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Extra centrosomes induce PIDD1-mediated inflammation and immunosurveillance

Irmina García-Carpio, Vincent Braun, Elias Weiler, Marina Leone, Sergio Niñerola, Angel Barco, Luca Fava, and Andreas Villunger
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Corresponding author(s): Andreas Villunger (andreas.villunger@i-med.ac.at) , Irmina García-Carpio (Irmina.Garcia@i-med.ac.at)

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Pre-submission Editorial Decision **19th Jan 2023**

Dear Andreas,

Thank you again for enquiring about the potential suitability of your new manuscript for The EMBO Journal, and apologies for not getting back to you earlier - things are a bit chaotic at the moment. But I did now have a chance to read the draft in detail, and to look back at the centrosomes/PIDD papers as well as the original PIDDosome/NF-kB papers. In this light, I found your new results quite interesting and potentially important, and would therefore be happy to consider the work for in-depth review at EMBO J. While I realize that a number of the individual connections have been reported before (and NF-kB also implicated in non-cell-autonomous clearing of aneuploid cells), I appreciate that these pathways had previously not been linked up, and that you nicely show the causal connection from supernumerary centrosomes via the NEMO-PIDDosome towards NK-mediated clearance. Also notable that DNA damage or the prominent micronuclei/cGAS/STING axis appear not to be involved.

In conclusion, I am inviting you to utilized the link below to formally submit your manuscript as soon as you have finalized the draft. No specific format required at this point, except that you'd need to enter all contributing authors into the system.

Thanks again and kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

To submit your manuscript, please follow this link.

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Please note that for technical reasons, this link will take you to a page for uploading revised manuscripts - once we receive the submission, it will be formally turned into an original submission.

Prof. Andreas Villunger
Medical University of Innsbruck
Insitute of Dev. Immunology
Biocenter
Innrain 80
Innsbruck A-6020
Austria

16th Feb 2023

Re: EMBOJ-2023-113510
Extra centrosomes induce PIDD1-mediated inflammation and immunosurveillance

Dear Andreas,

Thank you again for formally submitting your full manuscript on PIDDosome links to inflammation in the presence of extra centrosomes. I have now heard back from three referees with expertise in aneuploidy, centrosome amplification, and PIDD1/inflammation, respectively. As you will see from their comments copied below, they all acknowledge the interest and timeliness of this work, and would in principle support publication, pending satisfactory revision of a number of specific concerns raised in the three reports.

I would therefore like to invite you to revise the study according to their comments. Please note that it is our policy to allow only a single round of (major) revision, making it important to adequately clarify all issues by the time of resubmission. In this light, I would therefore encourage you to contact me with a tentative response letter and revision plan already during the early stages of the revision. On the basis of this, I would be happy to discuss the revision further with you via online call, as well as the possibility of an extended resubmission deadline if needed. Our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) would of course remain valid even during extended revision.

Detailed information on preparing, formatting and uploading a revised manuscript can be found below and in our Guide to Authors. Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to your revision!

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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1) Every manuscript requires a Data Availability section (even if only stating that no deposited datasets are included). Primary datasets or computer code produced in the current study have to be deposited in appropriate public repositories prior to resubmission, and reviewer access details provided in case that public access is not yet allowed. Further information: embopress.org/page/journal/14602075/authorguide#dataavailability

2) Each figure legend must specify
- size of the scale bars that are mandatory for all micrograph panels
- the statistical test used to generate error bars and P-values
- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

4) Each main and each Expanded View (EV) figure should be uploaded as individual production-quality files (preferably in .eps, .tif, .jpg formats). For suggestions on figure preparation/layout, please refer to our Figure Preparation Guidelines: http://bit.ly/EMBOPressFigurePreparationGuideline

5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.

6) Please complete our Author Checklist, and make sure that information entered into the checklist is also reflected in the manuscript; the checklist will be available to readers as part of the Review Process File. A download link is found at the top of our Guide to Authors: embopress.org/page/journal/14602075/authorguide

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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (17th May 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

The manuscript by Garcia-Carpio and colleagues elegantly links the presence of extra centrosomes to activation of NF-kB signalling and subsequent activation of sterile inflammation. This in turn leads to an increase in immunogenicity and increased immune clearance in vitro. This study is extremely well done and the results of many experiments often validated with multiple, orthogonal approaches, thus reinforcing their findings. Given the impact and the novelty of their main findings, this manuscript would be a good fit for The EMBO Journal, pending one important experiment outlined below.

The novel aspect of the paper is the link between extra centrosomes, activation of NF-kB and increased immunogenicity. For this reason, it is important to show that the phenotype downstream of PIDD1 activation is actually dependent on NF-kB. For this, key experiments, including cytokine secretion and immune clearance, should be repeated in a background in which NF-kB activity is inhibited through a chemical approach or, even better, through a genetic approach by knocking-out RelA/RelB.

Minor point:
In Figure 2C and S2E, the colours of Parental and sgControl are very similar. It would be better to change one of them.

Referee #2:

Centrosome abnormalities, and in particular centrosome amplification, are commonly detected in human tumours, albeit at low

frequency. These abnormalities can impact mitosis, promote aneuploidy and induce a secretory phenotype, suggesting that the presence of extra centrosomes could alter cell physiology in multiple ways.

In this manuscript, Garcia-Carpio et al describe that amplification of centrosomes could drive an inflammatory response due to NF-kb activation, which makes cells carrying these abnormalities more susceptible to NK killing. This mechanism could provide a way to keep normal tissues 'free' of such aberrant and detrimental cells. The work presented here stems from Villunger lab previous work that demonstrated for the first time that too many centrosomes activate the PIDDosome pathway leading to p53 stabilisation to prevent the proliferation of cells with amplified centrosomes. Here, activation of the same PIDDosome pathway is responsible for NF-kb activation and pro-inflammatory cytokine secretion. This study sheds some light into the secretory phenotype displayed by cells with amplified centrosomes and it is generally well conducted with interesting findings that will appeal to a broad readership. However, there are several issues/concerns that should be addressed/clarified by the authors prior to publication.

#1. One of the main concerns I have is regarding the % of tetraploid and centrosome amplification obtained in these experiments. It is very unclear to me how centrosome amplification and % of tetraploid is assessed throughout the manuscript. I cannot find the data for how much tetraploid cells the authors get from the different treatments. This is important to compare experiments (mentioned below as well in several more specific points).

#2. It is unclear how the analyses of gene expression of cells treated with Aurora kinase inhibitor is performed. Is it done in purified tetraploid cells? So that it can be compared to diploid cells? What is the % of tetraploid cells obtained upon this treatment?

#3. Gene expression analyses of FACS-sorted tetraploid cells and 'evolved' tetraploid cells that lost amplified centrosomes has been previously reported by Ganem, Cornils et al 2014 (Cell). Did the authors check if some of their hits have been previous found in tetraploid cells with extra centrosomes? I also think this work needs to be referenced better in the manuscript.

#4. CRISPR KO efficiency needs to be shown to all cell lines used here.

#5. The Aurora kinase inhibitor ZM447439 inhibits both Aurora A and B. Do the authors known if it affects centrosome function? Since Aurora A is required for centrosome maturation?

#6. I was intrigued by why U2OS cells data on figure 3E is so different between PLK4 overexpressing cells and ZM treated cells? It seems that ZM treated cells have a stronger phenotype (A549 cell also seem to have a lower response to just centrosome amplification). It would be good to show centrosome amplification data in these different conditions. Otherwise, this suggests that tetraploid cells have a stronger NF-kb activation phenotype and thus other things apart from amplified centrosomes are contributing to this, which is not a problem but needs to be assessed as the current emphasis of this work in on centrosome amplification (including title of the manuscript).

#7. Because this work is focused on centrosome amplification, could the authors validate some of the findings in the PLK4 overexpression cells they already have? For example, if the formation of PIDD1 complex shown in figure 4G is due to extra centrosomes, could this be repeated in diploid cells with extra centrosomes (e.g. PLK4 overexpression)? Or since it has been previously demonstrated that 'evolved' tetraploid cells that lost extra centrosomes can be isolated, this could be a control as well to demonstrate specificity. (see Ganem, Cornils et al 2014 paper).

#8. When gene expression profile of pidd1-/- cells is assessed, it would be great to demonstrate that the level of tetraploidy and centrosome amplification obtained is similar between KO MEFs and control cells as a control.

#9. On the last page of the results section, the authors state that "Like parental A549EGFP,
CRISPR control cells were more efficiently eliminated by NK-92 cells and displayed a reduction of polyploid cell confluence over time with respect to euploid controls (Fig. 6 D and F)". However, it is unclear what is the starting polyploid rate for each treatment/cell?

#10. In the discussion, the authors state that: "The results included in this article strongly support the idea that supernumerary centrosomes are one of the key triggers of this cellular immunogenicity." However, the data presented here regarding immunogenicity only applies to polyploid cells treated with ZM or DHCB. The authors should repeat these experiments with the PLK4 cells as well. Otherwise, controls are missing to make such a statement.

#11. Could the authors discuss what happens with tissues that are polyploid with extra centrosomes, e.g. liver hepatocytes? Aren't these cells targeted by NK? Is PIDDosome-mediated NF-kb activation a general response in cells? Seems a very obvious question that should be at least discussed.

Referee #3:

This manuscript by Garcia-Carpio et al describes the role of PIDD1 in activating NFkB and NK cell clearance in response to supernumerary centrioles. This is a strong study and a good natural expansion of the groups prior work showing caspase-2 activation in response to extra centrioles. It is significant because it shows a clear role for PIDD1 in the inflammatory response and this may have an important role in signaling to cells for NK-cell mediated clearance of polyploidy cells that could otherwise become cancerous. The experiments supporting this model are generally clear and well done. My specific comments are below:

Major points
1. Most of the confocal images lack quantitation. In particular, figure 3A, 3C, 3F, S3B (right panel)
2. Figure 4F-G is presented as evidence that PIDD binds RIP following ZM treatment. While reasonably convincing, the majority of this is done using a FLAG-tagged PIDD. IPs with endogenous PIDD following ZM treatment would strengthen this argument.
3. In Figure 4G, Only PIDD FL and not the non-cleavable mutant binds to RIPK1 and Nemo. This suggests that the N-term is required for binding to RIPK1 but this construct was not included in the experiment. The authors should test for binding to the N-term on its own to determine this. Following on from Point 2, the S17 PIDD antibody (Santa Cruz) recognizes the N-term and can be used to IP endogenous PIDD. This can be compared to an antibody that only binds the C-term to more thoroughly characterize the binding. Given, as the authors state, it has long been assumed that PIDD binds RIPK1 through the DD, it is really important to properly characterize this interaction in the context of polyploidy.
4. In figure 4F binding of PIDD1 to RAIDD appears to be dominant and constant even when PIDD1 is binding to RAIDD. While testing for what regulates caspase-2 activation vs NFkB activation in response to polyploidy is, in my opinion, not required for this study, it should be included in the discussion. For example, would ATM or ATR mediated phosphorylation be expected to play a role here?
5. This role of PIDD is proposed to promote NK cell mediated clearance of potential cancer cells. However, knockout of PIDD in mouse models has been shown to block tumorigenesis suggesting a potential oncogenic role for PIDD. How this fits with the model presented here should be included in the discussion.

Minor points
1. Page numbers would be helpful
2. Figure 2A - images lack scale bars
3. Figure S2B, which appears to be the quantitation of S2A, seems to be only one experiment (lacks error bars)
4. Some of the figures are not labeled correctly or are not cited in order which makes reading the results a little more difficult. Some figures, like S3B have two panels. These would be easier to follow if they were labelled with different letters. E.g Figure S2C is labeled as S2B in the text
5. Figure S3A - the drug used to treat the cells is not labeled (presumably Dox?).

Point to point reply: EMBOJ_2023-113510

Referee #1:

The manuscript by Garcia-Carpio and colleagues elegantly links the presence of extra centrosomes to activation of NF-kB signalling and subsequent activation of sterile inflammation. This in turn leads to an increase in immunogenicity and increased immune clearance in vitro. This study is extremely well done and the results of many experiments often validated with multiple, orthogonal approaches, thus reinforcing their findings. Given the impact and the novelty of their main findings, this manuscript would be a good fit for The EMBO Journal, pending one important experiment outlined below.

The novel aspect of the paper is the link between extra centrosomes, activation of NF-kB and increased immunogenicity. For this reason, it is important to show that the phenotype downstream of PIDD1 activation is actually dependent on NF-kB. For this, key experiments, including cytokine secretion and immune clearance, should be repeated in a background in which NF-kB activity is inhibited through a chemical approach or, even better, through a genetic approach by knocking-out RelA/RelB.

Response: We appreciate the overall very supportive comments of this referee. Indeed, demonstrating NF-kB dependence of the phenomena observed will strengthen the overall conclusions made. After quite some time and optimization, we have managed to establish REL/B double mutant A549 reporter cells but also made use of the IkBα Super-repressor (SR), blunting canonical NF-kB signaling. All relevant quality controls are presented in Fig EV5C, documenting loss of NF-kB signaling in response to TNF or TWEAK in RELA/B cells while the SR blocked only TNF signaling-induced canonical reporter activity. Using this model, we have repeated the key experiment addressing the question if the increased immunogenicity and subsequent NK cells recognition depends solely on NF-kB activation that we propose to be PIDD1 dependent. Indeed, loss of RELA/B or overexpression of the SR phenocopies the effect of loss of PIDD1 or RIPK1 and blunts NK cell killing (Fig 6C and Fig EV5D). In a set of additional experiments, we were also able to show that human macrophages induce a set of chemokines only when treated with supernatants from wt A549 cells that fail cytokinesis, but not supernatants harvested from A549 cells lacking PIDD1 or overexpressing the SR (Fig EV5E). Due to time constrains for revision, we were unable to repeat these experiments with supernatants from RELA/B deficient cells. However, we hope that these efforts will be appreciated none-the-less, as we expand our data to human monocyte-derived macrophages, in addition to mouse bone-marrow derived macrophages (Figure 5; EV4)

Minor point:
In Figure 2C and S2E, the colours of Parental and sgControl are very similar. It would be better to change one of them.

Response: We actually made use of a color scheme that is legible to color-blind people and we hope that this argument is acceptable. Moreover, similar colors identify parental cells and CRISPR controls, that are behaving near identical.

Referee #2:

Centrosome abnormalities, and in particular centrosome amplification, are commonly detected in human tumours, albeit at low frequency. These abnormalities can impact mitosis, promote aneuploidy and induce a secretory phenotype, suggesting that the presence of extra centrosomes could alter cell physiology in multiple ways.

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#1. One of the main concerns I have is regarding the % of tetraploid and centrosome amplification obtained in

these experiments. It is very unclear to me how centrosome amplification and % of tetraploid is assessed throughout the manuscript. I cannot find the data for how much tetraploid cells the authors get from the different treatments. This is important to compare experiments (mentioned below as well in several more specific points).

Response: We appreciate this concern and hence now also provide a detailed assessment of the levels of ploidy induced by the different treatments. This analysis revealed that ploidy increases way beyond tetraploidy and > 90% of our cells are harboring a DNA content of 4c or higher at the time of analysis. The corresponding data can be found in Fig. 2C; Fig EV2E; Fig EV3E,F; Fig EV4C,D; Fig EV5B; Appendix Fig 1). Centrosome counts after treatment or PLK4 overexpression are also provided now in Fig EV3A,E,F; Fig EV4F and the Appendix Fig 2).

#2. It is unclear how the analyses of gene expression of cells treated with Aurora kinase inhibitor is performed. Is it done in purified tetraploid cells? So that it can be compared to diploid cells? What is the % of tetraploid cells obtained upon this treatment?

Response: From this comment, we do understand that or wording was not clear enough. To clarify, we did not specifically isolate polyploid cells from wt or PIDD1 mutant cultures to compare transcriptomes to diploid counterparts. As can also be seen from our ploidy analysis provided and mentioned above, many cells have a DNA content that is higher as 4c, which is even more pronounced in the absence of PIDD1. We have compared the transcriptomes of wt and PIDD1-null MEF treated for 72h with ZM. At this time, less than 25% of cells were still diploid in both genotypes analyzed (Fig EV4C,D).

#3. Gene expression analyses of FACS-sorted tetraploid cells and 'evolved' tetraploid cells that lost amplified centrosomes has been previously reported by Ganem, Cornils et al 2014 (Cell). Did the authors check if some of their hits have been previous found in tetraploid cells with extra centrosomes? I also think this work needs to be referenced better in the manuscript.

Response: we appreciate this comment and the study conducted by Ganem et al. that brought up a set if interesting findings and was actually already cited in our first submission. Yet, in contrast to their work, we did not study evolved tetraploids and do not believe that such cells contribute to the effects reported in our study. In the referenced paper, the authors create “evolved tetraploids” by repeat flow cytometric isolation of cells with a polyploid DNA content to expand the few cells within that pool of tetraploids that maintain proliferative capacity. This seems to have been done in up to 4 weekly spaced out intervals, from what we could extract in the text and methods section. These cells were then found to proliferate, show reduced p53/p21 activity and carry two centrioles. The gene expression changes in our experiments were analyzed after 72h where the majority of cells was polyploid and carried extra centrosomes, as shown in Fig 2C; Fig EV2E; Fig EV3E,F; Fig EV4C,D; Fig EV5B; Appendix Fig 1 for ploidy and in Fig EV3A,E,F; Fig EV4F and the Appendix Fig 2 for centrosome numbers.

A quick side-by-side comparison of the top 30 genes induced or repressed in MEF after ZM treatment did not identify common candidates beyond p53 targets and RELA, suggesting that the inflammatory response we see may contribute to maintain growth arrest. However, this needs to be confirmed and overall, the experimental set up and times of analyses are hard to compare between the two studies.

#4. CRISPR KO efficiency needs to be shown to all cell lines used here.

Response: Next to confirming loss of PIDDosome function, we now also provide western blot analysis of PIDD1 and RAIDD mutant cells that were newly generated during this study. The corresponding data is shown in (Fig 2D; Fig EV2F; Fig EV3C; Appendix Fig. 3). In addition, we refer to our past publications where the additional cell lines lacking Caspase-2 or RAIDD used here were already characterized in detail (PMID: 28130345, PMID: 33350486).

#5. The Aurora kinase inhibitor ZM447439 inhibits both Aurora A and B. Do the authors known if it affects centrosome function? Since Aurora A is required for centrosome maturation?

Response: We have not found such information. It is also not clear which centrosomal function is referred to here. Regarding maturation it can be said that this inhibitor does not affect the microtubule nucleation capacity of centrosomes, which we believe is evidence that it does not interfere significantly with their maturation (PMID: 15616188).

#6. I was intrigued by why U2OS cells data on figure 3E is so different between PLK4 overexpressing cells and ZM treated cells? It seems that ZM treated cells have a stronger phenotype (A549 cell also seem to have a lower

response to just centrosome amplification). It would be good to show centrosome amplification data in these different conditions. Otherwise, this suggests that tetraploid cells have a stronger NF-kb activation phenotype and thus other things apart from amplified centrosomes are contributing to this, which is not a problem but needs to be assessed as the current emphasis of this work in on centrosome amplification (including title of the manuscript).

Response: This point is well taken and we also wondered about the underlying reason. While IncuCyte analyses shown in Fig 3E suggest a weaker signal induced by PLK4 OE compared to ZM treatment, our luciferase based assay in Fig 3D does not suggest major differences when analyzed at the same time point in parallel. However, based on our newly added ploidy and centrosome quantification data, we do see that DHCB is least efficient in causing ploidy increases and differences between cell lines exist. We have added the following to the discussion on page 15. *It is worth mentioning that across many of our experiments the effects induced by PLK4 overexpression were weaker in U2OS cells compared to A549 cells, presenting the mildest increase in centrosome numbers in our hands, that could potentially explain the differences in the amplitude of NF-κB reporter activity noted (Fig 3, Fig EV3 and Appendix Fig 2). In general, ZM-treatment appeared more potent in inducing NF-κB activation but this did not lead to enhanced NK cell killing, when compared to PLK4 overexpression or DHCB treatment (Fig 6). Yet, we cannot fully exclude that additional effects triggered by Aurora kinase inhibition may add to the signal strength observed.*

#7. Because this work is focused on centrosome amplification, could the authors validate some of the findings in the PLK4 overexpression cells they already have? For example, if the formation of PIDD1 complex shown in figure 4G is due to extra centrosomes, could this be repeated in diploid cells with extra centrosomes (e.g. PLK4 overexpression)? Or since it has been previously demonstrated that 'evolved' tetraploid cells that lost extra centrosomes can be isolated, this could be a control as well to demonstrate specificity (see Ganem, Cornils et al 2014 paper).

Response: Indeed, we document that PLK4 OE causes a similar phenotype as do ZM or DHCB treatment on several occasion, including NF-kB activation in A549 cells, U2OS cells and MEF, as well as in our macrophage polarization assays (Fig 5D). In addition, we now also show that PLK4 OE itself increases susceptibility to NK cell killing, while centrinone pretreatment reduces this response (Fig 6F,G). Hence, we believe our conclusions are justified.

Regarding documentation of complex formation, despite loving to see such a result, we anticipated several hurdles that held us back form actively pursuing this experiment. We hope this reviewer will appreciate the complexity and technical challenge of the suggested experiments and the high chances that this will not pin out as aimed for:

a) we would need to introduce a second conditional transgene, ie. PLK4, in 293TRex cells lacking PIDD1 to be induced along with FLAG-tagged PIDD1 for IP analyses; **b)** we would need to identify time points where both (re)-expressed genes are functional; **c)** we would need to determine the time point when centrosomes are amplified in the majority of cells and **d)** establish conditions where most of these cells with extra centrioles passage through mitosis to trigger PIDDosome pathway activation. With all due respect, we believe these are too many variables to control. Moreover, based on our analyses provided, complex formation appears also to be transient. Hence, we believe that the right timing of all these events will be extremely tricky to achieve and the risk of failure is high. Given the list of additional experiments that needed to be conducted, we considered these experiments to be beyond the scope of this study.

#8. When gene expression profile of pidd1-/- cells is assessed, it would be great to demonstrate that the level of tetraploidy and centrosome amplification obtained is similar between KO MEFs and control cells as a control.

Response: We have assessed the levels of ploidy in Pidd1-mutant vs. wt MEF after ZM treatment. As shown in Fig EV4F, levels of ploidy start to differ in MEF lacking PIDD1 only after 72h and centrosome amplification is comparable (Fig EV4F), hence, we cannot identify a confounding factor that may compromise the comparison made. If the assumption was that differences in gene copy number affect relative expression of mRNAs, we want to emphasize that PIDD1-mutant cells actually show a reduction in the expression of relevant target genes.

#9. On the last page of the results section, the authors state that "Like parental A549EGFP, CRISPR control cells were more efficiently eliminated by NK-92 cells and displayed a reduction of polyploid cell

confluence over time with respect to euploid controls (Fig. 6 D and F)". However, it is unclear what is the starting polyploid rate for each treatment/cell?

Response: As suggested, we have assessed ploidy levels of A549 cells after ZM or DHCB treatment, indicating that after 72h of treatment (i.e. the start of our co-cultures with NK cells), ploidy levels in CRISPR control cells range between 4c and 8c while the majority of PIDD1-mutant cells reaches 16c and this does not change until the end of our experiments after 120h (Fig EV5B).

#10. In the discussion, the authors state that: "The results included in this article strongly support the idea that supernumerary centrosomes are one of the key triggers of this cellular immunogenicity." However, the data presented here regarding immunogenicity only applies to polyploid cells treated with ZM or DHCB. The authors should repeat these experiments with the PLK4 cells as well. Otherwise, controls are missing to make such a statement.

Response: As we have now been able to demonstrate that PLK4 overexpression induces the expression and secretion of inflammatory mediators in a PIDD1-dependent manner that promote macrophage polarization (Fig 5D) and we can also show that it triggers NK-cell recognition (Fig 6G), while loss of centrosomes ablates this effect (Fig 6F), we are confident this statement is justified.

#11. Could the authors discuss what happens with tissues that are polyploid with extra centrosomes, e.g. liver hepatocytes? Aren't these cells targeted by NK? Is PIDDosome-mediated NF-kb activation a general response in cells? Seems a very obvious question that should be at least discussed.

Response: this point is well taken, we have added to the discussion on page 16, stating: This begs the question how naturally polyploid cells, such as hepatocytes or cardiomyocytes that experience centrosome amplification during development avoid sterile inflammation. At least for liver, we documented that Pidd1 levels are repressed by inhibitory E2F7/8 in mature hepatocytes (PMID: 31983631), while mature rat cardiomyocytes were reported to dissolve their centrosomes that, extrapolating this observation to mice, would also disarm PIDD1 (PMID: 26247711).

Referee #3:

This manuscript by Garcia-Carpio et al describes the role of PIDD1 in activating NFkB and NK cell clearance in response to supernumerary centrioles. This is a strong study and a good natural expansion of the groups prior work showing caspase-2 activation in response to extra centrioles. It is significant because it shows a clear role for PIDD1 in the inflammatory response and this may have an important role in signaling to cells for NK-cell mediated clearance of polyploidy cells that could otherwise become cancerous. The experiments supporting this model are generally clear and well done. My specific comments are below:

Response: we are thankful for this referee´s positive evaluation and constructive criticism.

Major points

1. Most of the confocal images lack quantitation. In particular, figure 3A, 3C, 3F, S3B (right panel)

Response: this critique aligns with concerns brought up by referee #2, who asked to provide quantification of centrosome number and ploidy levels. The corresponding data can be found in Fig. 2C; Fig EV2E; Fig EV3E,F; Fig EV4C,D; Fig EV5B, Appendix Fig 1). Centrosome counts after treatment or PLK4 overexpression are also provided now in Fig EV3A,E,F; Fig EV4F and the Appendix Fig 2). We hope that this thorough analysis will satisfy also this referee.

2. Figure 4F-G is presented as evidence that PIDD binds RIP following ZM treatment. While reasonably convincing, the majority of this is done using a FLAG-tagged PIDD. IPs with endogenous PIDD following ZM treatment would strengthen this argument.

Response: this point is well taken, and, in fact, we have made several attempts to document interaction of endogenous proteins prior our first submission using anti-PIDD1 (clone Anto-1). Unfortunately, this failed to provide convincing results, leading us to compromise on the semi-endogenous set up shown, where FLAG-tagged PIDD1 is use to pull down endogenous interactors, RAIDD, RIPK1 and NEMO. Commenting on point 3, the antibody S17 formerly distributed by Santa Cruz is no longer commercially available and could not be tested.

3. In Figure 4G, only PIDD FL and not the non-cleavable mutant binds to RIPK1 and Nemo. This suggests that the N-term is required for binding to RIPK1 but this construct was not included in the experiment. The authors should test for binding to the N-term on its own to determine this. Following on from Point 2, the S17 PIDD antibody (Santa Cruz) recognizes the N-term and can be used to IP endogenous PIDD. This can be compared to an antibody that only binds the C-term to more thoroughly characterize the binding. Given, as the authors state, it has long been assumed that PIDD binds RIPK1 through the DD, it is really important to properly characterize this interaction in the context of polyploidy.

Response: We appreciate this suggestion, but, as mentioned, clone S17 has been discontinued by the provider and is no longer commercially available. The suggested replacement antibody (B-5) has been raised against C-term epitopes and is hence not suitable for what this referee proposes. As an alternative, we have transfected 293T cells with the following constructs: GFP (negative control), PIDD1-FL with a C-terminal FLAG tag (as used already in the initial manuscript), a double-tagged construct encoding PIDD1-FL carrying an N-terminal HA-tag and a C-term FLAG tag, as well as PIDD1-N with an N-terminal HA-tag. Using this strategy, we observed in two independent experiments that the N-terminus of PIDD1 alone is able to interact with endogenous RIPK1 (Fig 4F) Together with the fact that pulling down a non-cleavable version of PIDD1-FLAG fails to co-elute RIPK1, we conclude that the N-terminus of PIDD1 is involved in the interaction, either in complex with PIDD1-C and/or -CC. Additional studies will be needed to define the exact mode of complex assembly. However, we believe it is fair to conclude that the N-terminus of PIDD1 is involved in complex formation after auto-processing. These findings are now introduced on page 11.

4. In figure 4F binding of PIDD1 to RAIDD appears to be dominant and constant even when PIDD1 is binding to RAIDD. While testing for what regulates caspase-2 activation vs NFkB activation in response to polyploidy is, in my opinion, not required for this study, it should be included in the discussion. For example, would ATM or ATR mediated phosphorylation be expected to play a role here?

Response: we assume this referee refers to binding to RIPK1?! We have added the following to the discussion on page 15.
It will be interesting to dissect what defines formation of the NEMO- vs. Caspase-2-PIDDosome under these conditions which, as suggested by our western analysis (Fig 1), seems to be sequential. In the context of DNA damage, ATM/ATR kinases have been implicated in regulating Caspase-2-PIDDosome function (Ando et al., 2012; Shah et al, 2021). As we did not find evidence for DNA damage in response to cytokinesis failure or PLK4 overexpression, a decisive contribution of these kinases in regulating the switch between NEMO- vs. Caspase-2-PIDDosome assembly in response to centrosome amplification seems rather unlikely.

5. This role of PIDD is proposed to promote NK cell mediated clearance of potential cancer cells. However, knockout of PIDD in mouse models has been shown to block tumorigenesis suggesting a potential oncogenic role for PIDD. How this fits with the model presented here should be included in the discussion.

Response: this point is well taken, we have added to the discussion on page 16, stating:
Hence, it appears surprising that Pidd1 has been shown to exert oncogenic effects, as its loss delayed the onset of MYC-driven B cell lymphomas (Manzl et al, 2012) or DEN-induce hepatocellular carcinoma in mice (Sladky et al, 2020b). While in the latter case an increase in hepatocyte ploidy, paralleled by an increase in tumor suppressor genes may account for the delay of tumor onset and dominate over impaired immune clearance, the situation in MYC-driven lymphomas is less clear and awaits clarification.

Minor points

1. Page numbers would be helpful

Introduced

2. Figure 2A - images lack scale bars

Introduced

3. Figure S2B, which appears to be the quantitation of S2A, seems to be only one experiment (lacks error bars)

Indeed this pilot was only done once to help define timing of our analyses

4. Some of the figures are not labeled correctly or are not cited in order which makes reading the results a little more difficult. Some figures, like S3B have two panels. These would be easier to follow if they were labelled with different letters. E.g Figure S2C is labeled as S2B in the text

We have corrected and optimized labelling and hope this improved legibility

5. Figure S3A - the drug used to treat the cells is not labeled (presumably Dox?).

Corrected

Prof. Andreas Villunger
Medical University of Innsbruck
Insitute of Dev. Immunology
Biocenter
Innrain 80
Innsbruck A-6020
Austria

18th Jul 2023

Re: EMBOJ-2023-113510R
Extra centrosomes induce PIDD1-mediated inflammation and immunosurveillance

Dear Andreas,

Thank you for submitting your revised manuscript for our consideration. The three original referees (see comments below) have now reviewed it once more, and all of them were fully satisfied with the revision. I am thus pleased to inform you that we have now accepted the article for publication in The EMBO Journal!

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements. You will also be provided with page proofs after copy-editing and typesetting of main manuscript and expanded view figure files.

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Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Yours sincerely,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

Referee #1:

The authors have addressed my comments/requests. I strongly support the publication of this paper in the EMBO Journal

Referee #2:

Overall, the authors did a great job in addressing my initial concerns with this study. The new experiments/discussion points are really helpful and improved clarity. Thus, I think this manuscript can be published as it is and I have no further comments that need clarification.

Referee #3:

The authors have addressed my concerns and have strengthened their manuscript with the additional experiments. This main conclusion of this paper is of high importance to the cell death/caspase field and I look forward to reading it in print!

EMBO Press Author Checklist

Corresponding Author Name: Andreas Villunger
Journal Submitted to: The EMBO Journal
Manuscript Number: 2023-113510R1

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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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Each figure caption should contain the following information, for each panel where they are relevant:
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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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