GRWD1 negatively regulates p53 via the RPL11-MDM2 pathway and promotes tumorigenesis

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1st Editorial Decision 26 April 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees acknowledge the potential interest of the findings. However, all referees have raised a number of concerns and have suggestions to improve the manuscript or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here, but in particular, co-immunoprecipitation experiments with endogenous proteins should be done, more cell lines should be used, the rescue experiment with a siRNA-resistant GRWD1 construct (first point of referee #1) should be done and more data on how (or if) endogenous GRWD1 (and its experimental manipulation) influences p53 levels and activity should be provided, as well as the correlation with the p53 status in the clinical samples (last point of referee #1 and point 7 by referee #2).

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns (as detailed in their reports) must be fully addressed in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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.

REFEREE REPORTS
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Referee #1:

In this Ms Kayama and colleagues describe the glutamate-rich WD40 repeat containing protein 1, GRWD1, as a new negative regulator of p53 activity under nucleolar stress. Authors show that GRWD1 binds to the Ribosomal protein L11 (RPL11) and thus compete for the interaction of RPL11 with MDM2 thus allowing MDM2 to degrade p53. Authors show that GRWD1 cooperates with E7 and Ras in conferring anchorage-independent growth and tumorigenic capacity to human fibroblast. Finally the authors show that GRWD1 expression is associated with poor prognosis in breast cancer.

The pathways inducing p53 response upon nucleolar stress are relevant for tumor suppression and are presently understudied, therefore the data presented in this MS are of interest for the field. At this stage however the MS seems to be preliminary and needs more experimental data to support the conclusions before publication.

In Fig 1a the result showing that silencing of GRWD1 leads to stabilization of p53 are not impressive. A rescue experiment with a GRWD1 siRNA resistant construct to make the evidence more robust is suggested. In fig 1a, right panel, in addition to caspase cleavage evaluation of the apoptosis is needed. Moreover neither in the figure legend nor in the text there are comments on pATM.

In Fig 2 the interaction between GRWD1 and RPL11 seems consistent, however CoIP experiments with endogenous proteins (Fig2a) should be done by with both proteins and also in other cell lines.

In Fig 4 authors evaluate the transforming capability of GRWD1 in cooperation with E7 and Ras oncogenes in human fibroblasts. According to the hypothesis and the results presented in figures 1 to 3, GRWD1 should bind to RPL11 and reduce the levels of p53. However in fig 4a, (or in other panels) p53 levels are not shown. In other words it is crucial for the paper to show that the in vivo transforming/oncogenic potential of GRWD1 depends on interference with the PRL11/MDM2 pathway.

Authors also show that GRWD1 expression correlates with poor prognosis in tumors in some datasets. How does this correlated with the status of p53 (wt or mutant) in the tumors analysed?

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Referee #2:

This manuscript presents evidence that GRWD1 (glutamine rich WD repeat containing 1) is a negative regulator of p53. The results indicate that GRWD1 sequesters ribosomal L11 protein and relieves its repression on MDM2, so that MDM2 can efficiently degrade p53. They also show that GRWD1 along with KRAS and HPV E7 are able to transform normal fibroblasts. Finally, by mining publicly available databases the authors show that GRWD1 overexpression is correlated with poor prognosis. Overall, the results are generally interesting and convincing. There are several issues, which the authors need to address as follows:

Major Points:

1. In the abstract, the authors mention that they found that GRWD1 was localized to nucleoli and was released into the nucleoplasm upon nucleolar stress. However, The authors do not provide data to support this conclusion and should do so.

2. The authors show that silencing GRWD1 increases p53 induction and that GRWD1 over expression of GRWD1 reduces p53 induction. No data are presented to correlate these results with
effects on p53 activity except for a western blot of p21 in Fig 1A. The authors should show that transcriptional activation/induction of well-established p53 target genes correlates with effects of GRWD1 manipulations on p53 levels.

3. The authors show the induction of apoptosis (PARP cleavage) with GRWD1 silencing. Is the observed phenotype p53-dependent? The authors should results in p53 wt and p53 null cells to rule in or rule out the role of p53 in such apoptosis induction.

4. Is p53 a substrate for GRWD1? In other words does GRWD1 function as an E3 ubiquitin ligase for p53?

5. It is well established that nucleolar stress activation of p53 is disabled in the MDM2C305F model system. What is the role/function of GRWD1 in such conditions? For example, does GRWD1 still bind to L11 in MDM2-C305F knock-in cells? Does GRWD1 modulate MDM2-C305F's E3 ligase function?

6. Neddylation of L11 has been shown to be crucial for its interaction with MDM2. The authors show that GRWD1 inhibits RPL11-MDM2 interactions. Do the authors have any idea concerning the effect of GRWD1 on L11 neddylation? Further, L11 recruitment on p53 target promoters makes MDM2 inactive at these sites. Is GRWD1 recruited on to the p53 target promoters?

7. The authors provide clinical data using publicly available expression profile studies indicating that over expression of GRWD1 correlates with poor prognosis. In this meta-analysis, have the authors considered p53 status (mutation, deletion etc.) and MDM2 status, such as 12q amplification? Without considering these parameters, this particular analysis does not appear relevant to the present manuscript.

8. Ribosomal protein mediated p53 activation in response to MDM2 inactivation is complicated. While p53 activation may help in tumor retardation, ribosomal dysfunction can lead to myelodisplastic syndromes. The authors indicate that over expression of GRWD1 correlates with poor prognosis (and functioning through ribosomal stress), but it is not at all clear what the mechanism might be and at the very least warrants some discussion.

Minor Point:

1. Authors use Actinomycin D to induce nucleolar stress. While it is a well-established drug for nucleolar stress induction, it would be helpful to use at least one more agent (like 5-FU or MPA) in some experiments, to make sure that the effects observed are not specific to one particular agent.

2. In Fig. 3A was the amount of p53 DNA construct used 7.5 micrograms instead of 7.5 nanograms?

Referee #3:

The paper from Kayama et al describes a possible role for GRWD1 in the regulation of p53, through binding to L11. GRWD1 is shown to interact with L11, and prevent L11 binding to MDM2. GRWD1 therefore prevents L11 dependent inhibition of MDM2 and stabilization of p53.

This is a novel story and the paper is clear and well written, and in general the data are convincing. The main problem with the paper is that it relies very heavily on overexpression systems and it is not clear whether endogenous GRWD1 functions to limit p53 activity and how this may be regulated during the stress response. Overall it would be important to have some insight into what GRWD1 is normally doing - why would the ability of L11 to activate p53 after nucleolar stress need to be restrained?

Specific points

1. The authors should show the effect of depletion of GRWD1 in other cell lines, and confirm that the loss of GRWD1 results in a more robust activation of the p53 response. Does depletion of
GRWD1 affect proliferation or survival, limit anchorage independent growth or tumorigenesis? The p53-dependence of changes in cell behaviour should also be confirmed.

2. The half-life study (Figure 1B) is not very convincing, and seems to have been done only once. Again, other cell lines would be helpful here. Does overexpression of GRWD1 increase the half-life of p53? Is the effect of GRWD1 depletion enhanced under conditions of nucleolar stress?

3. It is not clear to me whether the authors expect depletion of GRWD1 to stabilize p53 under non-stressed conditions, or whether they think GRWD1 only functions in response to stress.

4. Is the binding of GRWD1 to L11 regulated by stress? What is the normal physiological role of GRWD1 in this context?

5. The interaction between L11 and GRWD1 is clear in overexpression systems, but the evidence that the endogenous proteins interact is limited to Figure 2B. Again, I am not sure I understand the model. These are unstressed cells, yet GRWD1 is apparently binding to L11. Is this increased or decreased in response to stress?

We would like to thank you very much for your editorial expertise. We are very grateful to you for finding our study potentially interesting, providing useful comments, and inviting us to submit the revised manuscript. Please see enclosed our revised manuscript. We have addressed all of the concerns raised by the reviewers. Details of the changes made to the original version and our response to the reviewersí comments are described in separate sheets.

We think we have obtained several important new data. Especially, the data showing that high expression of GRWD1 is highly associated with poor prognosis in brain lower grade glioma patients with wild type p53 but not those with mutated p53 (new Figure 5G) are very impressive and therefore will be paid attention widely in the field, strongly stimulating future studies. We sincerely hope that you now find the manuscript acceptable for publication in EMBO Reports.

We would like to thank you for your consideration, and we eagerly await your response.

Referee #1

In this Ms Kayama and colleagues describe the glutamate-rich WD40 repeat containing protein 1, GRWD1, as a new negative regulator of p53 activity under nucleolar stress. Authors show that GRWD1 binds to the Ribosomal protein L11 (RPL11) and thus compete for the interaction of RPL11 with MDM2 thus allowing MDM2 to degrade p53. Authors show that GRWD1 cooperates with E7 and Ras in conferring anchorage-independent growth and tumorigenic capacity to human fibroblast. Finally the authors show that GRWD1 expression is associated with poor prognosis in breast cancer. The pathways inducing p53 response upon nucleolar stress are relevant for tumor suppression and are presently understudied; therefore the data presented in this MS are of interest for the field. At this stage however the MS seems to be preliminary and needs more experimental data to support the conclusions before publication.

Response: We thank the referee for the positive comments and constructive suggestions.

1. In Fig 1a the result showing that silencing of GRWD1 leads to stabilization of p53 are not impressive. A rescue experiment with a GRWD1 siRNA resistant construct to make the evidence more robust is suggested.

Response: Following the suggestion, we have performed rescue experiments using U2OS cells stably overexpressing siRNA-resistant HA-GRWD1, which were established using retrorviral vector pCLMSCVhyg-HA-GRWD1 (Sugimoto et al., 2015). Because our GRWD1 cDNA was codon-optimized (Sugimoto et al., 2008), expression of the exogenous HA-GRWD1 proteins was expected to be resistant to siRNAs targeting endogenous GRWD1 (please see Materials and Method; page 22). The cells were transfected with control (mixture of siDsS, siLuci and siGFP) or GRWD1 (mixture of siGRWD1-3&4) siRNAs for 36 h. As expected, immunoblotting revealed a reduction in the expression levels of endogenous GRWD1 but little change in the expression levels of exogenous HA-GRWD1 (new Figure 1B). We then investigated the dynamics of p53 and p21 levels in response to a low-dose actinomycin D. In control cells infected with the backborn vector, the hyperinduction
of p53 and p21 was observed upon GRWD1 depletion. In contrast, in U2OS cells overexpressing siRNA-resistant HA-GRWD1, the hyperinduction upon the nucleolar stress was alleviated (new Figure 1B), supporting that the effect of siRNA-mediated GRWD1 depletion is mostly specific. We couldn’t obtain HCT116 cells stably overexpressing GRWD1 as the population. Therefore, we couldn’t perform rescue experiments using HCT116 cells.

2. In fig 1a, right panel, in addition to caspase cleavage evaluation of the apoptosis is needed.

Response: We added the data showing that PUMA induction in response to actinomycin D-induced nucleolar stress is also enhanced by GRWD1 depletion (new Figure 1A and Figure EV1A). Since PUMA is a p53-induced pro-apoptotic protein, this also supports the notion that GRWD1 depletion enhances apoptosis induction by nucleolar stress.

In addition, we also showed that in p53-/- HCT116 and p53-null H1299 cells, little induction of PUMA and cleaved PARP is observed upon GRWD1 depletion and actinomycin D treatment, demonstrating that such hyperinduction of PUMA and cleaved PARP is largely dependent on the presence of p53 (new Figure 1A, right panel and Figure EV1C). In this regard, it would be notable that nucleolar stress can induce some p53-independent cell cycle arrest and senescence although the mechanism is unclear (Drygin et al., Cancer Res. 71, 1418, 2011; Holmberg Olausson et al., Cells 1, 774, 2012).

In association with this point, we now added the following new data and descriptions in the revised manuscript. During the experiments, we noticed that although GRWD1 depletion by siRNAs enhances activation of the p53 pathway in response to nucleolar stress, it per se somewhat induces p53 without actinomycin D treatment (e.g. the data for time 0 in Figure 1A). Also in U2OS cells, GRWD1 depletion by siRNAs per se induced up-regulation of p53 and accumulation of sub-G1 cells likely representing apoptotic cells (new Figure 2, A and B). It has been suggested that GRWD1 may be required for ribosome biogenesis (Gratenstein et al., 2005; Iouk et al., 2001; Schaper et al., 2001).

Therefore, we thought it possible that GRWD1 depletion per se induces nucleolar stress. To address this issue, we first revisited cellular localization of GRWD1. Although GRWD1 is present in nuclei and binds chromatin (Sugimoto et al., 2015), it tends to accumulate in nucleoli (Killian et al., 2004; Sugimoto et al., 2015). We examined the localization of GRWD1 by immunostaining after non-ionic detergent extraction of cells to remove nucleoplasmic proteins. This assay revealed that GRWD1 is enriched in nucleoli and co-localizes with fibrillarin, a well-known nucleolar marker (Figure 2C [previous Figure 2F]). Furthermore, nucleolar GRWD1, like fibrillarin, dispersed into nuclei upon nucleolar stress induced by actinomycin D (Figure 2C). We then investigated whether nucleolar integrity is affected by GRWD1 depletion. As shown in new Figure 2D, nucleolar fibrillarin dispersed when cells were treated with siRNAs targeting GRWD1, suggesting that its depletion impairs nucleolar integrity and thereby induces nucleolar stress response. Therefore, only with the data from endogenous GRWD1-depleted cells, it would be difficult to clarify whether endogenous GRWD1 actively suppresses p53 pathway in addition to maintaining nucleolar integrity, although the hyperinduction of p53 pathway by GRWD1 depletion in cells undergoing actinomycin D-induced nucleolar stress is in line with the idea. Nevertheless, following further data including the effects of GRWD1 overexpression on p53 pathway activation strongly support the model that GRWD1 actively suppresses p53 pathway.

3. Moreover, neither in the figure legend nor in the text there are comments on pATM.

Response: This is just to show that under our experimental condition to induce nucleolar stress with low-dose actinomycin D, remarkable DNA damage may not be induced. This explanation was added to the revised manuscript (page 5) with the data showing that pATM was actually induced by bleomycin as a positive control (new Figure EV1B).

4. In Fig2 the interaction between GRWD1 and RPL11 seems consistent, however CoIP experiments with endogenous proteins (Fig2a) should be done by with both proteins and also in other cell lines.

Response: In the revised manuscript, we have added new data showing that immunoprecipitation of endogenous GRWD1 co-precipitates endogenous RPL11 also in HCT116 cells (new Figure 3C).
Unfortunately, although we have examined several anti-RPL11 antibody available for us (in-house, Santa Cruz, etc), we couldn’t so far immunoprecipitate endogenous RPL11 efficiently with then. Thus, we couldn’t investigate whether endogenous RPL11 co-precipitates endogenous GRWD1. We would very much appreciate your understanding in this matter. On the other hand, the data shown in Figure 4B (previous Figure 3B) reveals that transfected FLAG-RPL11 co-immunoprecipitates endogenous GRWD1 even in the presence of competitor overexpressed MDM2.

5. In Fig 4 authors evaluate the transforming capability of GRWD1 in cooperation with E7 and Ras oncogenes in human fibroblasts. According to the hypothesis and the results presented in figures 1 to 3, GRWD1 should bind to RPL11 and reduce the levels of p53. However, in fig 4A, (or in other panels) p53 levels are not shown. In other words, it is crucial for the paper to show that the in vivo transforming/oncogenic potential of GRWD1 depends on interference with the PRL11/MDM2 pathway.

Response: Interestingly, it has been recently reported that RPL5/11-dependent nucleolar stress pathway also plays a crucial role in activated RAS-mediated p53 induction (Nishimura et al., 2015 cited in the revised manuscript). Therefore, we have carefully compared p53 levels under non-stressed, normally growing condition among HFF2/T cells transduced with HPV16 E7, activated KRAS, and GRWD1. To achieve highly accurate comparison, we prepared their cell lysates under rigorously same growth condition (seeding cells at same cell density and harvesting at 24 h after seeding) and investigated p53 levels. As a result, we found that basal p53 levels are up-regulated by E7 and activated RAS, as reported previously (Nishimura et al.), and the increase in p53 levels are repressed by GRWD1 overexpression (new Figure 5A, lower panel). The data provide further strong support to the notion that suppression of the KRAS-activated p53 by GRWD1 play a crucial role in its transforming activity.

As has been described in the original version, we think that the data presented mainly in Figure 6 (previous Figure 5) also support the notion that in vivo transforming/oncogenic potential of GRWD1 depends on interference with the PRL11/MDM2 pathway. Briefly, the ability of GRWD1 to inhibit p53 and promote tumorigenesis requires an interaction with RPL11 mediated by the N-terminal and acidic domains; GRWD1 D1-136 mutant, which cannot bind to RPL11, loses its functions to inhibit p53 and promote tumorigenesis (Figure 6C).

6. Authors also show that GRWD1 expression correlates with poor prognosis in tumors in some datasets. How does this correlate with the status of p53 (wt or mutant) in the tumors analyzed?

Response: We have performed additional meta-analysis using publicly available data to examine whether GRWD1 overexpression is associated poor prognosis especially in cancer patients harboring wild type p53. Since the databases we originally used don’t contain information of the p53 status in the cancer patients, we have now mined other databases. As a result, we found that in a cohort of brain lower grade glioma (for detail, please see Materials and Methods; page 23), high expression of GRWD1 is highly associated with poor prognosis in patients with wild type p53 but not those with mutated p53 (new Figure 5G). These data provide further support for our conclusion that GRWD1 promotes oncogenic transformation by inhibiting p53. Although future in depth clinical studies should be required to rigorously establish significance of GRWD1 overexpression in cancer diagnosis, our data will be paid attention and strongly stimulate such future studies.

Referee #2
This manuscript presents evidence that GRWD1 (glutamine rich WD repeat containing 1) is a negative regulator of p53. The results indicate that GRWD1 sequesters ribosomal L11 protein and relieves its repression on MDM2, so that MDM2 can efficiently degrade p53. They also show that GRWD1 along with KRAS and HPV E7 are able to transform normal fibroblasts. Finally, by mining publicly available databases the authors show that GRWD1 overexpression is correlated with poor prognosis. Overall, the results are generally interesting and convincing. There are several issues, which the authors need to address as follows.

Response: We are very grateful for the referee’s favorable and constructive comments.

1. In the abstract, the authors mention that they found that GRWD1 was localized to nucleoli and was released into the nucleoplasm upon nucleolar stress. However, the authors do not provide data to support this conclusion and should do so.
Response: The data showing GRWD1 enrichment in nucleoli and its dispersion into nucleoplasm upon nucleolar stress have been presented in Figure 2C (previous Figure 2F) and Figure 3G (previous Supplementary Figure S2), respectively.

2. The authors show that silencing GRWD1 increases p53 induction and that GRWD1 over expression of GRWD1 reduces p53 induction. No data are presented to correlate these results with effects on p53 activity except for a western blot of p21 in Fig 1A. The authors should show that transcriptional activation/induction of well-established p53 target genes correlates with effects of GRWD1 manipulations on p53 levels.

Response: We have now added the new data showing that induction of PUMA, a well-known p53 target (Nakano and Vousden, Mol. Cell 7, 683, 2001), by actinomycin D is also enhanced by GRWD1 depletion in HCT116 cells (new Figure 1A and Figure EV1A). This also provides additional evidence indicating that GRWD1 depletion promotes apoptosis.

3. The authors show the induction of apoptosis (PARP cleavage) with GRWD1 silencing. Is the observed phenotype p53-dependent? The authors should results in p53 wt and p53 null cells to rule in or rule out the role of p53 in such apoptosis induction.

Response: In the revised version, we have added new data showing that in p53-/- HCT116 cells, remarkable induction of PUMA and PARP cleavage is not observed upon GRWD1 silencing and actinomycin D treatment, clearly differing from WT HCT116 (new Figure 1A, right panel). Similar findings were also obtained with p53-null H1299 cells (new Figure EV1C). In this regard, it would be notable that nucleolar stress can induce p53-independent cell cycle arrest and senescence although the mechanism is unclear (Drygin et al., Cancer Res 71, 1418, 2011; Holmberg Olausson et al., Cells 1, 774, 2012).

4. Is p53 a substrate for GRWD1? In other words, does GRWD1 function as an E3 ubiquitin ligase for p53?

Response: When p53 and GRWD1 only are co-expressed in H1299 cells without MDM2, GRWD1 does not decrease p53 levels (new Figure EV2A). Therefore, although GRWD1 may also function as a component of several ubiquitin ligases (He et al., 2006; Higa et al., 2006), it may not directly control proteolysis of p53 (page 8).

5. It is well established that nucleolar stress activation of p53 is disabled in the MDM2C305F model system. What is the role/function of GRWD1 in such conditions? For example, does GRWD1 still bind to L11 in MDM2-C305F knock-in cells? Does GRWD1 modulate MDM2-C305F’s E3 ligase function?

Response: In our present studies, all experiments were performed based on human cells and genes. Therefore, we have created a human MDM2 C305F mutant and examined whether it is relieved from repression by RPL11. Strangely, the steady-state levels of MDM2 C305F and p53 were still increased by co-expression of RPL11 and this repression by RPL11 was reversed by GRWD1 (rebuttal Figure R1), suggesting that ubiquitin ligase activity of human MDM2 C305F mutant is suppressed by RPL11 in human cells like wild type MDM2.

Please note that, to our best knowledge, almost all experiments for MDM2 C305F have been based on murine MDM2. For example, Macias et al. (Cancer Cell 18, 231, 2010) showed that the MDM2 C305F mutant does not bind to RPL11 and thereby escapes from RPL11-mediated suppression of the activity in murine system. Xirodimas’s group has been used human cells but still used murine MDM2 C305F (Sundqvist et al., EMBO Rep 10, 1132, 2009; Mahata et al., Oncogene 31, 3060, 2012); seeing the original paper by Xirodimas et al. (Cell 118, 83, 2004), the MDM2 consists of 489 aa, showing it is murine MDM2.

It is so far unclear why the property of human MDM2 C305F is apparently different from that of murine MDM2 C305F. Of course, it remains possible that our experimental conditions are not optimized. On the other hand, it is also possible that the property of human MDM2 C305F is actually different from that of murine MDM2 C305F. It will be worthwhile to clarify this point for
biology of MDM2 and p53 but will take much more time. In addition, it would not be directly relevant to our present study to show novel GRWD1 functions and thus wouldn’t be essential for our study. We would very much appreciate your understanding in this matter.

**Figure R1.** H1299 cells were co-transfected with the indicated expression vectors (p53, 7.5 ng; HA-Ub, 0.5 µg; His-Xpress-MDM2, 2 µg; RPL11-FLAG, 1 µg; HA-GRWD1-FLAG, 1.5 µg; GFP, 0.04 µg) for 48 h and analyzed by immunoblotting with the indicated antibodies as in Figure 4A. GFP serves as a control protein to show equal transfection efficiencies.

6. Neddylation of L11 has been shown to be crucial for its interaction with MDM2. The authors show that GRWD1 inhibits RPL11-MDM2 interactions. Do the authors have any idea concerning the effect of GRWD1 on L11 neddylation? Further, L11 recruitment on p53 target promoters makes MDM2 inactive at these sites. Is GRWD1 recruited on to the p53 target promoters?

**Response:** We have been also interested in the point raised by the reviewer (i.e. whether GRWD1 can also inhibit p53 transcriptional activity on the target promoters). In this regard, we have obtained several data supporting this could be the case (e.g. exogenous GRWD1 overexpression can somewhat inhibit exogenous p53 activity in a reporter assay without affecting p53 levels in non-stressed cells).

However, further solid data will be required to reach the conclusion. Especially, we have failed to accurately do ChIP assay with our anti-GRWD1 antibody in p53-positive HCT116 cells, although we have obtained specific GRWD1 ChIP signals at replication origins in p53-suppressed HeLa cells (Sugimoto et al., 2015). Unfortunately, the reason for the difference is currently unknown. It would be also a difficult problem for investigating possible GRWD1 function on p53-responsive promoters that manipulation of endogenous GRWD1 inevitably affects p53 protein levels as shown here. In addition, possible involvement of RPL11 neddylation in the GRWD1-RPL11-MDM2 axis would be also interesting. It has been suggested that neddylation promotes its nucleolar localization of RPL11 and protects it from degradation (Sundqvist et al., 2009). Upon nucleolar stress, RPL11 neddylation decreases, resulting in its release from nucleoli to activate p53 pathway (Sundqvist et al., 2009).
Therefore, it is now thought that neddylation of RPL11 *per se* is not required for interaction with MDM2. Rather, de-neddylated RPL11 may be recruited to p53-regulated promoters through the MDM2 interaction to relieve p53 from MDM2-mediated transcriptional suppression (Mahata et al., 2012). The reason why knockdown of NEDD8 compromises p53 transcriptional activation is thought to be that de-neddylation decreases the RPL11 stability and thereby the protein levels (Sundqvist et al., 2009; Mahata et al., 2012).

In our experiments, bacterially-produced recombinant GRWD1 binds to bacterially-produced recombinant RPL11 in vitro (Figure 3D [previous Figure 2C]), the recombinant RPL11 inhibits MDM2 ubiquitin ligase activity in vitro, and the recombinant GRWD1 alleviates the RPL11-mediated suppression (Figure 4D [previous Figure 3D]). All these findings suggest that RPL11 neddylation may not be necessarily required for the GRWD1-RPL11-MDM2 regulatory pathway. This seems in line with the above findings. On the other hand, undoubtedly, the possibility that GRWD1 might affect RPL11 neddylation, especially in the context of regulation of p53 transcriptional activity, remains to be clarified.

Overall, it will take much time to completely clarify the points described above (i.e. possible roles of GRWD1 for regulation of p53 transcriptional activity on the target promoters and for regulation of RPL11 neddylation) and it would be beyond the main scope of our present manuscript. Therefore, in the revised manuscript, we have added the above discussions with referring to the papers by Dr. Xiropadimas’s group (pages 14-15). We would very much appreciate your understanding in this matter.

7. *The authors provide clinical data using publicly available expression profile studies indicating that over expression of GRWD1 correlates with poor prognosis. In this meta-analysis, have the authors considered p53 status (mutation, deletion etc.) and MDM2 status, such as 12q amplification? Without considering these parameters, this particular analysis does not appear relevant to the present manuscript.*

**Response:** We have performed additional meta-analysis using publicly available data to examine whether GRWD1 overexpression is associated poor prognosis especially in cancer patients harboring wild type p53. Since the databases we originally used don’t contain information of the p53 status in the cancer patients, we have now mined other databases. As a result, we found that in a cohort of brain lower grade glioma (for detail, please see Materials and Methods; page 23), high expression of GRWD1 is highly associated with poor prognosis in patients with wild type p53 but not those with mutated p53 (new Figure 5G). These data provide further support for our conclusion that GRWD1 promotes oncogenic transformation by inhibiting p53. Although future in depth clinical studies should be required to rigorously establish significance of GRWD1 overexpression in cancer diagnosis, our data will be paid attention and strongly stimulate such future studies.

8. *Ribosomal protein mediated p53 activation in response to MDM2 inactivation is complicated. While p53 activation may help in tumor retardation, ribosomal dysfunction can lead to myelodysplastic syndromes. The authors indicate that over expression of GRWD1 correlates with poor prognosis (and functioning through ribosomal stress), but it is not at all clear what the mechanism might be and at the very least warrants some discussion.*

**Response:** Several studies demonstrate that even if most of endogenous RPL11 (or RPL5) is depleted by siRNA, p53 is only slightly up-regulated, in clear contrast to the case for RPL23 and RPL 26, depletion of which induces remarkable p53 (e.g. Bursac et al., 2012). Obviously, depletion of RPL11 rather reduces p53 activation by drug-induced nucleolar stress. These are described in “Introduction.”

In principle, RPL11 protein levels decreases to about a half in Damond-Blackfan anemia (DBA) patients with RPL11 heterozygous mutation. Under such condition, RPL11 haploinsufficiency may lead to general impairment of the p53 responses rather than specific deficiency in p53 activation by nucleolar dysfunction (of course, RPL11 haploinsufficiency may lead to impairment of ribosome production and thereby cell growth defect). Actually, in mouse fibroblasts, even heterozygous deletion of RPL11 impairs the activation of p53 not only by nucleolar stress but also by DNA damage (Morgado-Palacin et al., 2015). In this regard, it would be also notable that in mouse
harboring the RPL11 binding-deficient MDM2 C305F mutant, p53 induction by overexpressed c-myc is impaired (Macias et al., 2010).

Interestingly, it has been recently reported that RPL5/11-dependent nucleolar stress pathway also plays a crucial role in activated RAS-mediated p53 induction (Nishimura et al., 2015 cited in the revised manuscript). Therefore, we have carefully compared p53 levels under non-stressed, normally growing condition among HFF2/T cells transduced with HPV16 E7, activated KRAS, and GRWD1. To achieve highly accurate comparison, we prepared their cell lysates under rigorously same growth condition (seeding cells at same cell density and harvesting at 24 h after seeding) and investigated p53 levels. As a result, we found that basal p53 levels are up-regulated by E7 and activated RAS, as reported previously (Nishimura et al.), and the increase in p53 levels are repressed by GRWD1 overexpression (new Figure 5A, lower panel). The data provide further strong support to the notion that suppression of the KRAS-activated p53 by GRWD1 play a crucial role in its transforming activity.

These new data and discussion (pages 13-14) have been added to the revised manuscript.

Minor Point:

1. Authors use Actinomycin D to induce nucleolar stress. While it is a well-established drug for nucleolar stress induction, it would be helpful to use at least one more agent (like 5-FU or MPA) in some experiments, to make sure that the effects observed are not specific to one particular agent.

Response: We have added the new data showing that p53 and p21 are hyperinduced by GRWD1 depletion also in HCT116 cells cells treated with bleomycin, which generates DNA double-strand breaks (new Figure EV1B). The data would be interesting because indicating that GRWD1 may play a role for suppression of p53 pathway not only activated by nucleolar stress but also by DSB.

Together with the new data showing that GRWD1 overexpression suppresses p53 levels up-regulated by E7 and activated KRAS (new Figure 5A, lower panel), it is strongly suggested that GRWD1 may contribute to suppression of the general p53 responses.

2. In Fig. 3A was the amount of p53 DNA construct used 7.5 micrograms instead of 7.5 nanograms?

Response: We have actually used 7.5 nanogram pCMV-p53. We found that the vector very efficiently expresses p53 proteins under our experimental conditions and this amount is optimal for our experimental conditions.

Referee #3

The paper from Kayama et al describes a possible role for GRWD1 in the regulation of p53, through binding to L11. GRWD1 is shown to interact with L11, and prevent L11 binding to MDM2. GRWD1 therefore prevents L11 dependent inhibition of MDM2 and stabilization of p53.

This is a novel story and the paper is clear and well written, and in general the data are convincing. The main problem with the paper is that it relies very heavily on overexpression systems and it is not clear whether endogenous GRWD1 functions to limit p53 activity and how this may be regulated during the stress response. Overall it would be important to have some insight into what GRWD1 is normally doing - why would the ability of L11 to activate p53 after nucleolar stress need to be restrained?

Response: We thank the referee for the positive comments and constructive suggestions.

1. The authors should show the effect of depletion of GRWD1 in other cell lines, and confirm that the loss of GRWD1 results in a more robust activation of the p53 response. Does depletion of GRWD1 affect proliferation or survival, limit anchorage independent growth or tumorigenesis? The p53-dependence of changes in cell behavior should also be confirmed.

Response: We have addressed the points raised by the referee by several approaches.

a) We have performed rescue experiments using U2OS cells stably overexpressing siRNA-resistant HA-GRWD1, which were established using retroviral vector pCLMSCVhyg-HA-GRWD1 (Sugimoto et al., 2015). Because our GRWD1 cDNA was codon-optimized (Sugimoto et al., 2008),
expression of the exogenous HA-GRWD1 proteins was expected to be resistant to siRNAs targeting endogenous GRWD1 (please see Materials and Method; page 22). The cells were transfected with control (mixture of siDsS, siLuc and siGFP) or GRWD1 (mixture of siGRWD1-3&4) siRNAs for 36 h. As expected, immunoblotting revealed a reduction in the expression levels of endogenous GRWD1 but little change in the expression levels of exogenous HA-GRWD1 (new Figure 1B). We then investigated the dynamics of p53 and p21 levels in response to a low-dose actinomycin D. In control cells infected with the backborn vector, the hyperinduction of p53 and p21 was observed upon GRWD1 depletion as well as HCT116 cells. In contrast, in U2OS cells overexpressing siRNA-resistant HA-GRWD1, the hyperinduction upon the nucleolar stress was alleviated (new Figure 1B), supporting that the effect of siRNA-mediated GRWD1 depletion is mostly specific.

b) We have also added the new data showing that p53 and p21 are hyperinduced by GRWD1 depletion also in HCT116 cells treated with bleomycin, which generates DNA double-strand breaks (new Figure EV1B). The data would be interesting because indicating that GRWD1 may play a role for suppression of p53 pathway not only activated by nucleolar stress but also by DSB. Together with the new data showing that GRWD1 overexpression decreases p53 levels up-regulated by E7 and activated KRAS (please see our new data, Figure 5A lower panel, and the description about them), it is strongly suggested that GRWD1 may contribute to suppression of the general p53 responses.

c) In addition, we have showed that in p53 -/- HCT116 and p53-null H1299 cells, little induction of PUMA and cleaved PARP is observed upon GRWD1 depletion and actinomycin D treatment, demonstrating that such hyperinduction of PUMA and cleaved PARP is largely dependent on the presence of p53 (new Figure 1A, right panel and Figure EV1C). In this regard, it would be notable that nucleolar stress can induce some p53-independent cell cycle arrest and senescence although the mechanism is unclear (Drygin et al., Cancer Res. 71, 1418, 2011; Holmberg Olausson et al., Cells 1, 774, 2012).

d) In association with this point, we now added the following new data and descriptions in the revised manuscript. During the experiments, we noticed that although GRWD1 depletion by siRNAs enhances activation of the p53 pathway in response to nucleolar stress, it per se somewhat induces p53 without actinomycin D treatment (e.g. the data for time 0 in Figure 1A). Also in U2OS cells, GRWD1 depletion by siRNAs per se induced up-regulation of p53 and accumulation of sub-G1 cells likely representing apoptotic cells (new Figure 2, A and B). It has been suggested that GRWD1 may be required for ribosome biogenesis (Gratenstein et al., 2005; Iouk et al., 2001; Schaper et al., 2001). Therefore, we thought it possible that GRWD1 depletion per se induces nucleolar stress. To address this issue, we first revisited cellular localization of GRWD1. Although GRWD1 is present in nuclei and binds chromatin (Sugimoto et al., 2015), it tends to accumulate in nucleoli (Killian et al., 2004; Sugimoto et al., 2015). We examined the localization of GRWD1 by immunostaining after non-ionic detergent extraction of cells to remove nucleoplasmic proteins. This assay revealed that GRWD1 is enriched in nucleoli and co-localizes with fibrillarin, a well-known nucleolar marker (Figure 2C [previous Figure 2F]). Furthermore, nucleolar GRWD1, like fibrillarin, dispersed into nuclei upon nucleolar stress induced by actinomycin D (Figure 2C). We then investigated whether nucleolar integrity is affected by GRWD1 depletion. As shown in new Figure 2D, nucleolar fibrillarin dispersed when cells were treated with siRNAs targeting GRWD1, suggesting that its depletion impairs nucleolar integrity and thereby induces nucleolar stress response. Therefore, only with the data from endogenous GRWD1-depleted cells, it would be difficult to clarify whether endogenous GRWD1 actively suppresses p53 pathway in addition to maintaining nucleolar integrity, although the hyperinduction of p53 pathway by GRWD1 depletion in cells undergoing actinomycin D-induced nucleolar stress is in line with the idea. Nevertheless, following further data including the effects of GRWD1 overexpression on p53 pathway activation strongly support the model that GRWD1 actively suppresses p53 pathway.

e) Since GRWD1 depletion by itself induces nucleolar stress, p53 activation, and apoptosis, as above, it is naturally deleterious for cell growth. Considering that the budding yeast homolog of GRWD1 is essential for growth and that GRWD1 may be required for ribosome biogenesis (Gratenstein et al., 2005; Iouk et al., 2001; Schaper et al., 2001), this would make sense. Therefore, we have not performed detailed quantitative analyses for GRWD1 depletion-induced growth defects.
2. The half-life study (Figure 1B) is not very convincing, and seems to have been done only once. Again, other cell lines would be helpful here. Does overexpression of GRWD1 increase the half-life of p53? Is the effect of GRWD1 depletion enhanced under conditions of nucleolar stress?

**Response:** For the half-life study of p53 in GRWD1-depleted HCT116 cells, we have obtained multiple data essentially similar to Figure 1C (previous Figure 1B). We have now presented another data set in new Figure EV1D in the revised manuscript. In addition, following the suggestion, we have investigated the effect of GRWD1 overexpression on the half-life of p53 in actinomycin D-treated HFF2/T cells. The data indicate that GRWD1 overexpression stabilizes p53 in actinomycin D-treated HFF2/T cells (new Figure 1E). We tried to examine the effect of GRWD1 depletion on half-life of p53 in cells undergoing nucleolar stress. Unfortunately, we found that the triple treatments (GRWD siRNAs, actinomycin D, and cycloheximide) severely damage cells and therefore it was difficult to obtain steady results in this matter.

3. It is not clear to me whether the authors expect depletion of GRWD1 to stabilize p53 under non-stressed conditions, or whether they think GRWD1 only functions in response to stress.

**Response:** Please see the response to “1” described above. Also, please see the response to “5” described below.

4. Is the binding of GRWD1 to L11 regulated by stress? What is the normal physiological role of GRWD1 in this context?

**Response:** Please see the response to “5” described below.

5. The interaction between L11 and GRWD1 is clear in overexpression systems, but the evidence that the endogenous proteins interact is limited to Figure 2B. Again, I am not sure I understand the model. These are unstressed cells, yet GRWD1 is apparently binding to L11. Is this increased or decreased in response to stress?

**Response:** In the revised manuscript, we have added new data showing that immunoprecipitation of endogenous GRWD1 co-precipitates endogenous RPL11 also in HCT116 cells (new Figure 3C). Unfortunately, although we have examined several anti-RPL11 antibody available for us (in-house, Santa Cruz, etc), we couldn’t so far immunoprecipitate endogenous RPL11 efficiently with them. Thus, we couldn’t investigate whether endogenous RPL11 co-precipitates endogenous GRWD1. We would very much appreciate your understanding in this matter. On the other hand, the data shown in Figure 4B (previous Figure 3B) reveals that transfected FLAG-RPL11 co-immunoprecipitates endogenous GRWD1 even in the presence of competitor overexpressed MDM2. We also investigated whether the co-immunoprecipitation efficacy between GRWD1 and RPL11 is changed by actinomycin D-induced nucleolar stress in HCT116 cells (new Figure 3C). The data suggest that they may interact even in unstressed cells and the binding efficacy may not be remarkably enhanced by nucleolar stress. It is generally thought that MDM2 ubiquitin ligase functions in nucleoplasm while RPL11 mainly exists in nucleoli. GRWD1 exists both in nuclei (and some bind to chromatin; Sugimoto et al., 2015) and in nucleoli (please see the description in page 6 and the related data). Therefore, even if GRWD1 binds to RPL11 in nucleoli, it may only subtly influence MDM2-p53 pathway. On the other hand, when the levels of ribosome-free RPL11 increase, for example by nucleolar stress and activated RAS (Nishimura et al., 2015; new Figure 5A lower panel), GRWD1 may have a significant effect on the pathway as a negative balancer. Especially, when overexpressed, GRWD1 may accelerate malignant transformation, as shown here. It is so far unclear whether the GRWD1-RPL11 interaction in nucleoli has a certain physiological role. In line with the previous findings that GRWD1 is required for ribosome biogenesis (Gratenstein et al., 2005; Iouk et al., 2001; Schaper et al., 2001), we found that GRWD1 depletion per se induces nucleolar stress, as described above (new Figure 2). Therefore, GRWD1-RPL11 interaction in unstressed cells could contribute to maintenance of ribosome biogenesis. The discussion is also added to the revised version (page 14).
Thank you for the submission of your revised manuscript to our editorial offices. We have now received the three referee reports that you will find enclosed below. As you will see, all three referees support now the publication of your manuscript in EMBO reports. Before we can proceed with formal acceptance, I have a final request:

Please add the data showing the identification of RPL5, RPL11 and RPL23 as candidate GRWD1 interactors by a combination of FLAG-GRWD1 immunopurification and mass spectrometric analysis (presently "data not shown") as EV figure (EV1) and mention this in the manuscript text. Please provide a figure legend, additional methods information and adjust the figure call-outs accordingly throughout the manuscript text.

REFEREE REPORTS

Referee #1:

The authors have addressed my concerns on their demonstration of the role of GRWD1 as a negative regulator of p53 activity under nucleolar stress. In particular, the effects of overexpressing RNAi-resistant GRWD1, together with amelioration of biochemical data and addition of analysis of the p53-target gene PUMA have added robustness to in vitro experiments. Moreover, the in vivo data provided and the analysis of new cancer datasets, now support the conclusions more convincingly. In consideration of this and of the overall amelioration of this revised version of the manuscript, I now recommend its publication in EMBO Reports.

Referee #2:

During the revision, the authors added sufficiently enough data that support their conclusions. This has substantially improved the quality of this manuscript and made suitable for publication.

Referee #3:

The authors have made a reasonable attempt to address my specific points, and I think the paper is suitable for publication in EMBO Reports.

2nd Revision - authors' response

We would like to thank you very much for your editorial expertise. Following your instruction, we have now fully revised the manuscript. The amendment made are described in a separate sheet. We sincerely hope that the manuscript will be formally accepted for publication in EMBO Reports. We are looking forward to hearing from you soon.

Please add the data showing the identification of RPL5, RPL11 and RPL23 as candidate GRWD1 interactors by a combination of FLAG-GRWD1 immunopurification and mass spectrometric analysis (presently "data not shown") as EV figure (EV1) and mention this in the manuscript text. Please provide a figure legend, additional methods information and adjust the figure call-outs accordingly throughout the manuscript text.

Response: We have now added the data showing the identification of RPL5, RPL11 and RPL23 as candidate GRWD1 interactors by a combination of FLAG-GRWD1 immunopurification and mass spectrometric analysis as Figure EV1 with the figure legend and description of the related materials and methods. Related corrections have been also made. In addition, in the previous version, we mentioned that iSpecificity of the anti-RPL11 antibody was confirmed by the data that RPL11 depletion by siRNAs diminished the signals detected by the antibody in immunoblotting without showing the data. Therefore, we have now presented the data as Figure EV5C.
3rd Editorial Decision: Acceptance

21 October 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.
**b. Figures**

The data shown in figure should satisfy the following conditions:

- The data are relevant and presented according to the figure’s best practice and are presented so that the results of the experiments are reproducible and controlled.
- The figure panels include data points, measurement or observations that can be compared in each other in a scientifically meaningful way.
- The statistical analysis of each treatment is adequately shown by bars or curves.
- The errors bars should be shown for statistical significance.
- If yes, the statistical data points for each experiment should be plotted and any statistical test employed should be justified.
- Source Data should be included to print the data underlying graphs. Please follow the guidelines set out in the authorship guidelines for Data Presentation.

**c. Captions**

Each figure caption should contain the following information, for each panel where it is relevant:

- A clear statement of the experimental system investigated (e.g., cell line, species name).
- The sample collection and study size.
- The number of biological or technical replicates.
- An explicit mention of the biological and chemical entity(ies) that are being measured.
- The assays and methods used to carry out the reported observations and measurements.
- The experimental system investigated (e.g., cell line, species name).
- The variance between the groups that are being statistically compared.
- The results of any blinding performed on the investigator.
- A statement about whether the test was performed independently or not.

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

Please ensure that the following questions are included in the narrative text. We encourage you to insert a specific citation to the methods section for statistics, imaging, animal models and human subjects.

- Is the variance between the groups that are being statistically compared?
- Are there any sample size estimates?
- Is there an explicit mention of the biological and chemical entity(ies) that are being measured?
- Is there a specification of the experimental system investigated (e.g., cell line, species name).
- Is the variance between the groups that are being statistically compared?
- Is the variance between the groups that are being statistically compared?
14. Report any restrictions on the availability (and/or on the use) of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

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.

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.

18. Provide accession codes for deposited data. See author guidelines, under ‘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

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 or in a Supplementary Document. See author guidelines, under ‘Reporting Guidelines’.

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).

21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.

**Examples:**

**Primary Data**


**Referenced Data**

Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26

AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208

22. 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 formats (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.

23. 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, please state whether it could.

**F-Data Accessibility**

**G-Dual use research**