EMBO reports

WDR63 inhibits Arp2/3-dependent actin polymerization and mediates the function of p53 in suppressing metastasis

Kailiang Zhao, Decai Wang, Xiaolong Zhao, Chenfeng Wang, Yongxiang Gao, Kaiyue Liu, Fang Wang, Xianning Wu, Xuejuan Wang, Linfeng Sun, Jianye Zang, and Yide Mei

Review timeline:

Submission date:	12 September 2019
Editorial Decision:	11 October 2019
Revision received:	9 December 2019
Editorial Decision:	28 January 2020
Revision received:	30 January 2020
Accepted:	7 February 2020

Editor: Deniz Senyilmaz-Tiebe

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

1st Editorial Decision 11 October 2019

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

As you can see, the referees express interest in the proposed role of WDR63 in regulation of cell migration downstream of p53. However, they also raise a number of concerns that need to be addressed to consider publication here.

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

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

REFEREE REPORTS

Referee #1:

Zhao et al reports that WDR63 is transcriptionally upregulated by P53 and that WDR63 negatively

regulates cell migration, invasion and extravasation. The authors provide evidence that WDR63 directly interacts with the Arp2 component of the Arp2/3 complex and somehow this interaction reduces binding of the Arp2/3 complex to the VCA domain of nucleation promoting factors. Overall, this study was performed to a high technical standard with most experiments properly controlled. The results that WDR63 is a direct inhibitor of the Arp2/3 complex and that it functions to inhibit cell migration are novel and exciting. Thus, I would be happy to support publication in EMBO Reports if my following major concerns have been addressed:

1.) Throughout the manuscript the wrong statistical analysis has been used: The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests. Furthermore, the normalization and statistical analysis of your data is flawed: If you artificially set your control to exactly 1, you loose the SD or SEM of your data and of course then it is more easy for it to be different to your experimental sample. If you want to normalise you can do that and keep the SD by anchoring 100% or 1 to the mean of your control sample. All experiments need to be repeated at least 3 times: As you state the following experiments shown in Figs 3G and H, EV3A and B, and EV4B, were only repeated twice.

2.) Fig. 4A is not at all convincing. Are you stating that the Arp2/3 complex is not recruited to existing lamellipodia or that lamellipodia formation is inhibited upon WDR63 expression? The former one is not convincing since in your images you don't see lamellipodia without FBS and with WDR63 expression. For the latter one you will need to quantify lamellipodia formation.

3.) Since the Arp2/3 complex is so essential for cell migration, it is not a very convincing conclusion from double knockdown studies that WDR63 function via the Arp2/3 complex. It would be more convincing if the exact binding site of the Arp2/3 complex in the WDR63 WD repeats (WDR63 (394-817) deletion is too big a deletion) would be mapped and mutated and overexpression of this construct would not show any effect on cell migration compared to WT WDR63.

Specific concerns:
Why were lung cancer cell lines used? What is the expression level and mutation rates of WDR66 compared to P53 in lung cancer?
Fig. 1 Total number of counted cells for each experiment should be specified in the figure legend.
Fig. 1A,E; EV1 D,F,J: The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests.
Fig. 1C,F and EV1 I the random migration data needs to be quantified and speed and persistence statistically analysed.
Fig. 1H; EV1 R: The box and wiskers plots used need to be specified in the figure legend. Please show all data points on the plots. Why is the data for the second shRNA cell line not normally distributed? The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests. Are the 6 mice each from 3 independent experiments? This assay only test extravasation ability and does not recapitulates full metastasis. Therefore, you should correctly describe the results in the text as such.
Fig. 2A,B,C,D,G,H,: The normalization and statistical analysis of your data is flawed: If you artificially set your control to exactly 1, you loose the SD or SEM of your data and of course then it is more easy for it to be different to your experimental sample. If you want to normalise you can do that and keep the SD by anchoring 100% or 1 to the mean of your control sample.
Fig. 3 D: Why does the WDR63 run at a wrong, higher molecular weight after IP? This is not convincing.
Fig 3G: You would expect if WDR63 interacts directly with Arp2 that it also coIPs with the entire Arp2/3 complex.

Figs 3G and H, EV3A and B, and EV4B, which were repeated twice. This is not acceptable and needs to be repeated at least 3 times.

Figs 3 H. Since FLAG-WDR63 FL and fragments were purified from HEK cells can you exclude that the Arp2/3 co-purified or was unspecifically bound already? As a control you should show FLAG-WDR63 FL and fragment pulldown without addition of Arp2/3 to show that this is not positive for Arp2/3.
Fig. 4A is not at all convincing. Are you stating that the Arp2/3 complex is not recruited to existing lamellipodia or that lamellipodia formation is inhibited upon WDR63 expression? The former one is

not convincing since in your images you don't see lamellipodia without FBS and with WDR63 expression. For the latter one you will need to quantify lamellipodia formation.
Fig. 4EF: Amounts of F-WDR63 in each lane needs used needs to be specified in figure legend.
Fig 4D: It needs to be specified what this percentage refers to. It is not at all clear from the figure legend how this was calculated.
Fig. 5A: The normalization and statistical analysis of your data is flawed: If you artificially set your control to exactly 1, you loose the SD or SEM of your data and of course then it is more easy for it to be different to your experimental sample. If you want to normalise you can do that and keep the SD by anchoring 100% or 1 to the mean of your control sample. : The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests.

Fig. 5: Since the Arp2/3 complex is so essential for cell migration, it is not a very convincing conclusion from double knockdown studies that WDR63 function through the Arp2/3 complex. It would be more convincing if the exact binding site of the Arp2/3 complex in the WDR63 WD repeats (WDR63 (394-817) deletion is too big a deletion) would be mapped and mutated and overexpression of this contruct would not show any effect on cell migration compared to WT WDR63.
Fig. 6A: The normalization and statistical analysis of your data is flawed: If you artificially set your control to exactly 1, you loose the SD or SEM of your data and of course then it is more easy for it to be different to your experimental sample. If you want to normalise you can do that and keep the SD by anchoring 100% or 1 to the mean of your control sample. : The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests.

Referee #2:

1. Overall rating.
Good, should probably be published in EMBO Reports after revision
2. Suitability for publication.
Interesting and novel, even though the exact molecular mechanisms are not explored in detail. Some points require confirmation at this point.
3. Clarity / figures.
The Figures are well organized and clearly present the main findings of the article.
4. Remarks to the Author.
The article by Zhao et al is dedicated to the functional characterization of WDR63, a novel inhibitor of cell migration in vivo and in vitro. This protein, positively regulated at the transcriptional level by p53, interacts with Arp2/3 complex and inhibits it in vitro. Consistently WDR63 appears to inhibit cell migration. Importantly, the authors show that manipulating the levels of Arp2/3 interacting domain of WDR63 in the cell can fully reverse the effects of p53 up- or down-regulation on cell migration. These are nice findings, but everything is not yet well demonstrated.
1. In Fig. 3, the authors show the interaction between WDR63 and various Arp2/3 subunits. However, it is unclear how any subunit of the Arp2/3 complex exists in the cell on its own. Based on the results of pyrene actin assays, WDR63 must bind directly to the whole Arp2/3 complex. This direct binding with purified Arp2/3 complex should be demonstrated.
2. The endogenous coIP is not convincing, because the bait protein is not even convincingly pulled down. It sounds logical that more Arp2/3 complex should be immunoprecipitated when WDR63 is induced by p53 (PCDH-p53 in Fig2A).
3. Fig.3G. The ability of various Arp2/3 subunits to bind to GFP-WDR63 is highly variable. Is it due to the efficiency of incorporation of the tagged subunit into the Arp2/3 complex? Several endogenous subunits of the Arp2/3 complex should be analyzed by WB.
4. The localization of WDR63 in the cell is not analyzed. Localization of both GFP-WDR63 and endogenous WDR63 should be shown. Is it a global inhibitor throughout the cell or is it localized at the cell cortex like Arpin, the previously demonstrated Arp2/3 inhibitory protein that inhibits cell migration ?
5. Fig. 4A is so poor that nothing can be concluded from the phalloidin staining. Staining of branched actin networks should be performed with cortactin antibodies. Cortactin is an accepted marker of branched actin networks in the field. Lamellipodia should be quantified.
6. The authors used several assays to analyze cell migration in vitro. Whereas wound healing, as well as transwell migration and invasion, are quantified and statistically evaluated, the cell trajectories are not. Migration persistence is a key parameter, specifically regulated by the Arp2/3

activity. Speed, persistence and MSD should be extracted from cell tracks. There are useful tools designed to this end (Gorelik and Gautreau, 2014).
7. Fig. 5 uses shRNA against Arp2 to show the role of Arp2/3 in WDR63-regulated cell migration. These experiments should be completed by using specific Arp2/3 inhibitors, such as CK666. This would allow the precise reversal of the shWDR63 effect, by carefully titrating the dose of the inhibitor.
8. Fig. 6 demonstrates that the role of p53 in regulating cell migration fully depends of WDR53. This is an important claim, and as such, it should be discussed in detail. In its current form, the Discussion section mainly repeats the Results of the article, instead of describing the state of the art in the field, and putting the current findings with relation to previously published articles on p53 and migration. For example, recent papers on the role of p53 in the migration of lung cancer cells (Tang et al., Oncotarget, 2017), (Basu et al, Genes&Dev, 2018), or breast cancer cells (Kim et al., Cancer Research, 2017) are directly relevant to the current study and provide alternative mechanisms to WDR63. How do the authors explain that WDR63 alone can antagonize the effects of p53 ? The Discussion should pay attention to previously performed studies in the field.
9. The mass spec analysis yielding Arp2 identification (Fig. 3A) is not described. How come only Arp2 has been identified ?
10. A careful proof-reading will improve the quality of the manuscript. For example, there is « upregualted » instead of « upregulated » as soon as in the Abstract, and such typos are to be found throughout the text...

Referee #4:

In the present manuscript entitled "WDR63 inhibits Arp2/3-dependent actin polymerization and mediates p53 function in suppressing metastasis" by Kailiang Zhao et al., the authors identified WDR63 as a novel bona fide transcriptional target of p53. Moreover, the authors characterized the functional activity of WDR63 by demonstrating its ability to prevent metastatization and invasion of cancer cells through the physical interaction with Arp2. They also demonstrated that WDR63 depletion impaired p53-dependent inhibition of cancer cell migration.
The manuscript is based on robust evidences obtained both in vitro and in vivo. The data are well presented and discussed.
Specific comment
The clinical relevance of the reported findings needs tbe investigated to further reinforce the overall message of the manuscript. Due to the important role of WDR63 in mediating p53 tumor suppressor activity the authors could validate the positive correlation between WDR63 and p53 in several available cohorts of cancer patients and WDR63 expression with the most relevant clinical-pathological features.

1st Revision - authors' response 9 December 2019

Response to Referee #1:

Zhao et al reports that WDR63 is transcriptionally upregulated by P53 and that WDR63 negatively regulates cell migration, invasion and extravasation. The authors provide evidence that WDR63 directly interacts with the Arp2 component of the Arp2/3 complex and somehow this interaction reduces binding of the Arp2/3 complex to the VCA domain of nucleation promoting factors. Overall, this study was performed to a high technical standard with most experiments properly controlled. The results that WDR63 is a direct inhibitor of the Arp2/3 complex and that it functions to inhibit cell migration are novel and exciting. Thus, I would be happy to support publication in EMBO Reports if my following major concerns have been addressed:

Response: We greatly appreciate the reviewer's positive and encouraging comments. Our detailed point-to-point response to the reviewer's comments is given below.

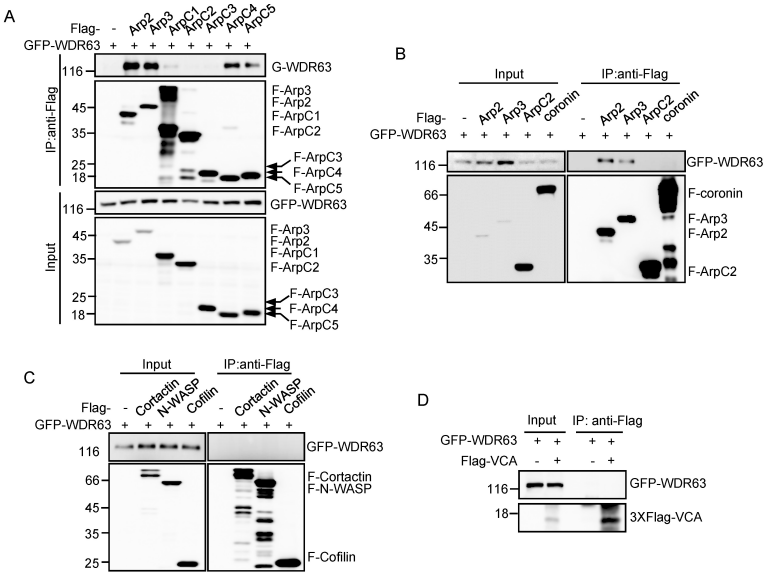
1.) Throughout the manuscript the wrong statistical analysis has been used: The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests. Furthermore, the normalization and statistical analysis of your data is flawed: If you artificially set

your control to exactly 1, you loose the SD or SEM of your data and of course then it is more easy for it to be different to your experimental sample. If you want to normalise you can do that and keep the SD by anchoring 100% or 1 to the mean of your control sample. All experiments need to be repeated at least 3 times: As you state the following experiments shown in Figs 3G and H, EV3A and B, and EV4B, were only repeated twice.

Response: According to the reviewer's comments, in the revised manuscript, one-way ANOVA has been used for comparing more than two groups to determine whether there are statistic significance between different groups (Figs 1A-H, 4A, 5A, C-E, and G, 6A, C-G, EV1A, B, D, F, I, J, P, R, U, and V, EV4A, C, and D-I, EV5A, C-E, and G). Our conclusions remain the same.

As suggested by the reviewer, by anchoring 1 to the mean of our control samples, error bars for control groups have been calculated and added accordingly (Figs 2A-D, G, and H, 5A, 6A, EV1B, EV2A, EV2D, EV4A, and EV5A).

In the revised manuscript, we have repeated the experiments shown in the previous Figs 3G and H, EV3A and B, and EV4B (Please see the following Figures, and the previous Fig 3H has been replaced by a new figure in the revised manuscript). Now, all the experiments were repeated independently at least three times with similar results.



(Figs A-D were repeats for the previous Figs 3G, EV3A and B, and EV4B (the revised EV3G), respectively)

2.) Fig. 4A is not at all convincing. Are you stating that the Arp2/3 complex is not recruited to existing lamellipodia or that lamellipodia formation is inhibited upon WDR63 expression? The former one is not convincing since in your images you don't see lamellipodia without FBS and with WDR63 expression. For the latter one you will need to quantify lamellipodia formation.

Response: Thanks for the comments. In Fig 4A, we were trying to state that ectopic expression of WDR63 was able to inhibit FBS-stimulated lamellipodia formation by impairing the recruitment of Arp2 to the leading edge of migrating cells. To better show the branched actin networks, we have re-performed immunofluorescence experiments with anti-Cortactin antibody instead of phalloidin staining. The lamellipodia formation has been quantified by measuring the length of the outer margin of lamellipodia in individual cell (n=20 for each condition), and expressed as the proportion of the total length of the cell perimeter (Fig 4A).

3.) Since the Arp2/3 complex is so essential for cell migration, it is not a very convincing conclusion from double knockdown studies that WDR63 function via the Arp2/3 complex. It would be more convincing if the exact binding site of the Arp2/3 complex in the WDR63 WD repeats (WDR63 (394-817) deletion is too big a deletion) would be mapped and mutated and overexpression of this

construct would not show any effect on cell migration compared to WT WDR63.

Response: We appreciate the reviewer's critical and thoughtful comments. We agree with the reviewer's point that it would be better to use an Arp2/3-binding defective mutant of WDR63 with minimal aa deletion and supposedly this mutant would not show any effect on cell migration compared to wild type WDR63. Unfortunately, through the mapping experiments, we found that aa 1-200 of WDR63 was the only region that cannot interact with Arp2/3, while all the other tested WDR63 mutants, such as WDR63 (aa 201-393), WDR63 (aa 394-891), WDR63 (ΔWD) and WDR63 (aa 394-817), showed strong binding to Arp2/3 (Fig 3H). These data suggest that multiple regions of WDR63 except the region of aa 1-200 mediate the interaction with the Arp2/3 complex.

To make our conclusion that WDR63 inhibits cell migration and invasion via the Arp2/3 complex more convincing, the Arp2/3-specific inhibitor, CK666, has been used in the revised manuscript. The results showed that knockdown of WDR63 consistently enhanced the migration and invasion of A549 cells, however, this promoting effect was greatly minimized by CK666 (Figs EV4G and H).

Specific concerns:
Why were lung cancer cell lines used? What is the expression level and mutation rates of WDR63 compared to P53 in lung cancer?

Response: The reason why lung cancer cell lines were used in this study is because WDR63 is shown to be down-regulated in both lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) (Figs EV1S and T). According to the reviewer's comments, we analyzed the mutation rates of WDR63 and p53 in LUAD and LUSC using cBioPortal database. The results showed that in 1053 TCGA lung cancer samples (566 LUAD and 487 LUSC samples), mutation frequencies of WDR63 and p53 were 1.7% and 66.4%, respectively.

Intriguingly, by analyzing the expression levels of WDR63 in LUAD and LUSC harboring wild-type or mutant *TP53* gene using TCGA database, we found that in each of these two tumor types, WDR63 was expressed at lower levels in tumors harboring mutant *TP53* gene (Figs EV2D and E). These data indicate the physiological importance of p53-regulated WDR63 expression.

Fig. 1 Total number of counted cells for each experiment should be specified in the figure legend.

Response: For the transwell migration and invasion assays, cells migrated/invaded to the lower surface were fixed and stained with 0.1% crystal violet. The stained cells were then photographed, and the numbers of cells counted in three randomly chosen field (200×) were averaged. This detailed information has been provided in the Material and Methods section of the revised manuscript. As the reviewer suggested, the number of migrated/invaded cells has been also shown in the corresponding figures (Figs 1B, D, E, and G, 5C-E, and G, 6C-F, EV1F and J, EV4C and F-H, EV5C and D).

Fig. 1A,E; EV1 D,F,J: The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests.

Response: In the revised manuscript, one-way ANOVA has been used for comparing more than two groups to determine whether there are statistic significance between different groups as the reviewer suggested. Our conclusions remain the same.

Fig. 1C,F and EV1 I the random migration data needs to be quantified and speed and persistence statistically analysed.

Response: Thanks for the comment. For the single cell tracking assay, cell migration speed and persistence have been quantified using an Excel macro described by Gorelik and Gautreau (Gorelik and Gautreau 2014 ***Nature Protoc*** 9: 1931-43) (Figs 1C, F and EV1I).

Fig. 1H; EV1 R: The box and wiskers plots used need to be specified in the figure legend. Please show all data points on the plots. Why is the data for the second shRNA cell line not normally distributed? The appropriate statistics to use for comparing more than two groups is One-WAY

ANOVA and not repeated t-tests. Are the 6 mice each from 3 independent experiments? This assay only test extravasation ability and does not recapitulates full metastasis. Therefore, you should correctly describe the results in the text as such.

Response: We appreciate the reviewer for pointing this out. All the data points have now been shown on the plots (Figs 1H, 6G, EV1R and EV5G). As the reviewer can see, there are two data points that severely deviate from other data points (Fig 1H), and we think that is the reason why the data for the second shRNA cell line is not normally distributed. One-way ANOVA has been used to determine whether there are statistic significance between different groups. Our conclusions remain the same. Yes, the 6 mice for each group were indeed from 3 independent experiments. This information has been specified in the Material and Methods section of the revised manuscript. We agree with the reviewer's point that this assay does not recapitulate full metastasis. In the revised manuscript, we have changed the description accordingly in the text as the reviewer suggested.

Fig. 2A,B,C,D,G,H,: The normalization and statistical analysis of your data is flawed: If you artificially set your control to exactly 1, you loose the SD or SEM of your data and of course then it is more easy for it to be different to your experimental sample. If you want to normalise you can do that and keep the SD by anchoring 100% or 1 to the mean of your control sample.

Response: As suggested by the reviewer, by anchoring 1 to the mean of our control samples, error bars for control groups have been calculated and added accordingly (Figs 2A-D, G, and H).

Fig. 3 D: Why does the WDR63 run at a wrong, higher molecular weight after IP? This is not convincing.

Response: According to the reviewer's comments, we have repeated this endogenous immunoprecipitation experiment. The previous Fig 3D has been replaced by new figure (the revised Fig 3D).

Fig 3G: You would expect if WDR63 interacts directly with Arp2 that it also coIPs with the entire Arp2/3 complex.

Response: We agree with the reviewer's point that if WDR63 interacts directly with Arp2, WDR63 should be able to interact with the entire Arp2/3 complex. In Fig 3G, the ability of various Flag-tagged Arp2/3 subunits to bind to GFP-WDR63 seems variable, one possible reason for this could be that the efficiency of incorporation of the Flag-tagged subunits into the Arp2/3 complex is different.

To address the reviewer's concern, in the revised manuscript, we have re-performed endogenous immunoprecipitation experiments. The results showed that WDR63 was able to interact with the entire Arp2/3 complex, because multiple subunits of Arp2/3, including Arp2, Arp3, ArpC2 and ArpC3, were specifically present in anti-WDR63 immunoprecipitates (Fig 3D).

Figs 3G and H, EV3A and B, and EV4B, which were repeated twice. This is not acceptable and needs to be repeated at least 3 times.

Response: As suggested by the reviewer, in the revised manuscript, we have repeated the experiments shown in the previous Figs 3G and H, EV3A and B, and EV4B. Now, all the experiments were repeated independently at least three times with similar results.

Figs 3 H. Since FLAG-WDR63 FL and fragments were purified from HEK cells can you exclude that the Arp2/3 co-purified or was unspecifically bound already? As a control you should show FLAG-WDR63 FL and fragment pulldown without addition of Arp2/3 to show that this is not positive for Arp2/3.

Response: According to the reviewer's comments, in the binding experiments, we have included one additional control where Flag-WDR63 FL proteins purified from HEK293T cells were incubated without addition of Arp2/3 *in vitro* (Fig 3H). The results showed no Arp2 signal in this control lane, indicating that Flag-WDR63 proteins we purified were not contaminated by Arp2/3 from HEK293T cells.

Fig. 4A is not at all convincing. Are you stating that the Arp2/3 complex is not recruited to existing lamellipodia or that lamellipodia formation is inhibited upon WDR63 expression? The former one is not convincing since in your images you don't see lamellipodia without FBS and with WDR63 expression. For the latter one you will need to quantify lamellipodia formation.

Response: In Fig 4A, we were trying to state that ectopic expression of WDR63 was able to inhibit FBS-stimulated lamellipodia formation by impairing the recruitment of Arp2 to the leading edge of migrating cells. To better show the branched actin networks, we have re-performed immunofluorescence experiments with anti-Cortactin antibody instead of phalloidin staining. The lamellipodia formation has been quantified by measuring the length of the outer margin of lamellipodia in individual cell (n=20 for each condition), and expressed as the proportion of the total length of the cell perimeter (Fig 4A).

Fig. 4EF: Amounts of F-WDR63 in each lane needs used needs to be specified in figure legend.

Response: In the revised manuscript, the amounts of F-WDR63 in each lane have been specified in the legends for Fig 4E and F.

Fig 4D: It needs to be specified what this percentage refers to. It is not at all clear from the figure legend how this was calculated.

Response: The percentage of branched actin filaments was expressed as the ratio of the number of actin filaments with branch junctions to the total numbers of actin filaments. For each condition, over 150 actin filaments were counted. This detailed information has been provided in both Material and Methods section and legends for Fig 4D in the revised manuscript.

Fig. 5A: The normalization and statistical analysis of your data is flawed: If you artificially set your control to exactly 1, you loose the SD or SEM of your data and of course then it is more easy for it to be different to your experimental sample. If you want to normalise you can do that and keep the SD by anchoring 100% or 1 to the mean of your control sample. : The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests.

Response: For Fig 5A, by anchoring 1 to the mean of our control sample, error bar for the control group has been calculated and added. Also, as suggested by the reviewer, one-way ANOVA has been used to determine whether there are statistic significance between different groups. Our conclusions remain the same.

Fig. 5: Since the Arp2/3 complex is so essential for cell migration, it is not a very convincing conclusion from double knockdown studies that WDR63 function through the Arp2/3 complex. It would be more convincing if the exact binding site of the Arp2/3 complex in the WDR63 WD repeats (WDR63 (394-817) deletion is too big a deletion) would be mapped and mutated and overexpression of this construct would not show any effect on cell migration compared to WT WDR63.

Response: We agree with the reviewer's point that it would be better to use an Arp2/3-binding defective mutant of WDR63 with minimal aa deletion and supposedly this mutant would not show any effect on cell migration compared to wild type WDR63. Unfortunately, through the mapping experiments, we found that aa 1-200 of WDR63 was the only region that cannot interact with Arp2/3, while all the other tested WDR63 mutants, such as WDR63 (aa 201-393), WDR63 (aa 394-891), WDR63 (ΔWD) and WDR63 (aa 394-817), showed strong binding to Arp2/3 (Fig 3H). These data suggest that multiple regions of WDR63 except the region of aa 1-200 mediate the interaction with the Arp2/3 complex.

To make our conclusion that WDR63 inhibits cell migration and invasion via the Arp2/3 complex more convincing, the Arp2/3-specific inhibitor, CK666, has been used in the revised manuscript. The results showed that knockdown of WDR63 consistently enhanced the migration and invasion of A549 cells, however, this promoting effect was greatly minimized by CK666 (Figs EV4G and H).

Fig. 6A: The normalization and statistical analysis of your data is flawed: If you artificially set your

control to exactly 1, you loose the SD or SEM of your data and of course then it is more easy for it to be different to your experimental sample. If you want to normalise you can do that and keep the SD by anchoring 100% or 1 to the mean of your control sample. : The appropriate statistics to use for comparing more than two groups is One-WAY ANOVA and not repeated t-tests.

Response: For Fig 6A, by anchoring 1 to the mean of our control sample, error bar for the control group has been calculated and added. In addition, as suggested by the reviewer, one-way ANOVA has been used to determine whether there are statistic significance between different groups. Our conclusions remain the same.

Response to Referee #2:

1. Overall rating.
Good, should probably be published in EMBO Reports after revision
2. Suitability for publication.
Interesting and novel, even though the exact molecular mechanisms are not explored in detail. Some points require confirmation at this point.
3. Clarity / figures.
The Figures are well organized and clearly present the main findings of the article.
4. Remarks to the Author.
The article by Zhao et al is dedicated to the functional characterization of WDR63, a novel inhibitor of cell migration in vivo and in vitro. This protein, positively regulated at the transcriptional level by p53, interacts with Arp2/3 complex and inhibits it in vitro. Consistently WDR63 appears to inhibit cell migration. Importantly, the authors show that manipulating the levels of Arp2/3 interacting domain of WDR63 in the cell can fully reverse the effects of p53 up- or down-regulation on cell migration. These are nice findings, but everything is not yet well demonstrated.

Response: We greatly thank the reviewer for his/her positive and encouraging comments. Our detailed point-to-point response to the reviewer's comments is given below.

1. In Fig. 3, the authors show the interaction between WDR63 and various Arp2/3 subunits. However, it is unclear how any subunit of the Arp2/3 complex exists in the cell on its own. Based on the results of pyrene actin assays, WDR63 must bind directly to the whole Arp2/3 complex. This direct binding with purified Arp2/3 complex should be demonstrated.

Response: We agree with the reviewer's point that WDR63 must bind directly to the whole Arp2/3 complex. We have re-performed *in vitro* binding experiments to determine whether WDR63 was able to bind to the purified Arp2/3 complex. The results showed that WDR63 indeed strongly interacted with the purified Arp2/3 complex (Fig 3H).

2. The endogenous coIP is not convincing, because the bait protein is not even convincingly pulled down. It sounds logical that more Arp2/3 complex should be immunoprecipitated when WDR63 is induced by p53 (PCDH-p53 in Fig2A).

Response: According to the reviewer's comments, we have re-performed endogenous immunoprecipitation experiments. Consistent with our previous finding, WDR63 strongly interacted with Arp2 at the endogenous level (Fig 3D). In addition, several other subunits of Arp2/3, such as Arp3, ArpC2 and ArpC3, were also specifically present in anti-WDR63 immunoprecipitates (Fig 3D). These data suggest that WDR63 is able to interact with the entire Arp2/3 complex. The previous Fig 3D has been replaced by a new figure.

3. Fig.3G. The ability of various Arp2/3 subunits to bind to GFP-WDR63 is highly variable. Is it due to the efficiency of incorporation of the tagged subunit into the Arp2/3 complex? Several endogenous subunits of the Arp2/3 complex should be analyzed by WB.

Response: We thank the reviewer for pointing this out. It is possible that the reason why the ability of various Flag-tagged Arp2/3 subunits to bind to GFP-WDR63 seems variable could be that the efficiency of incorporation of the Flag-tagged subunits into the Arp2/3 complex is different.

To address the reviewer's concern, we have re-performed endogenous immunoprecipitation experiments. The results showed that WDR63 was able to interact with the entire Arp2/3 complex, because multiple subunits of Arp2/3, including Arp2, Arp3, ArpC2 and ArpC3, were specifically present in anti-WDR63 immunoprecipitates (Fig 3D).

4. The localization of WDR63 in the cell is not analyzed. Localization of both GFP-WDR63 and endogenous WDR63 should be shown. Is it a global inhibitor throughout the cell or is it localized at the cell cortex like Arpin, the previously demonstrated Arp2/3 inhibitory protein that inhibits cell migration ?

Response: As suggested by the reviewer, we have performed immunofluorescence to determine the cellular localization of WDR63. The results showed that exogenously expressed GFP-WDR63 was predominantly and evenly distributed in the cytoplasm (Fig EV3D). To examine the localization of endogenous WDR63, we used a fractionation assay, because the anti-WDR63 antibodies in our hand are not suitable for immunofluorescence. The results showed that endogenous WDR63 was also localized in the cytoplasm (Fig EV3E). Taken together, these data suggest that unlike Arpin, WDR63 is distributed throughout the cytoplasm.

5. Fig. 4A is so poor that nothing can be concluded from the phalloidin staining. Staining of branched actin networks should be performed with cortactin antibodies. Cortactin is an accepted marker of branched actin networks in the field. Lamellipodia should be quantified.

Response: We appreciated the reviewer's constructive comments. As suggested by the reviewer, to better show the branched actin networks, we have re-performed immunofluorescence experiments with anti-Cortactin antibody instead of phalloidin staining. The lamellipodia formation has been quantified by measuring the length of the outer margin of lamellipodia in individual cell (n=20 for each condition), and expressed as the proportion of the total length of the cell perimeter (Fig 4A). The results consistently showed that ectopic expression of WDR63 was able to inhibit FBS-stimulated lamellipodia formation by impairing the recruitment of Arp2 to the leading edge of migrating cells.

6. The authors used several assays to analyze cell migration in vitro. Whereas wound healing, as well as transwell migration and invasion, are quantified and statistically evaluated, the cell trajectories are not. Migration persistence is a key parameter, specifically regulated by the Arp2/3 activity. Speed, persistence and MSD should be extracted from cell tracks. There are useful tools designed to this end (Gorelik and Gautreau, 2014).

Response: We thank the reviewer's comments. As suggested by the reviewer, for the single cell tracking experiments, cell migration speed, persistence and MSD have been extracted using an Excel macro described by Gorelik and Gautreau (Gorelik and Gautreau 2014 ***Nature Protoc*** 9: 1931-43) (Figs 1C, F and EV1I).

7. Fig. 5 uses shRNA against Arp2 to show the role of Arp2/3 in WDR63-regulated cell migration. These experiments should be completed by using specific Arp2/3 inhibitors, such as CK666. This would allow the precise reversal of the shWDR63 effect, by carefully titrating the dose of the inhibitor.

Response: According to the reviewer's suggestion, to make our conclusion that WDR63 inhibits cell migration and invasion via the Arp2/3 complex more convincing, the Arp2/3-specific inhibitor, CK666, has been used in the revised manuscript. The results showed that knockdown of WDR63 consistently enhanced the migration and invasion of A549 cells, however, this promoting effect was greatly minimized by CK666 (Figs EV4G and H).

8. Fig. 6 demonstrates that the role of p53 in regulating cell migration fully depends of WDR63. This is an important claim, and as such, it should be discussed in detail. In its current form, the Discussion section mainly repeats the Results of the article, instead of describing the state of the art in the field, and putting the current findings with relation to previously published articles on p53 and migration. For example, recent papers on the role of p53 in the migration of lung cancer cells (Tang et al., Oncotarget, 2017), (Basu et al, Genes&Dev, 2018), or breast cancer cells (Kim et al., Cancer Research, 2017) are directly relevant to the current study and provide alternative mechanisms to

WDR63. How do the authors explain that WDR63 alone can antagonize the effects of p53 ? The Discussion should pay attention to previously performed studies in the field.

Response: We thank the reviewer's comments. We agree with the reviewer's point that it is unlikely that WDR63 is the only determinant of the metastasis suppressive function for p53. In Fig 6, it seems that WDR63 alone can antagonize the effects of p53 knockdown. We think the reason behind this is that the levels of ectopically expressed WDR63 were higher than the control endogenous WDR63 levels. The references mentioned by the reviewer have been cited and discussed in the Discussion section. In the revised manuscript, we state that p53 may exert its function in suppressing metastasis through multiple mechanisms.

9. The mass spec analysis yielding Arp2 identification (Fig. 3A) is not described. How come only Arp2 has been identified ?

Response: To identify WDR63-interacting proteins, we employed a GST pull-down experiment. 1×10^7 A549 cells were lysed in IP lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol) supplemented with 1× protease inhibitor cocktail. Cell lysates were then incubated with purified GST or GST-WDR63 immobilized on glutathione beads. After extensive washing, beads-bound proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. The strong protein bands specifically present in GST-WDR63 pull-down complex were analyzed by mass spectrometry, and a ~44 kD protein band was identified as Arp2. This detailed information has been provided in the Material and Methods section of the revised manuscript.

The reason why only Arp2 was identified could be that only strong protein bands specifically present in GST-WDR63 pull-down complex were cut and analyzed by mass spectrometry. To confirm whether GST-WDR63 could also pull down other subunits of Arp2/3, we performed the similar GST pull-down experiment as above described. The results showed that purified GST-WDR63 was indeed able to pull down multiple subunits of endogenous Arp2/3, such as Arp2, Arp3, ArpC2 and ArpC3 (Fig EV3C).

10. A careful proof-reading will improve the quality of the manuscript. For example, there is « upregualted » instead of « upregulated » as soon as in the Abstract, and such typos are to be found throughout the text...

Response: We really appreciate the reviewer for his/her careful reading of our manuscript. In the revised manuscript, the typo errors have been corrected.

Response to Referee #4:

In the present manuscript entitled "WDR63 inhibits Arp2/3-dependent actin polymerization and mediates p53 function in suppressing metastasis" by Kailiang Zhao et al., the authors identified WDR63 as a novel bona fide transcriptional target of p53. Moreover, the authors characterized the functional activity of WDR63 by demonstrating its ability to prevent metastatization and invasion of cancer cells through the physical interaction with Arp2. They also demonstrated that WDR63 depletion impaired p53-dependent inhibition of cancer cell migration.
The manuscript is based on robust evidences obtained both in vitro and in vivo. The data are well presented and discussed.

Response: We really appreciate the reviewer's positive comments.

Specific comment
The clinical relevance of the reported findings needs to be investigated to further reinforce the overall message of the manuscript. Due to the important role of WDR63 in mediating p53 tumor suppressor activity the authors could validate the positive correlation between WDR63 and p53 in several available cohorts of cancer patients and WDR63 expression with the most relevant clinical-pathological features.

Response: We greatly thank the reviewer for his/her constructive comments. As suggested by the reviewer, we analyzed the expression levels of WDR63 in LUAD and LUSC harboring wild-type or mutant *TP53* gene using TCGA database. We found that in each of these two tumor types, WDR63 was indeed expressed at lower levels in tumors harboring mutant *TP53* gene (Figs EV2D and E). These data indicate the physiological importance of p53-regulated WDR63 expression.

We also analyzed whether the levels of WDR63 are correlated with the malignancy of lung cancer using TCGA database. The results showed that the levels of WDR63 were indeed correlated with the malignancy of LUSC (Fig EV1V). However, in LUAD, the correlation between WDR63 levels and the malignancy is not statistically significant (Fig EV1U).

2nd Editorial Decision 28 January 2020

Thank you for submitting the revised version of your manuscript. It has now been seen by all of the original referees. My apologies for this unusual delay in getting back to you. It took longer than anticipated to receive the referee reports due to the recent holiday season.

As you can see, the referees find that the study is significantly improved during revision and recommend publication here. Before I can accept the manuscript, I need you to address some minor points below:

REFEREE REPORTS

Referee #1:

The authors have substantially improved the manuscript and addressed all my major concerns.

Referee #2:

The authors have very conscientiously addressed all the major criticisms of this reviewer, as well as the others, and have considerably improved the quality of the manuscript, which was quite good to begin with. Therefore, publication in EMBO Reports is justified.

Referee #4:

The authors have adequately addressed to the comments previously raised by this reviewer.

2nd Revision - authors' response 30 January 2020

The authors performed all minor editorial changes.

3rd Editorial Decision 7 February 2020

Thank you for submitting your revised manuscript. I have now taken a look at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yide Mei
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49269

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The information has been provided in Materials and Methods section.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	Mice were used in the experiment at random. This information has been provided in Materials and Methods section.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For animal studies, the experimentalist was blinded to all samples during data collection and analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The experimentalist was blinded to all samples during data collection and analysis.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, we used two-tailed Student's t-test or one-way ANOVA as indicated.

Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

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