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ANKRD26 recruits PIDD1 to centriolar distal appendages to activate the PIDDosome following centrosome amplification

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1st Editorial Decision 22nd Apr 2020

Thank you again for submitting your manuscript on PIDD1 recruitment/activation by ANKRD26. It has now been assessed by three expert referees, in light of whose overall supportive comments we would be happy to consider this study further for EMBO Journal publication. As you will see from the reports copied below, there are however several important experimental points that would need to be satisfactorily addressed prior to publication, mainly related to experimental setup, controls, knock-outs and cell lines. In addition, the reviewers indicate that the proposed model for how extra centrosomes cause PIDDosome activation remains somewhat unclear and would benefit from some follow-up analyses, along the lines suggested in the reports.

REFEREE REPORTS

Referee #1:

Review report Evans/Holland

In the manuscript entitled: "ANKRD26 recruits PIDD1 to distal appendages to activate the PIDDosome following centrosome amplification" presented by Evans et al., the authors set out to unravel the mechanisms that causes cells with supernumerary centrosomes to cease proliferation. In order to identify how cells respond to extra centrosomes the authors perform genome-wide CRISPR knock-out screens in cells in which they can induce extra centrosomes through PLK4

overexpression. To simultaneously allow the identification of genes required for centrosome duplication the screen was performed in the background of TRIM37 or USP28 knock-out. The authors identify several genes and continue to validate whether these genes allow cell growth with extra centrosomes through competition assays. Then, the authors address whether the hits of unknown function are required for PIDD1 centrosome localization, and confirm that several hits are important for this. Using knock out cells, the authors show that PIDDosome activation in response to extra centrosomes requires ANKRD26, and through elegant deletion mutants the authors identify the coiled-coil region of ANKRD26 to interact with the c-terminus (c or cc) of PIDD1. Importantly, the authors show that although ANKRD26□CC itself localizes to the centrosome, it fails to recruit PIDD1 to the centrosome. Consistently, they show that the ANKRD26-cc is required for PIDDosome activation in response to supernumerary centrosomes and hence prevents the proliferation of cells with extra centrosomes. Thus, these data establish PIDD1 centrosome recruitment by ANKRD26 as a crucial event in the response to supernumerary centrosomes.
In general, the manuscript is nicely written, the data are presented pleasantly, and the experiments support the conclusions drawn by the authors. However, in my opinion, there are some potential pitfalls in the experimental design, that need to be addressed. Nonetheless, the data presented here further our understanding of the mechanism required for centrosome quantity surveillance. Therefore, I am supportive of publication in EMBO Journal provided the authors address the major concerns listed below.

Major comments

1. The authors use many different KO cell lines throughout their manuscript. However, it seems that these cell lines are polyclonal (cells are infected with sgRNAs, followed by 2 days selection). The generation of KO cell lines through such an approach will include cells that are a) infected yet not successfully genome edited or b) harbor in frame deletions that do not disrupt gene function. In my view, this is visible in figure EV4B, where 25% of ANKRD26 "knock-out"-cells retain ANKRD26 localization on the centrosome. Therefore, I have some concerns that should be resolved:
• First, it is of utmost importance to show the success of this approach by showing the efficiency of each guide. The authors should apply TIDE (https://tide.deskgen.com) for each guide to show the genome editing efficiency and the spectrum of indels in each respective polyclonal cell line, or alternatively, derive a monoclonal cell line with a well characterized inactivating mutation.
• Second, although the screen is done in Plk4dox cell lines that lack TRIM37 or USP28 (to allow for growth when centrosomes numbers are reduced, and thus allow for the identification of hits that are involved in centrosome duplication/stability), the control experiments to check whether hits have a role in these processes are performed in PLK4dox cell lines with a WT background (Fig 1 G,H). Since these cell lines are polyclonal knock-out for the respective hits, it is likely that when centriole assembly is affected, true KO cells will die, while unedited cells do not. Therefore, cells that are quantified might represent the cells that were heterozygous KO's or WT. This might explain why some hits could not be confirmed (while using the same gRNAs as used in the screen) and might overlook a role in centriole assembly. There are several solutions to this. Most favorably, these experiments (Fig 1 G,H) should be repeated in cells that have the TRIM37 or USP28 deletion in the background. Alternatively, KO levels or guide efficiency should be determined before and after the activation of PLK4 to show that representative KO cells are quantified and there is no selection bias for unedited alleles or specific in-frame deletions. Finally, the authors could generate KO cell lines with a similar approach for some of the known centriole assembly genes to serve as a positive control.

2. To show that recruitment of PIDD1 to the centrioles is only involved in activation of the PIDDosome in response to supernumerary centrosomes, they perform an experiment where they

inflict DNA damage by Etoposide (Fig EV3). They show that Caspase 2 is still cleaved, even in ANKRD26 KO cells. However, there are alternative ways by which CASP2 is cleaved/activated as PIDD1 KO mice are still capable of Caspase 2 processing and activation (Kim et al. 2009, Apoptosis). To exclude that the results the authors observe in figure EV3 are explained by a PIDDosome-independent activation of Caspase-2, they should repeat this experiment in a PIDD1 KO background.
3. Figure 4A, B are likely done in the absence of extra centrosomes (they use HEK293T cells but these assays are not clearly described in the material and methods). These assays should be repeated or done side by side in cells where PLK4 is overexpressed as that is the situation where PIDD gets activated. This might lead to important insights into the mechanism by which extra centrosomes activate the PIDDosome.
4. Even though the authors show that there are no distal appendages in ANKRD26 KO cells, the fact that different PIDD1 truncations bind to mCherry-ANKRD26 does not mean it does so at the centrosome. For this, an IF experiment needs to be performed to identify whether or not the truncations indeed localize to the centrosome.
5. Finally, based on Fig 4. the authors speculate (on page 15) that PIDD1-n prevents association of PIDD1-c with ANKRD26. This should be addressed directly addressed through Co-IPs in cells co-expressing PIDD1-n and PIDD1-c.

Minor points:
1. Figure 1D: HYLS1 is colored in blue in the graph while it is annotated as a 'response gene' which are depicted in green.
2. Figure 1F: Color legend is missing (shown in 1G).
3. Figure 2F: There are 6 replicates shown for ANKRD26 but only 3 for the WT. Did the authors not take a WT reference sample along in the 3 extra ANKRD26 experiments?
4. For Figures 2G, 3A and EV1C, can the authors show some representative example images?
5. On page 10 the authors state: " Previous work has established a hierarchy for centriole distal appendage assembly ... and finally ANKRD26". A reference to this previous work should be inserted.
6. On page 13 the authors state: "in contrast to TP53 loss, knockout of ANKRD26 did not prevent PIDDosome activation in cells that experienced DNA damage". I think this should say: "similar to TP53 loss,...". Or they mean expression of p21 and should adjust accordingly.
7. Figure 4A, B, the authors should show a longer exposure of this western blot. It is hard to observe if there is no, or maybe minimal binding of the CC-fragment.
8. Typo on page 14: "The 850-1320 amino acids. region of ANKRD26...". Remove . after acids
9. On page 18 and 19 there are some formatting inconsistencies regarding the references (not EMBO style; numerical versus text).
10. The first sentence of the abstract "Centriole number is maintained by once per cycle replication followed by the segregation of these organelles into the daughter cells during division." might benefit from rephrasing.
11. The authors state that H2O2 treated ANKRD26 KO cells entered senescence to similar levels as control cells (EV3C). These experiments measure proliferation over 24 hours post drug treatment and are no measure for senescence. This statement should be amended accordingly.

Referee #2:

Centrosome amplification is commonly observed in cancer. However, extra centrosomes are poorly tolerated by normal cells in part due to the activation of p53 pathway. Until recently, the

mechanisms leading to p53 stabilisation in response to extra centrosomes were elusive, and only recent work have begun to shed some light into this problem. In mammalian cells, two main mechanisms have been proposed to activate p53 downstream of centrosome amplification: Hippo pathways via LATS2 and the PIDDosome, suggesting that multiple mechanisms could exist to prevent the proliferation of cells with extra centrosomes.

In this manuscript, Evans et al performed an unbiased CRISPR-Cas9 screen to identify proteins required for the proliferation of cells with extra centrosomes. In this screen they identified several proteins, including components of the previously reported PIDDosome complex and several others that upon KO promoted the proliferation of cells with extra centrosomes. Some of these, C2CD3, SCLT1 and ANKRD26 localise to centriole distal appendages (DA) suggesting they may be involved in the recruitment of PIDD1 and PIDDosome activation. Indeed, the authors demonstrate that loss of these proteins impair PIDD1 recruitment and PIDDosome activation in response to centrosome amplification. Moreover, they found that ANKRD26 binds and recruits PIDD1 to the DA. This mechanism seems to be specific for centrosome amplification since the authors demonstrate that DNA damage induced p53 stabilisation is independent of ANKRD26. A mutation in ANKRD26 was observed in some cancers and it abrogates the ability of ANKRD26 to recruit PIDD1 to the DA and to activate PIDDosome. Thus, the authors' identified a mechanism by which PIDD1 is recruited to the centriole to activate the PIDDosome.

Overall, the work presented here is well controlled, clear and conclusive. The authors provide significant mechanistic detail on how PIDD1 triggers p53 stabilisation in response to centrosome amplification. In my opinion, this work will be of interest to the EMBO readership. There are however few comments that authors should address prior to publication.

#1. The main criticism I have with this work is that it is unclear how extra centrosomes promote PIDDosome activation. The published model suggests that PIDD1 in one mother centriole might interact with PIDD1 in a second mother centriole, as the authors also refer to. But, while extra centrosomes are usually clustered, how close are the mothers? Are they close enough to make this model a possibility? The authors suggest that this might be the case but it is unclear the distance proteins have to be for this to happen in vivo?

#2. In FigEV2C the effect of ANKRD26-/- on the proliferation of tetraploid cells seems rather subtle. Did the authors looked at this in PIDD1-/- KO cells as well? Or RAID-/- cells? Is it possible that tetraploid cells might have other impairments?

#3. The authors should provide evidence of efficient KO for the cell lines used here (western blotting would be the most appropriate).

#4. It was unclear to me if the tumours that contain the ANKRD26 mutation also possessed p53 mutation?

#. Clarify y axis in graphs that only say "fraction of cells" to make it easier for readers.

#. Page 14 - 1st paragraph: there is an extra . : "...amino acids. region..."

Referee #4:

In this manuscript Evans et al. perform a genome-wide CRISPR screen to identify factors required for arresting the cell cycle in untransformed RPE1 cells when centrioles become amplified. This process has previously been shown to rely on the activation of the PIDDosome, but the mechanism linking centriole amplification to PIDDosome activation is unclear. The authors show that several components of the centriole distal appendages are required for this activation, and that ANKRD26 normally recruits PIDD1 to the centriole distal appendages. This recruitment is required to allow extra centrioles to activate the PIDDosome and so trigger the TP53-dependent response that arrests cells with amplified centrioles.

The data is generally well presented and of high quality. This is a relatively straightforward story that will be of considerable interest to the centrosome and cell-senescence/cell cycle fields and so I strongly support publication in The EMBO J. As described below, I only have one significant issue that I hope the authors might address prior to publication, and a few other relatively minor points.

Major points:

(1) I was surprised that the authors did not try to assess the rate of turnover of ANKRD26 and PIDD1 at the distal appendages, and whether this rate changes when centrioles are amplified. This information would help in assessing their model, which is actually quite complicated and is described slightly superficially. ANKRD26 seems to recruit PIDD1 to all centrioles all of the time, so it is unclear how the presence of a few extra centrioles is sufficient to trigger a robust cell cycle arrest. The authors briefly discuss two possibilities: (1) that extra centrioles titrate out a limiting distal appendage component that normally keeps PIDD1 in an inactive conformation; (2) that clustering of the extra centrioles brings about a conformational change in PIDD1 that leads to PIDDosome activation. I was surprised that the authors didn't consider the idea that centrioles may constantly generate low-levels of active PIDD1, but this is below a threshold level required to activate the PIDDosome; the presence of extra centrioles simply helps push this level above threshold (perhaps explaining why cell cycle arrest in these cells is often gradual and stochastic). The ability of PIDD1 to quickly turn-over at centrioles (either all the time, or perhaps in response to centriole amplification) is central to all of these models, so some information about PIDD1 (and ANKRD26) dynamics would be very helpful.

Minor Points:

1. I may have missed it, but the authors should state whether they believe (or have done any experiments to address) whether FOPNL, a major hit in their screen, works via the same mechanism.

2. I think it would be useful to have the authors thoughts on the relevant functions of the other hits they identified in their screen (Figure 1E,F)-perhaps in a Table with links to any relevant references. I realise many of these will be obvious to experts, but this will be useful for non-experts, and there were some genes here that I did not immediately recognise.

3. The authors point out that the ANKRD26 mutation they highlight in certain cancer cells would only be expected to potentially give a growth advantage to cancer cells that retain an active p53 and that have amplified centrosomes. Do they know if this is the case for the patients that carry these mutations?

4. The authors claim that supernumary mature centrioles are often oriented with their distal appendages clustered in interphase, but they only show one image as evidence (FigEV1B). This is

potentially an important observation, so it should be properly documented.

5. The graphs in Figure EV4B,C lack statistics.

6. The referencing in the Discussion is in a different format to the rest of the paper.

Referee #1:

Review report Evans/Holland

In the manuscript entitled: "ANKRD26 recruits PIDD1 to distal appendages to activate the PIDDosome following centrosome amplification" presented by Evans et al., the authors set out to unravel the mechanisms that causes cells with supernumerary centrosomes to cease proliferation.

In order to identify how cells respond to extra centrosomes the authors perform genome-wide CRISPR knock-out screens in cells in which they can induce extra centrosomes through PLK4 overexpression. To simultaneously allow the identification of genes required for centrosome duplication the screen was performed in the background of TRIM37 or USP28 knock-out. The authors identify several genes and continue to validate whether these genes allow cell growth with extra centrosomes through competition assays. Then, the authors address whether the hits of unknown function are required for PIDD1 centrosome localization, and confirm that several hits are important for this. Using knock out cells, the authors show that PIDDosome activation in response to extra centrosomes requires ANKRD26, and through elegant deletion mutants the authors identify the coiled-coil region of ANKRD26 to interact with the c-terminus (c or cc) of PIDD1. Importantly, the authors show that although ANKRD26ΔCC itself localizes to the centrosome, it fails to recruit PIDD1 to the centrosome. Consistently, they show that the ANKRD26-cc is required for PIDDosome activation in response to supernumerary centrosomes and hence prevents the proliferation of cells with extra centrosomes. Thus, these data establish PIDD1 centrosome recruitment by ANKRD26 as a crucial event in the response to supernumerary centrosomes.

In general, the manuscript is nicely written, the data are presented pleasantly, and the experiments support the conclusions drawn by the authors. However, in my opinion, there are some potential pitfalls in the experimental design, that need to be addressed. Nonetheless, the data presented here further our understanding of the mechanism required for centrosome quantity surveillance. Therefore, I am supportive of publication in EMBO Journal provided the authors address the major concerns listed below.

Major comments

1. The authors use many different KO cell lines throughout their manuscript. However, it seems that these cell lines are polyclonal (cells are infected with sgRNAs, followed by 2 days selection). The generation of KO cell lines through such an approach will include cells that are a) infected yet not successfully genome edited or b) harbor in frame deletions that do not disrupt gene function. In my view, this is visible in figure EV4B, where 25% of ANKRD26 "knock-out"-cells retain ANKRD26 localization on the centrosome. Therefore, I have some concerns that should be resolved:

• First, it is of utmost importance to show the success of this approach by showing the efficiency of each guide. The authors should apply TIDE (https://tide.deskgen.com) for

each guide to show the genome editing efficiency and the spectrum of indels in each respective polyclonal cell line, or alternatively, derive a monoclonal cell line with a well characterized inactivating mutation.

Based on the reviewer's suggestion, we performed TIDEseq for each of the sgRNAs used to validate the hits in PLK4^{Dox} cells. This new data shows the editing efficiency achieved with each sgRNA (**Figure 1G)**. Experiments in **Figure 2D** and **Figure 1G,H** were performed in polyclonal knockout cell lines. The other experiments in the paper were carried out using monoclonal knockout cell lines. These include the experiments in **Figure 2F,G**, and all the experiments in **Figure 3** and **Figure 4**. These monoclonal knockout cell lines were validated by sequencing genomic DNA to characterize frame-shift mutations and loss of signal by either immunoblotting (in the case of ANKRD26 – see **Figure 4C**) or immunofluorescence (in the case of C2CD3, SCLT1, CEP83 and FOPNL - see **Figure EV1F,G**). We have now made it clear in the figure legends which experiments make use of polyclonal or monoclonal knockout cell lines, and we have added the details for how knockout lines were validated to the Materials and Methods section.

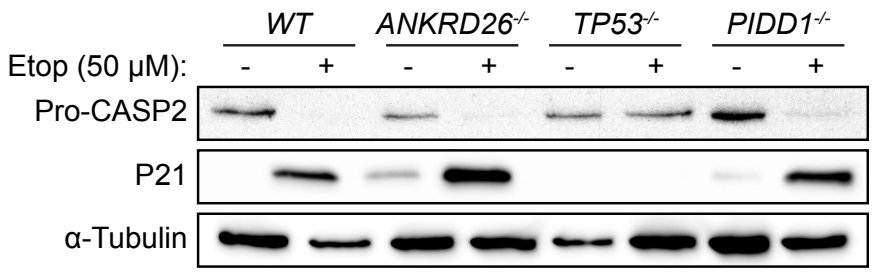
The reviewer is correct that the *ANKRD26*$^{-/-}$ cell line used in **Figure EV4B,C** had a weak ANKRD26 signal remaining at the centriole in some cells. Western blotting has shown that although this particular cell line has a strong decrease in ANKRD26 protein levels, it does have a small amount of ANKRD26 protein remaining. We apologize for not being clear about this in our original draft. In the revised version of the manuscript, we replaced this data with experiments performed in an *ANKRD26*$^{-/-}$ cell line that is a complete null for ANKRD26. As expected, we no longer observe residual ANKRD26 signal at the centriole in the *ANKRD26*$^{-/-}$ cells **(Figure EV4B,C)**.

• Second, although the screen is done in Plk4dox cell lines that lack TRIM37 or USP28 (to allow for growth when centrosomes numbers are reduced, and thus allow for the identification of hits that are involved in centrosome duplication/stability), the control experiments to check whether hits have a role in these processes are performed in PLK4dox cell lines with a WT background (Fig 1 G,H). Since these cell lines are polyclonal knock-out for the respective hits, it is likely that when centriole assembly is affected, true KO cells will die, while unedited cells do not. Therefore, cells that are quantified might represent the cells that were heterozygous KO's or WT. This might explain why some hits could not be confirmed (while using the same gRNAs as used in the screen) and might overlook a role in centriole assembly. There are several solutions to this. Most favorably, these experiments (Fig 1 G,H) should be repeated in cells that have the TRIM37 or USP28 deletion in the background. Alternatively, KO levels or guide efficiency should be determined before and after the activation of PLK4 to show that representative KO cells are quantified and there is no selection bias for unedited alleles or specific in-frame deletions. Finally, the authors could generate KO cell lines with a similar approach for some of the known centriole assembly genes to serve as a positive control.

Although we did not focus on proteins required for centriole assembly in this manuscript, it is plausible that some of the genes that failed to improve the proliferation of PLK4^{Dox} cells were hits in our screen because they lead to a failure of centriole duplication. To test this, we repeated the competition growth assays for all of the hits that did not have a known role in centriole duplication in the same PLK4^{Dox}; *TRIM37*$^{-/-}$ RPE1 cells used in the primary screen (**Figure EV1B, Table EV2)**. 15/16 sgRNAs tested increased the proliferation of the PLK4^{Dox}; *TRIM37*$^{-/-}$ cells. Only one sgRNA (NXT1) failed to show any increase in growth. Interestingly, a sgRNA targeting CEP350 that failed to increase the growth of the PLK4^{Dox} cells significantly increase the proliferation of PLK4^{Dox}; *TRIM37*$^{-/-}$ cells. This suggests that CEP350 emerged from our screen because of its requirement for centriole duplication/stability.

2. To show that recruitment of PIDD1 to the centrioles is only involved in activation of the PIDDosome in response to supernumerary centrosomes, they perform an experiment where they inflict DNA damage by Etoposide (Fig EV3). They show that Caspase 2 is still cleaved, even in ANKRD26 KO cells. However, there are alternative ways by which CASP2 is cleaved/activated as PIDD1 KO mice are still capable of Caspase 2 processing and activation (Kim et al. 2009, Apoptosis). To exclude that the results the authors observe in figure EV3 are explained by a PIDDosome-independent activation of Caspase-2, they should repeat this experiment in a PIDD1 KO background.

We thank the reviewer for raising this excellent point. To test whether activation of Caspase 2 in response to etoposide requires the PIDDosome, we monitored Caspase 2 cleavage in PIDD1 knockout cells treated with etoposide. As the review correctly deduced (and to our surprise), Caspase 2 cleavage still occurred in response to etoposide exposure even in the absence of PIDD1 **(Reviewer Figure 1)**. We contacted experts in the field to ask for their advice, and we were told that centriole amplification is the only stimulus where Caspase 2 activation unambiguously requires PIDD1. In the revised version of our manuscript, we have changed the text to reflect these new findings: we now conclude that ANKRD26 is not an obligate requirement for Caspase 2 (rather than PIDDosome) activation.

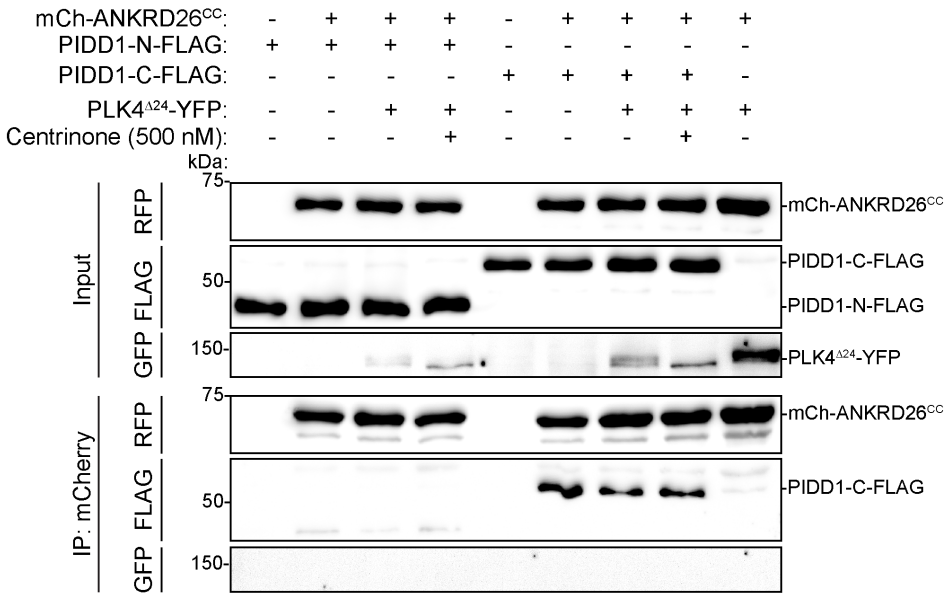


Reviewer Figure 1. Western blot showing expression of pro-CASP2 and P21 following treatment with etoposide. Experiments were performed in PLK4^{Dox} cells knocked out for the indicated genes. Pro-CASP2 processing still occurs in cells lacking the PIDDosome component PIDD1.

3. Figure 4A, B are likely done in the absence of extra centrosomes (they use HEK293T cells but these assays are not clearly described in the material and methods). These assays should be repeated or done side by side in cells where PLK4 is overexpressed as that is the situation where PIDD gets activated. This might lead to important insights into the mechanism by which extra centrosomes activate the PIDDosome.

The experiments in **Figure 4A,B** were carried out by transiently transfecting HEK293FT cells with the indicated expression constructs and then performing co-immunoprecipitation experiments. This has now been clarified in the Materials and Methods and Figure legend. Since ANKRD26^{CC} and PIDD1 are overexpressed in these experiments, the observed interactions are likely to be taking place in the cytosol and not at the centrosome. Unfortunately, we have been unable to immunoprecipitate sufficient endogenous ANKRD26 or PIDD1 to detect the proteins by western blotting.

To test if PLK4 directly influences the binding of PIDD1 to ANKRD26, we performed co-immunoprecipitation analysis of mCherry-ANKRD26^{CC} and PIDD1-N-FLAG or PIDD1-C-FLAG in the presence of absence of a stabilized version of PLK4 (PLK4$^{\Delta 24}$-YFP). Overexpression of PLK4 did not change the amount of PIDD1-C bound to ANKRD26^{CC}, suggesting this interaction is not directly regulated by PLK4 **(Reviewer Figure 2)**.



Reviewer Figure 2. HEK293FT cells were transfected with the indicated constructs, subjected to co- immunoprecipitation and immunoblotted with the indicated antibodies.

4. Even though the authors show that there are no distal appendages in ANKRD26 KO cells, the fact that different PIDD1 truncations bind to mCherry-ANKRD26 does not mean it does so at the centrosome. For this, an IF experiment needs to be performed to identify whether or not the truncations indeed localize to the centrosome.

To determine which region of PIDD1 is responsible for its recruitment to the centriole, we generated *PIDD1*$^{-/-}$ RPE1 cells stably expressing mCherry-PIDD1-N, or untagged full-length PIDD1, non-cleavable PIDD1, PIDD1-C, or PIDD1-CC. We evaluated the localization of full-length PIDD1, non-cleavable PIDD1, PIDD1-C, and PIDD1-CC using an antibody raised against the PIDD1 death domain. The PIDD1-N fragment lacked the death domain and was visualized using the mCherry tag. As expected, both full-length and non-cleavable PIDD1 localized to the mature parent centriole **(Figure EV4G,H)**. The PIDD1-C and PIDD1-CC fragments that are capable of binding to ANKRD26 were also recruited to the centriole, but at a diminished level compared with full-length PIDD1. By contrast, the PIDD1-N fragment that failed to interact with ANKRD26 showed no centriole localization **(Fig EV4G,H)**. Taken together, these data suggest that the PIDD1-CC fragment is responsible for binding to ANKRD26 and recruiting PIDD1 to the centriole.

5. Finally, based on Fig 4. the authors speculate (on page 15) that PIDD1-n prevents association of PIDD1-c with ANKRD26. This should be addressed directly addressed through Co-IPs in cells co-expressing PIDD1-n and PIDD1-c.

To test this hypothesis, we co-transfected PIDD1-N-MycGFP, mCherry-ANKRD26^{CC}, and PIDD1-C-FLAG into HEK293 cells. We then lysed cells and immunoprecipitated mCherry-ANKRD26^{CC} or PIDD1-N-MycGFP and analyzed the bound proteins by immunoblot. As previously reported, PIDD1-N-MycGFP interacted with PIDD1-C-FLAG. Notably, the interaction of PIDD1-C-FLAG with mCherry-ANKRD26^{CC} decreased when PIDD1-N-MycGFP was present. This is consistent with our proposal that PIDD1-C preferentially associates with PIDD1-N and suggests this binding inhibits the association of PIDD1-C with ANKRD26^{CC}. This data is shown in **Figure EV4F**.

Minor points:
1. Figure 1D: HYLS1 is colored in blue in the graph while it is annotated as a 'response gene' which are depicted in green.

The color of HYLS1 has been changed to green in **Figure 1D** to match the other centriole amplification response genes.

2. Figure 1F: Color legend is missing (shown in 1G).

The color legend was moved to **Figure 1F**

3. Figure 2F: There are 6 replicates shown for ANKRD26 but only 3 for the WT. Did the authors not take a WT reference sample along in the 3 extra ANKRD26 experiments?

We apologize for the error here. **Figure 2F** has been updated with all control samples for each experimental replicate.

4. For Figures 2G, 3A and EV1C, can the authors show some representative example images?

Representative images are now shown for each of the above quantifications in **Figure EV1H**, **Figure EV2A** and **Figure EV2C**.

5. On page 10 the authors state: " Previous work has established a hierarchy for centriole distal appendage assembly ... and finally ANKRD26". A reference to this previous work should be inserted.

We have added a reference to Bowler et al., 2019 and Tanos et al., 2013

6. On page 13 the authors state: "in contrast to TP53 loss, knockout of ANKRD26 did not prevent PIDDosome activation in cells that experienced DNA damage". I think this should say: "similar to TP53 loss,...". Or they mean expression of p21 and should adjust accordingly.

Thanks for pointing out this error. The sentence now reads: “While the loss *TP53* prevented P21 expression downstream of CASP2 activation, knockout of *ANKRD26* did not alter CASP2 activation or P21 induction in cells that experienced DNA damage”

7. Figure 4A, B, the authors should show a longer exposure of this western blot. It is hard to observe if there is no, or maybe minimal binding of the CC-fragment.

The FLAG-IP images in **Figure 4A,B** have been updated with a longer exposure. We didn’t detect any binding of the PIDD1-ΔCC-fragment even on the longest exposure.

8. Typo on page 14: "The 850-1320 amino acids. region of ANKRD26...". Remove . after acids

This error has been corrected.

9. On page 18 and 19 there are some formatting inconsistencies regarding the references (not EMBO style; numerical versus text).

Thanks for pointing out this error. The references have been changed to EMBO format.

10. The first sentence of the abstract "Centriole number is maintained by once per cycle replication followed by the segregation of these organelles into the daughter cells during division." might benefit from rephrasing.

The sentence has been changed to, “Centriole copy number is maintained by the once per cycle replication of these organelles.”

11. The authors state that H2O2 treated ANKRD26 KO cells entered senescence to similar levels as control cells (EV3C). These experiments measure proliferation over 24 hours post drug treatment and are no measure for senescence. This statement should be amended accordingly.

The reviewer makes a good point. The term “premature senescence” has been changed to “arrest”.

Referee #2:

Centrosome amplification is commonly observed in cancer. However, extra centrosomes are poorly tolerated by normal cells in part due to the activation of p53 pathway. Until recently, the mechanisms leading to p53 stabilisation in response to extra centrosomes were elusive, and only recent work have begun to shed some light into this problem. In mammalian cells, two main mechanisms have been proposed to activate p53 downstream of centrosome amplification: Hippo pathways via LATS2 and the PIDDosome, suggesting that multiple mechanisms could exist to prevent the proliferation of cells with extra centrosomes.

In this manuscript, Evans et al performed an unbiased CRISPR-Cas9 screen to identify proteins required for the proliferation of cells with extra centrosomes. In this screen they identified several proteins, including components of the previously reported PIDDosome complex and several others that upon KO promoted the proliferation of cells with extra centrosomes. Some of these, C2CD3, SCLT1 and ANKRD26 localise to centriole distal appendages (DA) suggesting they may be involved in the recruitment of PIDD1 and PIDDosome activation. Indeed, the authors demonstrate that loss of these proteins impair PIDD1 recruitment and PIDDosome activation in response to centrosome amplification. Moreover, they found that ANKRD26 binds and recruits PIDD1 to the DA. This mechanism seems to be specific for centrosome amplification since the authors demonstrate that DNA damage induced p53 stabilisation is independent of ANKRD26. A mutation in ANKRD26 was observed in some cancers and it abrogates the ability of ANKRD26 to recruit PIDD1 to the DA and to activate PIDDosome. Thus, the authors' identified a mechanism by which PIDD1 is recruited to the centriole to activate the PIDDosome.

Overall, the work presented here is well controlled, clear and conclusive. The authors provide significant mechanistic detail on how PIDD1 triggers p53 stabilisation in response to centrosome amplification. In my opinion, this work will be of interest to the EMBO readership. There are however few comments that authors should address prior to publication.

#1. The main criticism I have with this work is that it is unclear how extra centrosomes promote PIDDosome activation. The published model suggests that PIDD1 in one mother centriole might interact with PIDD1 in a second mother centriole, as the authors also refer to. But, while extra centrosomes are usually clustered, how close are the mothers? Are they close enough to make this model a possibility? The authors suggest that this might be the case but it is unclear the distance proteins have to be for this to happen in vivo?

To address this question, we measured the distance between the center of the two closest CEP164 rings in cycling (EdU+) and non-cycling (EdU-) cells with two or more mature parent centrioles. The average distance between the two closest mature parent centrioles in non-cycling cells was 579 nm **(Figure EV5B)**. The inner and outer diameter of PIDD1 at the centriole determined was 349 and 595 nm, respectively **(Figure 2C)**. Based on these measurements, we feel that it is feasible that the PIDD1 on adjacent mature parent centrioles could interact.

#2. In FigEV2C the effect of ANKRD26-/- on the proliferation of tetraploid cells seems rather subtle. Did the authors looked at this in PIDD1-/- KO cells as well? Or RAID-/- cells? Is it possible that tetraploid cells might have other impairments?

We repeated the cytokinesis failure assay using *PIDD1*$^{-/-}$, *CASP2*$^{-/-}$, *ANKRD26*$^{-/-}$, and *TP53*$^{-/-}$ cells. Knockout of *PIDD1*, *CASP2*, *ANKRD26,* and *TP53* increased the proliferation of tetraploid cells **(Figure EV2F)**. The less dramatic increase in growth in *ANKRD26*$^{-/-}$ cells observed in this assay compared to the competition growth assays shown elsewhere in the manuscript is likely explained by the difference in the duration of the two assays. The duration of the competition growth assay performed in cells with extra centrosomes is 5 days, whereas the duration of the cytokinesis failure assay is only 2 days. The longer duration in the competition growth assay allows for a larger difference between arrested WT cells and proliferating knockout cells. Alternatively, tetraploid cells may trigger other pathways to restrict cell proliferation that do not depend on ANKRD26 and PIDDosome activation. We now discuss both of these possibilities in the results section of the revised manuscript.

#3. The authors should provide evidence of efficient KO for the cell lines used here (western blotting would be the most appropriate).

Knockout cell lines were validated by sequencing genomic DNA to characterize the inactivating mutations and loss of signal by either immunoblotting (in the case of ANKRD26 – see **Figure 4C**) or immunofluorescence (in the case of C2CD3, SCLT1 and CEP83 - see **Figure EV1F**). We have added the details for how knockout lines were validated to the Materials and Methods section of the revised manuscript.

#4. It was unclear to me if the tumours that contain the ANKRD26 mutation also possessed p53 mutation?

This is an excellent point. To address this, we analyzed the tumors on the cBioPortal database with ANKRD26 K1234N mutation for any TP53 mutations and compiled the results in **Table EV3**. Of the 20 tumors containing K1234N mutations, 15% also contained an oncogenic TP53 alteration, and an additional 15% have a TP53 variant of unknown significance **(Table EV3)**. This fraction is lower than the overall frequency of TP53 alterations observed for each tumor subtype. However, since the numbers of tumors analyzed for each tumor subtype are small, no definitive conclusions can be drawn. We now discuss this in the results section of the revised manuscript.

#5. Clarify y axis in graphs that only say "fraction of cells" to make it easier for readers.

The y-axis labels in several figures have been changed to make interpretation easier.

#6. Page 14 - 1st paragraph: there is an extra . : "...amino acids. region..."

The error has been corrected.

Referee #4:

In this manuscript Evans et al. perform a genome-wide CRISPR screen to identify factors required for arresting the cell cycle in untransformed RPE1 cells when centrioles become amplified. This process has previously been shown to rely on the activation of the PIDDosome, but the mechanism linking centriole amplification to PIDDosome activation is unclear. The authors show that several components of the centriole distal appendages are required for this activation, and that ANKRD26 normally recruits PIDD1 to the centriole distal appendages. This recruitment is required to allow extra centrioles to activate the PIDDosome and so trigger the TP53-dependent response that arrests cells with amplified centrioles.

The data is generally well presented and of high quality. This is a relatively straightforward story that will be of considerable interest to the centrosome and cell-senescence/cell cycle fields and so I strongly support publication in The EMBO J. As described below, I only have one significant issue that I hope the authors might address prior to publication, and a few other relatively minor points.

Major points:

(1) I was surprised that the authors did not try to assess the rate of turnover of ANKRD26 and PIDD1 at the distal appendages, and whether this rate changes when centrioles are amplified. This information would help in assessing their model, which is actually quite complicated and is described slightly superficially. ANKRD26 seems to recruit PIDD1 to all centrioles all of the time, so it is unclear how the presence of a few extra centrioles is sufficient to trigger a robust cell cycle arrest. The authors briefly discuss two possibilities: (1) that extra centrioles titrate out a limiting distal appendage component that normally keeps PIDD1 in an inactive conformation; (2) that clustering of the extra centrioles brings about a conformational change in PIDD1 that leads to PIDDosome activation. I was surprised that the authors didn't consider the idea that centrioles may constantly generate low-levels of active PIDD1, but this is below a threshold level required to activate the PIDDosome; the presence of extra centrioles simply helps push this level above threshold (perhaps explaining why cell cycle arrest in these cells is often gradual and stochastic). The ability of PIDD1 to quickly turn-over at centrioles (either all the time, or perhaps in response to centriole amplification) is central to all of these models, so some information about PIDD1 (and ANKRD26) dynamics

would be very helpful.

We thank the reviewer for the thoughtful comments. We agree that the turnover of PIDD1 at the distal appendage is central to understanding how the PIDDosome is activated in cells with extra centrioles. To address this question, we set out to use Fluorescence Recovery After Photobleaching (FRAP) to monitor the turnover of a PIDD1 at the centriole of RPE1 cells. Unfortunately, this series of experiments proved to be more complicated than we anticipated.

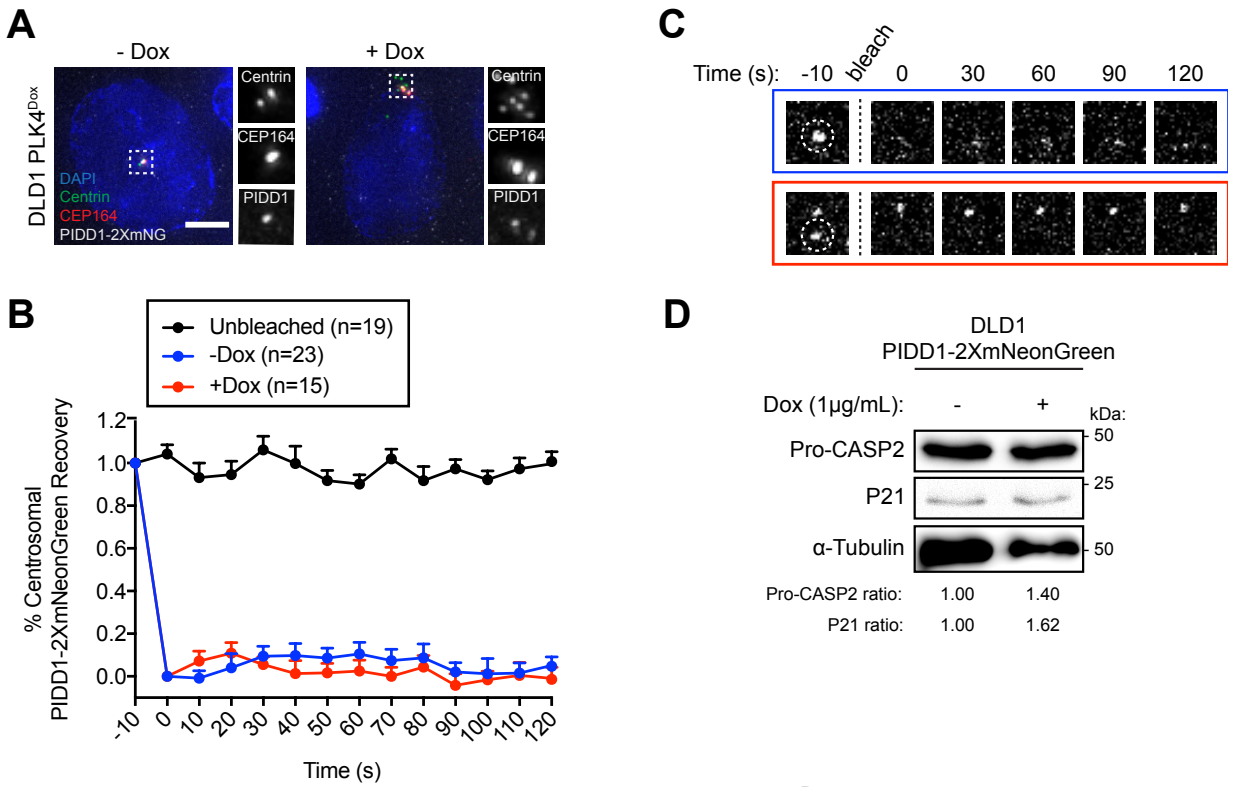
First, we integrated a PIDD1-mNeonGreen transgene into RPE1 cells. The transgene's high expression led to the formation of a large number of cytoplasmic foci and significantly impaired the proliferation of RPE1 cells. The cytoplasmic foci are likely to represent spontaneous PIDDosome assembly and made it difficult to identify the centriole-localized population of PIDD1.

To avoid PIDD1 overexpression problems, we chose to endogenously tag the C-terminus of the PIDD1 gene with a 2xmNeonGreen tag. Unfortunately, this targeting approach failed in RPE1 cells but was successful in the TP53 deficient human colon cancer cell line DLD-1. Endogenous PIDD1-mNeonGreen showed a highly specific localization to the centriole distal appendage in DLD-1 cells **(Reviewer Figure 3A)**. We therefore performed FRAP on the centriole-localized PIDD1-mNeonGreen. The DLD-1 cells we used also carried a doxycycline-inducible PLK4 transgene so that we could induce centrosome amplification. Surprisingly, we detected very little turnover of PIDD1-mNeonGreen, in cells with either one or multiple parent centrioles **(Reviewer Figure 3B,C)**. However, we later found out that DLD-1 cells also fail to activate the PIDDosome in response to centrosome amplification **(Reviewer Figure 3D)**. It is thus possible that the lack of PIDD1 turnover at the centriole distal appendage is the reason DLD-1 cells do not activate the PIDDosome in response to centrosome amplification. Alternatively, perhaps PIDD1 never turns over at the centriole distal appendage - a result that contradicts that observed by Luca Fava's lab using an overexpressed mutant of PIDD1-mNeonGreen that is defective in PIDDosome activation.

Since we cannot distinguish between these two possibilities at present, we have been unable to come to a conclusion on the turnover of PIDD1 and elected not to add this data to our paper. We acknowledge that defining the turnover of PIDD1 in RPE1 cells is a critical experiment. However, it will take several more months to engineer the RPE1 cell line needed for these experiments. COVID19 already significantly extended the timeframe of this revision, and an accompanying manuscript from the group of Luca Fava was resubmitted several weeks back. We hope the reviewer will understand that defining the turnover of PIDD1 now falls outside the timeframe of this revision.

We modified the Discussion to incorporate the model that the reviewer put forth. We state: 'Finally, it is plausible that a mature parent centriole produces a low-level of active PIDD1 that is below the threshold level required for PIDDosome assembly. The presence of extra mature centrioles may push the amount of active PIDD1 above the

threshold needed to trigger a cell cycle arrest. Identifying the turnover kinetics of endogenous PIDD1 at the centriole distal appendage is will be critical to test this model.



Reviewer Figure 3. (A) Representative images of PLK4^{Dox} PIDD1-mNeonGreen DLD1 cells treated with and without doxycycline for two days and immunostained with the indicated antibodies. Scale bar = 5 µM. (B) PIDD1-mNeonGreen centrosomal foci were photobleached, and fluorescence recovery was measured. The number of quantified photobleaching events is indicated. (C) Representative images from (B). Circle represents the area that was bleached. (D) Western blot showing expression of pro-CASP2 and P21 following treatment with doxycycline.

Minor Points:

1. I may have missed it, but the authors should state whether they believe (or have done any experiments to address) whether FOPNL, a major hit in their screen, works via the same mechanism.

FOPNL (also known as FOR20) has been characterized as a centriole satellite protein that plays a key role in ciliogenesis (Sedjai et al., 2010). To define the role of FOPNL in cells with extra centrosomes we created a monoclonal *FOPNL*$^{-/-}$ cell line. Our new data show that loss of FOPNL abolishes the recruitment of ANKRD26 and PIDD1 to the centriole distal appendage **(Figure 2G, Figure 3A, Figure EV2A)**. Therefore, FOPNL has a role in centriole distal appendage assembly and functions in the same pathway as the other genes identified in our study. This is now discussed in the Results section of the revised manuscript.

2. I think it would be useful to have the authors thoughts on the relevant functions of the other hits they identified in their screen (Figure 1E,F)-perhaps in a Table with links to any relevant references. I realise many of these will be obvious to experts, but this will be useful for non-experts, and there were some genes here that I did not immediately recognise.

Thanks for the helpful suggestion. We have organized the hits from the screen in a table that details each proteins' function and localization. Where appropriate, we also reference the first paper that identified the function of the protein in centriole assembly **(Table EV2)**.

3. The authors point out that the ANKRD26 mutation they highlight in certain cancer cells would only be expected to potentially give a growth advantage to cancer cells that retain an active p53 and that have amplified centrosomes. Do they know if this is the case for the patients that carry these mutations?

This is an excellent point. To address this, we analyzed the tumors on the cBioPortal database with ANKRD26 K1234N mutation for any TP53 mutations and compiled the results in **Table EV3**. Of the 20 tumors containing K1234N mutations, 15% also contained an oncogenic TP53 alteration, and an additional 15% have a TP53 variant of unknown significance **(Table EV3)**. This fraction is lower than the overall frequency of TP53 alterations observed for each tumor subtype. However, since the numbers of tumors analyzed for each tumor subtype are small, no definitive conclusions can be drawn. We now discuss this in the Results section of the revised manuscript.

4. The authors claim that supernumary mature centrioles are often oriented with their distal appendages clustered in interphase, but they only show one image as evidence (FigEV1B). This is potentially an important observation, so it should be properly documented.

To address this question, we measured the distance between the center of the two closest CEP164 rings in cycling (EdU+) and non-cycling (EdU-) cells with two or more mature parent centrioles. The average distance between two mature parent centrioles in non-cycling cells was 579 nm **(Figure EV5B)**. The inner and outer diameter of PIDD1 at the centriole determined from STORM images was 349 and 595 nm, respectively **(Figure 2C)**. Based on these measurements, we feel that it is feasible that the PIDD1 on adjacent mature parent centrioles could interact. Moreover, we observed that the average distance between the two closest mature parent centrioles is shorter in non-cycling (EdU-) cells compared with cycling (EdU+) cells **(Figure EV5B-C)**, but this difference did not reach statistical significance. This new data is presented in the Discussion when we discuss various models for how extra mature mother centrioles could trigger PIDDosome activation. Since we have not identified a way to prevent centriole clustering in interphase without perturbing other cell cycle functions, we cannot directly test whether the clustering of centriole distal appendages on adjacent mature parent centrioles is responsible for triggering the PIDDosome pathway.

5. The graphs in Figure EV4B,C lack statistics.

The *ANKRD26*$^{-/-}$ cell line used in **Figure EV4B,C** had weak ANKRD26 signal remaining at the centriole in some cells. Western blotting has shown that although this particular cell line has a strong decrease in ANKRD26 protein levels, it does have a small amount of ANKRD26 protein remaining. We apologize for not being clear about this in our original draft. In the revised version of the manuscript, we replaced this data with new experiments performed in an *ANKRD26*$^{-/-}$ cell line that is a complete null for ANKRD26. As expected, we no longer observe residual ANKRD26 signal remaining at the centriole in the *ANKRD26*$^{-/-}$ cells. The results in the *ANKRD26*$^{-/-}$ cells differ from those obtained previously in the hypomorphic ANKRD26 cell line: the new data reveal that the ANKRD26 N and C-terminal region are important for its centriole localization **(Figure EV4B,C)**. Nevertheless, as before, our new data also show that the 850-1320 amino acid region of ANKRD26 is required to recruit PIDD1 to the centriole distal appendage **(Figure EV4B,C)**. Statistics have been added to the new graphs, as requested.

6. The referencing in the Discussion is in a different format to the rest of the paper.

The references have all be changed to EMBO format.

2nd Editorial Decision 3rd Nov 2020

Thank you for submitting your revised manuscript on ANKRD26 in PIDDosome activation to our office. All three referees have now looked at it again, and generally found the study significantly improved and most concerns satisfactorily addressed. Nevertheless, referee 1 retains a several reservations regarding the answers to particular issues they had previously raised, as you will see from the comments copied below. Prior to publication, I would therefore like to ask you to clarify these remaining points in an exceptional round of further revision, by including additional explanations and discussion as well as (where needed) more control data. As proposed by referee 1, please also try to incorporate the Caspase-2 cleavage data (currently only shown to referees) in one of the main or EV figures.

During this additional revision round, please also take care of the following editorial points.

REFERE REPORTS

Referee #1:

Response to revisions Evans et al.

The revised manuscript by Evans and colleagues addresses most of the point raised. However, there are two main points that are not addressed sufficiently and require additional experiments and/or extensive discussion.

Major points
1. In our previous review report, we asked for the characterization of the KO efficiency in the polyclonal cell lines. The authors' reply to our question is incomplete and raises new questions.

a. Efficiency of some guides is really poor (p53, PIDD1, SCLT1, HYLS1, KIAA0753 have an efficiency below 25%). Some have an '#' indicating large/complex indels. What is this based on? How can this be extracted from TIDE analysis?
b. Can the authors quantify/display the out of frame deletions specifically instead of only showing the total INDEL frequency? This is important as some guides preferentially generate an in frame deletion and thus likely not result in a full KO.
c. Multiple guides have poor efficiency (and no '#') under which PIDD1, one of their most prominent hits (equally strong as its complex partners CASP2 and CRADD which have a very efficient INDEL generation). How do the authors explain this (please elaborate in text)?

2. In our initial report, we commented on the use of different genetic backgrounds for the screen and the validation experiments. This is important because in a WT background, cells with loss of centrosomes will stop growing in a TRIM37-dependent manner (and thus un-edited cells would enrich over time). Therefore, we predicted that all hits with a direct role in centrosome amplification would be overlooked, and therefore we asked to perform the validation experiment in the same cell line that was used for the screen (TRIM37 -/-). Indeed, their validation experiment in the TRIM37 -/- cells (new EV1B) shows that now all hits can be validated with the exception of 1 gene. This is in contrast with the number of hits that could be validated in the WT cells (4 hits were not significant in the WT background which are significant in the TRIM37 -/- cells). This shows that indeed these genes likely have role in centrosome amplification. While this is not the main focus of the paper, currently there is no discussion at all of these findings, and I think this requires at least some attention. In addition, the results in EV1B have implications for the analysis performed in figure 1H, as the experiment in 1H is performed in a WT background and cells that would have lost their centrosomes would have stopped growing. Therefore, the analysis of centrosome number should also be performed in a TRIM37 k.o. background. Finally, as it is unclear why the authors switched to

the WT background I would also suggest to move the validations in the TRIM37 -/- cells to the main text.

Minor points
1. In our original major point 2 we asked the authors to investigate the role of PIDD1 in CASP2 cleavage in response to DNA damage. The authors acknowledged the value of this point and have nicely addressed this question. However, they decided to leave out this figure for the manuscript. I would suggest to put this in the manuscript and not solely in the reviewer comments as this finding adds to our understanding of the role of the PIDDosome to Caspase 2 activation in response to other stimuli and further re-inforce that centrosome duplication is a process that solely depends on the PIDDosome while other p53 activating insults do not.
2. The authors conclude on their new figure EV4G that "these data suggest that the PIDD-CC fragment is responsible for the binding to ANKRD26 and recruiting PIDD1 to the centriole". However, the percentage of cells showing PIDD1 localization when expressing PIDD1-CC just slightly exceed those observed in the full KO (goes from ~10% to ~20%). Thus, recruitment of PIDD1-CC is still much lower as compared to the WT PIDD1 (~40%). Possibly, full-length PIDD1 is processed immediately as soon as it binds to the centriole, obstructing its detection. To overcome this, the authors could make deletion mutants in a non-cleavable variant. I understand for now this is maybe too much work but at least the authors should adjust their conclusions to make them align with the presented data, and discuss potential alternatives of to their own interpretation.
3. In our original report we raised the point that the interactions between ANKRD26CC and PIDD1-C are tested in cells lacking extra centrosomes. In their response, the authors now show that the interaction of overexpressed ANKRD26CC and PIDD1-C is not regulated by PLK4 by expressing a stabilized version of PLK4. While this could be addressing our issue, no controls for whether this indeed induced extra centrosomes in this specific setting is presented. Therefore, as is, this experiment does not address the issue whether or not the interaction between ANKRD26CC and PIDD1-C is affected by supernumerary centrosomes. Clearly, the interaction between ANKRD26 and PIDD1 seems required for pro-Casp2 cleavage. However, how this is affected by extra centrosomes remains elusive. A simple experiment could be done where the number of centrosomes is quantified in this setting but otherwise this experiment is not very telling. Again, since these data are only presented as a reviewer figure, the authors should point out the limitation of their system in their main text (i.e. that the experiment is performed in the absence of extra centrosomes).
4. Fig 1G typo: efficiency
5. EV4G/H: typo: non-clevable

Referee #2:

The authors fully addressed my main concerns in this reviewed version of their manuscript and I strongly support its publication in EMBO Journal.

Referee #4:

In their revised manuscript Evans et al. have done a nice job of addressing my concerns and so I strongly support publication in The EMBO J.

Referee #1:

Response to revisions Evans et al.

The revised manuscript by Evans and colleagues addresses most of the point raised. However, there are two main points that are not addressed sufficiently and require additional experiments and/or extensive discussion.

Major points
1. In our previous review report, we asked for the characterization of the KO efficiency in the polyclonal cell lines. The authors' reply to our question is incomplete and raises new questions.

a. Efficiency of some guides is really poor (p53, PIDD1, SCLT1, HYLS1, KIAA0753 have an efficiency below 25%). Some have an '#' indicating large/complex indels. What is this based on? How can this be extracted from TIDE analysis?

The referee previously suggested using TIDEseq to estimate the INDEL efficiency in the polyclonal $Plk4^{Dox}$ knockout cells used in Figure 1G,H. TIDESeq requires two parallel PCR reactions on a wildtype and knockout population of cells, followed by a pair of sequencing analyses. The two sequencing traces are then analyzed using a web-based software package (http://tide.nki.nl) to determine the frequency of INDELs in the polyclonal knockout population of cells. The reliability of TIDE depends on the purity of the PCR products and the quality of the sequence reads. We found that this varies depending on the genomic locus that is being amplified. A further limitation is that TIDEseq cannot accurately analyze sgRNAs that create large INDELs (>50bps) since they exceed the decomposition window's size for the sequence traces.

In light of the reviewer's comments, we re-analyzed all of our TIDEseq data and sequencing traces. For ten of the sgRNAs e analyzed, we were able to generate high-quality sequencing reads that resulted in a confident prediction of the INDEL frequency. However, five of the sgRNAs we analyzed (KIAA0753, STT3B, FOPNL, SCLT1, and TP53) generated sequencing reads in which the distance between the sequencing peaks was inconsistent. Although the INDEL region could clearly be identified in the sequencing traces, it was not possible to determine the relative abundance of nucleotides at each position in this region as the peaks did not always line up (see four examples below). Consequently, the TIDEseq analysis for these sgRNAs produced a low R^2 value, indicating the INDEL frequency is not accurately modeled and the TIDEseq analysis is unreliable. We could not resolve the issue with these particular genomic loci despite trying different primer combinations and using different sequencing primers. Therefore, we decided to only report the TIDEseq data for the ten sgRNAs with a reliable R^2 value of >0.5. In line with the reviewer's recommendation below, we also

modified the graph in Figure 1G to display the frequency of frameshift INDELs induced by each sgRNA.

b. Can the authors quantify/display the out of frame deletions specifically instead of only showing the total INDEL frequency? This is important as some guides preferentially generate an in frame deletion and thus likely not result in a full KO.

We performed the TIDEseq analysis again for all sgRNAs and modified the graph in Figure 1G to display the frequency of frameshift INDELs created by each sgRNA. In this analysis, INDELs in the +3, +6, +9, -3, -6, -9 reading frames were excluded from the final calculation.

c. Multiple guides have poor efficiency (and no '#') under which PIDD1, one of their most prominent hits (equally strong as its complex partners CASP2 and CRADD which have a very efficient INDEL generation). How do the authors explain this (please elaborate in text)?

The purpose of generating the polyclonal Plk4Dox knockout cells was to quickly validate the major hits that emerged from our genome-wide screen. The sgRNAs selected to validate these hits were the top-scoring sgRNAs that emerged from our genome-wide screen. As anticipated, the efficiency of INDEL generation for these sgRNAs varies depending on the locus. Two sgRNA in particular had relatively low frameshifting INDEL frequencies: PIDD1 and HYLS1. However, in the genome-wide screen, cells knocked out for target genes are positively selected for over three weeks of growth. Thus, even inefficient sgRNAs can become significantly enriched in the population. As with any CRISPR screen, ineffective sgRNAs may have led to false negatives.

Given the limitation of working with populations of cells with different knockout efficiencies, we created monoclonal knockout populations for all of the hits that we determined were involved in arresting the proliferation of cells with extra centrosomes. These monoclonal knockout cell lines were validated by sequencing genomic DNA to characterize frame-shift mutations and signal loss by either immunoblotting or immunofluorescence. For ANKRD26, we also rescued the knockout phenotype by the reintroduction of a sgRNA-resistant transgene. Monoclonal knockout populations of PIDD1, CRADD, and CASP2 behaved similarly and failed to activate the PIDDosome and arrest proliferation in response to centrosome amplification.

2. In our initial report, we commented on the use of different genetic backgrounds for the screen and the validation experiments. This is important because in a WT background, cells with loss of centrosomes will stop growing in a TRIM37-dependent manner (and thus un-edited cells would enrich over time). Therefore, we predicted that all hits with a direct role in centrosome amplification would be overlooked, and therefore we asked to perform the validation experiment in the same cell line that was used for the screen (TRIM37 -/-). Indeed, their validation experiment in the TRIM37 -/- cells (new EV1B) shows that now all hits can be validated with the exception of 1 gene. This is in contrast with the number of hits that could be validated in the WT cells (4 hits were not significant in the WT background which are significant in the TRIM37 -/- cells). This shows that indeed these genes likely have role in centrosome amplification. While this is not the main focus of the paper, currently there is no discussion at all of these findings, and I think this requires at least some attention. In addition, the results in EV1B have implications for the analysis performed in figure 1H, as the experiment in 1H is performed in a WT background and cells that would have lost their centrosomes would have stopped growing. Therefore, the analysis of centrosome number should also be performed in a TRIM37 k.o. background. Finally, as it is unclear why the authors switched to the WT background I would also suggest to move the validations in the TRIM37 -/- cells to the main text.

The analysis we performed of the top hits from our screen in Figure 1G, H offers an efficient means of identifying genes that function to arrest the proliferation of cells with extra centrosomes. Following the reviewer's suggestion, we repeated our analysis to determine if hits that failed to score in Plk4^{Dox} cells would have scored positive in the Plk4^{Dox}; *TRIM37*$^{-/-}$ genetic background used in our screen. This new analysis identified four hits that were not significant in Plk4^{Dox} cells but were significant in Plk4^{Dox}; *TRIM37*

$^{/-}$ cells. We agree that these four hits could represent proteins required for centrosome duplication (and thus centrosomes amplification). However, we feel that further analysis of hits required for centrosome duplication falls outside our manuscript's scope, which is focused on how cells arrest in response to centrosome amplification. In addition, sgRNAs targeting all four of these hits increased the proliferation of Plk4^{Dox} cells with extra centrosomes. Therefore, it is possible that these four proteins represent weak hits that only have a modest impact on the growth of cells with extra centrosomes and thus, do not reach statistical significance.

The knockout of TRIM37 leads to the generation of abnormal centriole protein assemblies (Balestra et al., BioRxiv, 2020: Balestra et al., Dev Cell, 2013). Such assemblies could complicate the analysis of how cells respond to centrosome amplification. Thus, we feel it is most useful to examine this response in Plk4^{Dox} cells where centrosome amplification can be induced in the presence of wildtype TRIM37.

Minor points

1. In our original major point 2 we asked the authors to investigate the role of PIDD1 in CASP2 cleavage in response to DNA damage. The authors acknowledged the value of this point and have nicely addressed this question. However, they decided to leave out this figure for the manuscript. I would suggest to put this in the manuscript and not solely in the reviewer comments as this finding adds to our understanding of the role of the PIDDosome to Caspase 2 activation in response to other stimuli and further re-inforce that centrosome duplication is a process that solely depends on the PIDDosome while other p53 activating insults do not.

Thanks for the helpful suggestion. As recommended, we have now moved this data to Figure EV3C and describe the data in the Results section of our manuscript. We state: 'Notably, CASP2 processing still occurred in *PIDD1*$^{-/-}$ cells treated with etoposide, suggesting that etoposide-induced CASP2 activation is independent of the PIDDosome **(Figure EV3C)**.'

2. The authors conclude on their new figure EV4G that "these data suggest that the PIDD-CC fragment is responsible for the binding to ANKRD26 and recruiting PIDD1 to the centriole". However, the percentage of cells showing PIDD1 localization when expressing PIDD1-CC just slightly exceed those observed in the full KO (goes from ~10% to ~20%). Thus, recruitment of PIDD1-CC is still much lower as compared to the WT PIDD1 (~40%). Possibly, full-length PIDD1 is processed immediately as soon as it binds to the centriole, obstructing its detection. To overcome this, the authors could make deletion mutants in a non-cleavable variant. I understand for now this is maybe too much work but at least the authors should adjust their conclusions to make them align with the presented data, and discuss potential alternatives of to their own interpretation.

The fragments of PIDD1 used in Figure EV4G represent the fragments of PIDD1 that are produced following PIDD1 self-cleavage. Therefore, it is not possible to generate a non-cleavable PIDD1-N or PIDD1-CC fragment since the fragments themselves lack

auto-cleavage sites. We agree that the recruitment of the PIDD1-CC fragment is much lower than that of WT PIDD1 and feel that this is adequately reflected in our discussion of this data. In the Results, we state: 'The PIDD1-C and PIDD1-CC fragments that are capable of binding to ANKRD26 were also recruited to the centriole, but at a significantly diminished level compared with full-length PIDD1.'

3. In our original report we raised the point that the interactions between ANKRD26CC and PIDD1-C are tested in cells lacking extra centrosomes. In their response, the authors now show that the interaction of overexpressed ANKRD26CC and PIDD1-C is not regulated by PLK4 by expressing a stabilized version of PLK4. While this could be addressing our issue, no controls for whether this indeed induced extra centrosomes in this specific setting is presented. Therefore, as is, this experiment does not address the issue whether or not the interaction between ANKRD26CC and PIDD1-C is affected by supernumerary centrosomes. Clearly, the interaction between ANKRD26 and PIDD1 seems required for pro-Casp2 cleavage. However, how this is affected by extra centrosomes remains elusive. A simple experiment could be done where the number of centrosomes is quantified in this setting but otherwise this experiment is not very telling. Again, since these data are only presented as a reviewer figure, the authors should point out the limitation of their system in their main text (i.e. that the experiment is performed in the absence of extra centrosomes).

Following the reviewer's recommendation, we now mention in the Results section of our manuscript that our binding experiments were performed by overexpression constructs in cells with a normal centrosome number. In the Results, we state: 'To establish if this coiled-coil region is responsible for binding to PIDD1, we overexpressed in cells with a normal centrosome content mCherry-ANKRD26 coiled-coil (mCherry-ANKRD26^{CC}) and wild-type PIDD1-FLAG.'

4. Fig 1G typo: efficiency

This has been corrected.

5. EV4G/H: typo: non-clevable

This has been corrected.

Accepted **10th 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.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Andrew J Holland
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-10510R

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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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?	Experiments were performed in at least 3 biological replicates where ever possible.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was 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.	Experiments were performed using populations of manipulated cell lines and therefore randomization was not appropriate.
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.	Investigators were not blinded to the experimental conditions used during most experiments. The data reported are not subjective but rather based on quantititve analusis of phenotypes such as cell growth.
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.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All data sets were assessed assuming a normal distribution.
Is there an estimate of variation within each group of data?	No.
Is the variance similar between the groups that are being statistically compared?	Yes.

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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).	Rabbit-PAX6 (Covance, PRB-278P, 1:500), Chicken-TBR2 (EMD Millipore, AB15894, 1:250), Rat-a-Tubulin (Pierce Antibodies, MA1-80017, 1:500), Goat-γ-Tubulin (homemade, polyclonal, raised against the peptide CDEYHAATRPDYISWGTQEQ, 1:500), Rabbit-Ki67 (D3B5) (Cell Signaling, 9129, 1:500), Mouse-pH3 (Cell Signaling, 9701, 1:100), Rabbit-CC3 (Asp175) (Cell Signaling, 9661, 1:500), Rabbit-Centrin (in house, raised against human Centrin2 (a.a. 1-172) ;1:500), Rabbit-γ-H2AX (p-Ser139) (Cell Signaling, 2577, 1:250), Mouse-TP53 (1C12) (Cell Signaling, 2524S, 1:250), Mouse-TBR1 (Proteintech, 66564-1-Ig, 1:250), Mouse-PAX6 (Thermo Fisher Scientific, MA1-109, 1:250), Rat-CTIP2 (Abcam, ab18465, 1:1000) and Rabbit-CUX1 (ProteinTech, 11733-1-AP, 1:1000), Mouse-TUJ1 (Covance, MMS-435P, 1:1000).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were deteremined to be free from mycoplasma contamination using DAPI staining. RPE1 and DLD1 cells were validated by STR genotyping.

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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
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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
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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	N/A
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