-709].

There is hardly a reasonable comparison with DNA input. We have now rearranged the format of data presentation to reflect the procedures in Figure 3A. In addition, we have used p21 promoter region as a positive control for p53 binding. The data are shown in Figure 3A.
Throughout our manuscript, we found "HERG" only in the references list. There is no such a label as "HERG" in the text and legends.

"b. The authors try to fortify the function of the claimed p53-responsive element in the PRGK1 promoter. In their rebuttal letter, they say that they have now included a mutated sequence in their EMSA experiments, and they talk about the right panel of Fig. 3B. Unfortunately, this experiment is not described at all in the figure legend. It is neither clear what the faint bands in these panels should tell the reader, nor is it clear what kind of mutation was introduced into the oligo used in this study."

We have conducted additional experiments using newly purchased anti-p53 antibody to improve the data quality. A new image is now presented in Fig. 3B to show a clear supershift band. And detailed description of the data is also provided in the Figure legend.
The mutant decoy ODN for luciferase assay was/is described in the subsection entitled "Preparation of decoy ODNs" (page 15, para 1). The sequences of mutant p53 cis-element in the PRGK1 promoter are provided under the subsection entitled "Electrophoresis mobility shift assay (EMSA)" (page 16, para 2).

"2 Mir-605 appears to induce apoptosis when overexpressed. Fig. 4B also argues that antagonizing miR-605 reduces doxorubicin-induced apoptosis. But does miR-605-induced apoptosis work through p53 activation? It might, since p53-inducible miR-34 appears necessary for miR-605-induced apoptosis, and since pftalpha also impairs miR-605-induced apoptosis. On the other hand, it is difficult to understand why antagonizing miR-605 also reduces Nutlin-3-induced apoptosis. After all, Nutlin-3 abolishes the interaction between p53 and Mdm2, and thus should be rendering the effects of miR-605 on Mdm2 irrelevant. But this is not what is seen in Fig. 4B, raising the suspicion that miR-605 induces apoptosis also by p53-independent mechanisms. The use of an entirely different, p53-mutant cell line is not really helpful, since many properties (including transfection efficiency) are may differ between these cell lines on top of the p53 status. Isogenic HCT116 cells with or without p53 are available from public depositories or from B. Vogelstein (Johns Hopkins, Baltimore), against the statements of the authors in their rebuttal letter. Similarly, it is very curious that they declare the regulation of p53 levels by Mdm2 as irrelevant in their rebuttal letter, despite the overwhelming literature on Mdm2 as a ubiquitin ligase for p53. Their failure to detect changes in p53 levels in response to miR-605 should at least be discussed in the text."

Figure 4B is not about apoptosis; we believe that the reviewer is referring to Fig. 5B. One possible explanation for the data showing the ability of AMO-605 to reduce nutlin-3-induced apoptosis is that the concentration of nutlin-3 used in our study might not be sufficient to inhibit the Mdm2 function completely, and the upregulation of Mdm2 protein by AMO-605 could thus enhance Mdm2 function to abrogate apoptosis in the presence of nutlin-3. This is indeed supported by our experiments demonstrating a failure of AMO-605 to reverse the nutlin-3-induced apoptosis when the concentration of nutlin-3 was elevated from 1 M to 3 M, as shown in the revised Figure 5B. Also important to note that Mdm2 is a p53 responsive geneóthat is, its transcription can be activated by p53. Thus when p53 is stabilized by nutlin-3, the transcription of Mdm2 is also induced, resulting in higher Mdm2 protein levels to counteract the inhibitory effect of nutlin-3.

We would like to thank the reviewer for his/her direction for getting the isogenic HCT116 cells from Dr. Vogelstein. We have repeated the same set of experiments shown in the original Figure 5 using these isogenic cells. The original data presented in Figure 5 in the old version have now been replaced by the new data collected from isogenic HCT116 cells. As expected, the results in our original data set were overall reproduced in the isogenic HCT116 cells. Additionally, we compared the luciferase activities of the pGL3 vector carrying the putative cis-elements for p53 in the promoter region of PRGK1 gene between isogenic colorectal carcinoma HCT116 cells (p53+/+ and p53 /), and we found substantially higher luciferase activities in p53+/+ than in p53 / HCT116 cells, an additional evidence for validity of the cis-elements as binding sites for p53. The result is added to text (page 7, lines 8-10) and Fig. 2F.

We have never declared in our rebuttal letter or anywhere in the manuscript that the regulation of p53 levels by Mdm2 is irrelevant. Our previous response is quoted below for information. Clearly, we stated that the data presentation should be more rational and logical if the data on this issue are presented in Figure 3 instead of in Figure 1.

[The focus of Figure 1 is on the role of miR-605 in repressing Mdm2. The consequences of Mdm2 repression (changes of p53 activity and level) are presented in Figure 3 and in the text under the section subtitled "miR-605 enhances transactivation activity of p53". Our results showed that repression of Mdm2 by miR-605 did not cause significant upregulation of p53 level though there is a tendency of increase. This is consistent with the common view that Mdm2 primarily inhibit the activation of p53 but not the expression of p53. We feel that addition of p53 data to Figure 1 would create an "early jump" and break the logical flow of the article. We therefore decided to place the p53 data in Supplementary Figure 6S].

As far as the regulation of p53 levels by Mdm2 concerned, it should be kept in mind that regulation between Mdm2 and p53 is mutual; there is a negative feedback between them. While p53 protein levels may be upregulated by miR-605 through reducing Mdm2 protein level to weaken its proteasome-dependent action, Mdm2, as a p53-responsive gene, may also be upregulated, which in turn can feedback to downregulate p53 protein level through the enhanced E3 ubiquitin ligase activity. Thus, whether an increase in p53 level by miR-605 can be seen may depend upon the time frame and other particular conditions for detection. Indeed, as in our manuscript, there is a tendency of increase in p53 protein level in the presence of miR-605. Moreover, we have carried out additional experiments in HCT116 cells by measuring p53 levels 24 h after nutlin-3 treatment (instead of 48 h after treatment as in our other experiments) and we indeed observed upregulation of p53 in p53+/+ HCT116 cells. The data are shown in Supplementary Figure 6C.

"3) How does miR-605 regulate Mdm2? The authors identified a number of putative binding sites for miR-605 within the 3' UTR of Mdm2. They decided not to test the relevance of any of them by mutational analysis, since they feel this is too much of a complicated issue (as said in their rebuttal letter). It is doubtful whether other high-ranking journals would accept such a statement. After all, even combining a limited number of candidate target sites would be very doable, and it would definitely improve the specificity and credibility of the model proposed by the authors."

It is often and easily oversimplified that each of the multiple binding sites for a given miRNA act independently; the reality is most likely that the sites interplay or crosstalk to produce either positively or negatively cooperative effects. For a single-site miRNA-mRNA interaction, mutagenesis may be able to provide precise information. But for multi-site interactions like the case for miR-605:Mdm2, it becomes a complicated issue not only for experimental approaches but also for data interpretations, and the suggested mutagenesis may not provide a clear picture for the interactions. This was why we tried to avoid touching the issue. However, to follow the suggestion raised by the reviewer, we have conducted additional experiments. We selected four sites out of 12 predicted sites for mutagenesis with the highest scoring candidate and the lowest scoring ones and performed luciferase assay with these constructs. Our results demonstrated differential decreases in luciferase activities induced by miR-605 with the vector carrying different binding sites. Mutation of the sites either eliminated or weakened the effects of miR-605 on the luciferase activities. These data provide evidence for the direct interactions between miR-605 and its binding sites at the 3íUTR of Mdm2 mRNA. The data are added to the text (page 5, last para and page 6, 1st para) and Figure 1D.

"4). Minor point: The model in the last figure should also have an "inhibitor"-like arrowhead (-----/) at the end of the arrow going from Mdm2 to p53."

We have revised the graph according to the reviewerís suggestion (Figure 6).

Response to Referee #2's Comments

"The paper has been strengthened substantially by new experimental data. It is now more convincing and better controlled than before.
Publication is recommended."
We thank the reviewer for his/her appreciation of our work.

Response to Referee #3's Comments

"The authors have addressed most of the previous questions verbally and experimentally. The manuscript has been improved significantly."
We thank the reviewer for his/her positive feedback on our revision.

"However, it is necessary to make the mutants of miR-605 targeting site of Mdm2 3UTR, and to check the effect of miR-605 on these mutants, since the main finding of this study is that miR-605 targets mdm2 directly."
It is often and easily oversimplified that each of the multiple binding sites for a given miRNA act independently; the reality is most likely that the sites interplay or crosstalk to produce either positively or negatively cooperative effects. For a single-site miRNA-mRNA interaction, mutagenesis may be able to provide precise information. But for multi-site interactions like the case for miR-605:Mdm2, it becomes a complicated issue not only for experimental approaches but also for data interpretations, and the suggested mutagenesis may not provide a clear picture for the interactions. This was why we tried to avoid touching the issue. However, to follow the suggestion raised by the reviewer, we have conducted additional experiments. We selected four sites out of 12 predicted sites for mutagenesis with the highest scoring candidate and the lowest scoring ones and performed luciferase assay with these constructs. Our results demonstrated differential decreases in luciferase activities induced by miR-605 with the vector carrying different binding sites. Mutation of the sites either eliminated or weakened the effects of miR-605 on the luciferase activities. These data provide evidence for the direct interactions between miR-605 and its binding sites at the 3íUTR of Mdm2 mRNA. The data are added to the text (page 5, last para and page 6, 1st para) and Figure 1D.

3rd Editorial Decision 01 December 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-74108R1 to the EMBO Journal. It has now been seen again by referee 1, whose comments are enclosed below. As you will see, he/she remains disappointed at the lack of clinical data, but has no other objections to publication at this time. Given that we had already agreed that such data - while clearly valuable - would not be essential for publication here, I am therefore pleased to tell you that we will be able to accept your manuscript for publication in EMBOJ.

However, I do have a couple of remaining issues from the editorial side. Firstly, and most importantly, the data presented in Figure 3A are somewhat confusing and poorly described/labelled. From the text, I guess that the lanes show the following:

1. Input: PRKG1 primers
2. PRKG1: p53 ChIP, PRKG1 primers
3. NFAT: p53 ChIP, NFAT primers (or is this NFAT ChIP, PRKG1 primers?)
4. Lamin A: lamin A ChIP, PRKG1 primers.

5. Input: p21 primers
6. p21: p53 ChIP, p21 primers
7. IgG: It's not at all clear what this lane represents
8. Lamin A: lamin A ChIP, p21 primers.

However, this is not clearly labelled on the figure or described in the legend. In addition, I'm unclear as to why you are using the lamin A qPCR as a control (particularly since you appear to see lamin A binding to the p21 promoter - lane 8), as opposed to a negative control using the p53 ChIP but primers amplifying a region to which p53 doesn't bind. Before publication, this clearly needs to be clarified, and the figure appropriately modified.

Secondly, please can I ask you to include Author Contributions and Conflict of Interest statements at the end of the manuscript text?

Please can you get back to me with a clarification of panel 3A (and including the new figure) before uploading the final version of the manuscript - so that we can check everything is okay before you formally submit? Once we have resolved this remaining issue, we should then be able to accept the study for publication.

REFEREE REPORT

Referee #1 (Remarks to the Author):

The authors successfully eliminated a number of technical concerns. Using HCT116 cells lacking p53 is an improvement, and also the mutational analysis of the microRNA targets was helpful. I am still not happy with the idea of publishing a p53-regulatory miRNA without evidence for its clinical relevance. At least expression data in cancer vs. normal tissue would really have been helpful. But since the editor made it clear that she does not find such evidence necessary for publishing the paper in EMBO J, I won't object any longer.

Additional Correspondence 02 December 2010

Thank you so very much for your favorable editorial decision.

We have addressed the concerns you raised, as listed below.

Response to Editor's Concerns

"Firstly, and most importantly, the data presented in Figure 3A are somewhat confusing and poorly described/labelled. From the text, I guess that the lanes show the following:
1. Input: PRKG1 primers
2. PRKG1: p53 ChIP, PRKG1 primers
3. NFAT: p53 ChIP, NFAT primers (or is this NFAT ChIP, PRKG1 primers?)
4. Lamin A: lamin A ChIP, PRKG1 primers.
5. Input: p21 primers
6. p21: p53 ChIP, p21 primers
7. IgG: It's not at all clear what this lane represents
8. Lamin A: lamin A ChIP, p21 primers

However, this is not clearly labelled on the figure or described in the legend. "

Yes, you are right. We have provided detailed description in Fig 3A legend. In addition, to avoid confusion, we have changed the labeling in Fig 3A.

"In addition, I'm unclear as to why you are using the lamin A qPCR as a control (particularly since you appear to see lamin A binding to the p21 promoter - lane 8), as opposed to a negative control using the p53 ChIP but primers amplifying a region to which p53 doesn't bind. Before publication, this clearly needs to be clarified, and the figure appropriately modified."

We initially used IgG as a negative control; but there was nearly zero amplification, which yielded a huge number of p21 signal when normalized to IgG. We then used added anti-lamin A as a second negative control, which gave a background signal after PCR amplification. We decided to use lamin A for normalization of the data, which actually gave similar results as in the case for PRKG1. There is a background signal for Lamin A which can be tuned down by adjusting the exposure of the image. If this is not satisfied we are willing to run a new experiment under identical conditions.

"Secondly, please can I ask you to include Author Contributions and Conflict of Interest statements at the end of the manuscript text?"
The sections Author Contributions and Conflict of Interest statements have now been added to the end of the manuscript after the ACKNOWLEDGEMENTS section

The modified manuscript (Figure 3A legend) and Figure 3 are atatched to this e-mail.

We thank you again for your highly efficient editorial process.

Best regards,

Additional Correspondence 02 December 2010

Many thanks for your clarification about figure 3A - the labelling on the panels is much clearer now. I would say, though, that I think the middle panel is now over-contrasted - it's not a problem that you have some amplification with the laminA antibody, but I don't think it's appropriate to try and hide this, and I would suggest that you revert to the initial settings, with a brief note in the legend to explain that this band is background. However, the figure legend is still somewhat confusing: you have added an appropriate explanation of each lane, but have not deleted the initial description of the panel, which still doesn't accurately describe the figure. I therefore suggest that you adjust the figure legend by deleting the sentences following "Left panel" and replacing with the detailed explanation of what each lane shows. In addition, you state in the legend that lane 7 is PRKG1 primers, but I ssume it's actually p21 - this needs to be changed.

Otherwise, I think it's fine now, so please make those changes and submit the final version of the manuscript through our online system. We should then be able to accept the study for publication without further delay.

Thanks and best wishes.

3rd Revision - authors' response 02 December 2010

Attached please find the revised manuscript that we would like to have your further consider for publication in EMBO Journal. The paper has been revised in response to your comments and suggestions. Specifically, we have corrected the mistakes in the Figure 3 legend and reverted the ChIP image back to the original version in Fig 3A.

We hope the manuscript is now acceptable for publication in the EMBO J. We would like to thank you sincerely for your favorable consideration and proof-reading editing of our manuscript.

Accepted 03 December 2010

Many thanks for submitting the final version of your manuscript. Everything looks fine, so I am pleased to tell you that we can now accept the study for publication in the EMBO Journal. You should receive the formal acceptance message shortly.

