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Centriolar distal appendages activate the centrosome-PIDDosome-p53 signaling axis via ANKRD26

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1st Editorial Decision 22nd Apr 2020

Thank you again for submitting your manuscript on ANKRD26-dependent PIDDosome activation at centrosomes. We have now received a complete set of comments from three expert referees, copied below for your information. Given their overall positive comments, we would be interested in pursuing this study further for EMBO Journal publication. Nevertheless, the reports also raise a number of substantive concerns that would need to be satisfactorily addressed prior to acceptance, necessitating in my view also additional experimental work. As you will see, a key concern shared by all referees is the lack of data quantification, statistical analyses and sufficient replicates; they further request several additional controls, including the validation of specific direct interaction. Finally, all referees agree that the study would benefit from some deeper insight into the exact mechanism of how supernumerary centrosomes lead to PIDDosome activation, and more direct support for the proposed model.

REFEREE REPORTS

Referee #1:

Dear editor,

In the presented manuscript, entitled 'Centrosome number surveillance requires ANKRD26-mediated recruitment of PIDD1 to distal appendages', the authors describe the requirement of

distal appendages and ANKRD26 for the recruitment of PIDD1 to centrosomes, as a mechanism to monitor centrosome number. This manuscript builds on the authors' previous finding that the PIDDosome is activated in response to supernumerary centrosomes.
Here, using several knockout cell lines, the authors nicely elucidate which distal appendage proteins are required for the localization of PIDD1 to the centrosome, and continue to show that PIDD1-localization to the distal appendage is required for PIDDosome activation in response to supernumerary centrosomes. They show that PIDD1 localization to the distal appendage is dispensable for PIDD1 autocleavage, yet they show that the recruitment of PIDD1 to DA's, as well as the autocleavage of PIDD1 (specifically the formation of PIDD1-cc) are both required for PIDDosome activity. Next, the authors show that PIDD1 is removed from the centrosome during mitosis and re-localizes in an asymmetrical manner during telophase. However, when cells become binucleated, resulting in the confinement of both centrosomes in a single cell, both centrosomes recruit PIDD1 in G1. Finally, it is shown that the clustering of the extra centromeres after mitosis is required for activation of the PIDDosome.
Overall, this represents an elegant piece of work and a well written manuscript. However, the authors' findings, especially in figures 4-7, raise questions regarding the exact mechanism of the sensing of supernumerary centromeres and PIDDosome activation. In my view, the manuscript would benefit from addressing these questions. In summary, I am supportive of publication in EMBO Journal, provided the authors address the questions mentioned below.

Major comments
1. Figure 3A shows that CEP83, SCLT1, ANKRD28 and PIDD1 itself are required for PIDDosome activity following binucleation. An important control that is lacking is whether the induction of binucleation and centrosome number is the same in every KO background. If KO cells proliferate with different speeds, dissimilar amounts of cells will reach binucleation in such assays. This is an important control to exclude that the observed reductions in PIDDosome activation are due to indirect effects on centrosome duplication or binucleation efficiency. For this, it would be required to analyze the percentage of cells that are binucleated and in addition, count the number of centrosomes in these cells.
2. Is PIDDosome-mediated Caspase-2 activation in the DAP-deficient cell lines also perturbed when different activation stimuli are imposed; i.e. DNA damage. This would shed light on the potential role of the centrosome in PIDDosome activation in response to other stimuli.
3. It is still unclear why the recruitment of PIDD1 to centrosomes is required while autoproteolysis seems to be independent of this recruitment. The authors suggest it might be the local priming of autoproteolytic PIDD1 fragments for PIDDosome activation. To test at which level PIDDosome activation is in fact perturbed, the authors could test if PIDDosome complex formation still occurs in the ANKRD26KO by performing Immunoprecipitation experiments or by size exclusion chromatography.
4. In telophase, PIDD1 initially appears on only 1 parental centriole (in 1 daughter cell, Fig. 6A). This should be accurately quantified, as it raises some interesting questions. How long is this asymmetric recruitment normally sustained throughout G1? And how does cytokinesis failure affect these dynamics (and does the addition of nocodazole/preventing clustering affect this)? The authors could investigate this by filming the GFP-tagged version of PIDD1. Furthermore, the authors show that PIDD1 levels are higher in cells treated with DHCB in Fig. S5E. How does nocodazole treatment affect these values? These findings could contribute to the understanding of how clustered centrosomes can induce PIDD1osome activity, in contrast to non-clustered centrosomes. An important control that is missing in the nocodazole experiments is to assess the effect of nocodazole addition after centrosomes have clustered. This to exclude that the PIDDosome inactivity in Figure 7D is due to direct effects of nocodazole on PIDDosome activation.

Minor comments:
1. On page 9 the authors state: "Thus, PIDDosome formation is preceded by PIDD1 recruitment to DAs and the two events can be uncoupled by the L828E mutation. Whether PIDD1 localization is a prerequisite for autoproteolysis, however, remains to be answered." Does the fact that the CEP83, ANKRD26 and SCLT1 KO cell lines still display high levels of autoproteolysis (Fig S4) not proof that recruitment to the DA's is in fact not a prerequisite for autoproteolysis?
2. Why is the FRAP done with L828 mutant? Why not with WT PIDD1?
3. The FRAP analysis shows slightly lower turnover of PIDD1 L828E after cytokinesis failure. The authors conclude that this could potentially contribute to the activation of the PIDDosome but they do not propose a model/hypothesis for this. How could this mechanistically work and how could this relatively minor difference in turnover result in such fast activation of the PIDDosome in response to the inheritance of too many centrosomes?
4. The model in Fig 7E shows a line between the centrosome and the CC fragment of PIDD1. However, there is no evidence provided that there is such a direct link. In fact, PIDD1-c and PIDD1-cc still form in lack of PIDD1-localization to the DA. The arrow should thus derive from the full length PIDD1. Similarly, in the right panel, the arrow to the CC fragment should derive from PIDD1 and not from the centrosomes directly. The authors should adjust the model accordingly.
5. The Hoechst staining in Fig 7 C looks very odd. The authors could show a better representative image.
6. Fig. 1A What are the bottom panels of each figure? Why can we not observe 9 structures in those panels?
7. Page 5. The authors use Cas9 to interfere with protein functions. Cas9 results in full gene KO's so the authors should use the word deletion instead of depletion. Also, western blots showing lack of the protein are only shown for some knockouts. This should also be done for the other KO cell lines.
8. As the authors draw strong conclusions about figures 6A and B, PIDD1 centrosome localization should be quantified.
9. There are no statistical analyses performed on any of the quantifications shown. These should be added to support the authors' conclusions.
10. PIDD1 autocleavage occurs in absence of centrosome localization (Fig. S4A). However, in Figure 7 the authors show that PIDDosome activation requires supernumerary clustered centrosomes. I agree with the authors that this suggests the presence of an additional que for PIDDosome activity. Would forcing a high local concentration of PIDD1 to another cellular structure lead to PIDDosome activation (in the presence or absence of supernumerary centrosomes). This will address whether there are additional signals originating from clustered supernumerary centrosomes, or if a high local concentration of PIDD1 sufficient for PIDDosome activation. In line with this question, what is the fraction of endogenous PIDD1 that localizes to the centrosome (normally and following clustering)? We realize that the points raised here (#10) represent a rather large effort. As these experiments would significantly clarify the mechanism of PIDDosome activation, we highly recommend the authors to address these issues, but we feel the manuscript can also be published in its absence.

Referee #2:

The presence of amplified centrosomes is commonly observed in cancer. However, as shown by several labs, extra centrosomes are poorly tolerated by normal cells. This is in part due to the activation of p53 pathway downstream of centrosome amplification, limiting the proliferation of cells with extra centrosomes. The mechanisms leading to p53 stabilisation in response to extra

centrosomes have remained elusive, and only recent work have begun to shed some light into this problem. In particular, work from L. Fava suggested that activation of the PIDDosome in cells with extra centrosomes led to MDM2 cleavage and p53 stabilisation, providing some evidence for a role of the PIDDosome in this process.

Here, the authors go further into the mechanism of PIDDosome recruitment to the centrosomes and activation in cells with extra centrosomes. They use superresolution microscopy to demonstrated that PIDD1 does localise to the distal appendages (DA) of the mother centriole. PIDD1 is recruited to the DA via ANKRD26 and its loss leads to failure in activating PIDDosome in cells with extra centrosomes. Just like ANKRD26, PIDD1 does not seem to be required for cilia formation, suggesting a function for DA outside ciliogenesis. Moreover, the authors found that PIDDosome autoproteolysis is important for p53 stabilisation in cells with extra centrosomes. Furthermore, centrosome clustering in G1 is essential for PIDDosome activation, favouring the authors current model that PIDD1 associated with different Das from extra mother centrioles is important to activate the complex. Although how this happens is still unclear.

Overall, the data presented and experiments conducted are of high quality and clear. The findings, in line with the last author previous publication, go beyond what was currently known and adds more mechanistic insight. While the findings presented here will be of interest to the readership of EMBO, it will be important for the authors to address the comments below prior to publication.

#1. My main criticism is regarding the model presented in Fig7E. the author proposed, as before, that the presence of extra mother centrioles is require to elicit PIDDosome activation. Here the authors show that disruption of clustering of extra centrosomes upon cytokinesis failure prevents MDM2 cleavage downstream of extra centrosomes. While this suggests that close proximity between mother centrioles may be required the question of how this happens remains unanswered. How the authors explain the model that PIDD1 needs to be in different mother centrioles to elicit proteolysis and PIDDosome activation? Why can't that be achieved with PIDD1 molecules in the same centriole? Is there a minimum distance required for these molecules to form a complex? I do not imagine that the mother centrioles are that close to each other that molecules from different DAs will interact?

#2. According to the author's model and data in Fig7D showing that centrosome clustering is required for PIDDosome activation, the prediction is that cells after noc treatment would not remain arrested in G1. Did the authors check that? It seems to me this validation would be important to demonstrate the clustering requirement to promote efficient G1 arrest.

#3. The authors need to show the efficiency of KO for all cell lines. Centriole localisation is not sufficiently quantitative to demonstrate KO. Can the authors perform western blot analyses?

#4. Localisation studies are usually not a reliable way to infer direct interaction. Do the authors have evidence of interaction between ANKRD26 and PIDD1 in human cells?

#5. On FigS5D it is difficult to see binucleation, which is expected if this cell is in G1. Otherwise it will be difficult to distinguish it from a 2N cells in G2, which would also have 2 centrosomes. Is it a matter of imaging?

#6. The authors propose that centrosome amplification leads to a very small decrease in PIDD1 turnover at the centrosomes. However, it is unclear if this is significant? What about in cells where centrosome clustering was prevented for example? It would also be interesting to know what

happens if more extra centrosomes are induced (e.g. PLK4 OE or repeated cytokinesis failure). If it is a matter of numbers, does that lead to increase stabilisation of PIDD2 at the centrosomes?

#7. I do not think n=3 cells for the live cell imaging experiment presented in Fig6 is acceptable for publication.

#8. Page 2. The work from Sercin et al and Coelho et al did not demonstrate that extra centrosomes by themselves are sufficient to induce spontaneous tumours.

Referee #3:

Burigotto and colleagues have investigated the underlying mechanisms triggering activation of the PIDDosome, one of several major pathways restraining cell cycle progression via p53. This manuscript follows up on recent work showing that the PIDDosome can be activated in response to supernumerary centrosomes, as occurs in various contexts including normal development of the liver, as well as in many human cancers. In the prior work, it was shown that the PIDDosome component, PIDD1, localizes to centrosomes (specifically, to "mother" centrioles); however, it has remained unclear how centrosome number is counted in the cell, and how this information is transduced into a biochemical signal for PIDDosome activation.

In the present study, the authors used CRISPR knockouts to demonstrate that PIDDosome activation requires the distal appendage structures found on "mother" centrioles, and that PIDD1 localization to distal appendages depends on centrosomal protein ANKRD26. Furthermore, a series of add-back experiments was performed in PIDD1 knockout cells to define the functional requirements of PIDD1 autoproteolytic processing for its centrosomal localization and for PIDDosome activation. Finally, an effort was made to address the mechanism of PIDDosome activation using FRAP experiments to assess PIDD1 dynamics at centrosomes, as well as timed disruption of microtubules to prevent supernumerary centrosome clustering.

Overall, the core findings of the paper define PIDD1 as a distal appendage protein whose localization at supernumerary mother centrioles is required for PIDDosome activation. This constitutes a significant advance on an important problem. However, one major issue is that there is a general lack of quantification throughout that should be addressed in order to consider these findings conclusive. In addition, some aspects of the paper, particularly on the mechanism of PIDDosome activation, suggest an interesting model but fall short of providing definitive evidence to support some of the authors' conclusions. Either additional experiments will be needed or the text should be edited to qualify some of the conclusions, as detailed in the specific comments below. Provided that these points are addressed, I would strongly support publication of this manuscript in EMBO Journal.

Specific Comments

1. Many of the major conclusions of the paper lack supporting quantification and/or statistical analyses.
a. For immunofluorescence assays, simply showing one or two example images is not sufficient to demonstrate the generality of the findings. In all cases, appropriate quantification (fluorescence intensity measurements) will need to be performed (at a minimum, images could be scored visually) and the number of cells analyzed has to be reported. This concerns Figures 1A, 1D, 3B, 3D, 4C, 4H,

5A, 5D, 5E, 6A, 6B, 7B and all similar data in the Supplement.
b. In cases where quantification has been done, please perform appropriate statistical tests and report P-values to compare control and experimental conditions. This concerns Figures 1B, 1F, 2B, 2C, 5E, 7B and the Supplement.
c. Please report the number of independent biological replicates that were performed for each Western blot (Figures 3A, 3C, 3E, 4B, 4F, 4G, 5B, 7D and Supplement).
2. The relationship between PIDD1 centrosome localization and PIDDosome activation remains unclear and some conclusions of the paper should be qualified accordingly.
a. Full-length PIDD1 is the only species the authors have been able to directly visualize at the centrosome, but this form of the protein is not competent for PIDDosome activation. On the other hand, autoproteolysis of PIDD1 into its active form (PIDD1-CC) is constitutive and does not require centrosome localization. Therefore, using FRAP to study the dynamics of full-length PIDD1 at the centrosome (Fig. 5E) does not clearly address the question of how the PIDDosome is activated-nor do the results indicate a compelling difference in PIDD1 dynamics. The data in Fig. 5E should be moved to the Supplement and the related conclusions in the main text should be qualified.
b. In general, a double-exponential will always provide a better fit than a single-exponential with this kind of FRAP data. However, common practice is to use the fewest terms required to achieve a reasonable fit to the data. There exist commonly accepted methods for determining goodness-of-fit, which can help assess when it is suitable to invoke additional terms-for example, by analyzing the residuals for single- versus double-exponential curve fits to each experimental dataset. In Fig. S5C, the authors report R-squared values (for double-exponential fits only), but R-squared is only valid for linear regression and should not be used to assess goodness-of-fit in nonlinear regression analysis.
3. The idea that centrosome clustering is required for PIDDosome activation is intriguing, but has not been tested rigorously enough to support strong claims made in the manuscript (e.g. the heading of this section in Results is "Extra centrosomes generate clusters necessary for PIDDosome activation"). The issue is that nocodazole treatment could block PIDDosome activation for any number of reasons not directly related to centrosome clustering. The first and most obvious reason could be disrupted localization of microtubule-associated proteins due to depolymerization of microtubules. Furthermore, treatment with microtubule poisons is well known to affect the structure, composition, and activities of centrioles/centrosomes (for example, see Vorobjev, et al., Membr Cell Biol 2000; Farina, et al. Nat Cell Biol 2016; Kuriyama J Cell Sci 1982; Cavazza, et al., Mol Biol Cell 2016; Le Clech PLoS One 2008). Even though PIDD1 localization appears preserved upon nocodazole treatment, this readout does not indicate competency for PIDDosome activation for reasons described above in point #2a. Therefore, the authors should tone down their conclusions and acknowledge these caveats, or they must perform additional experiments using orthogonal methods to interfere with centrosome clustering. One possible approach could be to use PJ-34, a PARP inhibitor that has been shown to affect interphase centrosome clustering (Castiel, et al. BMC Cancer 2011; Pannu, et al., Cell Death Dis 2014).
4. Data shown in Figure 2 seem ancillary to the overall message of the paper and could be moved to the supplement. For Fig. 2B-C, the authors should explain why ANKRD26 KO is plotted separately from the other conditions, and in the case of panel C, with a different y-axis range.

We thank you and the Referees for the very positive assessment of our work and for the constructive contributions. As you will see, during the revisions we have substantially strengthened our manuscript. To your three key points:

1) we increased the number of replicates, performed quantitative assessments of our data and utilized the appropriate statistical tools, thereby providing compelling support to our findings;
2) we have thoroughly validated the functional relevance of the PIDD1-ANKRD26 interaction originally found by yeast-two-hybrid by exploiting genetic complementation of non-transformed *ANKRD26*$^{-/-}$ human cells with ANKRD26 variants (-/+ PIDD1-interacting domain);
3) aiming to provide more mechanistic insight into the centrosome's capacity to trigger PIDDosome activation, we now reveal that physiologically elevating PIDD1-levels (by promoting p53 activation) also results into ANKRD26-dependent PIDDosome activation. Strikingly, as this stimulus bypasses the requirement for supernumerary centrosomes, this datum lends further support to the model that the centrosome acts as PIDD1 centralizer. In fact, different means to augment PIDD1 local concentration (i.e. proximity of two PIDD1-positive centrosomes following cytokinesis failure or increased overall PIDD1 levels after p53 activation) invariably rely on the scaffolding function of centrosomal ANKRD26 for PIDDosome activation.

Taken together, by addressing all key reviewer's points, we have not only improved the soundness of our work, but we also broadened the scope of our manuscript by demonstrating that the centrosome can also shape the DNA damage response via the ANKRD26-PIDDosome axis even when present in a single copy per cell.
Thus, we hope that our work will now match the standards for publication of the EMBO Journal. Please find our point-by-point rebuttal below.

Referee #1:

Dear editor,

In the presented manuscript, entitled 'Centrosome number surveillance requires ANKRD26-mediated recruitment of PIDD1 to distal appendages', the authors describe the requirement of distal appendages and ANKRD26 for the recruitment of PIDD1 to centrosomes, as a mechanism to monitor centrosome number. This manuscript builds on the authors' previous finding that the PIDDosome is activated in response to supernumerary centrosomes.
Here, using several knockout cell lines, the authors nicely elucidate which distal appendage proteins are required for the localization of PIDD1 to the centrosome, and continue to show that PIDD1-localization to the distal appendage is required for PIDDosome activation in response to supernumerary centrosomes. They show that PIDD1 localization to the distal appendage is dispensable for PIDD1 autocleavage, yet they show that the recruitment of PIDD1 to DA's, as well as the autocleavage of PIDD1 (specifically the formation of PIDD1-cc) are both required for PIDDosome activity. Next, the authors show that PIDD1 is removed from the centrosome during mitosis and re-localizes in an asymmetrical manner during telophase. However, when cells become binucleated, resulting in the confinement of both centrosomes in a single cell, both centrosomes recruit PIDD1 in G1. Finally, it is shown that the clustering of the extra centromeres after mitosis is required for activation of the PIDDosome.
Overall, this represents an elegant piece of work and a well written manuscript. However, the authors' findings, especially in figures 4-7, raise questions regarding the exact mechanism of the sensing of supernumerary centromeres and PIDDosome activation. In my view, the manuscript would benefit from addressing these questions. In summary, I am supportive of publication in EMBO Journal, provided the authors address the questions mentioned below.

Reply: We thank this reviewer for acknowledging the relevance of our work, for the constructive criticism and for precisely suggesting further experiments that significantly broadened the scope of our manuscript.

Referee #1, major comment 1: *Figure 3A shows that CEP83, SCLT1, ANKRD28 and PIDD1 itself are required for PIDDosome activity following binucleation. An important control that is lacking is whether the induction of binucleation and centrosome number is*

the same in every KO background. If KO cells proliferate with different speeds, dissimilar amounts of cells will reach binucleation in such assays. This is an important control to exclude that the observed reductions in PIDDosome activation are due to indirect effects on centrosome duplication or binucleation efficiency. For this, it would be required to analyze the percentage of cells that are binucleated and in addition, count the number of centrosomes in these cells.

Reply: We thank this reviewer for pointing out the lack of this control. We induced cytokinesis failure with DHCB for three times in all relevant KO cell lines, now including visual scoring of binucleation and centrosome abundance for all replicates. The newly acquired data (presented in Fig. 3B-D) completely rule out the possibility that a lack of PIDDosome activation is due to a decrease in the rate at which cells become binucleated or acquire supernumerary centrosomes.

Referee #1, major comment 2: *Is PIDDosome-mediated Caspase-2 activation in the DAP-deficient cell lines also perturbed when different activation stimuli are imposed; i.e. DNA damage. This would shed light on the potential role of the centrosome in PIDDosome activation in response to other stimuli.*

Reply: Yes, PIDDosome-mediated CASP2 activation is completely abolished in DAP-deficient cell lines when the DNA damaging agent camptothecin is used to activate the PIDDosome. We also assessed the presence of supernumerary centrosomes in the same experimental conditions, realizing that, to our surprise, cells did not induce supernumerary centrosomes in DNA damaging conditions leading to PIDDosome activation, revised Fig. 8A-B and EV5B-C.

To effectively integrate these data in the manuscript, we also addressed the contribution of p53 to the abovementioned phenomena (note that PIDD1 is a known p53 target (Lin et al, 2000), thus, it seemed plausible that PIDD1 transactivation could concur to the process). Conceivably, non-genotoxic p53 activation triggered by the MDM2 inhibitor Nutlin-3a was sufficient to transcriptionally elevate PIDD1 mRNA levels independently of DA integrity. Strikingly, however, Nutlin-3a triggered PIDDosome activation only in wild type cells, while *TP53* and relevant DAP KO cells were unable to activate the PIDDosome (Fig. 8C-D). Taken together, these data suggest that the concomitant elevation of PIDD1 protein levels and its localization at DAs are necessary to trigger PIDDosome activation in the absence of extra centrosomes.

Collectively, we provide an entire additional figure (Fig. 8) with important implications on a general role of the centrosome-PIDDosome axis in shaping the p53 response, not only in response to supernumerary centrosomes but also upon p53 activation within the “classical” DNA damage response. The PIDDosome contribution in the latter context has been also emphasized in a paper recently published by the Lahav lab (Tsabar et al, 2020). Thus, our new data clearly involve the centrosome in shaping the p53 response via the PIDDosome. In a mechanistic perspective, the same figure also provides further support to the notion that the centrosome activates the PIDDosome by augmenting the local concentration of the PIDD1 active species. These aspects have now been extensively discussed and incorporated into the final model (Fig. 8E).

Referee #1, major comment 3: *It is still unclear why the recruitment of PIDD1 to centrosomes is required while autoproteolysis seems to be independent of this recruitment. The authors suggest it might be the local priming of autoproteolytic PIDD1 fragments for PIDDosome activation. To test at which level PIDDosome activation is in fact perturbed, the authors could test if PIDDosome complex formation still occurs in the*

ANKRD26KO by performing Immunoprecipitation experiments or by size exclusion chromatography.

Reply: We have attempted to infer PIDDosome assembly in parental cells using both endogenous Co-IP and proximity ligation assays, while we did not have access to a refrigerated FPLC enabling size exclusion chromatography of lysates containing an active PIDDosome. Unfortunately, our attempts with the few available antibodies proved inconclusive so far, as we were not able to detect PIDDosome assembly in WT cells treated with cytokinesis inhibitors. In order to avoid a significant delay in resubmission of our manuscript, we opted for discussing the possibility that the lack of PIDD1 localization, as alternative to preventing PIDDosome assembly, might also lead to the formation of an inactive PIDDosome (page 17).

Referee #1, major comment 4: *In telophase, PIDD1 initially appears on only 1 parental centriole (in 1 daughter cell, Fig. 6A). This should be accurately quantified, as it raises some interesting questions. How long is this asymmetric recruitment normally sustained throughout G1?*

Reply: We have now accurately quantified the localization of PIDD1 at the two (parent and non-parent) centrioles throughout the cell cycle. From our quantification we can conclude that an asymmetry in PIDD1 recruitment to the two centrioles is maintained throughout the cell cycle. The asymmetry is however less pronounced during late telophase (Fig. 6A-B), as suggested by our previous non-quantitative analysis.

And how does cytokinesis failure affect these dynamics (and does the addition of nocodazole/preventing clustering affect this)? The authors could investigate this by filming the GFP-tagged version of PIDD1. Furthermore, the authors show that PIDD1 levels are higher in cells treated with DHCB in Fig. S5E. How does nocodazole treatment affect these values? These findings could contribute to the understanding of how clustered centrosomes can induce PIDD1osome activity, in contrast to non-clustered centrosomes.

Reply: While we agree that time lapse analysis of fluorescently tagged PIDD1 could elegantly answer this question, due to technical difficulties to acquire PIDD1 fluorescence concomitantly to a reference marker allowing to independently track the centrosomes in 4D, we opted for a simpler IF-based assessment, in continuity with the same experimental approach used above. Our analysis demonstrates that nocodazole treatment (with a concentration sufficient to fully de-cluster centrosomes and to prevent PIDDosome activation) does not affect PIDD1 fluorescence intensity at the centrosome (Appendix Fig. S6C).
Of note, while our original Fig. S5E reported a slight increase of PIDD1 fluorescence at the centrosome in response to DHCB, a more thorough analysis performed across a variety of assays and experimental conditions (Fig. 6D, EV5A, EV5D, Appendix Fig. S6C) has revealed that PIDD1 levels at individual centrioles do not vary in PIDDosome activating conditions. We have corrected the text accordingly.

An important control that is missing in the nocodazole experiments is to assess the effect of nocodazole addition after centrosomes have clustered. This to exclude that the PIDDosome inactivity in Figure 7D is due to direct effects of nocodazole on PIDDosome activation.

Reply: Provided that our most reliable readout for PIDDosome activation is the appearance of N-terminal MDM2 cleavage fragments by immunoblotting, a protein species with a >4h half-life (not shown), we argue that the proposed experiment could be inconclusive regarding a potential direct impact of nocodazole on PIDDosome activation unless protracted for long times, potentially confounding the interpretation of the data.

To address this point, we exploited the notion that DNA damage triggered DA-dependent PIDDosome activation. Briefly, we exposed cells to camptothecin in the absence/presence of the same dose of nocodazole used in the old Fig. 7D (1 µM). Strikingly, nocodazole had no measurable impact on PIDDosome activation (Appendix Fig. S6B), suggesting that neither an altered structure of the centriole nor a negative influence on PIDDosome activation caused by nocodazole account for the lack of PIDDosome activation presented in the old Fig. 7D (now Fig. 7E).

Referee #1, minor comment 1: *On page 9 the authors state: "Thus, PIDDosome formation is preceded by PIDD1 recruitment to DAs and the two events can be uncoupled by the L828E mutation. Whether PIDD1 localization is a prerequisite for autoproteolysis, however, remains to be answered." Does the fact that the CEP83, ANKRD26 and SCLT1 KO cell lines still display high levels of autoproteolysis (Fig S4) not proof that recruitment to the DA's is in fact not a prerequisite for autoproteolysis?*

Reply: Yes, we agree. We have removed the sentence. The data concerning the L828E mutant are now shown in Fig. EV4A-C, in support of the FRAP data, see next comment.

Referee #1, minor comment 2: *Why is the FRAP done with L828 mutant? Why not with WT PIDD1?*

Reply: We noticed that ectopic expression of PIDD1, albeit performed in *PIDD1* KO cells, leads to unscheduled PIDDosome activation in some individual RPE1 cells (used in the FRAP analysis). Thus, the L828E mutant (not proficient for PIDDosome formation) enabled us to study PIDD1 turnover at the centrosome while uncoupling it from PIDDosome assembly (i.e. we studied the upstream events in isolation). We have now added one extra sentence in the main text to motivate our choice (page 12).

Referee #1, minor comment 3: *The FRAP analysis shows slightly lower turnover of PIDD1 L828E after cytokinesis failure. The authors conclude that this could potentially contribute to the activation of the PIDDosome but they do not propose a model/hypothesis for this. How could this mechanistically work and how could this relatively minor difference in turnover result in such fast activation of the PIDDosome in response to the inheritance of too many centrosomes?*

Reply: We thank this reviewer for raising this concern, in line with comments raised by other referees (see Referee #2 point 6 and Referee #3 point 2). Given the minor difference observed when perturbing the number of centrosomes, we decided to present this experiment in Fig. EV4D-E and to discuss this phenomenon as something unlikely to account for PIDDosome assembly.

Referee #1, minor comment 4: *The model in Fig 7E shows a line between the centrosome and the CC fragment of PIDD1. However, there is no evidence provided that there is such a direct link. In fact, PIDD1-c and PIDD1-cc still form in lack of PIDD1-localization to the DA. The arrow should thus derive from the full length PIDD1. Similarly,*

in the right panel, the arrow to the CC fragment should derive from PIDD1 and not from the centrosomes directly. The authors should adjust the model accordingly.

Reply: We have now modified the model (Fig. 8E), also incorporating elements deriving from the new data shown in revised Fig. 8.

Referee #1, minor comment 5: *The Hoechst staining in Fig 7 C looks very odd. The authors could show a better representative image.*

Reply: We have now replaced the image originally shown in Fig. 7C with a high-quality image (revised Fig. 7F).

Referee #1, minor comment 6: *Fig. 1A What are the bottom panels of each figure? Why can we not observe 9 structures in those panels?*

Reply: For the sake of clarity, we modified Fig. 1A and 1E, providing two different STED acquisitions (top and side view of mature centrioles) for every staining.

Referee #1, minor comment 7: *Page 5. The authors use Cas9 to interfere with protein functions. Cas9 results in full gene KO's so the authors should use the word deletion instead of depletion.*

Reply: Considering that the production of frameshifting INDELs within the coding sequence of any protein might still allow translation initiation downstream of the target site or produce gene products partially retaining functionality via alternative splicing, we have decided to refer to our CRISPR derivatives using the more cautious “knock-out” rather than “deletion”.

Also, western blots showing lack of the protein are only shown for some knockouts. This should also be done for the other KO cell lines.

Reply: We have now included in Appendix Fig. S1 a systematic characterization of all the loss of function CRISPR derivatives presented in this study. As suitable antibodies for Western blot were not available for all proteins of interest, sequencing of the corresponding genomic DNA was also performed (Appendix Fig. S1).

Referee #1, minor comment 8: *As the authors draw strong conclusions about figures 6A and B, PIDD1 centrosome localization should be quantified.*

Reply: We have now accurately quantified the PIDD1 signal across the cell cycle and in the presence/absence of DHCB (revised Fig. 6A-D).

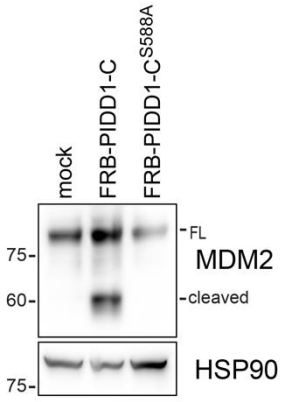
Referee #1, minor comment 9: *There are no statistical analyses performed on any of the quantifications shown. These should be added to support the authors' conclusions.*

Reply: We have now performed quantitative/statistical analyses of all imaging-based experiments shown in our manuscript.

Referee #1, minor comment 10: *PIDD1 autocleavage occurs in absence of centrosome localization (Fig. S4A). However, in Figure 7 the authors show that PIDDosome activation requires supernumerary clustered centrosomes. I agree with the authors that this suggests*

the presence of an additional que for PIDDosome activity. Would forcing a high local concentration of PIDD1 to another cellular structure lead to PIDDosome activation (in the presence or absence of supernumerary centrosomes). This will address whether there are additional signals originating from clustered supernumerary centrosomes, or if a high local concentration of PIDD1 sufficient for PIDDosome activation. In line with this question, what is the fraction of endogenous PIDD1 that localizes to the centrosome (normally and following clustering)? We realize that the points raised here (#10) represent a rather large effort. As these experiments would significantly clarify the mechanism of PIDDosome activation, we highly recommend the authors to address these issues, but we feel the manuscript can also be published in its absence.

Reply: We thank this reviewer for suggesting an elegant experiment with great potential. We attempted cloning PIDD1-C (either capable of autoproteolytically generate PIDD1-CC or carrying the S588A mutation to prevent auto-cleavage) as fusion with FRB in a lentiviral backbone. It seemed plausible to exploit this tool to dock a PIDD1-CC artificial precursor to distinct subcellular localizations in a chemically inducible manner (relying on co-expression with a FKBP-tagged decoy protein in the presence of rapamycin). Unfortunately, the sole ectopic expression of this construct was sufficient to trigger PIDDosome activation (Rebuttal Fig. 1). Tuning our system to the appropriate expression levels to carry out the desired experiment could indeed represent a large effort.



Rebuttal Figure 1. A549 *PIDD1*-KO cells were either left untransduced (mock) or transduced with lentiviral vectors expressing an FRB-PIDD1-C fusion (either wild type or S588A).

Nonetheless, we feel the fact that the non-genotoxic elevation of PIDD1 levels using Nutlin-3a (revised Fig. 8) leads to DAP-dependent PIDDosome activation provides further support to the notion that additional signals originating from clustered extra centrosomes are dispensable for PIDDosome activation.

Referee #2:

The presence of amplified centrosomes is commonly observed in cancer. However, as shown by several labs, extra centrosomes are poorly tolerated by normal cells. This is in part due to the activation of p53 pathway downstream of centrosome amplification, limiting the proliferation of cells with extra centrosomes. The mechanisms leading to p53 stabilisation in response to extra centrosomes have remained elusive, and only recent work have begun to shed some light into this problem. In particular, work from L. Fava suggested that activation of the PIDDosome in cells with extra centrosomes led to MDM2 cleavage and p53 stabilisation, providing some evidence for a role of the PIDDosome in this process.

Here, the authors go further into the mechanism of PIDDosome recruitment to the centrosomes and activation in cells with extra centrosomes. They use superresolution microscopy to demonstrated that PIDD1 does localise to the distal appendages (DA) of the mother centriole. PIDD1 is recruited to the DA via ANKRD26 and its loss leads to failure in activating PIDDosome in cells with extra centrosomes. Just like ANKRD26, PIDD1 does not seem to be required for cilia formation, suggesting a function for DA outside ciliogenesis. Moreover, the authors found that PIDDosome autoproteolysis is important for p53 stabilisation in cells with extra centrosomes. Furthermore, centrosome clustering in G1 is essential for PIDDosome activation, favouring the authors current model that PIDD1 associated with different Das from extra mother centrioles is important to activate the complex. Although how this happens is still unclear.

Overall, the data presented and experiments conducted are of high quality and clear. The findings, in line with the last author previous publication, go beyond what was currently known and adds more mechanistic insight. While the findings presented here will be of interest to the readership of EMBO, it will be important for the authors to address the comments below prior to publication.

Reply: We thank this reviewer for the fair assessment and for positioning our work into the broader context. By addressing all comments below, we believe that we have now significantly improved our manuscript.

Referee #2, comment 1: *My main criticism is regarding the model presented in Fig7E. the author proposed, as before, that the presence of extra mother centrioles is require to elicit PIDDosome activation. Here the authors show that disruption of clustering of extra centrosomes upon cytokinesis failure prevents MDM2 cleavage downstream of extra centrosomes. While this suggests that close proximity between mother centrioles may be required the question of how this happens remains unanswered. How the authors explain the model that PIDD1 needs to be in different mother centrioles to elicit proteolysis and PIDDosome activation? Why can't that be achieved with PIDD1 molecules in the same centriole? Is there a minimum distance required for these molecules to form a complex? I do not imagine that the mother centrioles are that close to each other that molecules from different DAs will interact?*

Reply: In light of a specific request of Referee #1, major comment 2 (i.e. testing the contribution of DAs to PIDDosome activation in response to genotoxic stress), we now reveal that the PIDDosome is activated in a centrosome-dependent fashion also in experimental conditions that lead to p53-dependent *PIDD1* transactivation. In this latter condition, however, the presence of supernumerary centrosomes was not required to trigger PIDDosome activation (Fig. 8B, Fig. EV5C and EV5F).

On this basis, we have revised and extended our model, suggesting that DAs work as centralizer of PIDD1: an increase in the local concentration of PIDD1 and autoproteolytic fragments thereof represent the cue leading to PIDDosome activation. This can be achieved either by elevating PIDD1 levels (e.g. in a p53-dependent manner) or by promoting the proximity of two mature centrosomes. Collectively, our data suggest that a critical PIDD1 local concentration threshold must be surpassed to achieve PIDDosome activation, without necessarily relying on physical contacts between PIDD1 species associated with distinct centrosomes.

Referee #2, comment 2: *According to the author's model and data in Fig7D showing that centrosome clustering is required for PIDDosome activation, the prediction is that cells after noc treatment would not remain arrested in G1. Did the authors check that? It seems to me this validation would be important to demonstrate the clustering requirement to promote efficient G1 arrest.*

Reply: Yes, we agree, and we thank this reviewer for raising this important point. The experimental design that we have exploited to de-cluster centrosomes in a telophase specific fashion was not suited to interrogate subsequent cell-cycle progression: RPE1 cells display a robust G1 arrest following nocodazole shake-off and release (not shown). Likely, this PIDDosome-independent phenotype relies on 53BP1:USP28 as a result of spending >90 min in mitosis (Uetake & Sluder, 2010; Meitinger et al, 2016; Lambrus et al, 2016; Fong et al, 2016).

To address this point, we have titrated nocodazole in conditions leading to cell cycle arrest in A549 cells, namely the concomitant inhibition of Aurora B kinase (to ensure cell division failure) and Mps1 kinase (to ensure the rapid mitotic traverse irrespectively of the presence/absence of nocodazole). Clearly, the same nocodazole concentration promoting effective de-clustering of extra centrosomes led to the override of the cell cycle arrest following cell division failure (Fig. 7A-C). Taken together, our data demonstrate that centrosome de-clustering conditions are equally effective in abrogating PIDDosome activation and PIDDosome-dependent cell cycle arrest, as expected.

Referee #2, comment 3: *The authors need to show the efficiency of KO for all cell lines. Centriole localisation is not sufficiently quantitative to demonstrate KO. Can the authors perform western blot analyses?*

Reply: We thank this reviewer for pinpointing the lack of a systematic characterization of all the KO derivatives presented in our manuscripts. We have now included in Appendix Fig. S1 a systematic characterization of all the cell lines utilized. As suitable antibodies for Western blot were not available for all proteins of interest, sequencing of the corresponding genomic DNA was also performed (Appendix Fig. S1).

Referee #2, comment 4: *Localisation studies are usually not a reliable way to infer direct interaction. Do the authors have evidence of interaction between ANKRD26 and PIDD1 in human cells?*

Reply: While the yeast-two-hybrid interaction shown in the original manuscript was already strongly suggesting a direct interaction between PIDD1 and ANKRD26, we now present a thorough validation of this result. Firstly, we have performed a follow up yeast-two-hybrid study to pinpoint the minimal ANKRD26 region sufficient to bind to PIDD1 in this assay, thereby defining residues 911-1181 as the PIDD1 Minimal Interaction Domain (PMID). Secondly, we show that while the PMID of ANKRD26 is not sufficient to localize to DAs in *ANKRD26* KO cells, ANKRD26-ΔPMID localized to DAs in a manner comparable to ANKRD26-WT. Thirdly, while exogenously expressed ANKRD26 was able to restore the ability of endogenous PIDD1 to localize to DAs in *ANKRD26* KO cells, exogenously expressed ANKRD26-ΔPMID failed to do so (revised Fig. 2). Lastly, the ability of PIDD1 to localize to DAs across the abovementioned conditions invariably correlated with the ability of the cells to activate the PIDDosome in response to supernumerary centrosomes (revised Fig. 3F). Taken together, our new results demonstrate that a direct ANKRD26-PIDD1 interaction recruits PIDD1 to DAs, thereby enabling PIDDosome activation. We

have now changed the running title of our manuscript with “ANKRD26 is the PIDD1 centrosome receptor”.

Referee #2, comment 5: *On FigS5D it is difficult to see binucleation, which is expected if this cell is in G1. Otherwise it will be difficult to distinguish it from a 2N cells in G2, which would also have 2 centrosomes. Is it a matter of imaging?*

Reply: In order to address a specific request of Referee #1 (major comment 4), the experiment originally shown Fig. S5D is now presented in revised Fig. 6C. Importantly, the experiment has been repeated and a new high-quality image allowing to better appreciate both binucleation and the centrosome status upon DHCB treatment has been selected.

Referee #2, comment 6: *The authors propose that centrosome amplification leads to a very small decrease in PIDD1 turnover at the centrosomes. However, it is unclear if this is significant? What about in cells where centrosome clustering was prevented for example? It would also be interesting to know what happens if more extra centrosomes are induced (e.g. PLK4 OE or repeated cytokinesis failure). If it is a matter of numbers, does that lead to increase stabilisation of PIDD2 at the centrosomes?*

Reply: As also Referee #1 and #3 agreed that the minor difference in the PIDD1 turnover at the centrosome observed in cells bearing normal vs abnormal centrosome number is unlikely to account for PIDDosome assembly, we have now decided to move this datum to Expanded View Fig. 4D-E (as suggested by Referee #3). Provided that elevating PIDD1 levels from its endogenous locus by activating p53 is sufficient to trigger DA-dependent PIDDosome activation (revised Fig. 8C-D), it seems no longer necessary to invoke a centrosome-driven change in PIDD1 protein-protein interactions to account for PIDDosome assembly.

Referee #2, comment 7: *I do not think n=3 cells for the live cell imaging experiment presented in Fig6 is acceptable for publication.*

Reply: We agree, and we thank this reviewer for raising this point. We have now repeated the time lapse analysis imaging and quantified more cells. In the revised version of our manuscript we present n=10 cells and updated values for mean and standard deviation for the centrosome clustering time (revised Fig. 6F). Thereby, we have confirmed the conclusions originally based on n=3.

Referee #2, comment 8: *Page 2. The work from Sercin et al and Coelho et al did not demonstrate that extra centrosomes by themselves are sufficient to induce spontaneous tumours.*

Reply: We thank this reviewer for this comment. To better reflect the findings reported by Sercin and Coelho in our introduction, we have now rephrased our sentence in:
“Additionally, flies and mice engineered to carry supernumerary centrosomes display a higher incidence of spontaneous tumours (Basto et al, 2008; Levine et al, 2017), which is normally restrained by the activity of the p53 tumour suppressor (Serçin et al, 2016; Coelho et al, 2015), demonstrating the carcinogenic potential of this condition.”

Referee #3:

Burigotto and colleagues have investigated the underlying mechanisms triggering activation of the PIDDosome, one of several major pathways restraining cell cycle progression via p53. This manuscript follows up on recent work showing that the PIDDosome can be activated in response to supernumerary centrosomes, as occurs in various contexts including normal development of the liver, as well as in many human cancers. In the prior work, it was shown that the PIDDosome component, PIDD1, localizes to centrosomes (specifically, to "mother" centrioles); however, it has remained unclear how centrosome number is counted in the cell, and how this information is transduced into a biochemical signal for PIDDosome activation.

In the present study, the authors used CRISPR knockouts to demonstrate that PIDDosome activation requires the distal appendage structures found on "mother" centrioles, and that PIDD1 localization to distal appendages depends on centrosomal protein ANKRD26. Furthermore, a series of add-back experiments was performed in PIDD1 knockout cells to define the functional requirements of PIDD1 autoproteolytic processing for its centrosomal localization and for PIDDosome activation. Finally, an effort was made to address the mechanism of PIDDosome activation using FRAP experiments to assess PIDD1 dynamics at centrosomes, as well as timed disruption of microtubules to prevent supernumerary centrosome clustering.

Overall, the core findings of the paper define PIDD1 as a distal appendage protein whose localization at supernumerary mother centrioles is required for PIDDosome activation. This constitutes a significant advance on an important problem. However, one major issue is that there is a general lack of quantification throughout that should be addressed in order to consider these findings conclusive. In addition, some aspects of the paper, particularly on the mechanism of PIDDosome activation, suggest an interesting model but fall short of providing definitive evidence to support some of the authors' conclusions. Either additional experiments will be needed or the text should be edited to qualify some of the conclusions, as detailed in the specific comments below. Provided that these points are addressed, I would strongly support publication of this manuscript in EMBO Journal.

Reply: We thank this referee for acknowledging the relevance of the question we tackled, and the advance provided by our manuscript. We have now strengthened the manuscript by addressing all specific requests.

Referee #3, comment 1: *Many of the major conclusions of the paper lack supporting quantification and/or statistical analyses.*
a. For immunofluorescence assays, simply showing one or two example images is not sufficient to demonstrate the generality of the findings. In all cases, appropriate quantification (fluorescence intensity measurements) will need to be performed (at a minimum, images could be scored visually) and the number of cells analyzed has to be reported. This concerns Figures 1A, 1D, 3B, 3D, 4C, 4H, 5A, 5D, 5E, 6A, 6B, 7B and all similar data in the Supplement.

Reply: We have now performed fluorescence intensity measurements, distance measurements and counting based on visual scoring across biological replicates for the following panels:

IF Figure	Quantification in Fig.	Parameter assessed
1A	1D	co-localization

1C	1B	fluorescence intensity
1E	1D	co-localization
1F	1G	fluorescence intensity
2B	2C	fluorescence intensity
2E	2D	fluorescence intensity
3B	3C	nuclear numerosity
3B	3D	centrosomal numerosity
4C	4D	fluorescence intensity
4F	4E	fluorescence intensity
5D	S4C	fluorescence intensity
6A	6B	fluorescence intensity
6C	6D	fluorescence intensity
7F	S6D	parent centriole distance
EV1A	EV1B	presence / absence of cilia
EV1A	EV1E	ciliary length
EV2A	EV2B	fluorescence intensity
EV4B	EV4C	fluorescence intensity
S1C	S1D	fluorescence intensity
S2A	S2B	fluorescence intensity
S2C	S2D	fluorescence intensity
S6A	7A	parent centriole distance

b. In cases where quantification has been done, please perform appropriate statistical tests and report P-values to compare control and experimental conditions. This concerns Figures 1B, 1F, 2B, 2C, 5E, 7B and the Supplement.

Reply: We have now included appropriated statistical tests, as requested. Details of the statistical tests employed can be found in the Material and Methods section of the revised manuscript and in the relevant figure legend. Note that the exact p-values for each individual test are reported in Table EV1.

c. Please report the number of independent biological replicates that were performed for each Western blot (Figures 3A, 3C, 3E, 4B, 4F, 4G, 5B, 7D and Supplement).

Reply: We have now included the number of replicates performed for each Western Blot in the corresponding figure legend.

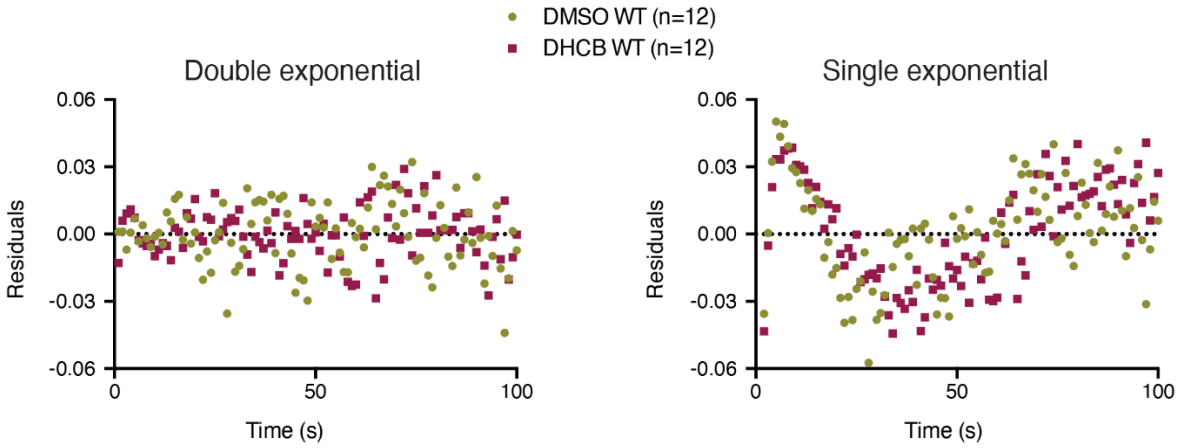
Referee #3, comment 2: *The relationship between PIDD1 centrosome localization and PIDDosome activation remains unclear and some conclusions of the paper should be qualified accordingly.*
a. Full-length PIDD1 is the only species the authors have been able to directly visualize at the centrosome, but this form of the protein is not competent for PIDDosome activation. On the other hand, autoproteolysis of PIDD1 into its active form (PIDD1-CC) is constitutive and does not require centrosome localization. Therefore, using FRAP to study the dynamics of full-length PIDD1 at the centrosome (Fig. 5E) does not clearly address the question of how the PIDDosome is activated-nor do the results indicate a compelling difference in PIDD1 dynamics. The data in Fig. 5E should be moved to the Supplement and the related conclusions in the main text should be qualified.

Reply: We thank this reviewer for raising this point. We agree that the FRAP per se does not explain how the PIDDosome is activated. Moreover, we recognize that the difference between the PIDD1 dynamic behaviour in the presence/absence of extra centrosomes is not compelling. Thus, we have moved the comparison to Expanded View 4D-E and partly amended the discussion relative to this datum, as suggested.

In light of the new data demonstrating that activating p53 is sufficient to trigger DA-dependent PIDDosome activation even in the absence of supernumerary centrosomes (see our reply to Referee #1, major point 2), we now postulate that the centrosome serves as a centralizer for PIDD1. Our new model suggests that in the vicinity of the centrosome a critical concentration of PIDD1-CC can be reached, enabling activation. This requires either the vicinity of two PIDD1-positive centrosomes or the overall elevation of PIDD1 levels. In this perspective, we suggest that the constitutively dynamic PIDD1 behavior we have observed by FRAP is likely a prerequisite enabling PIDDosome activation. In other words, in the absence of the observed dynamic behavior it would be impossible to explain how the recruitment of the PIDD1 precursor to the centrosome would enable PIDD1-CC activating the PIDDosome.

b. In general, a double-exponential will always provide a better fit than a single-exponential with this kind of FRAP data. However, common practice is to use the fewest terms required to achieve a reasonable fit to the data. There exist commonly accepted methods for determining goodness-of-fit, which can help assess when it is suitable to invoke additional terms-for example, by analyzing the residuals for single- versus double-exponential curve fits to each experimental dataset. In Fig. S5C, the authors report R-squared values (for double-exponential fits only), but R-squared is only valid for linear regression and should not be used to assess goodness-of-fit in nonlinear regression analysis.

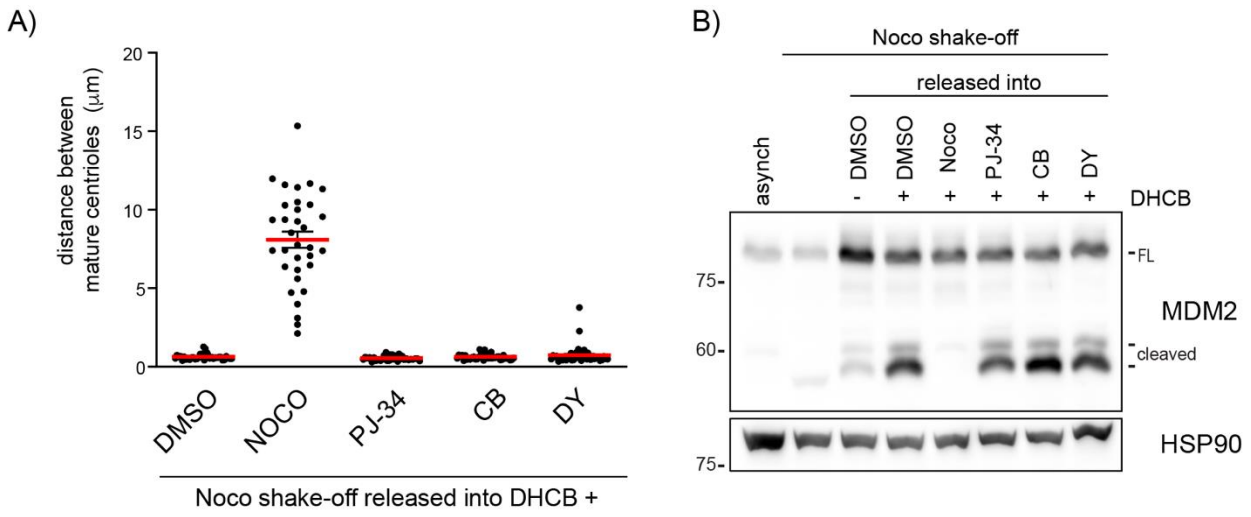
Reply: We agree with this reviewer that a model with more parameters would fit the data better, since having more inflection points will most likely minimise the sum-of squares. For this reason, given that the two models to compare are nested, the statistical choice between which model fits the data best has been operated using a statistical F-test and not by comparing the relative R-squared for the two possible models. We have now described this better in the Materials and Method section. In agreement with this, a comparison of the residuals' distributions for single versus double exponential clearly shows a non-random pattern for residuals of a single exponential. By contrast, residuals for a double exponential show no trend (Rebuttal Fig. 2). Together, this indicates that a double exponential model fits the dataset better than a single exponential. We also agree with the reviewer that, despite being commonly used, R-squared values are not an adequate measure of the goodness of fit for non-linear regressions. Thus, we removed this information from the summary table.



Rebuttal Figure 2. Residuals' distribution for double vs single exponential fitting.

Referee #3, comment 3: *The idea that centrosome clustering is required for PIDDosome activation is intriguing, but has not been tested rigorously enough to support strong claims made in the manuscript (e.g. the heading of this section in Results is "Extra centrosomes generate clusters necessary for PIDDosome activation"). The issue is that nocodazole treatment could block PIDDosome activation for any number of reasons not directly related to centrosome clustering. The first and most obvious reason could be disrupted localization of microtubule-associated proteins due to depolymerization of microtubules. Furthermore, treatment with microtubule poisons is well known to affect the structure, composition, and activities of centrioles/centrosomes (for example, see Vorobjev, et al., Membr Cell Biol 2000; Farina, et al. Nat Cell Biol 2016; Kuriyama J Cell Sci 1982; Cavazza, et al., Mol Biol Cell 2016; Le Clech PLoS One 2008). Even though PIDD1 localization appears preserved upon nocodazole treatment, this readout does not indicate competency for PIDDosome activation for reasons described above in point #2a. Therefore, the authors should tone down their conclusions and acknowledge these caveats, or they must perform additional experiments using orthogonal methods to interfere with centrosome clustering. One possible approach could be to use PJ-34, a PARP inhibitor that has been shown to affect interphase centrosome clustering (Castiel, et al. BMC Cancer 2011; Pannu, et al., Cell Death Dis 2014).*

Reply: We thank this reviewer for raising this concern. We followed the precise indication by attempting to achieve centrosome de-clustering with alternative pharmacological treatments. Unfortunately, neither the PARP1 inhibitor suggested, nor Ciliobrevin D or Dynarrestin were effective in achieving centrosome de-clustering in our hands (Rebuttal Fig. 3A). Unsurprisingly, PIDDosome activation induced by DHCB treatment occurred normally upon co-treatment with those drugs (Rebuttal Fig. 3B).



Rebuttal Figure 3. (A) RPE1 cells where subjected to synchronization as described in Fig. 7D and released in either DMSO or DHCB. Cells were then exposed to either nocodazole (1 μM), PJ-34 (25 μM), Ciliobrevin D (CB, 50 μM) or Dynarrestin (DY, 50 μM) during telophase to prevent centrosome clustering. Distance between mature centrioles in binucleated cells was measured in at least 30 cells. Data are shown as mean ± s.e.m. **(B)** Immunoblot of RPE1 cells treated as in (A).

At present, the only drug we found mimicking the effect of nocodazole (i.e. leading to centrosome de-clustering and compromising PIDDosome activation) is represented by griseofulvin (Rebacz et al, 2007), not shown. As griseofulvin impacts on microtubule nucleation as nocodazole, we deem this perturbation inappropriate to alleviate the concerns discussed here.

Importantly, however, we now show in Appendix Fig. S6B that nocodazole is not capable of preventing PIDDosome activation in response to camptothecin, a stimulus that promotes PIDDosome activation independently of the presence of extra centrosomes while still relying on PIDD1 localization to DAs (see our response to Referee #1, major comment 4 for a more detailed explanation). This demonstrates that nocodazole does not abrogate the centriole's competency for PIDDosome activation.

Finally, the new nocodazole titration shown in revised Fig. 7A-B demonstrates that the drug concentration sufficient to promote centrosome de-clustering corresponds to the concentration impacting on PIDDosome activation, lending additional support to our model. Thus, we have mildly softened our conclusions by including the following sentence (page 13):

"While the possibility that nocodazole perturbs PIDDosome activation independently of its de-clustering activity cannot be presently ruled out, our data demonstrate that the nocodazole effect shown here did not depend on direct PIDDosome inhibition or on altered centriolar competency to sustain PIDDosome activation."

Referee #3, comment 4: *Data shown in Figure 2 seem ancillary to the overall message of the paper and could be moved to the supplement. For Fig. 2B-C, the authors should explain why ANKRD26 KO is plotted separately from the other conditions, and in the case of panel C, with a different y-axis range.*

Reply: We agree with this reviewer on the marginal importance of the ciliogenesis data and in the revised manuscript and we moved them to Expanded View Fig. 1.

We have also pooled the datasets that were originally plotted separately within the same histogram (revised Fig. EV1B) and violin plot (revised Fig. EV1E).

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2nd Editorial Decision 16th Oct 2020

Thank you again for submitting your revised manuscript for our consideration. All three original referees have now reviewed it once more, and are generally satisfied with your revisions. Following additional minor textual revisions as requested by referee 1 (see below), we shall therefore be happy to accept this work for publication in The EMBO Journal.

During this final modification, please also address the following important editorial points.

REFEREE REPORTS

Referee #1:

In the revised manuscript Burigotto at al. have addressed most of the comments that were raised by the reviewers. The new data regarding PIDDosome activation in response to DNA damage are intruiging, and broaden the scope of the manuscript beyond the field of centrosome surveillance. Therefore, we are now supportive of publication in EMBO Journal. However, we strongly urge the authors to expand their comments regarding PIDD1 localization to centrioles during the cell cycle, as their quantification in figure 6B suggests active removal of PIDD1 from one of the two centrioles any time between telophase and G1-phase.

Referee #2:

The authors have addressed my main concerns in this revised version of their manuscript. I believe this work is now ready for it publication at EMBO Journal.

Referee #3:

The authors have done an outstanding job of responding to my criticisms in so far as was experimentally feasible. I support publication without delay.

Referee #1:

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Reply: We thank the reviewer for recognizing the value of the new experiments that have been incorporated in our revised manuscript and for supporting publication.
We realize that the bar chart presented in Fig. 6B might be misleading, due to a potential misperception of what “centriole 1” and “centriole 2” represent: while the two centrioles subjected to PIDD1 fluorescence measurement in telophase belonged to two distinct centrosomes, the two centrioles quantified in G1 cells belong to the same centrosome. In other words, both “centriole 1” and “centriole 2” of telophase cells are to be considered prospective “centriole 1s” of G1 daughter cells. To better reflect this experimental design, we marked with numbers (1 or 2) the centrioles subjected to quantification in the corresponding representative image in Fig. 6A. The figure legend and the main text describing this result have been also modified in tracking mode.

Accepted **4th Nov 2020**

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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: Luca L. Fava
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-104844R

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 iustified
- ➔ 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.

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?	No statistical approaches were used to define the sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No animals were used in this study.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For image-based quantifications presented in Figs. 1D, 1B, 1D, 1G, 2C, 2D, 3C, 3D, 4D, 4E, S4C, 6D, S6D, EV1B, EV1E, EV2B, EV4C, S1D, S2B, S2D, 7A exclusively interphase cells have been considered. In Fig. 6B each cell cycle phase (including mitosis) has been plotted separately (i.e. no exclusion critera).
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.	All samples were treated equally and quantified in a similar manner. Repeats of key experiments were performed by independent authors.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes, details on the used statistical tests are reported in the "Materials and Methods" section, as well as in every figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Before performing any analysis, the normality of each dataset was checked by performing a Shapiro-Wilk test. According to this, relevant parametric or non parametric tests were applied as described in the "Materials and Methods" section.

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Is there an estimate of variation within each group of data?	Yes, standard error of the mean, standard deviation or 95% confidence interval of the median are reported in every graph. The chosen estimate of variation is clearly pinpointed in the M&M or legend.
Is the variance similar between the groups that are being statistically compared?	No statistical tests were applied to compare the variance.

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).	We describe all antibodies used in the "Materials and Methods" section including catalogue number, clone number if monoclonal and dilution.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Done

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

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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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	All raw data presented in this study are available upon request.
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).	not relevant
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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.	N/A