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The mutant p53-ID4 complex controls VEGFA isoforms by recruiting lncRNA MALAT1

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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 21 October 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 high interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, which need to be addressed during the revision. As the reports are below, I will not detail them here, but all points by referees #1 and #3 are of particular importance, as are points 1 (proving that the RNA IP is specific under cross-linking conditions with each of the Abs employed would be sufficient), 2 and 5/11, 7 and 13 of referee #2.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be fully addressed in a complete point-by-point response. Acceptance of your 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.

REFEREE REPORTS

Referee #1:

In the manuscript ' The mutant p53-ID4 complex controls VEGFA isoforms production by recruiting lncRNA MALAT1' the authors identify a role for mutant p53 and ID4 in regulating SRSF1 and MALAT1 resulting in the formation of pro-tumourigenic VEGFA isoforms. Mutant p53 has previously been shown to regulate ID4 and to increase angiogenic potential. This manuscript builds upon these findings and describes in detail a role for lncRNA MALAT1 and SRSF1 in this process. The results are well described and presented.

Major comments:

Page 3, when introducing SRSF1, the authors should be a bit more specific about its effects. "modulates and influences" are too vague.

Page 6; The authors claim in the 3rd paragraph that p53 does not have an RNA binding domain. This is not correct as several reports have shown that p53 has an RNA binding capacity (review: Riley RNA. 2007 Nov;13(11):1825-3). This sentence should be revised.

Figure 2 I and supplemental figure 2G show an IP between mutant p53 and SRSF1. A band is seen in mutant p53 expressing cells and no band is seen in sh-p53 cells. The authors conclude that no interaction is seen in wt p53 expressing cells. However in both these figures a higher molecular weight band is seen in wtp53 expressing cells and not in the shp53 cells suggesting it is a product specifically coming down with p53. How are the authors certain that the product seen in mutant p53 cells is the SRSF1 band? Could the band identified in wt p53 expressing cells be an alternative SRSF1 isoform? Input and IP are on different gels and molecular weight markers are lacking so the reader cannot determine which band runs at the height of SRSF1 in the input.

The authors conclude from supplemental figure 2 that the interaction between ID4 and p53 is independent of SRSF1 using knockdown of SRSF1. However, the knockdown shown in suppl. figures D and E is only marginal, so perhaps no difference in the interaction is seen because the knockdown wasn't efficient enough. A better knockdown is pivotal to make this conclusion. Interestingly, in figure 1 G, the authors present a good knockdown, as shown by western blot without IP.

In order to verify the model shown in figure 2J (this model does not seem to be mentioned) it would be useful to show more IPs in vitro to determine which protein binds to which protein directly and indirectly.

The model in figure 2J suggests that loss of p53 or ID4 binding to SRSF1 leads to degradation of the MALAT1 RNA. In suppl. figure 1E, the authors show that the loss of SRSF1 leads to increased MALAT1 expression, but the expression levels after p53 or ID4 knockdown are not shown. If the authors want to present this model, such experiments should be included.

Minor comments:

Page 2, in the abstract, the authors should briefly mention the meaning of lncRNA for uninitiated readers.

Page 4, when talking about the peculiar character of Mtp53 proteins, the authors should be more specific when talking about "various" oncogenic properties.

Page 6, when first talking about MALAT1, the authors could briefly re-introduce "SR" splicing factors again.

Page 6, the authors claim to observe enrichment of MALAT1 "consistent with previously published data". A reference is needed here. In addition, they claim that NEAT1 is not enriched. What is this RNA? Is it used as a negative control against NEAT2/MALAT1?

The format between supplementary figure 3A and 3B is different and confusing when compared to the main figures of the text. This should be made more consistent.

Molecular weight markers should be included.

While the details given in the PLA material and methods (page 19) are welcome, they are not necessary in that context and do not fit the style of the other sections where this kind of information is lacking.

Some parts of the experimental procedures need refining, for example specifying vendors of the Duolink assay or specifying which Dynabeads were used.

Page 4, last third. Mtp53 are characterized by... "inability to recognize wt-p53 DNA binding sites. This statement should be moderated as not all mtp53 proteins have lost the ability to bind to wtp53 binding sites.

Images for figure 2A-D should be shown in the supplemental data

Referee #2:

The present manuscript attempts to describe the potential involvement of an RNP complex consisting of the abundant nuclear-localized Malat1 long noncoding RNA, SRSF1 splicing factor and mutant p53-ID4 complex in modulating angiogenesis in breast cancer cells by regulating the alternative splicing of VEGFA pre-mRNA. Authors have shown the interaction between mutant and not WT-p53 and ID4 protein with MALAT1 and SRSF1. Further, their data indicate that SRSF1 is required for the interaction between Malat1 and the mut-p53-ID4. Mut-p53 or ID4-depleted cells showed changes in the sub-cellular localization of Malat1, and also their data indicated that mut-p53-ID4 complex facilitates the association of Malat1 with chromatin. Finally, authors indicate that mut-p53-ID4-SRSF1-Malat1 complex facilitates the production of pro-angiogenic isoforms of VEGFA. Based on their results they propose a model, where in mutant p53 and ID4 expressing breast cancer cells these proteins form a stable complex with Malat1 and SRSF1 and the RNP complex regulates the alternative splicing of VEGFA pre-mRNA, resulting in the increased production pro-angiogenic version of the VEGFA protein. Their results implicate that mut-p53 along with other components enhances the angiogenic properties of breast cancer cells by regulating alternative splicing of pre-mRNA.

It is an interesting concept, and the authors have provided some technically sound data to support their claim. However, a large number of experiments lack proper controls that are required to completely interpret the data. Authors need to perform the experiments that are described below

Specific comments:

1. Based on what is mentioned in the materials and methods, authors have performed RNA immunoprecipitation (RIP) assays under non-crosslinking conditions. These assays, especially when done under native conditions are known to produce non-specific interactions between proteins and RNA. Therefore, it is recommended that authors perform all of RIP under crosslinking (formaldehyde or UV) conditions.

2. Malat1 KD efficiency is very poor (sup. Fig 2). Some of the key experiments to be reproduced in cells with better knock down of Malat1. Also, all of the experiments that are shown in the main figure of the manuscript should be reproduced with more than one siRNAs against the molecule of interest (Malat1, SRSF1, p53 and ID4). This is a major drawback of the paper. Authors should not make conclusions based on data obtained after using one siRNAs against each member of the RNP complex.

3. In general, the quality of the microscopy data is poor (for example see figure 2G, where p53 signal is saturated) and needs to be improved.

4. PLA data (Fig 2A-F) should be supplemented with endogenous IP experiment in control and Malat1-depleted cells.

5. Based on what is known in the literature, in most cell types most of the Malat1 is known to localize to nuclear speckles or SC35 domains. Authors should perform co-localization analyses of mut-p53 and ID4 with Malat1 to see their co-localization.

6. What are the two last lanes indicate in sup fig. 2D & E? Are they represents input samples? If it is, then the level of SRSF1 depletion achieved is poor and require to repeats the experiments under conditions of better KD. Surprisingly, sup. Fig. 3D shows better KD of SRSF1.

7. Quality of IP data in fig 2l & sup fig. 2G is poor (especially the SRSF1 immunoblot). Since both input and IP samples are run in separate blots, it is impossible to assess the percentage of interaction between the proteins. Actually, both input and IP should be run in parallel, and should be exposed together.

8. In addition to Malat1 KD, authors should also do IP to test interaction between SRSF1 and mut-p53 in ID4-depleted cells.

9. Authors should also test whether ID4 interacts with SRSF1 and MALAT1 in breast cancer cells expressing WT-p53

10. Several earlier studies, using different cell lines such as HeLa have shown positive interaction between SRSF1 and Malat1. Based on SRSF1 Clip-seq data from Sanford laboratory, Malat1 RNA contains several SRSF1 binding sites, that are distributed all over the length of the transcript. Based on that, this reviewer is not completely convinced with the authors' description (Fig 3A) that mut-p53/ID4 is required for stabilizing the interaction between SRSF1 and Malat1. In order to make it more convincing, authors need to do more direct experiments such as SRSF1 CliP in mut-p53 or ID4-depleted cells.

11. It is surprising to see that Malat1 shows speckle localization only in 10% of the breast cancer cells. Actually, even the so-called "diffused cells" (sup fig 4B) shows speckle staining of Malat1. I am also not convinced with staining shown in figure 4B (scr-si). In all these conditions, authors should do a co-staining of Malat1 with another known speckle marker such as SC35.

12. The experiment described in fig 4C-F is under the assumption that the diffused Malat1 fraction represents the chromatin-associated fraction of Malat1 (primarily based on its increased association with H3). However, these experiments require crucial controls. To be more quantitative, authors should do H3 ChIP and Malat1` interaction. They should treat the cells with RNA pol II inhibitors (amanitin or DRB), which are known to release MALAT1 from speckles (Bernard et al., 2010 EMBO J) and see if such treatments dramatically increase the association of Malat1 with H3.
13. In Fig 5, knock down of every component showed increased levels of anti-angiogenic VEGFA isoforms. These experiments requires negative control, where authors should deplete proteins such as hnRNP A1 (a known negative regulator of SRSF1) and see if that shows opposite effect with respect to alternative splicing of VEGFA. In addition, authors could test the potential changes in the alternative splicing of few house keeping gene mRNAs in cells that are depleted of the RNP constituents.

14. It is known that MALAt1 levels are high in breast cancer patient samples (Arun et al., 2016 G&D; Mahdiah et al., 2016 Oncotarget). In order to explain fig 5F better authors should catalog the patient samples with comparable levels of Malat1.

15. Again in fig 6 an important control experiment is missing. P53 Chip and RIP should be performed in BC cells expressing endogenous p53 to see potential interaction of WT p53 to these genomic regions. Similarly, authors should perform SRSF1 RIP (fig 6E-F) in control and MALAT1-depleted BC cells containing endogenous wild type p53, to see if this change happens only in cells containing mut-p53.

16. It is not clear why overexpression of VEGF165b alone did not reduce the mesh forming property of endothelial cells. In fig 7A, VEGF165b overexpressing data should be compared with control cell and not with VEGF165 overexpressing cells. In similar lines, I would have expected less effect on the mesh forming property in Malat1 or SRSF1-depleted cells compared to VEGF165b overexpressing cells. But the data presented in fig 7 speaks otherwise.

Referee #3:

In this manuscript, Pruszko et al. describe the existence of a quaternary complex made of 3 proteins (mutant p53, ID4 and SRSF1) and the long non-coding RNA (lncRNA) MALAT1. They provide evidence for and its pro-oncogenic role in basal-like breast cancer tumors. The authors have connected the oncogenic activity of this complex with its ability to shift the ratio of expression of VEGFA isoforms in favor of the pro-angiogenic VEGFA165 and VEGFA121 that include exon8a. They have verified the importance of each individual component of the complex, as well as the p53 status, for the predominant expression of exon8a containing isoforms. Furthermore, they corroborated their experimental data with analysis of publicly available data sets of breast cancer clinical samples. Long non-coding RNAs (lncRNAs) have recently been discovered to regulate

numerous intracellular processes. Consequently, their possible roles in carcinogenesis are also emerging. A number of publications have reported functional interactions between the p53 tumor suppressor and certain lncRNAs. Only a few of such studies, however, delivered sound and unbiased messages on the outcomes of p53 association with a specific RNA. The work described by this group is an exception in that it is solid and reasonably convincing. The authors have employed a number of techniques to verify involvement of each component of the complex, in the regulation of expression of anti-proliferative VEGFA isoforms. These include the proximity ligation assay (PLA), RNA FISH and several co-immunoprecipitations followed by either immunoblot or qRT-PCR analysis. Importantly, the authors did not simply document functional interactions between lncMALAT1 and either of the protein modules of the multicomponent complex that is involved in the regulation of VEGFA splicing, but also managed to connect (to a certain degree) the dots between each module and show their interdependence. In all this is an interesting and informative study that has some translational added benefit. I would recommend this study for publication in EMBO reports pending response of the authors to the following comments.

1. The levels of MALAT1 lncRNA and each of the protein components, namely, SRSF1, mutant p53 and ID4 should be verified in a comparative manner within both, MDA-468 and SkBr3 cell lines. Though the authors did the analysis of the expression of the individual components of the quaternary complex upon transient knock down of individual members of this complex, these data are scattered through the manuscript and difficult to follow. They should be provided next to the original experiment(s), as they represent important controls.

2. The authors did not observe interactions between wild-type p53 and lnc RNA MALAT1 in MCF-7 cells. What conditions did they use? Was wild-type p53 stabilized and present at comparable levels to mutant p53 levels in the respective MDA cell lines? It would also be good to show data with another breast tissue-derived cell line such as MCF10A cells that harbor wild-type p53.

3. In Figure1G the authors should include immunoblotting analysis of ID4 and mutant p53 protein levels upon siSRSF1 knockdown (see the first comment). The same should be done in Figure 2, where the levels of the corresponding interacting protein pairs upon knockdown of the RNA component of the complex are not shown. On Page 6, bottom, what is meant by the phrase "...despite comparable efficiency of protein immunoprecipitation in si-SCR vs si SRSF1 conditions."

4. On Page 7, bottom, the authors stated that replacing mutant p53 with wild-type p53 did not recover p53-SRSF1 interaction in MDA-468 cells. Did they try and perform the reciprocal substitution in MCF-7 cells and check if there is a mutant p53-SRSF1 complex in the MCF-7 background? Such an experiment would provide valuable data about the relevance of the cellular background for mutant p53-SRSF1 (indirect) interactions. In the co-IP results presented in Figure 2I, did the authors observe interactions after treatment with RNase?

5. In Figure 4D-G, the authors verified the association of MALAT1 with active chromatin by a ChIP assay followed by immunoblotting with anti H3 antibody. This analysis could be repeated to evaluate more specific marks of active chromatin such as probing for the H3 specific modifications H3K9Ac, H3K27Ac to prove their point.

Of lesser concern:

6. Many available breast cancer datasets show significant increase in expression of each of the 4 components of the quaternary complex described in the paper (c-bioportal website). In what percent of breast malignancies are all three, MALAT1, ID4 and SRSF1 upregulated and co-exist with mutant p53?

7. Is the ability of mutant p53 to interact with MALAT1 specific to cells of breast cancer origin? Do p53 mutants from (for example) colorectal cancer or ovarian cancers interact with MALAT1?

1st Revision - authors' response 26 February 2017

Point by point response shown on the following pages

Referee #1:

In the manuscript ' The mutant p53-ID4 complex controls VEGFA isoforms production by recruiting lncRNA MALAT1' the authors identify a role for mutant p53 and ID4 in regulating SRSF1 and MALAT1 resulting in the formation of pro-tumourigenic VEGFA isoforms. Mutant p53 has previously been shown to regulate ID4 and to increase angiogenic potential. This manuscript builds upon these findings and describes in detail a role for lncRNA MALAT1 and SRSF1 in this process. The results are well described and presented.

Major comments:

• Page 3, when introducing SRSF1, the authors should be a bit more specific about its effects. "modulates and influences" are too vague.
We agree with the reviewer and we have better detailed the activity of MALAT1 and SRSF1 in the Introduction.

• Page 6; The authors claim in the 3rd paragraph that p53 does not have an RNA binding domain. This is not correct as several reports have shown that p53 has an RNA binding capacity (review: Riley RNA. 2007 Nov;13(11):1825-3). This sentence should be revised.
We are sorry for this mistake; we have changed this sentence.

• Figure 2 I and supplemental figure 2G show an IP between mutant p53 and SRSF1. A band is seen in mutant p53 expressing cells and no band is seen in sh-p53 cells. The authors conclude that no interaction is seen in wt p53 expressing cells. However in both these figures a higher molecular weight band is seen in wtp53 expressing cells and not in the shp53 cells suggesting it is a product specifically coming down with p53. How are the authors certain that the product seen in mutant p53 cells is the SRSF1 band? Could the band identified in wt p53 expressing cells be an alternative SRSF1 isoform? Input and IP are on different gels and molecular weight markers are lacking so the reader cannot determine which band runs at the height of SRSF1 in the input.
To address this comment we have repeated IP p53>blot SRSF1 using different conditions/buffers but we continued to see the presence of various bands of uncertain specificity. We have then undertaken the reciprocal approach (IP SRSF1>blot p53) as IP of SRSF1 precipated a single well-defined SRSF1 protein product. This approach confirmed the interaction of endogenous (SKBR3-p53R175H) and exogenous (p53R273H transfected in p53-null H1299 cells) mutant p53 proteins with SRSF1 (**Figure 2H-J**).In H1299 we have also verified the interaction between endogenous ID4 and SRSF1 proteins (**Figure 2I**) and between endogenous ID4 and exogenous p53R273H (**Figure 2I**).

• The authors conclude from supplemental figure 2 that the interaction between ID4 and p53 is independent of SRSF1 using knockdown of SRSF1. However, the knockdown shown in suppl. figures D and E is only marginal, so perhaps no difference in the interaction is seen because the knockdown wasn't efficient enough. A better knockdown is pivotal to make this conclusion. Interestingly, in figure 1 G, the authors present a good knockdown, as shown by western blot without IP.
We agree with the reviewer's comment; we have repeated this experiments in SKBR3 and MDA-MB-468 cells with much more efficient SRSF1 depletion. The results are shown in **Expanded Figure 2E**.

• In order to verify the model shown in figure 2J (this model does not seem to be mentioned) it would be useful to show more IPs in vitro to determine which protein binds to which protein directly and indirectly. The model in figure 2J suggests that loss of p53 or ID4 binding to SRSF1 leads to degradation of the MALAT1 RNA. In suppl. figure 1E, the authors show that the loss of SRSF1 leads to increased MALAT1 expression, but the expression levels after p53 or ID4 knockdown are not shown. If the authors want to present this model, such experiments should be included.
We apologize for the lack of clarity of the model previously presented. We meant to represent that the depletion of MALAT1 (by interference) impairs the interactions SRSF1/mutant p53 and SRSF1/ID4, as assessed by PLA experiments. This model has been now removed, and a schematic summary figure has been provided, as from editor's request.

Minor comments:
• Page 2, in the abstract, the authors should briefly mention the meaning of lncRNA for uninitiated readers.
This has been included.
• Page 4, when talking about the peculiar character of Mtp53 proteins, the authors should be more specific when talking about "various" oncogenic properties.
A more detailed overview of the oncogenic properties of mutant p53 has been included in the Introduction.
• Page 6, when first talking about MALAT1, the authors could briefly re-introduce "SR" splicing factors again.
This has been included.

• Page 6, the authors claim to observe enrichment of MALAT1 "consistent with previously published data". A reference is needed here. In addition, they claim that NEAT1 is not enriched. What is this RNA? Is it used as a negative control against NEAT2/MALAT1?
We have included the reference for SRSF1-MALAT1 interaction and we have specified that NEAT1 has been used as negative control.
• The format between supplementary figure 3A and 3B is different and confusing when compared to the main figures of the text. This should be made more consistent.
As panels showing the steady state level of all the proteins of the complex, as well as of MALAT1, have been included close to the various experiments presented in this new version of the manuscript following the request of reviewer 3, we reasoned that this was sufficient to show the presence of the proteins immunoprecipitated in RIP experiments. Supplementary figure 3A and 3B of the previous version were then omitted in this new version.
• Molecular weight markers should be included
These have been included.
• While the details given in the PLA material and methods (page 19) are welcome, they are not necessary in that context and do not fit the style of the other sections where this kind of information is lacking.
This has been changed as from reviewer's comment.
• Some parts of the experimental procedures need refining, for example specifying vendors of the Duolink assay or specifying which Dynabeads were used.
This missing information has been included.
• page 4, last third. Mtp53 are characterized by... "inability to recognize wt-p53 DNA binding sites. This statement should be moderated as not all mtp53 proteins have lost the ability to bind to wtp53 binding sites.
This statement has been changed declaring that only some of the mutant p53 proteins lose the ability to recognize wt-p53 DNA binding sites
• Images for figure 2A-D should be shown in the supplemental data
Representative images of PLA have been included in the **Expanded Figure 2C**.

Referee #2:

The present manuscript attempts to describe the potential involvement of an RNP complex consisting of the abundant nuclear-localized Malat1 long noncoding RNA, SRSF1 splicing factor and mutant p53-ID4 complex in modulating angiogenesis in breast cancer cells by regulating the alternative splicing of VEGFA pre-mRNA. Authors have shown the interaction between mutant and not WT-p53 and ID4 protein with MALAT1 and SRSF1. Further, their data indicate that SRSF1 is required for the interaction between Malat1 and the mut-p53-ID4. Mut-p53 or ID4-depleted cells showed changes in the sub-cellular localization of Malat1, and also their data indicated that mut-p53-ID4 complex facilitates the association of Malat1 with chromatin. Finally, authors indicate that mut-p53-ID4-SRSF1-Malat1 complex facilitates the production of pro-angiogenic isoforms of VEGFA. Based on their results they propose a model, where in mutant p53 and ID4 expressing breast cancer cells these proteins form a stable complex with Malat1 and SRSF1 and the RNP complex regulates the alternative splicing of VEGFA pre-mRNA, resulting in the increased production pro-angiogenic version of the VEGFA protein. Their results implicate that mut-p53 along with other components enhances the angiogenic properties of breast cancer cells by regulating alternative splicing of pre-mRNA.

It is an interesting concept, and the authors have provided some technically sound data to support their claim. However, a large number of experiments lack proper controls that are required to completely interpret the data. Authors need to perform the experiments that are described below

Specific comments:

1. Based on what is mentioned in the materials and methods, authors have performed RNA immunoprecipitation (RIP) assays under non-crosslinking conditions. These assays, especially when done under native conditions are known to produce non-specific interactions between proteins and RNA. Therefore, it is recommended that authors perform all of RIP under crosslinking (formaldehyde or UV) conditions.
We agree with the reviewer and we have analysed the ability of mutant p53, ID4 and SRSF1 proteins to bind to MALAT1 RNA in cells crosslinked with formaldehyde (leading to protein-protein and protein-RNA covalent links) or with U.V. (covalently linking interacting proteins:RNAs and completely avoiding protein:protein cross-linking). We observed that SRSF1 binds to MALAT1 in both crosslinking conditions, according to what previously reported in literature; on the contrary, mutant p53 and ID4 interact with MALAT1 only in cells crosslinked with formaldehyde (and in native lysates as well), indicating that they are able to bind MALAT1 indirectly. Results have been included in **Figure**

1A-C-F.

2. Malat1 KD efficiency is very poor (sup. Fig 2). Some of the key experiments to be reproduced in cells with better knock down of Malat1. Also, all of the experiments that are shown in the main figure of the manuscript should be reproduced with more than one siRNAs against the molecule of interest (Malat1, SRSF1, p53 and ID4). This is a major drawback of the paper. Authors should not make conclusions based on data obtained after using one siRNAs against each member of the RNP complex.
Following the reviewer's request we have repeated the experiments (in MDA-MB-468 and SKBR3 cells) using a second interference for MALAT1 showing higher efficiency. Results have been included in **Figure 2A-E** and **Expanded Figure 2A-B**.
Moreover, an additional siRNA for each component of the RNP complex has been also employed for the majority of the experiments (Figures 1, 2, 3, Expanded Figure 2; more than 1 siRNA already reported for Figure 5/Expanded Figure 4).

3. In general, the quality of the microscopy data is poor (for example see figure 2G, where p53 signal is saturated) and needs to be improved.
Many of the microscopy images have been replaced with better ones obtained from repeated independent experiments, as for example the one indicated by the reviewer.

4. PLA data (Fig 2A-F) should be supplemented with endogenous IP experiment in control and Malat1-depleted cells.
In this new version of the manuscript we have verified the existence of the complexes by Co-IP experiments (**Figure 2H,I,J**). This approach confirmed the interaction of endogenous (SKBR3-p53R175H) and exogenous (p53R273H transfected in p53-null H1299 cells) mutant p53 proteins with SRSF1 (**Figure 2H-J**). We have also verified the interaction between endogenous ID4 and SRSF1 proteins (**Figure 2I**) and between endogenous ID4 and exogenous p53R273H (**Figure 2I**).
Amount of interactions have then been assessed by PLA as this is a more quantitative approach. Effects of MALAT1 depletion have been confirmed using a second, more efficient interference (**Figure 2 A-C** and **Expanded Figure 2A-C**).

5. Based on what is known in the literature, in most cell types most of the Malat1 is known to localize to nuclear speckles or SC35 domains. Authors should perform co-localization analyses of mut-p53 and ID4 with Malat1 to see their co-localization.
Co-localization of MALAT1 & mutant p53/ID4 has been analysed combining RNA FISH with IF (immunofluorescence). We confirmed the interaction of mutant p53 with MALAT1 RNA [presented in **Figure 1D-E**; Pearson's correlation coefficients R =0.68 (+/-0.11) in MDA-MB-468 & R=0.52 (+/-0.09) in SKBR3 cells].
Unfortunately, we encountered a technical problem with the detection of ID4. This protocol indeed includes IF followed by RNA FISH, requiring a formamide-containing buffer, and we observed that subsequent FISH caused a delocalization of ID4 protein from the nucleus. This was observed with 2 different antibodies.

6. What are the two last lanes indicate in sup fig. 2D & E? Are they represents input samples? If it is, then the level of SRSF1 depletion achieved is poor and require to repeats the experiments under conditions of better KD. Surprisingly, sup. Fig. 3D shows better KD of SRSF1.
We agree with the reviewer's comment; we have repeated this experiments in SKBR3 and MDA-MB-468 cells with much more efficient SRSF1 depletion and we confirmed that mutant p53-ID4 interaction is not affected by SRSF1 depletion. The results are shown in **Expanded Figure 2E**.

7. Quality of IP data in fig 2I & sup fig. 2G is poor (especially the SRSF1 immunoblot). Since both input and IP samples are run in separate blots, it is impossible to assess the percentage of interaction between the proteins. Actually, both input and IP should be run in parallel, and should be exposed together.
We have repeated IP p53>blot SRSF1 using different conditions/buffers but we continued to see the presence of various bands of uncertain specificity. We have then undertaken the reciprocal approach (IP SRSF1>blot p53) as IP of SRSF1 precipitated a single well-defined SRSF1 protein product. This approach confirmed the interaction of endogenous (SKBR3 cells carrying p53R175H) and exogenous (p53R273H transfected in p53-null H1299 cells) mutant p53 proteins with SRSF1 (**Figure 2H-J**).

8. In addition to Malat1 KD, authors should also do IP to test interaction between SRSF1 and mut-p53 in ID4-depleted cells.
ID4 contribution to the interaction between SRSF1 and mut-p53 has been evaluated performing Proximity Ligation Assay (PLA) in control and in ID4-depleted MDA-MB-468 and SKBR3 cells. Results, presented in **Figure 3E**, showed that

SRSF1-p53 interaction was severely impaired following ID4 depletion.

9. Authors should also test whether ID4 interacts with SRSF1 and MALAT1 in breast cancer cells expressing WT-p53
We have tested whether ID4 interacts with MALAT1 RNA in MCF7 breast cancer cells and in breast tissue derived MCF10A cells (both carrying wt-p53) in basal condition and upon treatment with a DNA damaging agent, which stabilizes p53. These experiments showed that ID4 protein doesn't interact with MALAT1 RNA in these cells (**Expanded Figure 1B-D**).

10. Several earlier studies, using different cell lines such as HeLa have shown positive interaction between SRSF1 and Malat1. Based on SRSF1 Clip-seq data from Sanford laboratory, Malat1 RNA contains several SRSF1 binding sites, that are distributed all over the length of the transcript. Based on that, this reviewer is not completely convinced with the authors' description (Fig 3A) that mut-p53/ID4 is required for stabilizing the interaction between SRSF1 and Malat1. In order to make it more convincing, authors need to do more direct experiments such as SRSF1 CliP in mut-p53 or ID4-depleted cells.
To address this reviewer's concern we have evaluated the recruitment of SRSF1 protein along MALAT1 RNA in control and ID4-depleted MDA-MB-468 cells. To this end, cells were crosslinked with formaldehyde and subsequently cell lysate was sonicated to obtain fragments ≤500 bp and used to immunoprecipitate SRSF1. We then evaluated recruitment of SRSF1 along MALAT1 RNA using 14 couples of primers covering the whole MALAT1 RNA. This analysis revealed that SRSF1 is recruited in several regions along MALAT1 RNA. A major site of enrichment was located in the 5' half of MALAT1. Additional regions significantly enriched were also observed along the RNA.
This is in agreement with what reported in HEK293T cells from Sanford JR et al., [Genome Research, 2009] and from Tripathi V et al., [Molecular Cell, 2010].
Depletion of ID4 caused reduction of SRSF1 binding to MALAT1 in all the enriched regions. Results have been included in **Figure 3D**.

11. It is surprising to see that Malat1 shows speckle localization only in 10% of the breast cancer cells. Actually, even the so-called "diffused cells" (sup fig 4B) shows speckle staining of Malat1.
We agree with the reviewer, speckles are indeed visible at fluorescence microscopy in the majority of cells, but many cells present also a diffused staining in MDA-MB-468 and SKBR3 cells. For that reason we changed the description of this result and the label in Figure 4A, indicating as "speckled+diffused" the cells previously indicated as "diffused".
During the revision we evaluated the MALAT1 RNA FISH in additional cell lines. Interestingly, we observed that MCF7 cells, carrying wt-p53, showed a staining similar to that reported in literature for example in HeLa cells (e.g. mainly localized in speckles). On the contrary, all the mutant p53-carrying cells presented a more diffused staining in addition to the speckled one. Images from this RNA FISH experiments have been included in **Expanded Figure 1E**.
I am also not convinced with staining shown in figure 4B (scr-si). In all these conditions, authors should do a co-staining of Malat1 with another known speckle marker such as SC35.
Visualization of images presented in Figure 4B has been performed by confocal microscopy and these images were only presented to qualitatively show the appearance of the MALAT1-positive speckles following interference of mutant p53 or ID4. We have replaced Figure 4B with new more representative images.
SC35-MALAT1 co-staining did not work in our experimental systems. However, we evaluated SC35 single staining in si-p53 or si-ID4 conditions and we didn't observe changes in SC35 distribution (data not shown).

12. The experiment described in fig 4C-F is under the assumption that the diffused Malat1 fraction represents the chromatin-associated fraction of Malat1 (primarily based on its increased association with H3). However, these experiments require crucial controls. To be more quantitative, authors should do H3 ChIP and Malat1` interaction. They should treat the cells with RNA pol II inhibitors (amanitin or DRB), which are known to release MALAT1 from speckles (Bernard et al., 2010 EMBO J) and see if such treatments dramatically increase the association of Malat1 with H3.
To address this concern we have performed immunoprecipitation with antibodies directed to H3, H3K36me3 [a modification specifically enriched in exons (Spies N et al., Molecular Cell 2009; Kolasinska-Zwierz P et al., Nature Genetics 2009) and that has been shown to interact with SRSF1 (Pradeepa MM et al., PLoS Genet. 2012)] and H3K27Ac (generally associated with active transcription) using lysates from control (si-SCR) and ID4/mut-p53 depleted cells (MDA-MB-468 and SKBR3) crosslinked with formaldehyde (as for ChIP protocol) and we have then analysed the amount of bound MALAT1 RNA by RT-qPCR. These experiments showed that a reduced amount of MALAT1 RNA is bound by H3 in cells depleted of ID4/mutp53 (**Figure 4G-J**). Interestingly, also H3K36me3 showed reduced interaction with MALAT1 after ID4/mut-p53 depletion in both cell lines, while H3K27Ac showed a reduction only in MDA-MB-468 cells.

13. In Fig 5, knock down of every component showed increased levels of anti-angiogenic VEGFA isoforms. These experiments requires negative control, where authors should deplete proteins such as hnRNP A1 (a known negative regulator of SRSF1) and see if that shows opposite effect with respect to alternative splicing of VEGFA. In addition, authors could test the potential changes in the alternative splicing of few house keeping gene mRNAs in cells that are depleted of the RNP constituents.
We thank the reviewer for the suggested additional controls. Analysis of VEGFA isoforms following hnRNP A1 depletion showed a significant reduction of VEGF 165b/165 ratio and increase in VEGF 121b/165b ratio, effects that are opposite to the ones observed upon SRSF1 depletion (**Figure 5B, 5E and Expanded Figure 4A**).
Concerning the analysis of alternative splicing of housekeeping genes, we have analysed the ratio between two isoforms of the Aldolase-A (ALDOA) gene, which differ for the inclusion of exon2, upon interference of the various components of the RNP complex. As shown in **Expanded Figure 4B**, depletion of SRSF1 significantly reduced the ratio between the two ALDOA isoforms while the other components did not affect this splicing event.

14. It is known that MALAT1 levels are high in breast cancer patient samples (Arun et al., 2016 G&D; Mahdiah et al., 2016 Oncotarget). In order to explain fig 5F better authors should catalog the patient samples with comparable levels of Malat1.
As indicated by the reviewer, MALAT1 expression levels are increased in various malignancies and its high expression has been related to decrease in disease-specific survival in lymph-node-negative triple-negative breast cancer (TNBC) patients. Unfortunately, probes for MALAT1 RNA were not present in the majority of the microarrays used to produce the dataset that we used for the survival analyses (Compendium cohort). The reference of the Oncotarget 2016 study from Jadaliha M et al., which was missing from our bibliography, has been included.

15. Again in fig 6 an important control experiment is missing. P53 Chip and RIP should be performed in BC cells expressing endogenous p53 to see potential interaction of WT p53 to these genomic regions. Similarly, authors should perform SRSF1 RIP (fig 6E-F) in control and MALAT1-depleted BC cells containing endogenous wild type p53, to see if this change happens only in cells containing mut-p53.
We have tested whether wt-p53 interacts with MALAT1 RNA in MCF7 breast cancer cells and in breast tissue-derived MCF10A cells (both carrying wt-p53) in basal condition and upon treatment with a DNA damaging agent, which stabilizes p53. These experiments showed that p53 protein doesn't interact with MALAT1 RNA in these cells (**Appendix Figure 1B-D**). We have also evaluated recruitment on VEGF pre-mRNA but this precursor was not detectable in both cell lines.

16. It is not clear why overexpression of VEGF165b alone did not reduce the mesh forming property of endothelial cells. In fig 7A, VEGF165b overexpressing data should be compared with control cell and not with VEGF165 overexpressing cells.
The VEGF165b is not anti-angiogenic when it is alone, but it works by competition with pro-angiogenic VEGF165. We can suppose that cancer cell may secret different cytokines, which stimulate mesh formation by endothelial cells, and in this circumstance I don't expect significant difference between Control (CM without overexpression) and CM with overexpressed 165b. On the contrary when endothelial cells are exposed to overexpressed 165 and 165b we can observe result of competition in comparison to stimulation with 165 alone.
In similar lines, I would have expected less effect on the mesh forming property in Malat1 or SRSF1-depleted cells compared to VEGF165b overexpressing cells. But the data presented in fig 7 speaks otherwise.
We apologize for having joined the controls (Figure 7A, four columns on the left) with the interference experiment (Figure 7A, five columns on the right). Samples CTR and siSCR were both set to 1, but they don't present the same basal level and comparison can't be done. We have created two graphs in the new version of the manuscript.

Referee #3:

In this manuscript, Pruszko et al. describe the existence of a quaternary complex made of 3 proteins (mutant p53, ID4 and SRSF1) and the long non-coding RNA (lncRNA) MALAT1. They provide evidence for and its pro-oncogenic role in basal-like breast cancer tumors. The authors have connected the oncogenic activity of this complex with its ability to shift the ratio of expression of VEGFA isoforms in favor of the pro-angiogenic VEGFA165 and VEGFA121 that include exon8a. They have verified the importance of each individual component of the complex, as well as the p53 status, for the predominant expression of exon8a containing isoforms. Furthermore, they corroborated their experimental data with analysis of publicly available data sets of breast cancer clinical samples.
Long non-coding RNAs (lncRNAs) have recently been discovered to regulate numerous intracellular processes.

Consequently, their possible roles in carcinogenesis are also emerging. A number of publications have reported functional interactions between the p53 tumor suppressor and certain lncRNAs. Only a few of such studies, however, delivered sound and unbiased messages on the outcomes of p53 association with a specific RNA. The work described by this group is an exception in that it is solid and reasonably convincing. The authors have employed a number of techniques to verify involvement of each component of the complex, in the regulation of expression of anti-proliferative VEGFA isoforms. These include the proximity ligation assay (PLA), RNA FISH and several co-immunoprecipitations followed by either immunoblot or qRT-PCR analysis. Importantly, the authors did not simply document functional interactions between lncMALAT1 and either of the protein modules of the multicomponent complex that is involved in the regulation of VEGFA splicing, but also managed to connect (to a certain degree) the dots between each module and show their interdependence. In all this is an interesting and informative study that has some translational added benefit. I would recommend this study for publication in EMBO reports pending response of the authors to the following comments.

1. The levels of MALAT1 lncRNA and each of the protein components, namely, SRSF1, mutant p53 and ID4 should be verified in a comparative manner within both, MDA-468 and SkBr3 cell lines. Though the authors did the analysis of the expression of the individual components of the quaternary complex upon transient knock down of individual members of this complex, these data are scattered through the manuscript and difficult to follow. They should be provided next to the original experiment(s), as they represent important controls.
In the new version of the manuscript we included analysis of all the components of the RNP complex near every experiment of interference (e.g. **Figures 1G, 2D-E, 3A-B**).

2. The authors did not observe interactions between wild-type p53 and lnc RNA MALAT1 in MCF-7 cells. What conditions did they use? Was wild-type p53 stabilized and present at comparable levels to mutant p53 levels in the respective MDA cell lines? It would also be good to show data with another breast tissue-derived cell line such as MCF10A cells that harbor wild-type p53.
As suggested by the reviewer we have analysed the interaction of wt-p53 (and ID4 protein as well) with MALAT1 in MCF7 and MCF10A cells treated or not with Adriamycin to stabilize p53 protein. In these conditions wt-p53 and ID4 did not interact with MALAT1. Results have been included in **Expanded Figure 1B-D**.

3. In Figure1G the authors should include immunoblotting analysis of ID4 and mutant p53 protein levels upon siSRSF1 knockdown (see the first comment). The same should be done in Figure 2, where the levels of the corresponding interacting protein pairs upon knockdown of the RNA component of the complex are not shown.
These controls have been included, as suggested.
On Page 6, bottom, what is meant by the phrase "...despite comparable efficiency of protein immunoprecipitation in si-SCR vs si SRSF1 conditions."
We meant that the observed differences in ID4-MALAT1 and mutp53-MALAT1 interactions upon SRSF1 depletion were not due to differences in IP efficiency between control and interference conditions. However, as now steady state levels for all the proteins have been presented near each RIP experiment, we omitted presenting this additional IP control in the Appendix material as maybe it is confusing.

4. On Page 7, bottom, the authors stated that replacing mutant p53 with wild-type p53 did not recover p53-SRSF1 interaction in MDA-468 cells. Did they try and perform the reciprocal substitution in MCF-7 cells and check if there is a mutant p53-SRSF1 complex in the MCF-7 background? Such an experiment would provide valuable data about the relevance of the cellular background for mutant p53-SRSF1 (indirect) interactions.
Concerning this replacement experiment, we had problems in performing exogenous expression of mutant p53 in MCF7 cells where endogenous wt-p53 was concomitantly depleted by using a siRNA directed to the 3'-UTR of p53 mRNA. To this end we performed exogenous expression of mutant p53 (R273H and R175H) in p53-null H1299 cells. Through this experiment we verified that mutant p53 interacts with SRSF1 (data shown in **Figure 2H**) also in H1299. In H1299 we have also verified interaction between: endogenous ID4 and exogenous p53R273H (**Figure 2I**); endogenous ID4 and SRSF1 proteins (**Figure 2I**).

In the co-IP results presented in Figure 2I, did the authors observe interactions after treatment with RNase?
To address this point we have performed Co-IP SRSF1-mutp53 after treatment with RNase. As shown in **Figure 2J**, a decreased interaction was detected after this treatment.

5. In Figure 4D-G, the authors verified the association of MALAT1 with active chromatin by a ChIP assay followed by

immunoblotting with anti H3 antibody. This analysis could be repeated to evaluate more specific marks of active chromatin such as probing for the H3 specific modifications H3K9Ac, H3K27Ac to prove their point.
Starting from this stimulating suggestion of the reviewer we reasoned on how to perform such analysis, as glutaraldehyde, which is the fixative needed in the ChIRP experiment, may compromise post-translational modifications of proteins. We have then applied a reciprocal approach, immunoprecipitating H3 (and some modified variants: H3K36me3, H3K27Ac, H3K9Ac) and evaluating the enrichment of MALAT1 RNA in control and ID4/mut-p53 depleted MDA-MB-468 and SKBR3 cells (results presented in Figure 4H-I-J).
These experiments showed that a reduced amount of MALAT1 RNA is bound by H3 in cells depleted of ID4/mutp53, compared to control cells (**Figure 4G-J**). Interestingly, also H3K36me3 [a modification specifically enriched in exons (Spies N et al., Molecular Cell 2009; Kolasinska-Zwierz P et al., Nature Genetics 2009) and that has been shown to interact with SRSF1 (Pradeepa MM et al., PLoS Genet. 2012)] showed reduced interaction with MALAT1 after ID4/mut-p53 depletion in both cell lines, while H3K27Ac showed a reduction only in MDA-MB-468 cells.
No significant enrichment of MALAT1 in samples immunoprecipitated with H3K9Ac was observed.

Of lesser concern.

6. Many available breast cancer datasets show significant increase in expression of each of the 4 components of the quaternary complex described in the paper (c-bioportal website). In what percent of breast malignancies are all three, MALAT1, ID4 and SRSF1 upregulated and co-exist with mutant p53?
Concerning this analysis, it is well accepted that SRSF1 is upregulated in many malignancies, but its activity is mainly modulated by post-translational modifications and we observed in our study that also MALAT1 RNA is controlled by ID4 and mutant p53 at the level of subnuclear localization. We aim to examine the staining of MALAT1 RNA and SRSF1 protein in basal-like breast cancer cases in the future development of this study.

7. Is the ability of mutant p53 to interact with MALAT1 specific to cells of breast cancer origin? Do p53 mutants from (for example) colorectal cancer or ovarian cancers interact with MALAT1?
We have addressed this concern by doing RIP analysis in ovarian cancer cells OVCAR-3, carrying mutant p53R273H. This experiment evidenced that mutant p53 binds to MALAT1 RNA also in this cell line. Data are shown in **Expanded Figure 1A**.

2nd Editorial Decision 31 March 2017

Thank you for the submission of your revised research manuscript to EMBO reports. We have now received reports from the three referees that were asked to re-asses your study, which can be found at the end of this email. As you will see, two referees still have concerns that preclude publication of the manuscript in its present form.

Nevertheless, given the constructive referee comments, I would like to invite you to provide a final revised version of your manuscript, addressing the remaining points of the referees. After cross-commenting with them, we think that it would be important to address the remaining point of referee #1, which overlaps with point 6 of referee #2. Also points 1 and 3 of referee #2 are important and should be addressed with additional data. However, we feel that it would be sufficient to address points 2, 4 and 5 of referee #2 in the rebuttal letter. Nevertheless, in case you have relevant additional data to address also these, you are very welcome to include these in the revised version.

In addition, I have a couple of editorial requests:

Please upload all figures (main figures and EV figures) as single editable TIFF or EPS-formatted figure files in high resolution.

The manuscript is currently very long (nearly 75000 character with spaces). Usually, articles in EMBO reports are not longer than 60000 characters (including M&M and references, excluding the figure legends). Please try to shorten and condense the manuscript, in particular the methods part.

In the legend for the EV figures, please name the figures according the nomenclature Figure EV1, Figure EV2 etc. For the Appendix figures and tables please follow the nomenclature Appendix Figure Sx (Appendix Table Sx) throughout the text and also label the figures according to this nomenclature in the Appendix.

Please add scale bars to all microscopy images (also requested by referee #2).

As most of the Western panels are cut and show only a fraction of the original gel, we would prefer to have source data files for these gels (also for the Western panels in the Appendix). Source data is published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please prepare source data files (containing scans of entire gels or blots) of your experiments including size markers. Please label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

Some of the Western panels in the Appendix seem to show pdf compression artifacts. Please use higher resolution sources for these and re-assemble the pdf.

Finally, it seems there is no call out for Appendix Table S4 in the manuscript.

REFEREE REPORTS

Referee #1:

The authors have changed the manuscript to large extents. Most concerns of this reviewer have been addressed. My only concern remaining is the interaction between SRSF1 and p53. In the new version the authors are only investigating the interaction between SRSF1 and p53 using a SRSF1 pulldown. The reciprocal IP is giving too much a-specific bands to be conclusive. As the IP was only done with IgG controls the evidence of this interaction is in my opinion still limited. An IP with SRSF1 knockdown or reciprocally with tagged proteins should be done to verify the specificity. P53 is notorious for binding to certain antibodies and beads, which is not always captured by using an IgG negative control.

Referee #2:

This is definitely a much-improved version of the ms. Authors have included more data to support the involvement of MALAT-1 and SRSF1 in the mut-p53/ID4-mediated pro-angiogenic activity in breast cancer cells.

However, the authors still need to clarify several more points in order to make the ms significant for publishing in EMBO Rep.

1.In the abstract the authors mentioned that SRSF1 bridges the interaction between mut-p53/ID4 and MALAT-1. UV-crosslinking IP experiments confirm that that SRSF1 and mut-p53 and ID4 do not directly bind to MALAT-1. To me it looks like that every component of the Malat-1/SRSF1/mtp53/ID4 RNP complex plays a part in stabilizing the complex. For example, SRSF1 depletion reduces the interaction between MALAT-1 and mtp53. Similarly Malat-1 depletion reduces the interaction between SRSF1 and mtp53. Finally, ID4 depletion altered the association between MALAT-1 and SRSF1. It is possible that SRSF1/mtp53/ID4 interaction is stabilized by yet another unknown factor that directly interacts with MALAT-1 and SRSF1. To supports authors claim, it is important to show direct interaction between SRSF1 and mtp53 or ID4. This piece of data would really strengthen the ms. And would support the model. Authors could perform an in vitro binding assay to test the direct interaction between SRSF1 and mtp53 or ID4.

2.Figure 1D, the RNA-FISH/IF figure quality is still poor. For example, mt-p53 signal is saturated and the merge figure is not in focus. Also, this data is still not convincing. Authors should pick up cells that show discrete speckle staining of MALAT-1 and test whether p53 mutant localizes to nuclear speckles. As a control, they could do the same experiment in MALAT-1 or SRSf1-depleted cells. MALAT-1 or SRSF1 depletion should disrupt the localization of mutant p53 from speckles.

3.Figure 1G. Since depletion of SRSF1 with only one of the siRNAs showed small but significant increase in MALAT-1 levels, it should not be included in the ms. Alternatively, authors could reproduce the data with another set of siRNA, or could try to rescue the changes in MALAT-1 levels by introducing exogenous SRSF1 (siRNA-resistant version) into siRNA-SRSF1-1-treated cells.

4.The data presented in Figure 3D is interesting. However, it is surprising to know that ID4 could regulate the direct binding of an RNA-binding protein such as SRSF1 to MALAT-1. Authors should provide more data or insights to explain this effect. Does ID4 change the confirmation of SRSF1 so that it could bind to MALAT-1 more efficiently? In general, RBPs show degenerate binding affinity. In that sense, an RBP normally does not need another protein to increase its binding affinity towards a target RNA. At least as a control experiment, authors should test whether ID4 also controls the binding of SRSF1 to one or two of its known target pre-mRNAs.

5.Figure 4 D-J. Does this mean that the modified or unmodified histones directly bind to MALAT-1? The H3 RIP data could possibly mean that histones directly interact with DNA regions on the chromatin that also interact with MALAT-1. In support of this, recent CHART and RAP seq data indicates association of MALAT-1 with transcriptionally active genes. It would ideal to determine whether the interaction between H3 and MALAT-1 is DNA-dependent. H3 crosslinking RIP followed by MALAT-1 RT-qPCR in DNase 1 treated extracts would answer this point.

6.In Figure 2J, p53 WB in no Ab IP lanes show a discrete band whereas the SRSF1 IP samples display two closely running bands. The input p53 band runs in parallel with the lower band. This data is not convincing to argue that endogenous mt-p53 interacts with SRSF1.

7.Please add magnification bar in all of the microscopy images.

Referee #3:

This is an interesting study, and the authors have responded appropriately to our critiques.

2nd Revision - authors' response 23 April 2017

Point by point response shown on the following pages

Referee #1 (Report for Author)

The authors have changed the manuscript to large extents. Most concerns of this reviewer have been addressed. My only concern remaining is the interaction between SRSF1 and p53. In the new version the authors are only investigating the interaction between SRSF1 and p53 using a SRSF1 pulldown. The reciprocal IP is giving too much a-specific bands to be conclusive. As the IP was only done with IgG controls the evidence of this interaction is in my opinion still limited. An IP with SRSF1 knockdown or reciprocally with tagged proteins should be done to verify the specificity. P53 is notorious for binding to certain antibodies and beads, which is not always captured by using an IgG negative control. To address this reviewer's concern we have now repeated this IP experiment in SKBR3 cells after SRSF1 knockdown. Result from this experiment has replaced panel J in Figure 2. As shown, we observed that reduced levels of SRSF1 impaired SRSF1-p53 Co-IP in these cells.

Referee #2 (Report for Author)

This is definitely a much-improved version of the ms. Authors have included more data to support the involvement of MALAT-1 and SRSF1 in the mut-p53/ID4-mediated pro-angiogenic activity in breast cancer cells.
However, the authors still need to clarify several more points in order to make the ms significant for publishing in EMBO Rep.

1.In the abstract the authors mentioned that SRSF1 bridges the interaction between mut-p53/ID4 and MALAT-1. UV-crosslinking IP experiments confirm that that SRSF1 and mut-p53 and ID4 do not directly bind to MALAT-1. To me it looks like that every component of the Malat-1/SRSF1/mtp53/ID4 RNP complex plays a part in stabilizing the complex. For example, SRSF1 depletion reduces the interaction between MALAT-1 and mtp53. Similarly Malat-1 depletion reduces the interaction between SRSF1 and mtp53. Finally, ID4 depletion altered the association between MALAT-1 and SRSF1. It is possible that SRSF1/mtp53/ID4 interaction is stabilized by yet another unknown factor that directly interacts with MALAT-1 and SRSF1. To supports authors claim, it is important to show direct interaction between SRSF1 and mtp53 or ID4. This piece of data would really strengthen the ms. And would support the model. Authors could perform an in vitro binding assay to test the direct interaction between SRSF1 and mtp53 or ID4. To address this concern we have tried to perform Co-IP assays using GFP-tagged SRSF1 protein and exogenously expressed p53 mutants. However, as GFP-SRSF1 protein overlapped with antibody free chains, we obtained results that are not clean enough to be presented in the paper. However, specificity of the interaction p53-SRSF1 has been provided through a Co-IP assay performed in SKBR3 cells upon SRSF1 knockdown. We observed that decreased SRSF1 protein level impaired the formation of the complex (Figure 2J).

2.Figure 1D, the RNA-FISH/IF figure quality is still poor. For example, mt-p53 signal is saturated and the merge figure is not in focus. Also, this data is still not convincing. Authors should pick up cells that show discrete speckle staining of MALAT-1 and test whether p53 mutant localizes to nuclear speckles. As a control, they could do the same experiment in MALAT-1 or SRSf1-depleted cells. MALAT-1 or SRSF1 depletion should disrupt the localization of mutant p53 from speckles. Concerning the IF/FISH experiments, pictures presented in panel 1D are just representative images to show the interaction. Most importantly, in these experiments the interactions have been evaluated using three different

colocalization analysis approaches [e.g. Pearson's correlation coefficient R, Manders correlation coefficient M2 (tM2) and Li intensity correlation quotient ICQ (Li)], described in detail in the Methods. All three analyses confirmed the interaction.
Of note, in these analyses we have observed that, in cells carrying mutant p53, a diffused staining of MALAT1 predominates compared to the speckled staining. Also mutant p53 protein shows a diffused nuclear staining.
Moreover, our data from ChIP/RIP/ChIRP experiments show that mutant p53 and MALAT1 are recruited on VEGFA genomic regions and pre-mRNA. As nuclear speckles do not represent major sites of transcription or splicing, but rather are considered sites from where splicing factors are recruited to active sites of transcription, we believe that dissecting the localization of mutant p53 into the speckles do not represent the main focus of the present manuscript but it would be anyway an interesting issue to address in the context of a more specific study.

3.Figure 1G. Since depletion of SRSF1 with only one of the siRNAs showed small but significant increase in MALAT-1 levels, it should not be included in the ms. Alternatively, authors could reproduce the data with another set of siRNA, or could try to rescue the changes in MALAT-1 levels by introducing exogenous SRSF1 (siRNA-resistant version) into siRNA-SRSF1-1-treated cells. As suggested by the reviewer we have evaluated MALAT1 levels after transfection of a third set of siRNA. Also this last siRNA caused an increase of MALAT1 RNA. Western blot of SRSF1 after this new siRNA transfection and analysis of MALAT1 levels after SRSF1 knockdown have been now included in Figure EV1 (panel F).

4.The data presented in Figure 3D is interesting. However, it is surprising to know that ID4 could regulate the direct binding of an RNA-binding protein such as SRSF1 to MALAT-1. Authors should provide more data or insights to explain this effect. Does ID4 change the confirmation of SRSF1 so that it could bind to MALAT-1 more efficiently? In general, RBPs show degenerate binding affinity. In that sense, an RBP normally does not need another protein to increase its binding affinity towards a target RNA. At least as a control experiment, authors should test whether ID4 also controls the binding of SRSF1 to one or two of its known target pre-mRNAs.
To evaluate whether a general effect on the ability of SRSF1 to bind to its target pre-mRNAs is obtained following ID4 depletion, we evaluated some of the SRSF1 well-known target pre-mRNAs in our RIP experiments +/- ID4 depletion. In particular, we have analysed the pre-mRNAs of *BCL2L1* (Leu S., et al. J Cell Sci 2012;125:3164–72) and that of *BIM* (Anczukow O., et al. Nat Struct Mol Biol 2012;19:220–8). As shown in Figure EV3 (panel A) these pre-mRNAs are bound by SRSF1 protein in MDA-MB-468 cells, but these interactions are not affected by ID4 depletion. This result indicates that ID4 expression is specifically required for interaction of SRSF1 with MALAT1.

5.Figure 4 D-J. Does this mean that the modified or unmodified histones directly bind to MALAT-1? The H3 RIP data could possibly mean that histones directly interact with DNA regions on the chromatin that also interact with MALAT-1. In support of this, recent CHART and RAP seq data indicates association of MALAT-1 with transcriptionally active genes. It would ideal to determine whether the interaction between H3 and MALAT-1 is DNA-dependent. H3 crosslinking RIP followed by MALAT-1 RT-qPCR in DNase 1 treated extracts would answer this point. Crosslinking procedures lead to stabilization of binding between macromolecules that are in close proximity (RNA:protein and protein:protein interactions); it is then reasonable that we observe interaction between MALAT1 and histone H3 because MALAT1 associates with active genes (as reported in literature).

6.In Figure 2J, p53 WB in no Ab IP lanes show a discrete band whereas the SRSF1 IP samples display two closely running bands. The input p53 band runs in parallel with the lower band. This data is not convincing to argue that endogenous mt-p53 interacts with SRSF1. To address this concern the experiment has been repeated and extracts from cells transfected with siRNAs to SRSF1 were also included in the analysis. Result from this experiment has replaced panel J in Figure 2. As shown, we observed that SRSF1-p53 Co-IP is affected by RNaseA treatment (as previously observed) and by SRSF1 depletion.

7.Please add magnification bar in all of the microscopy images. Magnification bars have been added.

Referee #3 (Report for Author)

This is an interesting study, and the authors have responded appropriately to our critiques.

3rd Editorial Decision 04 May 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from both referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, referee #1 now supports the publication of your manuscript in EMBO reports. Referee #2 has further minor concerns. However, we think the referee concern have been sufficiently addressed experimentally.

Nevertheless, please modify your manuscript text addressing points 1 and 3 of referee #2, i.e. please point out in the text the possibility that the interaction between SRSF1 and mut-p53/ID4/Malat-1 could also be indirect, and suggest that Malat-1 interacts with chromatin regions that contain H3. For Fig. 1D, in case you have this, or could obtain this in a timely manner, please provide better images.

It seems the "appendix" you now uploaded is only the source data for 3 appendix figures. Please upload the correct appendix file (named as such), and the source data for the Appendix as separate source data file. Please also be sure that in the final manuscript text the figures are labeled according the nomenclature Figure EV1, Figure EV2 etc. For the Appendix figures and tables please follow the nomenclature Appendix Figure Sx (Appendix Table Sx) throughout the text and also label the figures according to this nomenclature in the Appendix.

The panels in Fig. 1 are oddly arranged (non-alphabetically). Please fix this (i.e. swap E and F).

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the revised manuscript text
- the rearranged file for Fig. 1
- the final Appendix file

Please note that we now mandate that all corresponding authors list an ORCID digital identifier! As Giovanni Blandino is listed as corresponding author, please link his ORCID.

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 addressed my concerns regarding the specificity of the CO-IP sufficiently.

Referee #2:

In the revised version, authors have satisfactorily addressed only some but not several of the key questions.

Comment number1: In order to describe that SRSF1 acts as a bridge to facilitate interaction between mitp53/ID4 and Malat-1, authors need to show direct interaction between SRSF1 and mut-p53. As described by the authors, Co-IP experiments DO NOT support direct interaction.

Comment number 2: I still think that the microscopy image shown in Fig. 1D is of poor quality. It is not acceptable to show out of focus images to describe co-localization. I would suggest to remove this data from the ms, if authors are convinced that other experiments support direct interaction between Malat-1 and mutant p53.

Comment 5: Again, the experiments shown in fig. 4 do not support direct interaction between Malat1 and H3, especially without performing the suggested control experiments. In the absence of the experiments, authors could suggest that Malat-1 interacts with chromatin region that contains H3.

3rd Revision - authors' response 08 May 2017

The authors made the requested changes and submitted the final version of their manuscript.

4th Editorial Decision 16 May 2017

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.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
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http://www.selectagents.gov/	List of Select Agents

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.	NA
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.	NA
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.	YES, microscope images evaluation was performed independently and in blinded manner by two investigators (page 21, 23).
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	YES, for every experiment, in the relative figure legend, the statistical test applied has been indicated.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Pages 23, 26, 28 and all the Figure Legends
Is there an estimate of variation within each group of data?	Pages 21, 25, 27, 28
Is the variance similar between the groups that are being statistically compared?	YES

C- Reagents

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).	Pages 23, 26, 28
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Source: ATCC, Regularly Mycoplasma tested by PCR

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

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 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	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. 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** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **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	NA
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 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

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, provide a statement only if it could.	NO