nature portfolio

Peer Review File

Mdm2 requires Sprouty4 to regulate Focal Adhesion Formation and Metastasis in a p53-independent manner

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

MDM2 has previously been reported to support cancer cell migration and invasion, and several molecular mechanisms have been proposed. In the present paper, Muniz de Queiroz et al describe a new mechanism for this activity of MDM2. Specifically, they report that MDM2, in association with MDMX, downregulates the expression of the Sprouty4 (Spry4) gene, resulting in increased formation of focal adhesions via regulation of cofilin phosphorylation. This drives increased matrix attachment, migration, invasion and metastasis.

The findings reported in this study are potentially important, and add to the growing knowledge about p53-independent functions of MDM2. Nevertheless, they do not explain how MDM2 suppresses the expression of the Spry4 gene. In order for this study to qualify for publication in Nature Comm, at least some mechanistic elaboration would appear to be a must. Does the MDM2/MDMX complex bind to the Spry4 gene promoter? Or does it ubiquitinate a protein(s) that regulates Spry4 expression? And how is the effect of MDM2 on Spry4 RNA stability explained? Answering any of the above has the potential to yield truly novel mechanistic insights.

Major concerns:

1. As shown in Fig. 4, MDM2 depletion or inactivation results in a 2-fold increase in Spry4 protein. One wonders to what extent such moderate increase can truly account for the observed major changes in motility, invasion and metastasis. The experiments in Fig. 6 are impressive, but are based on almost complete ablation of Spry4 expression, and as such do not answer the above question. It will therefore be essential to generate cells in which Spry4 expression is only reduced about 2-fold, such that upon MDM2 depletion it will become similar to that of non-treated control cells, and test whether this is enough to make them behave like the non-treated control cells.

2. Since the proposed mechanism appears to involve suppression of transcription rather than degradation of the Spry4 protein, it begs to look for additional genes that are similarly affected by MDM2 depletion or inhibition and identify putative transcription factors that are shared by those genes. This might then identify the direct MDM2/MDMX target that is responsible for the reported observations. Such identification, if achieved, will provide the missing mechanistic novelty.

3. One of the main conclusions of the authors is that the E3 activity of the MDM2/MDMX complex is required for the observed effects. Although most probably correct, it is not rigorously proven: formally, it might still be argued that the complex binds to a target (e.g. specific chromatin region) and blocks protein-protein interactions, for example. To make a firm statement, it needs to be shown that the effects of MDM2 knockdown can be rescued by wild type MDM2 but not by E3 ligase-inactive mutants.

4. Fig 2H, I. At the very minimum, the images should be counterstained with DAPI. Better even would have been to perform DAB staining in order to see the histology of lungs with and without mets. In particular, it will be important to show Ki67 staining in Fig 2I, since larger mets could be due to increased invasion (as the authors conclude) or increased MDM2-dependent proliferation at the site of metastasis.

Minor comments:

1.Page 5: “correlated directly to interact” - please fix sentence.

2.Page 13: The message conveyed by the heading “Sprouty4 is required to promote FA formation” appears to be opposite to the authors’ data, which shows that Spry4 inhibits FA formation.

3. The authors may wish to comment on an earlier report (Guo et al, reference 61) that Spry4 reduces MDM2 expression, which together with the present study would suggest a negative feedback loop.

4. Fig 3A. How was FA area and number measured? Was this done in an automated manner? Also, please explain what are non-peripheral FA and what their abundance implies.

5.Fig 3A, B. Displaying a larger number of cells would be more helpful to get an impression of the generality of the phenotypic differences.

6.Fig. 4A. SPRY4 is “buried” in the Volcano plots, and should be highlighted or otherwise better visualized (arrow, perhaps?).

Reviewer #2 (Remarks to the Author):

In their manuscript, Muniz de Queiroz et al. demonstrate in cells lacking p53 expression that knockdown of Mdm2 or MdmX, or pharmacological inhibition of the Mdm2/MdmX complex, reduces cell migration and invasion, impairs cell spreading and focal adhesion formation, and decreases metastasis in vivo. The authors identify Sprouty4 to be required for the Mdm2-mediated effects and investigate its role regarding cell migration, focal adhesion formation and metastasis independent of p53.

The authors claim as unique aspects of their work that, in contrast to the majority of existing studies, they used TP53 deficient cells, i.e. a CRISPR-derived human HT1080 fibrosarcoma p53 knock-out cell line and the H1299 lung carcinoma-derived cell line that is endogenously p53-null. The second aspect is that instead of altering Mdm2/MdmX abundance, they inhibit the E3 ligase activity of the Mdm2/MdmX complex using a small molecule inhibitor of Mdm2, MEL23. Further claims are the use of 2D and 3D in vitro models which have not yet been employed in this setting

and the investigation of the role of post-translational modifications, of which the latter are not followed up to a greater extent.

The manuscript is well-written and the plethora of data demonstrating how Mdm2 p53-independently regulates migration and invasion in vitro and metastasis in vivo are presented comprehensively in almost all instances. A few open issues remain to be addressed.

A potential weakness is the lack of clarity regarding the identification/selection of the mechanistic candidates. After characterizing the effects of Mdm2/MdmX knockdown and pharmacological inhibition in 2D and 3D settings, a proteomics experiment is used to identify Spry4 as candidate protein. However, it is insufficiently explained how this candidate was selected. Similarly, in the last part of the results, cofilin is presented as a potential mediator of the Spry4/Mdm2 effect, but the alternative Ras/ERK signaling is a bit half-heartedly ruled out as candidate mechanism before cofilin is deduced from the literature.

Major issues:

- In Figure 1H the no coating control for MEL23 is missing. What is the reason for this omission?

- The authors point out that although Mdm2 modulation has been associated with EMT/MET before, there were no changes associated with different EMT markers (E-cadherin, N-cadherin, vimentin and Zeb1) in response to silencing Mdm2 (Supplementary Fig. 1F). However, a positive control for EMT markers, especially E-cadherin, is needed. EMT is also among the enriched processes in Supp Fig. 4E, indicating that EMT cannot be discarded completely.

- The authors state that assessment of cell motility and cell morphology in GFP-expressing cell lines derived from HT1080 p53KO cells that stably express shRNAs demonstrated that the HT1080 cell line with stable Mdm2 knockdown (shMdm2) displayed the same characteristics observed in cells transiently transfected with siRNA or in cells treated with MEL23 when compared to the shScramble cells. The comparability of these cell models seems to be a crucial prerequisite for this manuscript. Why did the authors not assess/present exactly the same parameters as for the siRNA knock-down (e.g. more relevant 3D culture etc.)?

 Integrin levels were assessed by mass spectrometry or by immunoblot but found to be not significantly affected by Mdm2 knockdown or inhibition. This might be explained by the limited power of the proteomic experiment (n=3), i.e. the ability to detect true positive differential abundance (see criticism below), as well as the fact that the presented experiment does not account for isoforms or PTMs. Due to the multitude of different integrins, this might also be true for the immunoblot, as there are no positive controls included.

- The proteomic experiment is a critical part of the manuscript as it produces the Spry4 candidate which is then evaluated. Unfortunately, this part is not well described and may suffer from a few shortcomings. The provided description including the two references is far from complete and it is unclear which parts of the experiment were performed as described in one of these two remotely related publications. It would be necessary to include in the (supplementary) methods section a complete description of all parameters of the experiment from sample preparation via LC-MS parameters to database search and statistics (e.g. p-value cut-offs are missing). A supplementary table should contain all identified proteins and abundance values in all samples. Such a table could be (with some limitations) reproduced from the jPOST submission but may not lead to the same results, even when using the same analysis software due to the lacking parameters.

- In the proteomic experiment the authors expect many proteins in both groups (siRNA and MEL23) to be significantly up- or downregulated after these treatments but it is not clear why that would be expected. It seems that there was no correction for multiple testing applied, potentially yielding many false positives. To estimate this biological noise, 3 vs 3 replicates of the Untreated or DMSO controls could be compared in the same way as the samples.

- The Volcano plots (hopefully) do not show all identified “peptides” (Fig. 4A).

- From looking at the marked proteins it is already obvious that there is inconsistent regulation, such as e.g., HSPA6 strongly downregulated in siRNA1#1 and not in the other comparisons, which is normal in an underpowered experiment. The Venn diagram in Supp Fig 4D should therefore differentiate siRNA#1 and #2 to see which proteins really overlapped in all three comparisons and the limitations of the minimum approach using three replicates should be discussed.

- The way the pathway enrichment is presented is insufficient and not suitable for this journal. It would be better to present a table instead of the figure, providing the statistical enrichment parameters (corrected pvalue, expected and observed frequencies or enrichment factor).

- Eventually, the authors assess 146 differentially expressed proteins (which ones? Supplementary table needed) and focus on on those associated with cell migration and motility (which ones? Please indicate in the same Supplementary table). Sprouty4 (Spry4) emerged as a viable candidate for further investigation, but the criteria for this selection as well as potential alternative candidates are not clearly described.

- Fig. 4B is redundant with Fig. 4A– instead Spry4 should be labeled in 4A (demonstrating fold change an p-value. Alternatively, in 4B as well as in Fig 4F, Supp Fig 5B and 5D (and similar figure

panels throughout the manuscript) individual data points should be visualized at least in addition to bars representing the mean

Minor issues:

- Intro: Redundant text: “harboring either wild-type or different mutant versions of p53”

- Results: “we found that the area of attaching cells was significantly reduced (Fig. 1E and F and Supplementary Fig 1D), while in solution (unattached) cell size did not change after such treatments (Supplementary Fig. 1E) demonstrating the changes in area are not due to cell volume

> I am not sure, if this transfer of results is really valid. After all the cell volume in adherent culture might be different, whereas in solution it might be not. I would therefore recommend to rephrase this passage.

- Results: “As expected, MdmX was coimmunoprecipitated with Mdm2, however Spry4 was not pulled down with Mdm2 and the reverse co-immunoprecipitation provided the same negative result (Fig. 4D).”

> The legend (“mix”, “DIV”) is not clear

- Results: “Surprisingly, both Spry4 transcription (as determined by the reporter assay) (Fig 4G) and mRNA stability, 13 as measured using two different transcription inhibitors (Fig. 4H and Supplementary Fig. 5F), were increased upon Mdm2 inhibition”

> Is Supp Fig 5F correctly referenced here?

- Results: “As seen in HT1080 cells, Mdm2 silencing induces Spry4 expression at the protein and mRNA levels (Fig. 5E and F) in H1299 cells, thereby extending our results to a cell line which had evolved naturally to lack expression of p53”

> line should be lines

- Results: “TCGA and GTEX databases revealed that Spry4 expression is decreased in lung cancers, both squamous and adenocarcinoma, compared to normal lung tissues (Supplementary Fig 5G).”

> should this be 5F?

- Supp Tab 1: “binging”

Reviewer #3 (Remarks to the Author):

Summary

This study explores the p53-independent role for Mdm2, and its binding partner MdmX, in cell migration, invasion and metastasis. While Mdm2 has already been implicated in the regulation of cell migration and metastasis, the authors here use 2D and 3D cell culture models, and in vivo models, to identify Mdm2-dependent expression of Sprouty4 as a regulator of these phenotypes in a p53-null setting, using a previously described Mdm2/MdmX inhibitor to implicate Mdm2's E3 ligase function. These are interesting findings further elaborating the link from the Mdm2/X complex to cell motility, although the intriguing link to Sprouty4 and cofilin could be strengthened and further experiments are suggested to determine precisely how Mdm2/X function is regulating cell–ECM attachment and cell migration.

Major comments

1. While the effect of the MEL23 compound on Mdm2 and MdmX protein levels is shown in Fig. 1, and a previous paper has characterized aspects of MEL23 activity/mechanism in certain cell types, does this compound inhibit Mdm2–MdmX complex formation and function in these cells and at the concentration used in the present manuscript?

2. Can the authors distinguish effects of Mdm2/X functional disruption via MEL23 from effects of alteration of protein expression levels (up-regulated upon MEL23 treatment)? Are these effects via a distinct mechanism from those effects observed upon depletion of Mdm2 levels?

3. Although relative ATP production of spheroids was measured, it appears that the HT1080 p53KO cells formed smaller spheroids upon Mdm2 knockdown and MEL23 treatment (Fig. 2). Was spheroid area quantified? Does this contribute to the apparent reduced capacity of these spheroids to invade?

4. It would be useful to include the primary tumor size measurement data for the tumor growth experiments, especially as this is commented on in the Results (p. 8).

5. Although total expression levels of some integrin subunits appear not to be affected by Mdm2/X disruption (Supplementary Fig. 3), the cell-surface expression levels of integrins should be monitored (e.g. using flow cytometry). Furthermore, is integrin activity (e.g. measured using an activation-state-reporting antibody) altered by Mdm2/X functional disruption? These readouts would help clarify how Mdm2/X is regulating cell–ECM attachment.

6. While focal adhesions appear to be dysregulated by disrupted Mdm2/X function, the suggestion that Mdm2 regulates maturation of focal adhesions and fibrillar adhesions (p. 10) is insufficiently supported by the present data.

a. Focal adhesions can mature into fibrillar adhesions, but fibrillar adhesions are not the same as focal complexes as stated in the Results (p. 10), so the text should be amended to correct this.

b. Non-peripheral vinculin-containing adhesions cannot be assumed to represent fibrillar adhesions, as central fibrillar adhesions have been reported to be largely devoid of vinculin staining. Instead, a fibrillar-adhesion-specific marker (e.g. active alpha5beta1 integrin) should be visualized and quantified to determine changes in fibrillar adhesions.

c. The claimed increased density of F-actin (p. 10) needs to be quantified, as it is not obvious from the images presented in Fig. 3. Is there a difference in specific forms of stress fibers upon Mdm2/X disruption (e.g. contractile ventral stress fibers that contain non-muscle myosin II or non-contractile dorsal stress fibers or transverse arcs that are not directly linked to focal adhesions)?

d. Can assembly of stress fibers and focal adhesions in Mdm2/X-disrupted cells be rescued by increasing actomyosin contractility via perturbation of the microtubule network (e.g. using nocodazole)?

7. The siSpry4-only controls shown in Supplementary Fig. 6F–K appear potentially contradictory to the observations of dual Mdm2 and Spry4 knockdown. Without quantification, it is difficult to conclude that there is no difference in focal adhesion formation upon Spry4 knockdown alone (Supplementary Fig. 6J).

8. The involvement of cofilin would benefit from further corroboration, as this appears to be a key link to the observed cell adhesion defects. It is difficult to tell from the actin images in Supplementary Fig. 6 whether there are substantial changes in actin stress fibers versus monomeric actin – this should be quantified. Does immunoblot analysis of G- and F-actin support this observation? Are there changes in the levels of free barbed ends of actin?

9. The presentation of the proteomics data would benefit from more detailed analysis to be more informative.

a. What is the justification for selecting Sprouty4 from the proteomics data, when the volcano plots show many other dysregulated hits with more substantial fold changes that may be more biologically relevant?

b. The Venn diagram in Supplementary Fig. 4 does not capture whether proteins within respective sets were differentially expressed in the same or different directions (i.e. up- or down-regulated). Could this information be displayed to clarify the concordance between treatments?

c. How were heatmaps clustered (there is no methodological information)? Were mean or median values used to summarize the replicates of each experimental condition? Combining replicates in this way removes information about variability between replicate analyses.

d. Why do the authors suppose that almost none of the same proteins were down-regulated in si#1 and si#2 (Supplementary Fig. 4C)?

e. The pathway analysis charts in Supplementary Fig. 4E have no axis labels, so the bar lengths cannot be interpreted. What are the false-discovery rates and enrichment ratios/gene-set overlaps for these analyses?

f. Supplementary Table 1 is inadequate for the reporting of mass spectrometry data. Protein names should be listed alongside corresponding gene names and database accessions. As a minimum, quantitative values should be provided for each protein for each experimental condition (including controls), and summary fold changes and adjusted p-values should be given for each protein. An additional supplementary dataset should be provided detailing all identified proteins (not just the selected integrins) presented with a similar level of information.

10. Mass spectrometry-based proteomics methods require further details. Was SDS cleaned from the peptide samples, and were they desalted prior to mass spectrometric analysis? What was the liquid chromatography setup, including column gradients? What were the acquisition settings for the mass spectrometer? How was the false-discovery rate controlled for protein identifications?

11. Statistical tests are not defined for analyses in any figures or supplementary figures. Were appropriate post-hoc corrections for multiple testing used where applicable? Units of n (cells, independent experiments?) are not specified throughout, particularly important where box-and-whisker plots do not show individual data points (e.g. Fig. 2A–D).

Minor comments

1. Supplementary Fig. 5A is difficult to interpret. There are no units for the expression color gradient – is it on a log scale? Many of the cancer type abbreviations are not obvious.

2. Focal adhesion parameters should be quantified for Fig. 5D.

3. Please correct the phrase “there is a newly literature” in the Introduction (p. 3).

4. Please correct the phrase “correlated directly to interact and bind to” in the Results (p. 5).

5. Please clarify what is meant by “biomechanically more challenging” in the Results (p. 6).

6. Please confirm the number of mice used in Fig. 2H – there are four data points shown in the figure but the legend states n = 5.

7. Please correct the spelling of fibrillar in the Results (p. 10).

8. Please confirm whether the volcano plot data points represent proteins rather than peptides as stated.

9. Correct the callouts to Supplementary Figs 5D–F in the Results text (pp. 12–13).

10. Clarify that siSpry4 “partially” rescued MEL23-treated cell migration (p. 13).

11. Please correct the spelling of mitomycin C in the Materials and Methods (p. 40).

Responses to the Reviewers' comments:

Reviewer #1 (Remarks to the Author):

MDM2 has previously been reported to support cancer cell migration and invasion, and several molecular mechanisms have been proposed. In the present paper, Muniz de Queiroz et al describe a new mechanism for this activity of MDM2. Specifically, they report that MDM2, in association with MDMX, downregulates the expression of the Sprouty4 (Spry4) gene, resulting in increased formation of focal adhesions via regulation of cofilin phosphorylation. This drives increased matrix attachment, migration, invasion and metastasis.
The findings reported in this study are potentially important, and add to the growing knowledge about p53-independent functions of MDM2. Nevertheless, they do not explain how MDM2 suppresses the expression of the Spry4 gene. In order for this study to qualify for publication in Nature Comm, at least some mechanistic elaboration would appear to be a must. Does the MDM2/MDMX complex bind to the Spry4 gene promoter? Or does it ubiquitinate a protein(s) that regulates Spry4 expression? And how is the effect of MDM2 on Spry4 RNA stability explained? Answering any of the above has the potential to yield truly novel mechanistic insights.

Major concerns:

1. As shown in Fig. 4, MDM2 depletion or inactivation results in a 2-fold increase in Spry4 protein. One wonders to what extent such moderate increase can truly account for the observed major changes in motility, invasion and metastasis. The experiments in Fig. 6 are impressive, but are based on almost complete ablation of Spry4 expression, and as such do not answer the above question. It will therefore be essential to generate cells in which Spry4 expression is only reduced about 2-fold, such that upon MDM2 depletion it will become similar to that of non-treated control cells, and test whether this is enough to make them behave like the non-treated control cells.

While we appreciate the reviewer's suggestion, we respectfully disagree that doubling the amount of detectably Spry4 protein is too moderate an increase. There are myriad examples in the literature where having twice the amount of a protein has a significant impact on the outcome of that protein. For example, please see examples our own studies Moon et al Fig 4I or Venkatesh et al, Fig 7G; and we note that a methods paper (Pillai-Kastoori et al, Analyt Biochem 2020) on quantitative Western blot analysis indicates that two-fold changes can indeed be meaningful.

As for lowering the concentration of Spry 4 we point out that in the double KD stable cell line (Spry4 and Mdm2) there is still detectable Spry4 protein that is approximately half the amount of Sprouty4 found in the control cells (please see Fig.6 panel G). Yet, when the double KD is compared to control cells they behave the same, which suggests that the boost in Spry4 caused by Mdm2 ablation is needed for the effects of Mdm2 KD in cell migration, FA regulation and metastasis.

Additionally, it would be technically very challenging using transient transfection of siRNA to precisely control how much we silence a protein on a per-cell basis such that cells individually express exactly a 50% decrease in a reproducible manner.

2. Since the proposed mechanism appears to involve suppression of transcription rather than degradation of the Spry4 protein, it begs to look for additional genes that are similarly affected by MDM2 depletion or inhibition and identify putative transcription factors that are shared by

those genes. This might then identify the direct MDM2/MDMX target that is responsible for the reported observations. Such identification, if achieved, will provide the missing mechanistic novelty.

We found that Spry4 mRNA levels were affected by knock-down or inhibition of Mdm2. What was unexpected is that both Spry4 mRNA transcription AND mRNA stability appear to be regulated by Mdm2. While it will eventually be interesting to determine the basis for the dual effects on Spry4 mRNA levels, this would be an extremely extensive undertaking. Should we be successful, this would undisputedly result in a huge amount of data that would be far too long to include in our already data-rich paper that we submitted. Indeed, to that end we pursued the OMICS data to look for clues and tested a couple of potential targets, such as LARP4 and PPAR based on the literature (which can be provided to the reviewer if needed), but the results were not informative and could not be built upon. Since there are two components involved in the regulation, mRNA stability and gene expression, we believe this question is a foundation for a whole new project and to answer this question appropriately would require a significant amount of data that would deviate from the main question of this manuscript, namely how does Mdm2 regulate migration and metastasis in cancer.

It is important to note as well that we cannot discard the regulation of protein levels of Spry4 by Mdm2. Although we found that proteasomal degradation of Spry4 is not affected by Mdm2 inhibition (which would be the expected mechanism regulated by Mdm2 as an E3-ligase) we do not rule out the possibility that other mechanisms for protein degradation can still play a role.

That said, we have new and interesting data regarding the main thrust of our paper, by further elucidating how Spry4 is mediating the Mdm2 response. We now show that Mdm2 control of Spry4 leads to RhoA signaling! Specifically, we found that RhoA protein (not mRNA) levels are decreased by Mdm2 knockdown or pharmacological inhibition. Strikingly, the lower levels of RhoA are rescued by co-ablation of Mdm2 and Spry4. To expand on this, we found that RhoA does not interact with Mdm2 directly and inhibition of RhoA prevents the rescue by co-knock-down of Mdm2 and Spry4 in migration.

3. One of the main conclusions of the authors is that the E3 activity of the MDM2/MDMX complex is required for the observed effects. Although most probably correct, it is not rigorously proven: formally, it might still be argued that the complex binds to a target (e.g. specific chromatin region) and blocks protein-protein interactions, for example. To make a firm statement, it needs to be shown that the effects of MDM2 knockdown can be rescued by wild type MDM2 but not by E3 ligase-inactive mutants.

That is a very good suggestion and we tried very hard to do the suggested experiments even though we were not optimistic (correctly) that they could be achieved. It is notable that the vast majority of experiments that document activities of ectopically expressed Mdm2 test its impact on transiently co- expressed proteins such as p53 or MdmX. In fact we and others have found that ectopic overexpressed Mdm2 can cause the degradation of ectopic p53 but not endogenous p53 even in the same cells (please see Ohkubo S et al, J.Biol Chem 2006; Kannemeier et al., Mol. Biol. Cell 2007) even though the impact of endogenous Mdm2 on p53 and other endogenously expressed proteins can easily be documented. Further, overexpression of exogenous Mdm2 is very hard to achieve since it acts as its own E3-ligase when so overexpressed and even high amounts of plasmid leads only to a very modest increase in ectopic Mdm2 protein levels. As shown in the representative images below, when HT1080 parental cells (harboring wild-type p53) are transfected with 1µg of 5 different Mdm2

expression plasmids, even when Mdm2 levels go up p53 levels are not changed (panel on the left), showing that even when overexpression is modestly achieved it does not increase the degradation of its endogenous targets. We used 5 different constructs with different tags (including flag, HA and myc) as well as untagged Mdm2 to make sure the problem was not the construct itself.

On the other hand, overexpression of MdmX can be easily achieved but it has the same functional problem as Mdm2. Transfection of 0.25 to 1 µg of MdmX construct leads to a robust increase in MdmX levels but it does not change the protein levels of p53, a target of the complex (panel on the right).

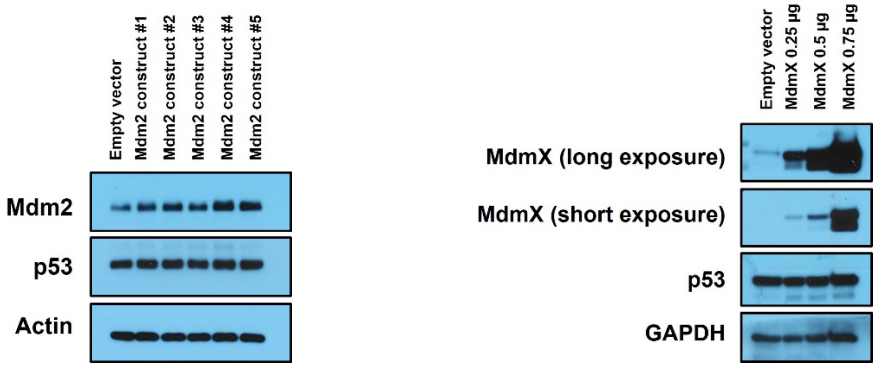

***Fig.1 (for reviewers)**: Overexpression of Mdm2 and MdmX in HT1080 parental cells (expressing wild type p53).* **Left:** protein levels of Mdm2 and p53 in cells transfected with empty vector or the indicated constructs for Mdm2 overexpression. Contruct#1: Mdm2-myc; construct#2: Mdm2 untagged; construct#3: Mdm2-flag; construct#4: Mdm2 untagged; construct#5: Mdm2-HAc. One microgram of each construct was transfected into cells using Mirus reagent. Cells were harvested 36h after transfection. β-actin was used as loading control for immunoblot. **Right:** protein levels of MdmX and p53 in cells transfected with empty vector or a construct for MdmX overexpression. The indicated amounts of plasmid were transfected into cells using Mirus reagent. Cells were harvested 36h after transfection. GAPDH was used as loading control for immunoblot.

The only other known way to increase Mdm2 levels in cells without exogenous expression is through stabilization of p53, but in our model we do not have the expression of the protein so we cannot do that. Even if we could, the activation of p53 might also affect migration and invasion independent of Mdm2 so that would not be a good strategy to use.

4. Fig 2H, I. At the very minimum, the images should be counterstained with DAPI. Better even would have been to perform DAB staining in order to see the histology of lungs with and without mets. In particular, it will be important to show Ki67 staining in Fig 2I, since larger mets could be due to increased invasion (as the authors conclude) or increased MDM2-dependent proliferation at the site of metastasis.

On panels H and I of Fig2 the images shown are from freshly harvested lungs from mice after euthanasia, those are not fixed samples therefore DAPI counterstaining cannot be performed in those samples. We do have formalin-fixed paraffin-embedded (FFPE) tissue sections and we stained it for Ki67. Data was added to **Supplementary Figure 2 panel F**.

We already know, however, that Mdm2 has also an impact in cell growth as shown in Supplementary Fig. 2D and by other papers in the literature. We tried to minimize the effect of

proliferation by using the tail vain injection, but unfortunately, there is no good way to uncouple those two events for the *in vivo* experiment. However, we here provide data showing that while silencing of Mdm2 decreases growth by inducing G2 cell cycle arrest, the double knockdown (shMdm2+shSpry4) does not significantly rescue the effects on cell cycle (see graphs below), but it does rescue markedly the effect of Mdm2 ablation on metastasis (Fig. 6H). This data has been added to the Supplementary Material (**Supplementary Fig. 7J**) and is mentioned in the Discussion section.

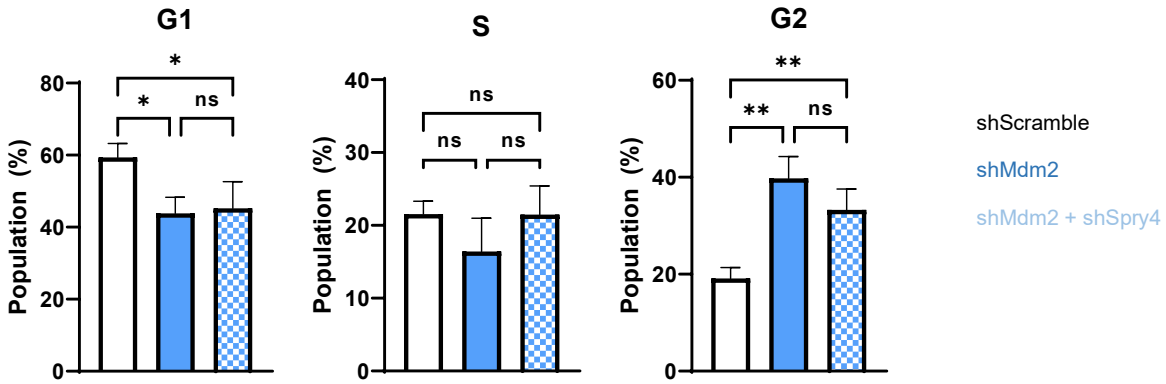

***Fig.2 (for reviewers)**: Cell cycle analysis of HT1080 p53KO cells stably expressing shRNAs against Mdm2 alone or Mdm2 and Spry4.* Graphs show percentage of population in each cell cycle phase for each stable cell line, n=3. *p<0.05, **p<0.01, n.s. not significant.

Additionally, in the wound scratch migration experiments shown throughout the manuscript cell proliferation is blocked by the use of mitomicyn-C and we still see a significant effect of Mdm2 depletion or inhibition on cell migration. Similarly, in the spheroid invasion experiments, although mitomycin-C could not be used, we show that for the duration of this experiment (48h), while there is not a significant change in cell proliferation in the spheroid, we see a striking effect in the invasion ability upon Mdm2 KD. These data suggest that the effects we obtained in the metastasis assay reflect defects in migration/invasion and not growth.

Minor comments:

1.Page 5: “correlated directly to interact” - please fix sentence. Thank you. We have corrected this sentence.

2.Page 13: The message conveyed by the heading “Sprouty4 is required to promote FA formation” appears to be opposite to the authors’ data, which shows that Spry4 inhibits FA formation. Thank you for the careful reading! We have fixed the heading.

3. The authors may wish to comment on an earlier report (Guo et al, reference 61) that Spry4 reduces MDM2 expression, which together with the present study would suggest a negative feedback loop. That is a very good suggestion, thank you! We have mentioned that in the Discussion.

4. Fig 3A. How was FA area and number measured? Was this done in an automated manner? Also, please explain what are non-peripheral FA and what their abundance implies. Clarification about the quantification of FA was added to Methods section.

5.Fig 3A, B. Displaying a larger number of cells would be more helpful to get an impression of the generality of the phenotypic differences. Due to the mesenchymal nature of these cells,

when plated in higher densities they sit/grow on top of each other, not allowing the discrimination of FAs of individual cells. For this reason, they need to be seeded in low density so we can identify the FAs of individual cells.

6.Fig. 4A. SPRY4 is "buried" in the Volcano plots, and should be highlighted or otherwise better visualized (arrow, perhaps?). Thank you for the good suggestion. Black arrows pointing to Spry4 in each Volcano Plot were added to **Figure 4A**.

Reviewer #2 (Remarks to the Author):

In their manuscript, Muniz de Queiroz et al. demonstrate in cells lacking p53 expression that knockdown of Mdm2 or MdmX, or pharmacological inhibition of the Mdm2/MdmX complex, reduces cell migration and invasion, impairs cell spreading and focal adhesion formation, and decreases metastasis in vivo. The authors identify Sprouty4 to be required for the Mdm2-mediated effects and investigate its role regarding cell migration, focal adhesion formation and metastasis independent of p53.

The authors claim as unique aspects of their work that, in contrast to the majority of existing studies, they used TP53 deficient cells, i.e. a CRISPR-derived human HT1080 fibrosarcoma p53 knock-out cell line and the H1299 lung carcinoma-derived cell line that is endogenously p53-null. The second aspect is that instead of altering Mdm2/MdmX abundance, they inhibit the E3 ligase activity of the Mdm2/MdmX complex using a small molecule inhibitor of Mdm2, MEL23. Further claims are the use of 2D and 3D in vitro models which have not yet been employed in this setting and the investigation of the role of post-translational modifications, of which the latter are not followed up to a greater extent.

The manuscript is well-written and the plethora of data demonstrating how Mdm2 p53-independently regulates migration and invasion in vitro and metastasis in vivo are presented comprehensively in almost all instances. A few open issues remain to be addressed. A potential weakness is the lack of clarity regarding the identification/selection of the mechanistic candidates. After characterizing the effects of Mdm2/MdmX knockdown and pharmacological inhibition in 2D and 3D settings, a proteomics experiment is used to identify Spry4 as candidate protein. However, it is insufficiently explained how this candidate was selected. Similarly, in the last part of the results, cofilin is presented as a potential mediator of the Spry4/Mdm2 effect, but the alternative Ras/ERK signaling is a bit half-heartedly ruled out as candidate mechanism before cofilin is deduced from the literature.

With respect to this referee's concern that we have not conclusively ruled out Ras-ERK signaling, we have now added more data to support this claim. We show in new **Supplementary Figure 7 panel D** that phosphorylation of c-Myc at Ser62, a site targeted by ERK for phosphorylation and activation of this transcription factor, is not altered in response to Mdm2 silencing.

Major issues:

- In Figure 1H the no coating control for MEL23 is missing. What is the reason for this omission?

Thanks for pointing this out. The control graph was added to **Supplementary Figure1 panel F**

- The authors point out that although Mdm2 modulation has been associated with EMT/MET before, there were no changes associated with different EMT markers (E-cadherin, N-

cadherin, vimentin and Zeb1) in response to silencing Mdm2 (**Supplementary Fig. 1F**). However, a positive control for EMT markers, especially E-cadherin, is needed. EMT is also among the enriched processes in **Supplementary Fig. 4E**, indicating that EMT cannot be discarded completely.

While we agree that we do not provide exhaustive evidence to completely discard the involvement of EMT. We tested all the markers that have been used in those papers that have reported EMT regulation by Mdm2 (Tang Y et al., Onco Targets Ther, 2019 and Ou M et al., Onc Rep, 2021). We assume that if EMT was a key process being regulated in our model we would have seen differences in those markers. To expand on this, we provide the data on other markers found in our proteomics analysis. We picked the genes among the 16-gene panel for EMT in cancer described by Gibbons and Creighton in 2018 that were detected by our proteomics analysis and some additional genes due to the low number of epithelial markers. As it can be seen in new **Supplementary Table 1**, we did not find any EMT marker significantly differentially expressed in all three conditions (siRNA#1, siRNA#2 or MEL23).

As pointed out by the reviewer, EMT does show up as a pathway regulated by Mdm2 modulation in our first pathway analysis, but this was based on differential expression of a single protein, FOXC2. Although it is significantly differentially expressed by Mdm2 modulation, the siRNA and pharmacological inhibitor conditions we used showed *inverse* changes in expression: MEL23 treatment led to less FOXC2 while silencing of Mdm2 increased the expression of FOXC2 (see graphs below). Thus, it is unlikely to be involved in the effects we see. Our stricture for considering any candidate is that all three conditions give us the same functional result which is not the case with FOXC2 that is inversely modulated by MEL and siRNAs.

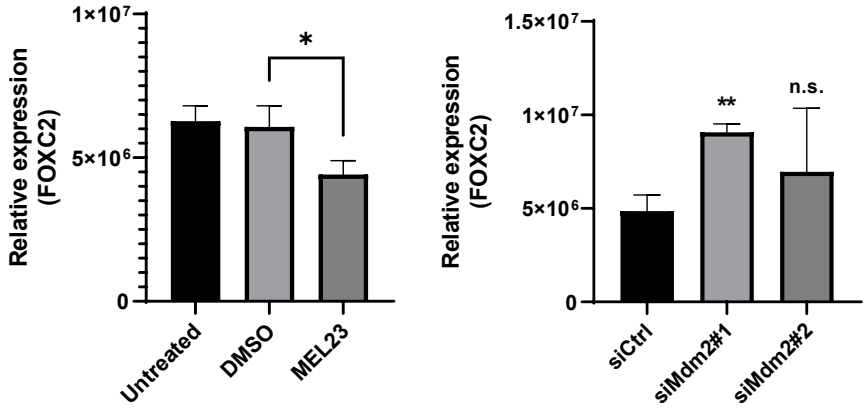

***Fig.3 (for reviewers)**: Expression of FOXC2 analyzed by mass spectrometry in cells treated with MEL23 (left) or silenced for Mdm2 using siRNAs (right)*, n=3. *p<0.05, **p<0.01, n.s. not significant.

Note as well that since both migration and metastasis are fully rescued by co-knockdown of Mdm2 and Spry4 either with siRNAs or the shRNA pool that was used to make the stable knock-down cells. Even if EMT is regulated in parallel with Spry4 in those cells it is not determinant in the effects we observe, since Spry4 silencing is sufficient to rescue those effects.

- The authors state that assessment of cell motility and cell morphology in GFP-expressing cell lines derived from HT1080 p53KO cells that stably express shRNAs demonstrated that

the HT1080 cell line with stable Mdm2 knockdown (shMdm2) displayed the same characteristics observed in cells transiently transfected with siRNA or in cells treated with MEL23 when compared to the shScramble cells. The comparability of these cell models seems to be a crucial prerequisite for this manuscript. Why did the authors not assess/present exactly the same parameters as for the siRNA knock-down (e.g. more relevant 3D culture etc.)?

Thank you for this suggestion. We have now added more validation experiments using the stable cell line. By comparing the shMdm2 stable cells line to the sh control line we now present (a) spheroid invasion assay results in **Supplementary Figure 2 panel D**, (b) the mRNA levels of Spry4 and Spry4 localization in **Supplementary Figure 6 panels E and I** as well as (c) protein and mRNA levels of RhoA in **Supplementary Figure 7 panel E and G**, respectively**.**

¬ Integrin levels were assessed by mass spectrometry or by immunoblot but found to be not significantly affected by Mdm2 knockdown or inhibition. This might be explained by the limited power of the proteomic experiment (n=3), i.e. the ability to detect true positive differential abundance (see criticism below), as well as the fact that the presented experiment does not account for isoforms or PTMs. Due to the multitude of different integrins, this might also be true for the immunoblot, as there are no positive controls included.

In addition to the proteomics (where is it acknowledged that there are limitations) the expression of integrins was determined by immunoblotting which showed that the levels of the evaluated integrins (including integrin alpha3, the only integrin close to significance in proteomics analysis) are not different in response to Mdm2 inhibition.

Regarding integrin's PTMs, for the purpose of their role in metastasis, one of the most important PTM for integrin function is glycosylation. In fact, we performed a glycocalix analysis shown below which showed that Mdm2 inhibition by MEL23 or siRNA does not interfere with membrane glycosylation patterns (this data can be included in the Supplementary Material section as needed). Investigating the PTM status of every expressed integrin in those cells, which would be necessary as we do not have a clear target to focus on, would not be practical since integrins are modified at many different sites by diverse types of PTMs.

Although we cannot definitively rule out changes in PTMs of all expressed integrins, the non-specificity in the ECM attachment experiment and the unchanged expression levels of integrins in cells and in the membrane together with our new data showing that a main component for the effects seems to be the cytoskeleton dynamics (**Fig. 7C-I**) points to integrins not being an active player in this signaling.

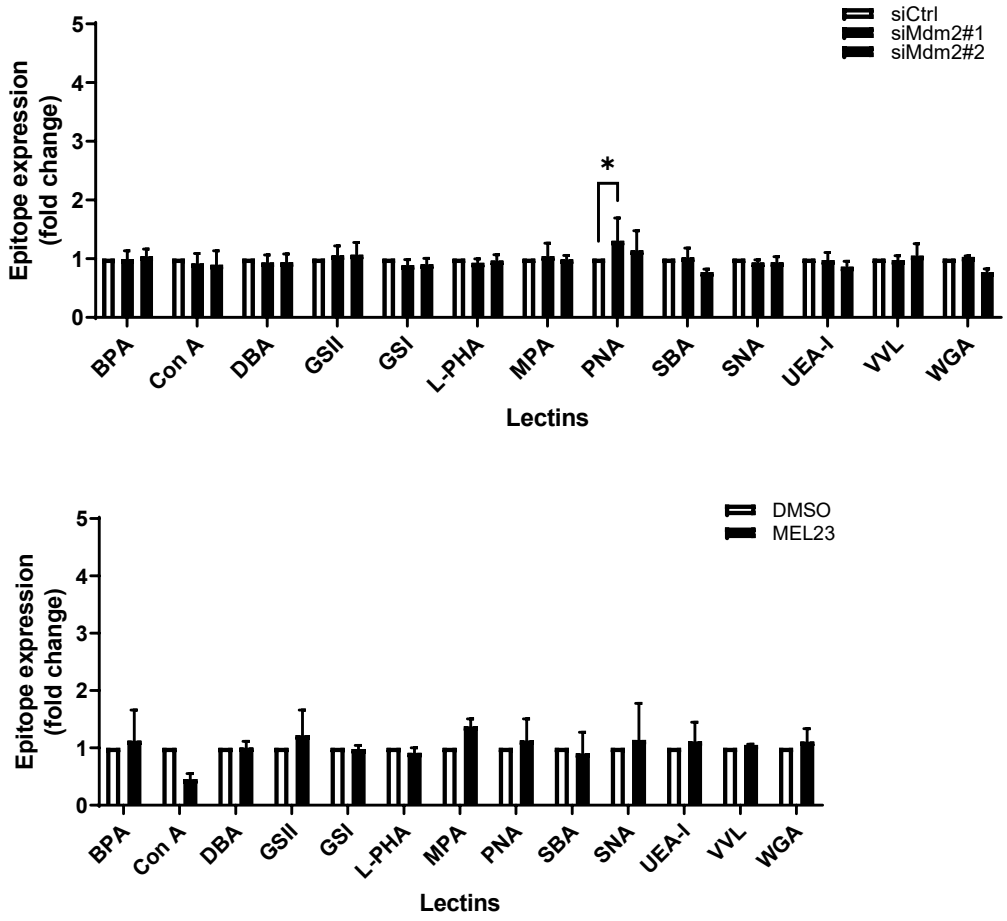

***Fig 4 (for reviewers)**: Expression of glycan epitopes analyzed by flow cytometry using a panel of lectins.* HT1080 p53KO cells were silenced for Mdm2 (top panel) or treated with MEL23 for 24h (bottom panel). Graphs show the fold change in the expression of each glycan epitope relative to control condition, n=3. *p<0.05.

The reviewer also states that there are no positive controls for the integrin blots. We are unsure of what controls the reviewer is referring to and would be grateful for clarification.

- The proteomic experiment is a critical part of the manuscript as it produces the Spry4 candidate which is then evaluated. Unfortunately, this part is not well described and may suffer from a few shortcomings. The provided description including the two references is far from complete and it is unclear which parts of the experiment were performed as described in one of these two remotely related publications. It would be necessary to include in the (supplementary) methods section a complete description of all parameters of the experiment from sample preparation via LC-MS parameters to database search and statistics (e.g. p-value cut-offs are missing). A supplementary table should contain all identified proteins and abundance values in all samples. Such a table could be (with some limitations) reproduced from the jPOST submission but may not lead to the same results, even when using the same analysis software due to the lacking parameters.

As correctly pointed out by the reviewer, our decision to focus on f Spry4 was not very clear in the text. We have now clarified in the text why Spry4 was chosen as the best candidate and

new data, suggested in revision, in **Supplementary Figure 4, panels D, E and F** help understanding this choice.

As requested, we increased the level of the detail in the **Methods section on proteomics**, including new citations and the parameters questioned. We also added as supplementary material .csv files containing the full list of identified proteins, fold changes and complete statistical analysis.

- In the proteomic experiment the authors expect many proteins in both groups (siRNA and MEL23) to be significantly up- or downregulated after these treatments but it is not clear why that would be expected. It seems that there was no correction for multiple testing applied, potentially yielding many false positives. To estimate this biological noise, 3 vs 3 replicates of the Untreated or DMSO controls could be compared in the same way as the samples.

We appreciate the reviewer's suggestion as this comment strengthens the quality of the data we produced. We have reanalyzed the proteomics data using false-discovery rate to correct for multiple testing. In new **Supplementary Figure 4D** (the Venn diagram) as well as **panels E and F** in the same figure we show the analysis in which we used a cutoff of FDR<0.2.

- The Volcano plots (hopefully) do not show all identified "peptides" (Fig. 4A).

Thank you for the attentive reading. It does not show all peptides; it shows all identified proteins. The text was corrected accordingly.

- From looking at the marked proteins it is already obvious that there is inconsistent regulation, such as e.g., HSPA6 strongly downregulated in siRNA1#1 and not in the other comparisons, which is normal in an underpowered experiment. The Venn diagram in Supp Fig 4D should therefore differentiate siRNA#1 and #2 to see which proteins really overlapped in all three comparisons and the limitations of the minimum approach using three replicates should be discussed.

Thank you for your suggestion. A new Venn diagram showing the proteins commonly significantly differentially expressed in all 3 experimental conditions (**Supplementary Fig. 4D**) as well as a table with the fold-change and FDR values for those proteins (**Supplementary Fig. E**) were added to new Supplementary Figure 4.

- The way the pathway enrichment is presented is insufficient and not suitable for this journal. It would be better to present a table instead of the figure, providing the statistical enrichment parameters (corrected pvalue, expected and observed frequencies or enrichment factor).

As suggested by the reviewer, a new pathway analysis was done using the reanalyzed data and a new list of proteins derived from the new Venn diagram (**Supplementary Fig. 4D**). The analysis now shows the statistical enrichment parameters (**Supplementary Fig. 4F**).

- Eventually, the authors assess 146 differentially expressed proteins (which ones? Supplementary table needed) and focus on those associated with cell migration and motility (which ones? Please indicate in the same Supplementary table). Sprouty4 (Spry4) emerged as a viable candidate for further investigation, but the criteria for this selection as well as potential alternative candidates are not clearly described.

As mentioned in a previous comment, the reason for choosing Spry4 is clarified in the text and reinforced by the new table found in **Supplementary Figure 4E**.

- Fig. 4B is redundant with Fig. 4A– instead Spry4 should be labeled in 4A (demonstrating fold change an p-value. Alternatively, in 4B as well as in Fig 4F, Supp Fig 5B and 5D (and similar figure panels throughout the manuscript) individual data points should be visualized at least in addition to bars representing the mean

Panel 4B was moved to Supplementary Material (**Supplementary Fig. 6A**).

Minor issues:

- Intro: Redundant text: "harboring either wild-type or different mutant versions of p53". We do not see redundancies. If the reviewer believes that is an important correction to make, please clarify further the redundancy so we can fix it accordingly.

- Results: "we found that the area of attaching cells was significantly reduced (Fig. 1E and F and Supplementary Fig 1D), while in solution (unattached) cell size did not change after such treatments (Supplementary Fig. 1E) demonstrating the changes in area are not due to cell volume
> I am not sure, if this transfer of results is really valid. After all the cell volume in adherent culture might be different, whereas in solution it might be not. I would therefore recommend to rephrase this passage. The sentence was rephrased.

- Results: "As expected, MdmX was coimmunoprecipitated with Mdm2, however Spry4 was not pulled down with Mdm2 and the reverse co-immunoprecipitation provided the same negative result (Fig. 4D).

> The legend ("mix", "DIV") is not clear Well pointed out. The figure legend was changed accordingly for clarification.

- Results: "Surprisingly, both Spry4 transcription (as determined by the reporter assay) (Fig 4G) and mRNA stability, 13 as measured using two different transcription inhibitors (Fig. 4H and Supplementary Fig. 5F), were increased upon Mdm2 inhibition"

> Is Supp Fig 5F correctly referenced here? It was wrongly referenced indeed! Thank you for your careful reading; the reference was fixed.

- Results: "As seen in HT1080 cells, Mdm2 silencing induces Spry4 expression at the protein and mRNA levels (Fig. 5E and F) in H1299 cells, thereby extending our results to a cell line which had evolved naturally to lack expression of p53"

> line should be lines. We extended the result to one cell line naturally lacking expression of p53, thus "lines" would not be correct.

- Results: "TCGA and GTEX databases revealed that Spry4 expression is decreased in lung cancers, both squamous and adenocarcinoma, compared to normal lung tissues (Supplementary Fig 5G)."

> should this be 5F? It was wrongly referenced indeed. Thank you for your careful reading, the reference was fixed.

- Supp Tab 1: “binging”. This table was changed to include statistical analysis for each integrin, please see new **Supplementary Table 2**.

Reviewer #3 (Remarks to the Author):

Summary

This study explores the p53-independent role for Mdm2, and its binding partner MdmX, in cell migration, invasion and metastasis. While Mdm2 has already been implicated in the regulation of cell migration and metastasis, the authors here use 2D and 3D cell culture models, and in vivo models, to identify Mdm2-dependent expression of Sprouty4 as a regulator of these phenotypes in a p53-null setting, using a previously described Mdm2/MdmX inhibitor to implicate Mdm2’s E3 ligase function. These are interesting findings further elaborating the link from the Mdm2/X complex to cell motility, although the intriguing link to Sprouty4 and cofilin could be strengthened and further experiments are suggested to determine precisely how Mdm2/X function is regulating cell–ECM attachment and cell migration.

Major comments

1. While the effect of the MEL23 compound on Mdm2 and MdmX protein levels is shown in Fig. 1, and a previous paper has characterized aspects of MEL23 activity/mechanism in certain cell types, does this compound inhibit Mdm2–MdmX complex formation and function in these cells and at the concentration used in the present manuscript?

MEL23 inhibits the E3 ligase activity of the Mdm2/Mdmx heterocomplex; it does not prevent the complex from forming or from interacting with a p53 as described by Herman et al 2011. Since Mdm2 can self-degrade and degrade MdmX the best way to access the outcome of treatment with this compound is through the quantification of its degradation targets, in our case Mdm2 and MdmX (since our cells do not express p53 whose levels also rise in MEL23-treated cells). In **Fig 1B** we show that MEL23 treatment leads to accumulation of both targets of the complex, Mdm2 and MdmX.

2. Can the authors distinguish effects of Mdm2/X functional disruption via MEL23 from effects of alteration of protein expression levels (up-regulated upon MEL23 treatment)? Are these effects via a distinct mechanism from those effects observed upon depletion of Mdm2 levels?

The fact that MEL23 treatment, which leads to accumulation of targets of the complex including Mdm2 and MdmX, leads to the same effects of the depletion of both proteins using siRNA points to the importance of the function of the Mdm2-X complex.

In our work we could not discriminate between increasing the protein levels of Mdm2 and inhibiting its function exactly because the E3-ligase function appears to be key to the effects we see. We show that both strategies (Mdm2 KD or inhibition of the activity of the Mdm2-X complex) lead to the same functional effects such as decreased cell attachment, decreased cell spreading, decreased focal adhesion formation and decreased migration and invasion.

3. Although relative ATP production of spheroids was measured, it appears that the HT1080 p53KO cells formed smaller spheroids upon Mdm2 knockdown and MEL23 treatment (Fig. 2). Was spheroid area quantified? Does this contribute to the apparent reduced capacity of these spheroids to invade?

This is a very good point. We did notice that spheroids look smaller when Mdm2 function is lost. Quantification of the spheroid area using the stable shMdm2 cell line reveals a trend of decreased size (~20%) but without significance (see graph below). Depending on what leads to those differences, it could be a contributing factor or a consequence of the changes induced by Mdm2, for example in the cytoskeleton machinery, which we found to be involved in the effects of Mdm2 inhibition (Fig. 7C-I). We did not perform experiments to further understand this process for this manuscript.

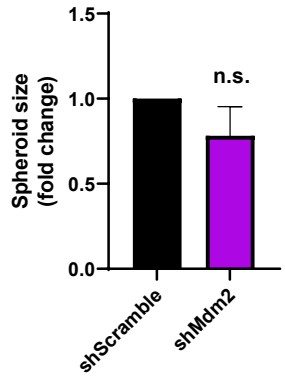

***Fig.5 (for reviewers)**: Quantification of spheroid size in collagen I matrix. Spheroids were formed using HT1080 p53KO stable cells lines.* One hour after implantation of spheroids into collagen I matrix spheroid area was quantified from micrographs. Graph shows the average and standard deviation of 3 biological replicates, in each biological replicate at least 8 spheroids were measured. n.s. not significant.

4. It would be useful to include the primary tumor size measurement data for the tumor growth experiments, especially as this is commented on in the Results (p. 8).

A table with tumor weight at the time of harvesting was added to **Supplementary Figure 2, panel E**.

5. Although total expression levels of some integrin subunits appear not to be affected by Mdm2/X disruption (Supplementary Fig. 3), the cell-surface expression levels of integrins should be monitored (e.g. using flow cytometry). Furthermore, is integrin activity (e.g. measured using an activation-state-reporting antibody) altered by Mdm2/X functional disruption? These readouts would help clarify how Mdm2/X is regulating cell–ECM attachment.

Thank you for the suggestion. We investigated the presence in the membrane of a panel of four integrins (a2, a5, b1, b2) and one integrin complex (aVb3) and observed no differences upon silencing of Mdm2. These data are added to **Supplementary Figure 3, panel B.** Since we showed that Mdm2 ablation reduces attachment to each of the three main ECM components similarly, this indicates that no specific integrin was affected, and thus it is unlikely that integrins are the key players in the mechanism that we found. Rather, based on our new

data with RhoA, it is mostly likely that events downstream of integrins (cytoskeleton reorganization and/or dynamics) are being regulated by Mdm2 in our model.

6. While focal adhesions appear to be dysregulated by disrupted Mdm2/X function, the suggestion that Mdm2 regulates maturation of focal adhesions and fibrillar adhesions (p. 10) is insufficiently supported by the present data.

a. Focal adhesions can mature into fibrillar adhesions, but fibrillar adhesions are not the same as focal complexes as stated in the Results (p. 10), so the text should be amended to correct this.
b. Non-peripheral vinculin-containing adhesions cannot be assumed to represent fibrillar adhesions, as central fibrillar adhesions have been reported to be largely devoid of vinculin staining. Instead, a fibrillar-adhesion-specific marker (e.g. active alpha5beta1 integrin) should be visualized and quantified to determine changes in fibrillar adhesions.
c. The claimed increased density of F-actin (p. 10) needs to be quantified, as it is not obvious from the images presented in Fig. 3. Is there a difference in specific forms of stress fibers upon Mdm2/X disruption (e.g. contractile ventral stress fibers that contain non-muscle myosin II or non-contractile dorsal stress fibers or transverse arcs that are not directly linked to focal adhesions)?
d. Can assembly of stress fibers and focal adhesions in Mdm2/X-disrupted cells be rescued by increasing actomyosin contractility via perturbation of the microtubule network (e.g. using nocodazole)?

Thank you for your comments. You are right in your concern about our conclusions and we will remove the claim of FA maturation from the results section due to lack of data specifically pointing to this process.

Yet, we do believe that even without the claim on FA maturation the manuscript would benefit from the experiment suggested in (d.). Thus, we performed the FA disassembly experiment with nocodazole washout and we have added it to **Supplementary Fig 3, panel E.** In short, we found that cells silenced of not for Mdm2 behave similarly during the experiment, suggesting it is the formation and not recycling of the FAs the process affected by Mdm2 modulation.

7. The siSpry4-only controls shown in Supplementary Fig. 6F–K appear potentially contradictory to the observations of dual Mdm2 and Spry4 knockdown. Without quantification, it is difficult to conclude that there is no difference in focal adhesion formation upon Spry4 knockdown alone (Supplementary Fig. 6J).

The experiment was quantified and is added to **Supplementary Figure 8, panel E**.

8. The involvement of cofilin would benefit from further corroboration, as this appears to be a key link to the observed cell adhesion defects. It is difficult to tell from the actin images in Supplementary Fig. 6 whether there are substantial changes in actin stress fibers versus monomeric actin – this should be quantified. Does immunoblot analysis of G- and F-actin support this observation? Are there changes in the levels of free barbed ends of actin?

We did try to run the F/G actin ratio analysis by immunoblot, but the experiments gave us very inconsistent results even using appropriate controls such as treatment with cytochalasin D. We are including the quantification of the G- F-actin staining found in **Figure 7I**.

9. The presentation of the proteomics data would benefit from more detailed analysis to be more informative.

a. What is the justification for selecting Sprouty4 from the proteomics data, when the volcano plots show many other dysregulated hits with more substantial fold changes that may be more biologically relevant?

We agree this matter needs to be better addressed. Please see our responses to Reviewer #2. The reason for choosing Spry4 is clarified in the text and reinforced by the new table found in **Supplementary Figure 4, panel E** that shows all proteins that were commonly differentially expressed by all 3 conditions (including statistical analysis).

b. The Venn diagram in Supplementary Fig. 4 does not capture whether proteins within respective sets were differentially expressed in the same or different directions (i.e. up- or down-regulated). Could this information be displayed to clarify the concordance between treatments?

Thank you for the suggestion. Indeed, we have proteins that although are differentially expressed in all 3 conditions, they vary inversely when comparing MEL23 and siRNAs. A table was added to **Supplementary Figure 4 (panel E)** containing the 8 proteins that significantly changed expression in all 3 conditions with respective fold change values and FDR values. This table is also going to help explain the choice of Spry4 for further experiments.

c. How were heatmaps clustered (there is no methodological information)? Were mean or median values used to summarize the replicates of each experimental condition? Combining replicates in this way removes information about variability between replicate analyses.

The values used were the means, but we modified the heatmaps in **Supplementary Figure 4 panel C** to show all 3 biological replicates of each experimental group for clarification. The clustering and distance measurement methods were added to the **Methods** section.

d. Why do the authors suppose that almost none of the same proteins were down-regulated in si#1 and si#2 (Supplementary Fig. 4C)?

It is interesting indeed that the downregulated proteins do not have a high overlap, however, understanding the function of Mdm2 as an E3-ligase complex, we expected and were more interested in the upregulated proteins since we expect that blocking a protein degradation complex would lead to accumulation of its direct targets.

Despite that, the lack of commonly downregulated proteins could be a matter of reaching significance, meaning while in one siRNA it was significantly downregulated the other siRNA shows a trend of downregulation, but it did not reach significance. This is the case, for example, of the following proteins: AP3M2, BICC1, GLUL, CA13, CDCA8 and TEC.

e. The pathway analysis charts in Supplementary Fig. 4E have no axis labels, so the bar lengths cannot be interpreted. What are the false-discovery rates and enrichment ratios/gene-set overlaps for these analyses?

All the proteomics data was reanalyzed using FDR and are in new **Supplementary Figure 4, panels D to F,** which now shows data using the cutoff of FDR<0.2 for significance. A new pathway analysis was done using the reanalyzed data and a new list of proteins derived from

the new Venn diagram (**Supplementary Fig. 4D**). The pathway analysis now shows the statistical enrichment parameters (**Supplementary Fig. 4F**).

f. Supplementary Table 1 is inadequate for the reporting of mass spectrometry data. Protein names should be listed alongside corresponding gene names and database accessions. As a minimum, quantitative values should be provided for each protein for each experimental condition (including controls), and summary fold changes and adjusted p-values should be given for each protein. An additional supplementary dataset should be provided detailing all identified proteins (not just the selected integrins) presented with a similar level of information.

We modified Supplementary Table1 (now **Supplementary Table 2**), and added the uniport ac#, gene name, fold change and FDR values for all identified integrins for all 3 experimental groups. We are also including as supplementary data .csv files containing the values for all identified proteins for each biological replicate of each group, including controls, as well as all the statistical parameters such as p-value and adjusted p-value.

10. Mass spectrometry-based proteomics methods require further details. Was SDS cleaned from the peptide samples, and were they desalted prior to mass spectrometric analysis? What was the liquid chromatography setup, including column gradients? What were the acquisition settings for the mass spectrometer? How was the false-discovery rate controlled for protein identifications?
As requested, we increased the level of the detail in the **Methods** section on proteomics including the parameters requested. We also added as supplementary material .csv files containing the full list of identified proteins, fold changes and complete statistical analysis.

11. Statistical tests are not defined for analyses in any figures or supplementary figures. Were appropriate post-hoc corrections for multiple testing used where applicable? Units of n (cells, independent experiments?) are not specified throughout, particularly important where box-and-whisker plots do not show individual data points (e.g. Fig. 2A–D).

The statistical tests used are described in Methods. The number of biological replicates is mentioned in the figure legends for each panel. More detailed information was added to the legend to **Figure 2, panels A to D**.

Minor comments

1. Supplementary Fig. 5A is difficult to interpret. There are no units for the expression color gradient – is it on a log scale? Many of the cancer type abbreviations are not obvious. Yes, it is a log scale of TPM+1. The axis label has been added to the panel for clarification.

2. Focal adhesion parameters should be quantified for Fig. 5D. The quantification of FA parameters was added to **Figure 5, panel D**.

3. Please correct the phrase “there is a newly literature” in the Introduction (p. 3). The sentence was modified.

4. Please correct the phrase “correlated directly to interact and bind to” in the Results (p. 5). The sentence was corrected.

5. Please clarify what is meant by “biomechanically more challenging” in the Results (p. 6). A sentence was included for clarification.

6. Please confirm the number of mice used in Fig. 2H – there are four data points shown in the figure but the legend states n = 5. The figure legend was corrected.

7. Please correct the spelling of fibrillar in the Results (p. 10). Corrected.

8. Please confirm whether the volcano plot data points represent proteins rather than peptides as stated. The Volcano plots represent proteins. The figure legend was corrected accordingly.

9. Correct the callouts to Supplementary Figs 5D–F in the Results text (pp. 12–13). Thank you for the attentive reading. The figure references were fixed.

10. Clarify that siSpry4 “partially” rescued MEL23-treated cell migration (p. 13). The sentence was corrected for clarification.

11. Please correct the spelling of mitomycin C in the Materials and Methods (p. 40). Thank you for the careful reading. The spelling was fixed.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have performed only a minority of the additional analyses requested by this reviewer. Nevertheless, they provided reasonable explanations for the failure to carry out the rest of the requested work. I find their explanations satisfactory. I therefore support acceptance of the revised MS.

Reviewer #2 (Remarks to the Author):

All open points refer to initial comments.

Original comment (excerpt): A potential weakness is the lack of clarity regarding the identification/selection of the mechanistic candidates. After characterizing the effects of Mdm2/MdmX knockdown and pharmacological inhibition in 2D and 3D settings, a proteomics experiment is used to identify Spry4 as candidate protein. However, it is insufficiently explained how this candidate was selected.

> This major point is not answered in the rebuttal or by changes in the manuscript. While the technical details of the proteomic analysis are now provided, the limitation of an n=3 proteomics experiment with very low power remains. Low statistical power means that the likelihood of overlooking true positive results is substantial.

We assume that if EMT was a key process being regulated in our model we would have seen differences in those markers. To expand on this, we provide the data on other markers found in our proteomics analysis. We picked the genes among the 16-gene panel for EMT in cancer described by Gibbons and Creighton in 2018 that were detected by our proteomics analysis and some additional genes due to the low number of epithelial markers. As it can be seen in new Supplementary Table 1, we did not find any EMT marker significantly differentially expressed in all three conditions (siRNA#1, siRNA#2 or MEL23).

> Please present a sample size / power calculation for the replicate number needed to detect a true positive change with a probability of e.g. 80%. This proteomics experiment is not powered to allow exclusion of effects by the fact that candidate proteins were not found to be significantly differentially abundant (alpha vs. beta levels). In the interpretation of the data it has to be stated more clearly that EMT was not found but cannot be ruled out due to the low replicate number in the proteomics experiment.

As pointed out by the reviewer, EMT does show up as a pathway regulated by Mdm2 modulation in our first pathway analysis, but this was based on differential expression of a single protein, FOXC2. Although it is significantly differentially expressed by Mdm2 modulation, the siRNA and pharmacological inhibitor conditions we used showed inverse changes in expression: MEL23 treatment led to less FOXC2 while silencing of Mdm2 increased the expression of FOXC2 (see graphs below). Thus, it is unlikely to be involved in the effects we see. Our stricture for considering any candidate is that all three conditions give us the same functional result which is not the case with FOXC2 that is inversely modulated by MEL and siRNAs.

> Shouldn't this criterion (all three conditions give the same functional result) also apply to the proteins in Supp Figure 4E? How do the authors explain so very similar functional effects by siRNA knockdown or pharmacological intervention while on the protein level, there seems to be very little overlap between the 2 siRNAs targeting the same protein and huge differences to the effect of MEL23 (#1600 significantly regulated proteins with less 0,5% overlap to the regulation patterns induced by the siRNAs)?

As correctly pointed out by the reviewer, our decision to focus on f Spry4 was not very clear in the text. We have now clarified in the text why Spry4 was chosen as the best candidate and new data, suggested in revision, in Supplementary Figure 4, panels D, E and F help understanding this choice.

> The critical part of the manuscript now reads: "As mentioned, three proteins were significantly similarly regulated by all conditions: the lysine demethylase KDM3A, the deubiquitinase OTUD1 and the RTK signaling antagonist protein Sprouty4. Among those 3 hits, and considering their correlation to migration and metastasis described in the literature"

Given the heterogeneity of the observed effects by MEL23 and siRNA #1 and #2 on the protein level, were any attempts made to devalidate the other 2 highly significant candidates having similar effects, other than deduction from literature?

As requested, we increased the level of the detail in the Methods section on proteomics, including new citations and the parameters questioned. We also added as supplementary material .csv files containing the full list of identified proteins, fold changes and complete statistical analysis.

> The description of the methods has been greatly improved and is now sufficient to understand the workflow. What is still missing is the reviewer access to the dataset PXD033789 which currently is not publically available in jPOST or ProteomeXchange.

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> I would strongly suggest to have scatter plots overlaid to the barcharts (representing the mean) which can be highly misleading due to leverage effects of individual outliers.

Minor:

Intro: Redundant text: “harboring either wild-type or different mutant versions of p53”. We do not see redundancies. If the reviewer believes that is an important correction to make, please clarify further the redundancy so we can fix it accordingly.

> Indicated by brackets: One additional confounding issue of previous studies characterizing pro-oncogenic activities of Mdm2 has been that for the most part researchers have used cell lines that [[[either harbor wild-type or mutant versions of p53]]]to make their observations. While in such cases modulation of Mdm2 levels in different cell lines [[[harboring either wild-type or different mutant versions of p53]]] leads to changes in cell migration15, 20, 21, 24-27, the presence of either form of p53 might impact cellular responses, however, and thereby complicate interpretation of data.

Reviewer #3 (Remarks to the Author):

The authors have addressed my comments, thank you.

However, for original major point 11, units of n still need to be provided in the figure legends to make it clear what “n” represents. For example, the legend for Fig. 1E states n = 3, but there are clearly more than three data points that were presumably used for the statistical comparison between samples (so this needs to be “n = x cells from n = 3 independent experiments” or similar). For Fig. 2A-D, why are spheroid numbers not declared for all experimental conditions?

Minor issues introduced in revision:

- The new legend text for Fig. 2F is confusing: were the data from each biological replicate summarised as a median and then each biological replicate median value used to determine a mean ± SD for the bar chart? Or does the bar chart represent median ± error?

- Error bars and whiskers are not defined for any of the charts in Fig. 2.

- Setting FDR < 0.2 (20%) is a generous cut-off for differentially regulated proteins in the proteomics experiment and should be justified.

- Typos on p. 16: “HT12080 p53KO” and “e hypothesized”.

- Typo on p. 38: “phosphor-ERK”.

- Typo on p. 47: “beta1” is mentioned twice; presumably one occurrence should read “beta2”.

Response to reviewers' comments on NCOMMS-23-25997A-Z

Our responses to the reviewers are in red text and changes noted in our revised manuscript are in blue text.

Reviewer #1 (Remarks to the Author):

The authors have performed only a minority of the additional analyses requested by this reviewer. Nevertheless, they provided reasonable explanations for the failure to carry out the rest of the requested work. I find their explanations satisfactory. I therefore support acceptance of the revised MS.

We are grateful to Reviewer #1 for their understanding.

Reviewer #2 (Remarks to the Author):

All open points refer to initial comments.

1. Original comment (excerpt): "A potential weakness is the lack of clarity regarding the identification/selection of the mechanistic candidates. After characterizing the effects of Mdm2/MdmX knockdown and pharmacological inhibition in 2D and 3D settings, a proteomics experiment is used to identify Spry4 as candidate protein. However, it is insufficiently explained how this candidate was selected."

This major point is not answered in the rebuttal or by changes in the manuscript. While the technical details of the proteomic analysis are now provided, the limitation of an n=3 proteomics experiment with very low power remains. Low statistical power means that the likelihood of overlooking true positive results is substantial.

Thank you for pointing out this important and difficult issue. We understand that the statistical power of this proteomics analysis is limited and thereby we cannot rule out the possibility that we may have missed true positive results or other important groups of molecules. On the other hand, it should be noted that we performed proteomics analysis with the aim of searching for candidate molecules and pathways involved in underlying mechanisms. In such cases, we believe that even under limited sample size conditions, the potential molecules we identified as statistically significant and differentially expressed proteins are likely to have biological significance, making it worthwhile to further perform functional validation. It is worth mentioning that another group's exploratory proteomic analysis similar to our experimental conditions with sample size (n=3) led them to find and functionally characterize significant molecules (Li, Y., Watanabe, E., Kawashima, Y., et al. Identification of trypsin- Nature 609, 2022. https://doi.org/10.1038/s41586-022-05181-3).

Although the detection sensitivity of our experimental conditions may not be high, we believe that it is not a contradictory approach to consider that the group of molecules identified, including Sprouty4 (Spry4) (Supplementary Figures 4D and 4E), may have biological significance as an initial target for functional screening. To address the

reviewer's concerns, we now acknowledge the limitations of our proteomics power analysis based on three biological replicates in a new section of the Discussion on page 20 and21.

As to why Spry4 was picked among our list of hits, we further clarify our reasoning, as follows. Briefly, we looked for a target that was similarly regulated by all 3 conditions (MEL23 treatment, siRNA#1, and siRNA#2) in a significant manner. Our proteomics analysis provided us with 3 potential hits fitting this requirement. Among those 3 hits, we chose Spry4 to start with because its function in cells is directly associated with migration/cell motility while the other two potential targets do not have that close of an association. We mention it in the manuscript (page 12, first full paragraph):

"As mentioned, three proteins were significantly similarly regulated by all conditions: the lysine demethylase KDM3A, the deubiquitinase OTUD1 and the RTK signaling antagonist protein Sprouty4. Among those 3 hits and considering their correlation to migration and metastasis described in the literature, Sprouty4 (Spry4) emerged as a viable candidate for further investigation."

Following our validation of Spry4 results, we confirmed that it was indeed the protein responsible for our effects, as seen in the rescue experiments we performed.

In light of the reviewer's concern, to provide a clearer explanation, we modified the afore-mentioned text and added a reference. Now it reads:

"As mentioned, three proteins were significantly similarly regulated by all conditions: the lysine demethylase KDM3A, the deubiquitinase OTUD1 and the RTK signaling antagonist protein Sprouty4. Among those 3 hits, Sprouty4 (Spry4) emerged as the most promising candidate for further investigation since one of the main functions of this protein (and other members of the Sprouty/Spred family) in cancer cells is the regulation of cell motility and migration[40]"

2. "We assume that if EMT was a key process being regulated in our model we would have seen differences in those markers. To expand on this, we provide the data on other markers found in our proteomics analysis. We picked the genes among the 16-gene panel for EMT in cancer described by Gibbons and Creighton in 2018 that were detected by our proteomics analysis and some additional genes due to the low number of epithelial markers. As it can be seen in new Supplementary Table 1, we did not find any EMT marker significantly differentially expressed in all three conditions (siRNA#1, siRNA#2 or MEL23)."

Please present a sample size / power calculation for the replicate number needed to detect a true positive change with a probability of e.g. 80%. This proteomics experiment is not powered to allow exclusion of effects by the fact that candidate proteins were not found to be significantly differentially abundant (alpha vs. beta levels). In the interpretation of the data it has to be stated more clearly that EMT was not found but cannot be ruled out due to the low replicate number in the proteomics experiment.

First, to address this constructive suggestion, we have retrospectively evaluated the number of replicates required to detect a true positive change with an appropriate probability based on the protein expression levels of the targeted molecules determined by our proteomics analysis. For this, we utilized the G*Power, which is a tool to compute

statistical power analyses for many different *t* tests, *F* tests, χ2 tests as well as effect sizes (Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*. 2007).

The predicted sample size was determined using G*Power 3.1.9.7. For the test family, t test was selected, and for the statistical test, difference between two independent means (two groups) was selected, and for the type of power analysis, compute required sample size (with given α, power and effect size) was selected. For parameters setting, we set up Tail(s)=Two (two-sided test), α error probability=0.05, Power (1-β error probability) = 0.8, and allocation ratio N2/N1=1. In addition, as for the input parameter, effect size d, it was calculated from actual proteomic data. Specifically, the calculation method used the mean and standard deviation of Sample1 and Sample2 using our proteomics data normalized by TCC.

This calculation showed that the sample size (to detect a true positive change with α err prob=0.05, Power (1-β err prob)=0.8 of the eight molecules including SPRY4 identified in our proteomics analysis ranged from 2 to 4 (Reviewer Table 1).

Although we cannot deny the possibility that we were unable to detect other important groups of molecules due to the experimental conditions with this limited number of samples in our proteomics analysis, we believe that the identification of Spry4, which has previously reported relevant biological significance in combination with our subsequent functional analysis, provided what we were looking for from an exploratory perspective. If the referee wishes us to include this added information to the Supplementary Information section, we are happy to do so.

MEL	DMSO1	DMSO2	DMSO3	Mean	SD	Calculated Sample size	MEL23_1	MEL23_2	MEL23_3	Mean	SD	Calculated Sample size
BIRC5	14549009.49	15645213.09	12780881.45	14325034.68	1180034.597	4	19540985.17	18796850.27	16155106.37	18164313.94	1452841.003	4
CIT	145490094.9	136895614.6	127808814.5	136731508	7219285.162	3	119417131.6	118716949.1	118470780.1	118868286.9	400892.8745	3
DHX8	190955749.5	185786905.5	186797498.1	187846717.7	2236795.833	3	173697646	158289265.4	161551063.7	164512658.4	6629873.322	3
GSE1	14549009.49	16623038.91	14747170.9	15306406.43	934507.9971	4	11941713.16	13850310.72	11847078.01	12546367.3	922836.3132	4
KDM3A	100024440.2	97782581.83	97331327.94	98379449.99	1177681.935	3	119417131.6	108823870	107700709.2	111980570.3	5278396.593	3
OTUD1	872940.5692	977825.8183	589886.836	813551.0745	163848.5023	3	1519854.402	1187169.491	1400109.219	1369044.371	137582.8989	3
SPRY4	30916645.16	30312600.37	33426920.71	31552055.41	1348470.105	3	40167580.63	38583008.45	43080283.66	40610290.91	1862501.074	3
ZCCHC3	29098018.97	27379122.91	21629183.99	26035441.96	3193742.096	4	21712205.75	19786158.18	18309120.56	19935828.16	1393328.875	4

si1	siControl1	siControl2	siControl3	Mean	SD	Calculated Sample size	si1_1	si1_2	si1_3	Mean	SD	Calculated Sample size
BIRC5	15708050.49	16542672.9	15134215.1	15794979.5	578276.6539	2	8986031.522	8734290.875	10883073.16	9534465.186	959131.8782	2
CIT	92400297	91536123.39	96210367.44	93382262.61	2030653.705	2	146710718.7	152342282.7	128618137.4	142557046.3	10120886.44	2
DHX8	138600445.5	132341383.2	129721843.7	133554557.5	3724803.022	2	201727238.3	182810739.2	187980354.7	190839444.1	7982868.263	2
GSE1	6652821.384	6837638.133	7134701.406	6875053.641	198497.7398	2	12837187.89	13202997.83	11872443.45	12637543.06	561241.0558	2
KDM3A	75768243.54	67273536.47	74590060.15	72543946.72	3757654.16	2	110033039	111717674	98937028.77	106895913.9	5669649.515	2
OTUD1	498961.6038	430109.4954	443216.2995	457429.1329	29851.37275	2	916941.9921	812492.1744	840964.7446	856799.637	44087.02733	2
SPRY4	19404062.37	18748362.62	24863353.38	21005259.46	2741186.128	3	30259085.74	28437226.11	33638589.78	30778300.54	2154953.057	3
ZCCHC3	17556056.43	15439828.04	16215230.47	16403704.98	874165.3659	3	32092969.72	32499686.98	24734257.19	29775637.96	3568659.394	3

si2	siControl1	siControl2	siControl3	Mean	SD	Calculated Sample size	si2_1	si2_2	si2_3	Mean	SD	Calculated Sample size
BIRC5	15509833.92	16332489.24	14944401.95	15595575.04	569918.2605	2	9499406.859	9732530.232	10644389.79	9958775.627	494055.808	2
CIT	91234317.19	90373107.13	95003698.13	92203707.48	2010867.376	2	122572991.7	115020811.8	117088287.6	118227363.7	3186636.271	2
DHX8	136851475.8	130659913.9	128094873.9	131868754.5	3675639.715	2	173645071.6	159259585.6	159665846.8	164190168	6687683.386	2
GSE1	6568870.838	6750762.219	7045218.063	6788283.707	196269.4852	3	13278740.77	14156407.61	10644389.79	12693179.39	1492364.765	3
KDM3A	74812140.09	66418789.58	73654552.48	71628494.05	3714006.621	2	96015510.18	97325302.32	93670630.11	95670480.87	1511829.17	2
OTUD1	492665.3128	424644.7202	437657.4857	451655.8396	29480.67941	2	1327874.077	1150208.118	1383770.672	1287284.289	99577.49018	2
SPRY4	19159206.61	18510154.47	24551517.49	20740292.86	2707937.97	3	31664689.53	38045345.45	32997608.34	34235881.11	2748112.331	3
ZCCHC3	17334520.27	15243656.62	16011859.23	16196678.71	863537.8316	2	33707572.72	32736692.6	26610974.46	31018413.26	3141633.294	2

Reviewer Table 1: Sample size calculated using TCC normalized data of proteomics analysis.

Regarding EMT, in this technical context, we agree with the reviewer that solely based on the proteomics analysis, this process cannot be ruled out in our experimental system. However, we did not exclude it only based in the proteomics; the omics data corroborates our experimental data pointing to EMT not being responsible for the effects we see, which includes:

a. Assessment of the main EMT markers (including proteins that in other reports are regulated by Mdm2) showed no change in response to Mdm2 modulation, as determined by immunoblotting.

b. The fact that preventing the increase of Spry4 levels alone was able to fully rescue the decrease in migration and metastasis formation upon Mdm2 silencing.

To clarify, we did not intend to completely exclude any participation of EMT in the processes we describe. Our intention was to demonstrate that in our system it does not play a significant functional role, which we have mentioned in the second paragraph of the Discussion:

"Second, Mdm2 is required for robust FA formation in order to maintain the attachment of cells to the ECM and this likely does not involve regulation of EMT."

We do mention in the Discussion as well that although it does not seem like EMT plays an important role in our model, we do not exclude it being important in other models (page 20, second paragraph):

"Although in our model EMT/MET was not induced in response to Mdm2 modulation and thus is apparently not involved in the mechanism by which Mdm2 regulates migration, invasion and metastasis, we do not discard the possibility that in other cell types it may be a significant contributing factor."

However, to address the reviewer's concern and soften our claim, we have excluded EMT from the title of the first result subhead (page 4), and we have extended the explanation found in the Discussion as to why we believe EMT does not play an important functional role in our model (page 20, second paragraph) which now reads as follows:

"Based on the lack of change in expression of relevant EMT markers, as well as the fact that Spry4 silencing fully rescued the impact of Mdm2 inhibition, EMT/MET does not seem to be required for Mdm2 regulation of migration, invasion and metastasis in our model. That said, we do not discard the possibility that in other cell types EMT/MET may be a significant contributing factor."

3. "As pointed out by the reviewer, EMT does show up as a pathway regulated by Mdm2 modulation in our first pathway analysis, but this was based on differential expression of a single protein, FOXC2. Although it is significantly differentially expressed by Mdm2 modulation, the siRNA and pharmacological inhibitor conditions we used showed inverse changes in expression: MEL23 treatment led to less FOXC2 while silencing of Mdm2 increased the expression of FOXC2 (see graphs below). Thus, it is unlikely to be involved in the effects we see. Our stricture for considering any candidate is that all three conditions give us the same functional result which is not the case with FOXC2 that is inversely modulated by MEL and siRNAs."

Shouldn't this criterion (all three conditions give the same functional result) also apply to the proteins in Supp Figure 4E?

We completely agree that it should, and it does! That is what helped us choose Spry4 as a potential target to validate and test, as mentioned in the manuscript. We color-coded Supp. Fig.4E to point out that, although in a general analysis we obtained eight hits, when the direction of the regulation is considered (i,e. same direction, up or down, in all three conditions), there were only three common hits.

We previously commented on those differences in the main text of the manuscript in the Results section (page 11, third paragraph). To make it clearer we have added the description of the color code to the figure legend as well.

4. How do the authors explain so very similar functional effects by siRNA knockdown or pharmacological intervention while on the protein level, there seems to be very little overlap between the 2 siRNAs targeting the same protein and huge differences to the effect of MEL23 (#1600 significantly regulated proteins with less 0,5% overlap to the regulation patterns induced by the siRNAs)?

Thank you for that comment! We were initially surprised that the two siRNAs directed against Mdm2 had so many unique targets and that there were unrelated changes seen with MEL23. This prompted a brief perusal of the literature, which confirmed that this is not uncommon. The use of siRNAs does result in a significant number of off-target effects. Further, from our own experiments we know the use of MEL23 also results in off-target effects. For instance, treatment of cells with this drug for over 48 hours impacts cell survival while the siRNAs do not affect cell viability (hence our ability to generate the stable cell lines used in this paper in this work).

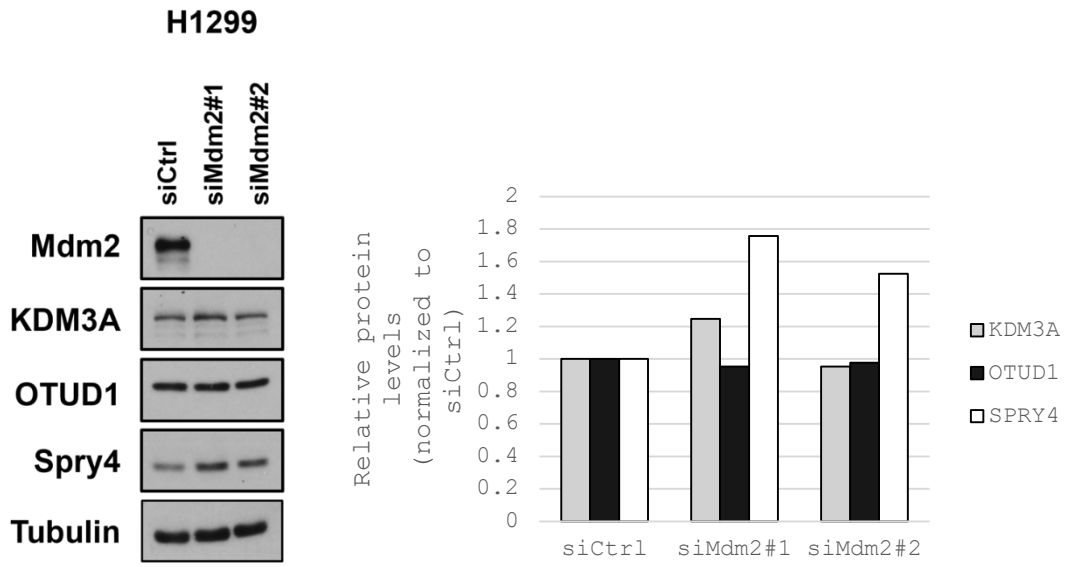
We agree that this is an interesting point and have added a new section to the Discussion (page 20 and 21). We have cited a couple of reviews on the topic of siRNA off-target effects (Neumeier and Meister, Front Plant Sci.,2020; Jackson and Linsley, Nature Reviews Drug Discovery, 2010) along with a sentence acknowledging the magnitude of off-target effects of different treatments that nevertheless form the basis for discovery of valid targets (page 21 first paragraph). In that regard, we would like to also point out that MEL 23 might function more like a true knock-out of Mdm2 rather than a knock-down by siRNAs, where the residual Mdm2 however small might be sufficient for maintaining the effects of the complex in some (but not all) cellular processes.

5. "As correctly pointed out by the reviewer, our decision to focus on f Spry4 was not very clear in the text. We have now clarified in the text why Spry4 was chosen as the best candidate and new data, suggested in revision, in Supplementary Figure 4, panels D, E and F help understanding this choice."

The critical part of the manuscript now reads: "As mentioned, three proteins were significantly similarly regulated by all conditions: the lysine demethylase KDM3A, the deubiquitinase OTUD1 and the RTK signaling antagonist protein Sprouty4. Among those 3 hits, and considering their correlation to migration and metastasis described in the literature". Given the heterogeneity of the observed effects by MEL23 and siRNA #1 and

#2 on the protein level, were any attempts made to devalidate the other 2 highly significant candidates having similar effects, other than deduction from literature?

This is a good point. We eliminated the other two targets not only by their apparent weak involvement with the cellular process we were studying, but also based on their expression in H1299 cells. As we show in the manuscript, H1299 cells, a naturally p53 null cancer cell line, recapitulate the findings we made with HT1080 p53 knock-out cells upon p53 inhibition (Fig. 5). We have measured the expression of the 3 hits (KDM3A, OTUD1 and Spry4) in H1299 cells in response to Mdm2 silencing and show that only the levels of Spry4 are changed and not the other 2 targets, as can be seen in the immunoblot below (Reviewers Fig.1).



Reviewer Fig. 1: Expression of KDM3A, OTUD1 and Spry4 in H1299 cells upon Mdm2 silencing using 2 different siRNAs. Left: Immunoblot of the indicated proteins. Right: Densitometry of the bands shown on the left normalized to tubulin, the loading control.

6. "As requested, we increased the level of the detail in the Methods section on proteomics, including new citations and the parameters questioned. We also added as supplementary material .csv files containing the full list of identified proteins, fold changes and complete statistical analysis."

The description of the methods has been greatly improved and is now sufficient to understand the workflow. What is still missing is the reviewer access to the dataset PXD033789 which currently is not publically available in jPOST or ProteomeXchange.

We apologize for this mistake; the dataset is now open access, and the availability of the data is described on page 53 (first paragraph) and in the Data Availability statement (newly added).

7. Original comment (excerpt): "throughout the manuscript) individual data points should be visualized at least in addition to bars representing the mean"

I would strongly suggest to have scatter plots overlaid to the barcharts (representing the mean) which can be highly misleading due to leverage effects of individual outliers.

We appreciate the suggestion. The single data points have been added to every bar graph in the main figures and supplementary figures.

Minor:

1. Intro: “Redundant text: “harboring either wild-type or different mutant versions of p53”. We do not see redundancies. If the reviewer believes that is an important correction to make, please clarify further the redundancy so we can fix it accordingly.”

Indicated by brackets: One additional confounding issue of previous studies characterizing pro-oncogenic activities of Mdm2 has been that for the most part researchers have used cell lines that [[[either harbor wild-type or mutant versions of p53]]]to make their observations. While in such cases modulation of Mdm2 levels in different cell lines [[[harboring either wild-type or different mutant versions of p53]]] leads to changes in cell migration15, 20, 21, 24-27, the presence of either form of p53 might impact cellular responses, however, and thereby complicate interpretation of data.

The sentence mentioned in the Introduction has been changed as per the reviewer’s suggestion.

Reviewer #3 (Remarks to the Author):

The authors have addressed my comments, thank you.

However, for original major point 11, units of n still need to be provided in the figure legends to make it clear what “n” represents. For example, the legend for Fig. 1E states n = 3, but there are clearly more than three data points that were presumably used for the statistical comparison between samples (so this needs to be “n = x cells from n = 3 independent experiments” or similar). For Fig. 2A-D, why are spheroid numbers not declared for all experimental conditions?

In Figure 1 the legends for panels E and F were changed to satisfy the reviewer’s suggestion. The quantification plot in Figure1E previously showed a representative quantification of one biological replicate of the experiment. To follow the pattern of the other panels we have substituted quantification of the combined biological replicates.

Regarding Figure 2, the exact number of spheroids for all conditions in Fig. 2 A-D were added to the figure legend as requested.

Minor issues introduced in revision:

- The new legend text for Fig. 2F is confusing: were the data from each biological replicate summarised as a median and then each biological replicate median value used to determine a mean ± SD for the bar chart? Or does the bar chart represent median ± error?

Because the legend for this figure is already very long, we have added clarifying information about the quantification of cell circularity to the Methods section on page 56 (first full paragraph).. We hope that makes it clearer.

- Error bars and whiskers are not defined for any of the charts in Fig. 2.

Clarification about the box and whiskers plot was added to the Figure 2 legend, as requested.

- Setting FDR < 0.2 (20%) is a generous cut-off for differentially regulated proteins in the proteomics experiment and should be justified.

Thank you so much for the thoughtful suggestion and we apologize for the lack of sufficient explanation in setting the FDR value. A previous paper reported that the setting of the FDR value can be adjusted according to the purpose of the experiment (Benjamini Y. Hochberg Y. Controlling the false discovery rate: a pratical and powerful approach to multiple testing. J. R. Statist. Soc. ser.B, 57(1): 298-300. 1995). In particular, it is acceptable to set FDR < 0.25 for exploratory analyses. This is because more stringent FDR cutoffs may miss significant results. Furthermore, since most expression data sets are inconsistent and there is a need to keep the proteomics sample size relatively small for cost-effectiveness, the FDR values were set to less stringent conditions in order to identify putative candidate molecules for subsequent functional screening in our specific case.

- Typos on p. 16: “HT12080 p53KO” and “e hypothesized”. Fixed

- Typo on p. 38: “phosphor-ERK”. Fixed

- Typo on p. 47: “beta1” is mentioned twice; presumably one occurrence should read “beta2”. That is correct. Thank you for the careful reading. The typo is fixed.

REVIEWERS' COMMENTS

Reviewer #2:

I would like to thank the authors for the diligent discussion of the raised issues. Despite arising from a somewhat underpowered proteomic experiment, the authors made it clear that they investigated alternative explanations for the role of Sprouty4 and thoroughly tested their hypothesis. My comments are sufficiently covered to recommend publication.

Reviewer #3:

The authors have addressed my further comments.

However, in Supplementary Table 2, ITGB1BP1 and ITGBL1 are not integrin subunits so should be removed from the table.

In the new data availability statement, “excel” should be capitalized.

Data analysis software and code package/app versions (e.g. for Scaffold DIA, EncyclopeDIA, Enrichr, etc.) should be provided to support reproducibility.