EMBO reports

Regulation of the Mdm2-p53 pathway by the ubiquitin E3 ligase MARCH7

Kailiang Zhao, Yang Yang, Guang Zhang, Chenfeng Wang, Decai Wang, Mian Wu and Yide Mei

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Transaction Report:

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

1st Editorial Decision 9 June 2017

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

I am sorry to say that the evaluation of your manuscript is not a positive one. As you will see, while all three referees acknowledge that the findings are potentially interesting, they all raise a number of largely overlapping concerns and point out a number of experiments and controls that should be done to substantiate the reported findings. All referees are concerned that no data are provided on the biological relevance of the observed regulation of Mdm2/p53 by MARCH7. Moreover, functional data from normal cell lines or data on endogenous proteins are missing.

Given these concerns, the amount of work required to address them, the uncertain outcome of these experiments, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from a majority of referees, I am sorry to say that we cannot offer to publish your manuscript.

However, given the potential interest of the findings, we would have no objection to consider a new manuscript on the same topic if at some time in the near future you obtained data that would considerably strengthen the message of the study and that address the referees concerns in full. To be completely clear, however, I would like to stress that if you were to send a new manuscript this would be treated as a new submission rather than a revision and would be reviewed afresh, also with respect to the literature and the novelty of your findings at the time of resubmission.

I am sorry to disappoint you on this occasion, and hope that the referee comments will be helpful in your continued work in this area.

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REFEREE REPORTS

Referee #1:

Comments
In this manuscript, Mei et al showed that MARCH7 as a critical regulator of MDM2 and define an important function of MARCH7 in the regulation of the MDM2-p53 pathway. MARCH7 interacts with MDM2 and is essential for maintaining the stability of MDM2. And also, MARCH7 promotes MDM2-dependent degradation of p53. Furthermore, MARCH 7 regulates cell proliferation, anchorage-independent cell growth, and apoptosis in a p53-dependent manner. These findings are potentially interesting. The manuscript could be further strengthened with a few additional experiments denoted below.

1) The authors should re-check the manuscript from page 6 line number 16-21. The contents are not concordance with figures. Interaction between endogenous MARCH1 and MDM2 should be explained about figure 1E, not 1D. And also, please explain in a little more detail how MARCH7 mutant works in cancer cells.

2) In figure 2B, there are mismatches between Flag-MDM2 (1-199) and Flag-MDM2 (300-491). Please re-label the bands.

3) In figure 3B and D, it is better to quantify the data same done for Figure 3A.

4) In figure 3F and G, the authors need to repeat these experiments. In figure 3F, Flag-MARCH7 expression is too low compared with other data on this manuscript, such as Figure 3B even though the same cell line were used for these figures. Also, transfection efficiency looks different between Flag-MARCH7 (-) and Flag-MARCH7 (+) group. For figure 3G, MARCH7 band is not clear on sh-control group. The authors need repeat this experiment as well.

5). It seems that MARCH7 inactivation of p53 might be cell specific, as this is not so apparent in U2OS cells (Figure 4A). This needs to be discussed.

6). It remains incompletely clear if MARCH7 regulation of cell proliferation and apoptosis is p53-dependent. It would be better to use a couple of paired cells with or without endogenous p53 to test this remaining question, though knockdown of p53 did rescue partially apoptosis that was caused by knockdown of MARCH7 in HCT116 cells (Figure 6).

7). There are several places that incorrectly or inaccurately describe the literature information on the p53-MDM2 field. Some sentences need to be re-written as they do not exactly reflect what is going on the field of p53-MDM2 research. For example, the sentence of "However, the regulation of Mdm2 remains largely unknown" is very inaccurate, as so much has been shown to regulate MDM2 in the field, even including some of the articles the authors have cited.

8). It would be more significant if biological relevance of the regulation of MDM2 by MARCH7 in cancer development can be addressed either using xenograft or clinical relevance, such as by searching the cancer genomic database.

Referee #2:

This work by Zhao et al describes a novel interaction between Mdm2 and MARCH7, a RING domain-containing ubiquitin E3 ligase, involved in the regulation of neurological development and the immune system. The authors claimed that MARCH7 directly interacts with, and stabilizes Mdm2 via Lys63-linked polyubiquitination, thereby regulating the Mdm2-p53 pathway. The overall idea of this work points to further elucidation of Mdm2 regulation, and ultimately, novel protein networks involved in the p53 degradation pathway that influence biological responses to DNA damage and other insults. The addition of MARCH7 to this network that includes numerous E3's, E4's and DUB's all acting on MDM2, p53, or both, speaks to incredible complexity.

As such, the finding of stable interaction of MARCH7 with MDM2, likely effect on MDM2 stability and possible interference with MDM2 degradation by K63-linked polyubiquitination suggests that this new finding could add significantly to our understanding of the regulation of p53 by MDM2. However, numerous deficiencies in the submitted manuscript detract from the potential significance, and reflect an underappreciation for the sophistication and evidence required to add yet another layer of regulation to this already complex system. Though detailed below, the paper focuses almost solely on examination of exogenously expressed MDM2 (and MARCH7), 2 cancer cell lines, and no normal/primary cells, a lack of explanation of confounding results (partial rescue of MARCH7 effects by p53 knockdown), and a general lack of rigor in arriving at conclusions (lack of protein analysis in Figs. 6E-F, lack of testing MARCH7 mutants that don't interact with MDM2 for MDM2 effects, etc.)
Based on the aforementioned concerns, and additional concerns mentioned below, this article cannot be recommended for publication due to lack of sufficient significance and impact for the general readership of EMBO reports.

Major concerns:
• Even if other hits from the Mass Spec analysis cannot be shown, the peak count and ranking within the hit list should be shown for MARCH 7.
• In Figure 2B, why does HA-MARCH band appear as doublets in the IP but not in the inputs? The arrow heads indicating Mdm2(1-199) and Mdm2(300-491) bands have been switched, making it confusing in interpreting the results. Also, is it Flag-Mdm2 1-199 or Flag-Mdm2 1-199aa? Authors called it deletion of the first 199 amino acids of Mdm2 but the schematic in Fig 2A and results in Fig 2B suggest the construct is Flag- Mdm2 1-199aa and not Flag-Mdm2 1-199. Please clarify.
• How can we tell the effect of MARCH7 on MDM2 is due to interaction? What is the effect on MDM2 ubiquitination and stability of a MARCH7 mutant that does not bind MDM2?
• Fig. 3B and 3C do not indicate dose-dependent increase in Mdm2 levels by MARCH7 as stated by the authors. Need to quantify bands?
• Authors need to show ubiquitination status and half-life of p53 with MARCH7 depletion/overexpression as well as with MARCH7:Mdm2 double depletion, to strengthen their claim that MARCH7 promotes Mdm2-dependent p53 degradation.
• Authors need to provide more evidence for the role of MARCH7 in p53-dependent apoptosis. Need to show activation of apoptotic markers such as PARP, cleaved caspases, and also, p53-dependent apoptotic genes such as PUMA, NOXA etc.
• What happens to p53 levels and ubiquitination after DNA damage with high or low MARCH7? Oddly, the authors show DNA damage induced apoptosis with no accompanying blots for p53, MDM2, MARCH7, etc.
• Again only one cancer cell line is used for the apoptosis assay so hard to generalize to other cancer types, and importantly no normal/primary cells are included in the analysis.
• What role does MARCH7 play in the known DNA damage induced destabilization of MDM2 that occurs after it is phosphorylated by ATM? This seems critical to understanding the biology of MARCH7 in this system.

Minor concerns:
• MARCH7 is never spelled out in unabbreviated form.
• Authors need to pay close attention to proper labeling of results. Fig. 1D and 1E have been switched. Endogenous interaction between MARCH7 and Mdm2 is Fig. 1E and not Fig. 1D as referred to under the result section in the text.
• Typological errors
• Labeling errors
• Fig. 1B shows expression of HA-MARCH7 alone or together with Flag-Mdm2, whereas under the result section in the text, authors described this as expression of Flag-Mdm2 alone or together with HA-MARCH7.

Referee #3:

The manuscript by Zhao et al., reports the regulation of Mdm2 by a novel interacting partner, MARCH7.
Mdm2 is a crucial regulator of the oncosuppressor p53. As a consequence, modification of Mdm2 levels impacts on p53 function and potentially human tumorigenesis. MARCH7 is a ubiquitin ligase

with few reports about its function.
Here, the authors show that MARCH7 interacts with Mdm2 (Fig 1, 2), enhances Mdm2 stabilization by promoting K63 ubiquitination (Fig 3, 5) and as a consequence impairs in vitro p53 function (Fig. 4, 6).
The experiments are clear as well as the flow of the study. The main molecular findings are well documented using independent lines of experimental evidence. However, some efforts should be made to evidence the ubiquitination of endogenous Mdm2 at K63 by MARCH7 (the data are provided only for an exogenously expressed mutant Mdm2), especially when considering that this activity has not previously reported for this protein.
My major concern is about the relevance of this relationship as such. There is no evidence of a functional link between the two proteins in normal or tumor cells. If MARCH7 is relevant for Mdm2/p53 control, we could expect a change in the Mdm2/MARCH7 binding and/or MARCH7 levels in different cell growth conditions when p53 activation is required. In addition, MARCH7 is overexpressed in ovarian cancer, a tumor that expresses mutant p53 in a high percentage of cases. This data seems to contradict the model proposed by the authors. Finally, MARCH7 is expressed in most embryonic tissues up to E15.5 (that can explain its presence in MEFs) and in the nervous/immune system of the adult mouse. The authors do not show functional data about these normal cells.
In the absence of this data, I think this study is of limited interest to the general tumor/molecular biology community.

Major concerns:
1) The authors should analyse the binding between endogenous proteins under different cell growth conditions. Alternatively, the authors could analyse MARCH7 levels correlated to Mdm2 in human tumors, especially in those tumors where Mdm2 is overexpressed but not amplified.
2) An important factor in Mdm2 function is its intracellular localization. In which compartment, does the binding between Mdm2 and MARCH7 occur?
3) There is not definitive proof that the activity of MARCH7 on endogenous p53 function is mediated by Mdm2. Silencing of Mdm2 or utilization of p53/Mdm2 interaction inhibitor (i.e. Nutlin) should be used to demonstrate this. Accordingly, the increased levels of exogenous p53 in Mdm2/p53 -/- MEFS (experiment Fig.4D) should be completed by analysis of the effect of MARCH7 on p53 transcriptional activity, p53 target genes, and p53-mediated function.
In addition, increased ubiquitination of p53 by MARCH7-stabilized Mdm2 should be shown to further demonstrate the Mdm2-mediated activity of MARCH7.
4) Some efforts should be done to evidence the ubiquitination of endogenous Mdm2 at K63 by MARCH7 (antibodies specific for the two ubiquitin chain types have been described).

Minor points:
- It is assumed that the authors have repeated each single experiment different times. However, this is not mentioned in the figure legends. Accordingly, quantification in Fig 3F, 3G, 4A, 4B, 4C, 4D should be shown as mean+SD of n experiments.

- The efficiency of MARCH7 silencing appears highly variable as well as its effect on MDM2 levels (in Fig. 6B, and 6D, Mdm2 levels are unaffected by siMARCH7). Can the authors provide an explanation for this? Did they check whether MARCH7 levels or its interaction with Mdm2 change during cell growth and/or stress conditions?

- The authors should mention and comment about the MARCH7 KO mouse model.

- The authors should mention the existence of other mechanisms that stabilize Mdm2 levels (i.e. the expression of aberrant forms of MDMX)

- Page 6, lane 18 (Figure 1D should be replaced by 1E)

- Please check typos (Page 7, line 12, "Fiugre", page 8 line 23 "dificient", page 10, line 3 "conjucated"...)

Response to Referee #1:

In this manuscript, Mei et al showed that MARCH7 as a critical regulator of MDM2 and define an important function of MARCH7 in the regulation of the MDM2-p53 pathway. MARCH7 interacts with MDM2 and is essential for maintaining the stability of MDM2. And also, MARCH7 promotes MDM2-dependent degradation of p53. Furthermore, MARCH 7 regulates cell proliferation, anchorage-independent cell growth, and apoptosis in a p53-dependent manner. These findings are potentially interesting. The manuscript could be further strengthened with a few additional experiments denoted below.

We greatly appreciate the reviewer's critical comments. Our detailed point-to-point response to the reviewer's comments is given below.

1) The authors should re-check the manuscript from page 6 line number 16-21. The contents are not concordance with figures. Interaction between endogenous MARCH1 and MDM2 should be explained about figure 1E, not 1D. And also, please explain in a little more detail how MARCH7 mutant works in cancer cells.
We thank the reviewer for his/her careful reading of our manuscript. In the revised manuscript, the labeling errors have been corrected (Figures 1D and 1E).

2) In figure 2B, there are mismatches between Flag-MDM2 (1-199) and Flag-MDM2 (300-491). Please re-label the bands.
In the revised manuscript, we have corrected the labeling errors (Figure 2B).

3) In figure 3B and D, it is better to quantify the data same done for Figure 3A.
According to the reviewer's comment, the previous Figures 3B and 3D have been replaced by new figures. Also, we have quantified the protein bands on these blots by using ImageJ software. The ratio of Mdm2 to GFP was then calculated. The data are represented as mean ± SD of three independent experiments (Figures EV2B and EV2E).

4) In figure 3F and G, the authors need to repeat these experiments. In figure 3F, Flag-MARCH7 expression is too low compared with other data on this manuscript, such as Figure 3B even though the same cell line were used for these figures. Also, transfection efficiency looks different between Flag-MARCH7 (-) and Flag-MARCH7 (+) group. For figure 3G, MARCH7 band is not clear on sh-control group. The authors need repeat this experiment as well.
As suggested by the reviewer, we have re-performed the experiment to evaluated the effect of MARCH7 on the stability of Mdm2. The result consistently showed that ectopic expression of MARCH7 markedly prolonged the half-life of Mdm2 (Figure 3F). We have also repeated the experiment for Figure 3G. The result was similar to the previous one, where MARCH7 was only detected in the CHX-untreated sample (0 time point), but not in the samples treated with CHX for 30, 60, and 90 minutes as indicated (Figure 3G). Therefore, the previous Figure 3G was not replaced in the revised manuscript. We reasoned that the half-life of endogenous MARCH7 are less than 30 min, which has been indicated in the revised manuscript.

5). It seems that MARCH7 inactivation of p53 might be cell specific, as this is not so apparent in U2OS cells (Figure 4A). This needs to be discussed.
As we mentioned in the Materials and Methods section of the manuscript, all the data have been repeated at least three times. Therefore, we are sure that the effect of MARCH7 on p53 expression is not cell specific. To avoid any possible misinterpretation of our presented data, in the revised manuscript, we have replaced the previous Figure 4A by the new figure (Figure 4A).

6). It remains incompletely clear if MARCH7 regulation of cell proliferation and apoptosis is p53-dependent. It would be better to use a couple of paired cells with or

without endogenous p53 to test this remaining question, though knockdown of p53 did rescue partially apoptosis that was caused by knockdown of MARCH7 in HCT116 cells (Figure 6).
We appreciate the reviewer's critical comments. To make our conclusions more persuasive, we evaluated the effect of MARCH7 on cell proliferation and apoptosis in the paired HCT116 human colorectal cancer cell lines (*p53*+/+ and *p53*-/-). The results showed that knockdown of MARCH7 effectively inhibited the proliferation of HCT116 *p53*+/+ cells, but not HCT116 *p53*-/- cells (Figures 6B and EV5D). In addition, MARCH7 knockdown dramatically sensitized HCT116 *p53*+/+ cells, but not HCT116 *p53*-/- cells, to doxorubicin-induced apoptosis (Figures 6D and EV6E).

In the revised manuscript, we have also used the normal mouse embryo fibroblast (MEF) cells to determine the p53-dependent effect of MARCH7 on cell proliferation and apoptosis. knockdown of MARCH7 greatly inhibited the proliferation of MEF cells (Figures 6C and EV5E). MARCH7 knockdown also led to a dramatic increase in doxorubicin-induced apoptosis in MEF cells (Figures EV6F and EV6G). However, both of these phenotypes, caused by MARCH7 knockdown, could be reversed by the simultaneous knockdown of p53 (Figures 6C, EV5E, EV6F and EV6G). Taken together, these data consistently suggest that MARCH7 regulates cell proliferation and apoptosis in a p53-dependent manner.

7). There are several places that incorrectly or inaccurately describe the literature information on the p53-MDM2 field. Some sentences need to be re-written as they do not exactly reflect what is going on the field of p53-MDM2 research. For example, the sentence of "However, the regulation of Mdm2 remains largely unknown" is very inaccurate, as so much has been shown to regulate MDM2 in the field, even including some of the articles the authors have cited.
According to the reviewer's suggestion, we have rephrased some sentences in the revised manuscript. For instance, the sentence "However, the regulation of Mdm2 remains largely unknown" has been changed to "However, the regulation of Mdm2 remains not well understood".

8). It would be more significant if biological relevance of the regulation of MDM2 by MARCH7 in cancer development can be addressed either using xenograft or clinical relevance, such as by searching the cancer genomic database.
To determine the biological relevance of the MARCH7-Mdm2-p53 axis in cancer development, we used a xenograft mouse model. MARCH7 knockdown greatly suppressed tumorigenecity of HCT116 cells (Figures 6H-6K), which was accompanied by the decreased levels of Mdm2 and the increased levels of p53 (Figure 6K). However, knockdown of MARCH7 had no obvious effect on tumorigenecity of p53-knockdown HCT116 cells (Figures 6H-6K). These data indicate that the MARCH7-Mdm2-p53 axis indeed plays an important role in the regulation of tumorigenesis.

Response to Referee #2:

This work by Zhao et al describes a novel interaction between Mdm2 and MARCH7, a RING domain-containing ubiquitin E3 ligase, involved in the regulation of neurological development and the immune system. The authors claimed that MARCH7 directly interacts with, and stabilizes Mdm2 via Lys63-linked polyubiquitination, thereby regulating the Mdm2-p53 pathway. The overall idea of this work points to further elucidation of Mdm2 regulation, and ultimately, novel protein networks involved in the p53 degradation pathway that influence biological responses to DNA damage and other insults. The addition of MARCH7 to this network that includes numerous E3's, E4's and DUB's all acting on MDM2, p53, or both, speaks to incredible complexity.
As such, the finding of stable interaction of MARCH7 with MDM2, likely effect on MDM2 stability and possible interference with MDM2 degradation by K63-linked polyubiquitination suggests that this new finding could add significantly to our

understanding of the regulation of p53 by MDM2. However, numerous deficiencies in the submitted manuscript detract from the potential significance, and reflect an underappreciation for the sophistication and evidence required to add yet another layer of regulation to this already complex system. Though detailed below, the paper focuses almost solely on examination of exogenously expressed MDM2 (and MARCH7), 2 cancer cell lines, and no normal/primary cells, a lack of explanation of confounding results (partial rescue of MARCH7 effects by p53 knockdown), and a general lack of rigor in arriving at conclusions (lack of protein analysis in Figs. 6E-F, lack of testing MARCH7 mutants that don't interact with MDM2 for MDM2 effects, etc.)
Based on the aforementioned concerns, and additional concerns mentioned below, this article cannot be recommended for publication due to lack of sufficient significance and impact for the general readership of EMBO reports.

We really appreciate the reviewer's critical comments. Our detailed point-to-point response to the reviewer's comments is given below.

Major concerns:
• Even if other hits from the Mass Spec analysis cannot be shown, the peak count and ranking within the hit list should be shown for MARCH 7.
According to the reviewer's suggestion, the list of proteins in anti-Mdm2 immunoprecipitates identified by mass spectrometry has been shown in the revised manuscript (Figure EV1A).

• In Figure 2B, why does HA-MARCH band appear as doublets in the IP but not in the inputs? The arrow heads indicating Mdm2(1-199) and Mdm2(300-491) bands have been switched, making it confusing in interpreting the results. Also, is it Flag-Mdm2 1-199 or Flag-Mdm2 1-199aa? Authors called it deletion of the first 199 amino acids of Mdm2 but the schematic in Fig 2A and results in Fig 2B suggest the construct is Flag- Mdm2 1-199aa and not Flag-Mdm2 1-199. Please clarify.
Since both anti-Flag and anti-HA antibodies used in this study are derived from mouse, we believe that the band migrating above the HA-MARCH7 band shown in Figure 2B is a non-specific band. In support of this, there are always non-specific bands with molecular weights around 116 kD appeared in the immunoprecipitates when we use anti-Flag antibody for immunoprecipitation (Figures 1B, 1C and 2B). In the legends of the corresponding Figures, we have indicated this upper band as a non-specific band (Figures 1B, 1C and 2B).

We thank the reviewer for his/her careful reading of our manuscript. The arrow heads indicating Mdm2 (1-199) and Mdm2 (300-491) bands were indeed switched in the previous version of our manuscript. The labeling errors have been corrected in the revised manuscript. Also, the reviewer was right. For the mapping experiments, Flag-Mdm2 1-199aa, but not Flag-Mdm2 1-199, was used. This has been clarified in the revised manuscript.

• How can we tell the effect of MARCH7 on MDM2 is due to interaction? What is the effect on MDM2 ubiquitination and stability of a MARCH7 mutant that does not bind MDM2?
To address the reviewer's concern regarding whether the effect of MARCH7 on Mdm2 is due to interaction, an Mdm2 binding-defective mutant of MARCH7 (aa 543-616) was used (Figure 2D). The results showed that, unlike wild-type MARCH7, MARCH7 (aa 543-616) failed to increase Mdm2 levels (Figure EV2D). In addition, this MARCH7 mutant had no effect on Mdm2 polyubiquitination (Figure EV4B). Together, these data indicate that the interaction with Mdm2 is essential for MARCH7 to stabilize Mdm2.

• Fig. 3B and 3C do not indicate dose-dependent increase in Mdm2 levels by MARCH7 as stated by the authors. Need to quantify bands?
According to the reviewer's comments, the previous Figures 3B and 3C have been replaced by new figures. We have also quantified the protein bands on these blots by

using ImageJ software. The ratio of Mdm2 to GFP was then calculated. The data are shown as mean ± SD of three independent experiments (Figures EV2B and EV2C).

• *Authors need to show ubiquitination status and half-life of p53 with MARCH7 depletion/overexpression as well as with MARCH7:Mdm2 double depletion, to strengthen their claim that MARCH7 promotes Mdm2-dependent p53 degradation.*
We appreciate the reviewer's constructive comments. According to the reviewer's comments, we first determined whether the effect of MARCH7 on p53 levels is mediated by Mdm2. The Mdm2-p53 interaction inhibitor Nutlin-3 was utilized. Knockdown of MARCH7 consistently led to a great increase in the levels of p53 in HCT116 cells (Figure 4D, lanes 1 and 2; Figure EV3D). However, when these cells were treated with Nutlin-3, MARCH7 knockdown no longer showed any regulatory effect on p53 levels (Figure 4D, lanes 3 and 4; Figure EV3D).

we next evaluated the effect of MARCH7 on the polyubiquitination of p53. The results showed that knockdown of MARCH7 greatly decreased, whereas ectopic expression of MARCH7 markedly increased the polyubiquitination of p53 (Figures 4E and 4F). More importantly, when the Mdm2-p53 interaction was disrupted by Nutlin-3, MARCH7 failed to enhance the polyubiquitination of p53 (Figure 4F). Taken together, these data further support our claim that MARCH7 promotes Mdm2-dependent degradation of p53.

• *Authors need to provide more evidence for the role of MARCH7 in p53-dependent apoptosis. Need to show activation of apoptotic markers such as PARP, cleaved caspases, and also, p53-dependent apoptotic genes such as PUMA, NOXA etc.*
To further confirm the role of MARCH7 in p53-dependent apoptosis, we have performed several additional experiments. First, we determined the effect of MARCH7 on p53-dependent apoptosis in HCT116 cells. Consistent with our previous findings in U2OS cells (Figures EV6C and EV6D), knockdown of MARCH7 dramatically increased the sensitivity of HCT116 cells to doxorubicin-induced apoptosis, however, which was reversed by simultaneous p53 knockdown (Figures EV6A and EV6B). Second, the paired HCT116 cell lines (*p53*+/+ and *p53*-/-) were used. MARCH7 knockdown dramatically sensitized HCT116 *p53*+/+ cells, but not HCT116 *p53*-/- cells, to doxorubicin-induced apoptosis (Figures 6D and EV6E). Third, the normal mouse embryo fibroblast (MEF) cells were used to determine the effect of MARCH7 on apoptosis. MARCH7 knockdown also led to a dramatic increase in doxorubicin-induced apoptosis in MEF cells, which could be reversed by the simultaneous knockdown of p53 (Figures EV6F and EV6G). Together, these data suggest that MARCH7 regulates DNA damage-induced apoptosis in a p53-dependent manner.

Also, we have shown expression levels of Puma, and activation of apoptotic markers such as caspase-3 activation and PARP cleavage in the indicated figures (Figures 6D, EV6B, EV6D and EV6F).

• *What happens to p53 levels and ubiquitination after DNA damage with high or low MARCH7? Oddly, the authors show DNA damage induced apoptosis with no accompanying blots for p53, MDM2, MARCH7, etc.*
To determine the effect of MARCH7 on the p53 response to DNA damage, U2OS cells stably expressing either MARCH7 shRNA or ectopic MARCH7 protein were treated with doxorubicin for 0, 1, 2, or 3 hours, followed by western blot analysis to examine levels of p53 and p21. Knockdown of MARCH7 resulted in an earlier and stronger p53 accumulation and p21 induction in response to doxorubicin treatment (Figure EV5F). Conversely, ectopic expression of MARCH7 led to a decreased p53 response to doxorubicin treatment, as evidenced by a slower p53 accumulation and a weaker induction of p21 in MARCH7 over-expressing U2OS cells (Figure EV5G). These data suggest that MARCH7 regulates the p53 response to DNA damage.

According to the reviewer's comments, in the revised manuscript, we have shown the western blots for p53, Mdm2, MARCH7, Puma, PARP and caspase-3 in the indicated

figures (Figures 6D, EV6B, EV6D and EV6G).

• *Again only one cancer cell line is used for the apoptosis assay so hard to generalize to other cancer types, and importantly no normal/primary cells are included in the analysis.*
According to the reviewer's comments, in the revised manuscript, we have included more cell lines for both cell proliferation and apoptosis analyses. First, we determined the effect of MARCH7 on p53-dependent apoptosis in HCT116 cells. Consistent with our previous findings in U2OS cells (Figures EV6C and EV6D), knockdown of MARCH7 dramatically increased the sensitivity of HCT116 cells to doxorubicin-induced apoptosis, however, which was reversed by simultaneous p53 knockdown (Figures EV6A and EV6B). Second, the paired HCT116 cell lines (*p53*+/+ and *p53*-/-) were also used. The results showed that knockdown of MARCH7 effectively inhibited the proliferation of HCT116 *p53*+/+ cells, but not HCT116 *p53*-/- cells (Figures 6B and EV5D). In addition, MARCH7 knockdown dramatically sensitized HCT116 *p53*+/+ cells, but not HCT116 *p53*-/- cells, to doxorubicin-induced apoptosis (Figures 6D and EV6E). Third, the normal mouse embryo fibroblast (MEF) cells were used to determine the effect of MARCH7 on cell proliferation and apoptosis. The results showed that knockdown of MARCH7 greatly inhibited the proliferation of MEF cells (Figures 6C and EV5E). MARCH7 knockdown also led to a dramatic increase in doxorubicin-induced apoptosis in MEF cells (Figures EV6F and EV6G). However, both of these phenotypes, caused by MARCH7 knockdown, could be reversed by the simultaneous knockdown of p53 (Figures 6C, EV5E, EV6F and EV6G). Together with our previous findings, these data suggest that MARCH7 regulates cell proliferation and DNA damage-induced apoptosis in a p53-dependent manner.

• *What role does MARCH7 play in the known DNA damage induced destabilization of MDM2 that occurs after it is phosphorylated by ATM? This seems critical to understanding the biology of MARCH7 in this system.*
To determine whether MARCH7 regulates DNA damage-induced destabilization of Mdm2, U2OS cells stably expressing either MARCH7 shRNA or ectopic MARCH7 protein were treated with doxorubicin for 0, 1, 2, or 3 hours, followed by western blot analysis to examine levels of Mdm2. The results showed that the decreased expression of Mdm2 upon doxorubicin treatment was not affected by either knockdown or ectopic expression of MARCH7 (Figures EV5F and EV5G), indicating that MARCH7 does not contribute to DNA damage-induced destabilization of Mdm2. Despite of this, MARCH7 is indeed able to regulate the p53 response to DNA damage as we mentioned above (Please see our detailed response to the reviewer's major concern 7); Figures EV5F and EV5G). Together with our findings that MARCH7 is able to stabilize Mdm2 (Figure 3), these data suggest that MARCH7 controls the p53 response to DNA damage via regulating the basal levels of Mdm2.

Minor concerns:
• *MARCH7 is never spelled out in unabbreviated form.*
In the revised manuscript, MARCH7 has been spelled out in unabbreviated form on its first appearance.

• *Authors need to pay close attention to proper labeling of results. Fig. 1D and 1E have been switched. Endogenous interaction between MARCH7 and Mdm2 is Fig. 1E and not Fig. 1D as referred to under the result section in the text.*
• *Typological errors*
• *Labeling errors*
Again, we thank the reviewer for his/her careful reading of our manuscript. We have corrected these errors according to the reviewer's comments.

• *Fig. 1B shows expression of HA-MARCH7 alone or together with Flag-Mdm2, whereas under the result section in the text, authors described this as expression of Flag-Mdm2 alone or together with HA-MARCH7.*
In the revised manuscript, the sentence "we expressed Flag-Mdm2 alone or together

with HA-MARCH7 in HEK293T cells" has been corrected to "we expressed HA-MARCH7 alone or together with Flag-Mdm2 in HEK293T cells".

Response to Referee #3:

The manuscript by Zhao et al., reports the regulation of Mdm2 by a novel interacting partner, MARCH7.
Mdm2 is a crucial regulator of the oncosuppressor p53. As a consequence, modification of Mdm2 levels impacts on p53 function and potentially human tumorigenesis. MARCH7 is a ubiquitin ligase with few reports about its function. Here, the authors show that MARCH7 interacts with Mdm2 (Fig 1, 2), enhances Mdm2 stabilization by promoting K63 ubiquitination (Fig 3, 5) and as a consequence impairs in vitro p53 function (Fig. 4, 6).
The experiments are clear as well as the flow of the study. The main molecular findings are well documented using independent lines of experimental evidence. However, some efforts should be made to evidence the ubiquitination of endogenous Mdm2 at K63 by MARCH7 (the data are provided only for an exogenously expressed mutant Mdm2), especially when considering that this activity has not previously reported for this protein.
My major concern is about the relevance of this relationship as such. There is no evidence of a functional link between the two proteins in normal or tumor cells. If MARCH7 is relevant for Mdm2/p53 control, we could expect a change in the Mdm2/MARCH7 binding and/or MARCH7 levels in different cell growth conditions when p53 activation is required. In addition, MARCH7 is overexpressed in ovarian cancer, a tumor that expresses mutant p53 in a high percentage of cases. This data seems to contradict the model proposed by the authors. Finally, MARCH7 is expressed in most embryonic tissues up to E15.5 (that can explain its presence in MEFs) and in the nervous/immune system of the adult mouse. The authors do not show functional data about these normal cells.
In the absence of this data, I think this study is of limited interest to the general tumor/molecular biology community.

We greatly appreciate the reviewer's critical comments. Our detailed point-to-point response to the reviewer's comments is given below.

Major concerns:
1) The authors should analyse the binding between endogenous proteins under different cell growth conditions. Alternatively, the authors could analyse MARCH7 levels correlated to Mdm2 in human tumors, especially in those tumors where Mdm2 is overexpressed but not amplified.
We understand the reviewer's point that given the important role of MARCH7 in the regulation of the Mdm2-p53 axis, one could expect a change in the Mdm2/MARCH7 binding and/or MARCH7 levels in different cell growth conditions when p53 activation is required such as DNA damage.

According to the reviewer's comments, we have performed an immunoprecipitation experiment to examine whether the MARCH7-Mdm2 binding is subjected to the regulation by DNA damage. The results showed that the MARCH7-Mdm2 binding was not affected by DNA damage (Figure EV5H). In addition, levels of MARCH7 were not obviously changed upon doxorubicin treatment (Figures EV5F and EV5G). However, we showed that MARCH7 is indeed able to regulate the p53 response to DNA damage (Figures EV5F and EV5G). Knockdown of MARCH7 resulted in an earlier and stronger p53 accumulation and p21 induction in response to doxorubicin treatment (Figure EV5F). In contrast, ectopic expression of MARCH7 led to a decreased p53 response to doxorubicin treatment, as evidenced by a slower p53 accumulation and a weaker induction of p21 in MARCH7 over-expressing U2OS cells (Figure EV5G). Together with our findings that MARCH7 is able to stabilize Mdm2 (Figure 3), these data suggest that MARCH7 controls the p53 response to DNA damage via regulating the basal levels of Mdm2.

We agree with the reviewer's point that in order to determine the biological significance of the MARCH7-Mdm2-p53 axis, we could analyze MARCH7 levels correlated to Mdm2 in human tumors, especially in those tumors where Mdm2 is overexpressed but not amplified. We actually tried several anti-MARCH7 antibodies from different companies (Santa Cruz, Cat.# sc-166945; Santa Cruz, Cat.# sc-49275; Abcam, Cat.# ab84130; Origene, Cat.# AP52607PU-N; Sigma, Cat.# SAB2101435; Abgent, Cat.# AT2801a) for immunohistochemistry (IHC), nonetheless, none of them worked for IHC. Therefore, we here used a xenograft mouse model as an alternative strategy to determine the biological relevance of the MARCH7-Mdm2-p53 axis in cancer development. MARCH7 knockdown greatly suppressed tumorigenecity of HCT116 cells (Figures 6H-6K), which was accompanied by the decreased levels of Mdm2 and the increased levels of p53 (Figure 6K). However, knockdown of MARCH7 had no obvious effect on tumorigenecity of p53-knockdown HCT116 cells (Figures 6H-6K). These data indicate that the MARCH7-Mdm2-p53 axis indeed plays an important role in the regulation of tumorigenesis.

2) An important factor in Mdm2 function is its intracellular localization. In which compartment, does the binding between Mdm2 and MARCH7 occur?
As suggested by the reviewer, we have performed an immunofluorescence experiment to determine whether Mdm2 and MARCH7 could co-localize in the same compartment. The results showed that ectopically expressed Mdm2 and MARCH7 were co-localized in the nucleus (Figure EV1B), indicating that the Mdm2-MARCH7 interaction occurs in the nucleus.

3) There is not definitive proof that the activity of MARCH7 on endogenous p53 function is mediated by Mdm2. Silencing of Mdm2 or utilization of p53/Mdm2 interaction inhibitor (i.e. Nutlin) should be used to demonstrate this. Accordingly, the increased levels of exogenous p53 in Mdm2/p53 -/- MEFS (experiment Fig.4D) should be completed by analysis of the effect of MARCH7 on p53 transcriptional activity, p53 target genes, and p53-mediated function.
In addition, increased ubiquitination of p53 by MARCH7-stabilized Mdm2 should be shown to further demonstrate the Mdm2-mediated activity of MARCH7.
We appreciate the reviewer's constructive suggestions. To further determine whether the effect of MARCH7 on endogenous p53 is mediated by Mdm2, the Mdm2-p53 interaction inhibitor Nutlin-3 was utilized. Knockdown of MARCH7 consistently led to a great increase in the levels of p53 in HCT116 cells (Figure 4D, lanes 1 and 2; Figure EV3D). However, when these cells were treated with Nutlin-3, MARCH7 knockdown no longer showed any regulatory effect on p53 levels (Figure 4D, lanes 3 and 4; Figure EV3D).

To further confirm that MARCH7 promotes Mdm2-dependent p53 degradation, we evaluated the effect of MARCH7 on the polyubiquitination of endogenous p53. The results showed that knockdown of MARCH7 greatly decreased, whereas ectopic expression of MARCH7 markedly increased the polyubiquitination of p53 (Figures 4E and 4F). More importantly, when the Mdm2-p53 interaction was disrupted by Nutlin-3, MARCH7 failed to enhance the polyubiquitination of p53 (Figure 4F).

In the exogenous experiments by using *Mdm2-/-p53-/-* MEF cells, MARCH7 was shown to enhance Mdm2-mediated p53 polyubiquitination in a dose-dependent manner (Figure 4H, lanes 4-6). In these experiments, MARCH7 did not affect p53 polyubiquitination in the absence of Mdm2 (Figure 4H, lanes 1-3).

As suggested by the reviewer, we have also examined the effect of MARCH7 on p53 transcriptional activity in *Mdm2-/-p53-/-* MEF cells. In accordance with the findings that MARCH7 enhanced Mdm2-dependent polyubiquitination and degradation of p53 (Figures 4G and 4H), MARCH7 decreased p53 transcriptional activity in the presence, but not in the absence of Mdm2 (Figure EV3F).
Taken together, these data further support our claim that the effect of MARCH7 on p53 is mediated by Mdm2.

4) Some efforts should be done to evidence the ubiquitination of endogenous Mdm2 at K63 by MARCH7 (antibodies specific for the two ubiquitin chain types have been described).
To determine whether MARCH7 promotes Lys63-linked polyubiquitination of endogenous Mdm2, we performed an *in vivo* ubiquitination experiment in HCT116 cells stably expressing either control or MARCH7 proteins. The results showed that ectopic expression of MARCH7 was indeed able to increase Lys63-linked polyubiquitination of endogenous Mdm2 (Figure 5F).

Minor points:
- It is assumed that the authors have repeated each single experiment different times. However, this is not mentioned in the figure legends. Accordingly, quantification in Fig 3F, 3G, 4A, 4B, 4C, 4D should be shown as mean+SD of n experiments.
As we mentioned in the Materials and Methods section of the manuscript, all the data have been repeated at least three times. This has been indicated in the legends of the corresponding figures. As suggested by the reviewer, we have quantified the protein bands on the blots in Figures 3A-3D, 3F, 3G, 4A-4D, and 4G by using ImageJ software. The data are shown as mean ± SD of three independent experiments (Figures EV2A-EV2C, EV2E-EV2H, and EV3A-EV3E).

- The efficiency of MARCH7 silencing appears highly variable as well as its effect on MDM2 levels (in Fig. 6B, and 6D, Mdm2 levels are unaffected by siMARCH7). Can the authors provide an explanation for this? Did they check whether MARCH7 levels or its interaction with Mdm2 change during cell growth and/or stress conditions?
Since all the experiments in this study have been repeated at least three times, we are sure that the inconsistency in the effect of MARCH7 on Mdm2 levels is caused by the experimental variations. To avoid any possible misinterpretation of our presented data, in the revised manuscript, we have replaced the previous Figures 6B and 6D by new figures (Figures EV5A and EV5C).

As we mentioned above, the MARCH7-Mdm2 binding was not affected by DNA damage (Figure EV5H). In addition, levels of MARCH7 were not obviously changed upon doxorubicin treatment (Figures EV5F and EV5G). However, we showed that MARCH7 is indeed able to regulate the p53 response to DNA damage (Figures EV5F and EV5G). For the detailed information, please see our above response to the reviewer's major concern 1).

- The authors should mention and comment about the MARCH7 KO mouse model.
According to the reviewer's suggestion, the MARCH7 KO mouse model has been mentioned and commented in the revised manuscript.

- The authors should mention the existence of other mechanisms that stabilize Mdm2 levels (i.e. the expression of aberrant forms of MDMX)
As suggested by the reviewer, the existence of other mechanisms that stabilize Mdm2 has been mentioned in the revised manuscript.

- Page 6, lane 18 (Figure 1D should be replaced by 1E)
This error has been corrected in the revised manuscript.

- Please check typos (Page 7, line 12, "Fiugre", page 8 line 23 "dificient", page 10, line 3 "conjucated"...).
We thank the reviewer for his/her careful reading of our manuscript. These errors have been corrected in the revised manuscript.

2nd Editorial Decision 17 October 2017

Thank you for the re-submission of your research manuscript to our journal. We have sent the study back to the referees who also evaluated the earlier version of your study and we have now received

their reports (see below).

As you will see, the referees acknowledge that the study has been significantly improved. However, referees 2 and 3 also point out several remaining concerns that should be addressed prior to publication. As far as the xenograft experiments are concerned (referee 3, point 2) it is not a prerequisite to analyze additional mice. Upon further discussion with the referees we suggest to clearly indicate the limitation of the small sample size (n=4) and to discuss the relative limitation of the results obtained from this small sample size in the most appropriate manner as also outlined by referee 3.

Given these constructive comments, we would like to invite a final revision of your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: Please note that only up to 5 images can be submitted as Expanded View. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, can you please specify the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

****
REFEREE REPORTS

Referee #1:

The authors have carefully addressed all of the previous concerns.

Referee #2:

Manuscript Number: EMBOR-2017-44465V2-Q

The authors have responded to a prior review of this work under prior submission adequately with substantial revision, but there are a few additional points to address before acceptance:

1. Figure 4: Why is such a huge difference in p53 ubiquitination in the control lanes in 4E and 4F? Also were lysates normalized for p53 as there is no difference in p53 in the inputs with knockdown

or overexpression of MARCH7? The different Ub intensity in control lanes may reflect loading/exposure differences and actually the input blots lack loading controls that allow comparison between blots for loading. The reasons should be commented upon. Please update figure legend to explain exactly how experiment was done, including any normalization for native p53 to allow fair comparison of ubiquitinated species (if that was done).

2. Also Fig. 4, due to use of p53 and not Ub blotting antibody, blots in panels E, F, H show only multiple monoUb species (<110 kDa) and no polyUb species which require use of a Ub antibody or cotransfected HA-Ub. Ideally the blots should also be probed for Ub to show the poly species or accompanied by a transfected HA-Ub experiment. Otherwise authors must explain why it is sufficient to show the multiple mono species of p53 and not the polyUb species if they are arguing that stability of p53 is being regulated?

3. In the discussion section, the sentence "The observation that p53 is mainly controlled by a single master regulator Mdm2" should be rewritten, as there are other known regulators of p53.
4. No detailed method/ primers sequences for qPCR

Referee #3:

The authors have greatly improved the manuscript and detailed in a sufficient way the mechanism of MDM2 stabilization by MARCH7.

My major concerns are about the effect of MARCH7 on p53.
1) The data on p53-/- and p53+/+ HCT116 are rather convincing indicating that MARCH7 is ineffective in the absence of p53 in p53-/- HCT116. Conversely, the data in U2OS, HCT, and MEFs demonstrate that shMARCH7 is effective also in the absence of p53 (the effect of shMARCH7 on cell proliferation and colony formation in the absence of p53 - i.e. between shMARCH7/shp53 and shp53 cells - is really strong although the efficient silencing of p53. Similar observation for cell apoptosis in U2OS). Maybe, a possible explanation could be due to the published effect of MDM2 on p21. However, p21 is not analysed in these cells.
2) The data deriving from 4 tumors obtained in two mice do not possess sufficient statistical power.

On the basis of these results, I'm not sure that the oncogenic properties of MARCH7 are p53-dependent.

The authors should attenuate the relative sentences and/or provide clarification. I think they should mention the potential existence of p53-independent activity too.

specific points:
Abstract: MARCH7 regulates cell proliferation, DNA
11 damage-induced apoptosis, and tumorigenesis in a p53-dependent manner. Based on previous comments, this sentence should be re-formulated.

Pg 6 line 24 : MARCH7 and Mdm2 were co-localized in the nucleus, indicating that the 25 MARCH7-Mdm2 interaction occurs in the nucleus. Since the analysis is not apparently by a confocal microscopy (In the Mat & Met I could not find details about it), I think "suggesting" is more appropriate

Pg 8 line 9: Conversely, when MARCH7 was knocked down in U2OS cells, the half-life of Mdm2 was greatly shortened (Figures 3G and EV2G), This word is not reflecting what is shown. From the reported graph, I think the data are not significant. The authors should perform statistics of this triplicate. Similarly, the authors should perform statistics about p53

Page 11 line 29: which could be restored by the simultaneous knockdown of p53. I would say partly restored. The data are not so strong.

Response to Referee #1:

The authors have carefully addressed all of the previous concerns.

We appreciate the reviewer's positive comments on our manuscript.

Response to Referee #2:

Manuscript Number: EMBOR-2017-44465V2-Q

The authors have responded to a prior review of this work under prior submission adequately with substantial revision, but there are a few additional points to address before acceptance:

1. Figure 4: Why is such a huge difference in p53 ubiquitination in the control lanes in 4E and 4F? Also were lysates normalized for p53 as there is no difference in p53 in the inputs with knockdown or overexpression of MARCH7? The different Ub intensity in control lanes may reflect loading/exposure differences and actually the input blots lack loading controls that allow comparison between blots for loading. The reasons should be commented upon. Please update figure legend to explain exactly how experiment was done, including any normalization for native p53 to allow fair comparison of ubiquitinated species (if that was done).

The difference in p53 ubiquitination in the control lanes in the previous Figures 4E and 4F was due to the different exposure time. For the previous Figure 4E, a longer exposure time was applied to better see the difference in p53 ubiquitination between sh-control and sh-MARCH7 groups.

For the previous Figures 4E and 4F, we did not normalize cell lysates for p53 in the inputs with knockdown or overexpression of MARCH7. The reason why there is no difference in p53 levels in the inputs with knockdown or overexpression of MARCH7 is because cells were treated with the proteasome inhibitor MG132 for additional 6 h. Treatment with MG132 stabilized and eventually equalized the levels of p53 in the inputs. This information has been indicated in the legends for the revised Figures 4E and 4F.

According to the reviewer's comments, we have re-performed the *in vivo* ubiquitination experiments by using anti-ubiquitin antibody to evaluate the effect of MARCH7 on the polyubiquitination of p53. The previous Figures 4E and 4F have been replaced by new figures in the revised manuscript. The results consistently showed that knockdown of MARCH7 decreased, whereas ectopic expression of MARCH7 increased the polyubiquitination of p53 (Revised Figures 4E and 4F). In addition, when the Mdm2-p53 interaction was disrupted by Nutlin-3, MARCH7 failed to enhance the polyubiquitination of p53 (Revised Figure 4F). In the revised Figures 4E and 4F, GAPDH was also included as a loading control as suggested by the reviewer.

2. Also Fig. 4, due to use of p53 and not Ub blotting antibody, blots in panels E, F, H show only multiple monoUb species (<110 kDa) and no polyUb species which require use of a Ub antibody or cotransfected HA-Ub. Ideally the blots should also be probed for Ub to show the poly species or accompanied by a transfected HA-Ub experiment. Otherwise authors must explain why it is sufficient to show the multiple mono species of p53 and not the polyUb species if they are arguing that stability of p53 is being regulated?

We appreciate the reviewer's critical comments. To make our data more conclusive, we have re-performed the *in vivo* ubiquitination experiments by using anti-ubiquitin antibody as mentioned above. The results consistently showed that knockdown of MARCH7 decreased, whereas ectopic expression of MARCH7 increased the polyubiquitination of p53 (Revised Figures 4E and 4F). More importantly, when the Mdm2-p53 interaction was disrupted by Nutlin-3, MARCH7 failed to enhance the polyubiquitination of p53 (Revised Figure 4F).

3. In the discussion section, the sentence "The observation that p53 is mainly controlled by a single master regulator Mdm2" should be rewritten, as there are other known regulators of p53.

As suggested by the reviewer, the sentence "The observation that p53 is mainly controlled by a single master regulator Mdm2" has been changed to "The observation that p53 is controlled largely by its master regulator Mdm2".

4. No detailed method/ primers sequences for qPCR

According to the reviewer's comment, the method and primer sequences for qPCR have been provided in the revised manuscript.

Response to Referee #3:

The authors have greatly improved the manuscript and detailed in a sufficient way the mechanism of MDM2 stabilization by MARCH7.

My major concerns are about the effect of MARCH7 on p53.
1) The data on p53-/- and p53+/+ HCT116 are rather convincing indicating that MARCH7 is ineffective in the absence of p53 in p53-/- HCT116. Conversely, the data in U2OS, HCT, and MEFs demonstrate that shMARCH7 is effective also in the absence of p53 (the effect of shMARCH7 on cell proliferation and colony formation in the absence of p53 - i.e. between shMARCH7/shp53 and shp53 cells - is really strong although the efficient silencing of p53. Similar observation for cell apoptosis in U2OS). Maybe, a possible explanation could be due to the published effect of MDM2 on p21. However, p21 is not analysed in these cells.

We appreciate the reviewer's comments. During the experiments, we have also noticed that p53 knockdown could not completely reverse the effect of MARCH7 on cell proliferation and DNA damage-induced apoptosis. We think there are two possible explanations. One is that although the p53 knockdown efficiency appears good based on our western blot analysis, p53 knockdown cells may still have a small amount of residual p53. Another possibility is that, as suggested by the reviewer, there is the potential existence of p53-independent activity of MARCH7. These have been mentioned in the discussion section of the revised manuscript.

2) The data deriving from 4 tumors obtained in two mice do not possess sufficient statistical power.

We apologize for the confusion that has caused to the reviewer. Actually, in each group, 4 tumors were obtained from the indicated four mice. Left and Right indicate that, respectively, cells were injected into the left and right flanks of the indicated mice. This information has been provided in the legends for Figure 6H. Also, in the discussion section of the revised manuscript, we have mentioned the relative small sample size (n=4) for our xenograft experiments.

On the basis of these results, I'm not sure that the oncogenic properties of MARCH7 are p53-dependent.
The authors should attenuate the relative sentences and/or provide clarification. I think they should mention the potential existence of p53-independent activity too.

As suggested by the reviewer, in the revised manuscript, we have mentioned the potential existence of p53-independent activity of MARCH7.

specific points:
Abstract: *MARCH7 regulates cell proliferation, DNA*
11 damage-induced apoptosis, and tumorigenesis in a p53-dependent manner. Based on previous comments, this sentence should be re-formulated.

In the revised manuscript, the sentence "MARCH7 regulates cell proliferation, DNA damage-induced apoptosis, and tumorigenesis in a p53-dependent manner" has been changed to "MARCH7 is able to regulate cell proliferation, DNA damage-induced apoptosis, and tumorigenesis via a p53-dependent mechanism".

Pg 6 line 24 : MARCH7 and Mdm2 were co-localized in the nucleus, *indicating* that the 25

MARCH7-Mdm2 interaction occurs in the nucleus. Since the analysis is not apparently by a confocal microscopy (In the Mat & Met I could not find details about it), I think "suggesting" is more appropriate

For the immunofluoresence analysis, the images were taken with a fluorescence microscope (Olympus IX73). This information has been indicated in the legend for Figure EV1B. In the revised manuscript, the sentence "MARCH7 and Mdm2 were co-localized in the nucleus, indicating that the MARCH7-Mdm2 interaction occurs in the nucleus" has been changed to "MARCH7 and Mdm2 were co-localized in the nucleus, suggesting that the MARCH7-Mdm2 interaction occurs in the nucleus".

Pg 8 line 9: Conversely, when MARCH7 was knocked down in U2OS cells, the half-life of Mdm2 was *greatly* shortened (Figures 3G and EV2G), This word is not reflecting what is shown. From the reported graph, I think the data are not significant. The authors should perform statistics of this triplicate. Similarly, the authors should perform statistics about p53

In the revised manuscript, the sentence "Conversely, when MARCH7 was knocked down in U2OS cells, the half-life of Mdm2 was greatly shortened" has been changed to "Conversely, when MARCH7 was knocked down in U2OS cells, the half-life of Mdm2 was shortened". As suggested by the reviewer, the data from the triplicate experiments have been statistically analyzed to determine the half-life of Mdm2 and p53 (Figure EV2G).

Page 11 line 29: which could be restored by the simultaneous knockdown of p53. I would say partly restored. The data are not so strong.

As suggested by the reviewer, in the revised manuscript, the sentence "which could be restored by the simultaneous knockdown of p53" has been changed to "which could be partially restored by the simultaneous knockdown of p53".

3rd Editorial Decision 30 November 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have asked former referee 2 to evaluate the new ubiquitination data and as you will see, also this referee is very positive about the study and supports publication in EMBO reports.

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

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REFEREE REPORT

Referee #2:

The authors have adequately addressed my concerns regarding Fig. 4 ubiquitination data.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yide Mei
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44465V2-Q

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The information has been provided in Materials and Methods section. In page 21.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Mice were used in the experiment at random. This information has been provided in Materials and Methods section. In page 21.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	During testing the tumors' weight, the experimentalists were blinded to the information of tumor tissues. This information has been provided in the Materials and Methods section. In page 21.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, we used two-tailed t-test. In page 22.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The information has been provided in Materials and Methods section. In page 17.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The information has been provided in Materials and Methods section. In page 17.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	The information has been provided in Materials and Methods section. In page 21.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The information has been provided in Materials and Methods section. In page 21.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes, we confirm compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA