THE
EMBO
JOURNAL

# Inhibition of CPAP-tubulin interaction prevents proliferation of centrosome amplified cancer cells

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**Review timeline:**

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|---|---|
| Submission date: | 22 May 2018 |
| Editorial Decision: | 13 June 2018 |
| Revision received: | 17 September 2018 |
| Editorial Decision: | 4 October 2018 |
| Revision received: | 11 October 2018 |
| Accepted: | 18 October 2018 |

Editor: Daniel Klimmeck

## Transaction Report:

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

1st Editorial Decision 13 June 2018

Thank you for the submission of your manuscript (EMBOJ-2018-99876) to The EMBO Journal. Your manuscript has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #3 states that your claims about a competition model for CPAP and CCB02 are not well supported by the current data. Further this referee is concerned that the microtubule phenotypes are not sufficiently explored in his/her view, and asks you to employ complementary regrowth assays. Referees #1 and 2 are overall more positive, but agree in that more work is needed to corroborate the details of how interfering with CPAP-tubulin interaction affects microtubule nucleation and centrosome fragmentation. In addition, the referees point to issues related to experimental design, methods documentation and integration of literature that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I agree that it would be critical to increase the mechanistic insights into the nature of microtubule alterations elicited by impairment of CPAP-tubulin interactions. I also concur that it would be helpful to reorganize the findings presented in supplemental versus main figures.

 

REFEREE REPORTS

Referee #1:

In this manuscript Jay Gopalakrishnan and colleagues identified and characterized a drug, CCB02, which prevents extra-centrosome clustering and selectively alters the proliferation and invasion of the corresponding cells. The drug was found to inhibit the interaction between free tubulin and CPAP and thus impact the nucleation of microtubules from the extra-centrosome. How this impacts extra-centrosome clustering and cell cycle arrest is not shown but the final effect on cancer cell survival is clear and most important. The results are clearly presented and data are convincing. Considering the importance of finding a drug that selectively impact cancer cells with extra-centrosome, I strongly support the publication of this work.

I was mostly convinced by the following points:
- cells with extra-centrosomes are delayed in mitosis when they express a mutated version of CPAP (Fig 1A)
- extra-centrosomes can't cluster in cells expressing a mutated version of CPAP (Fig 1B-E)
- tumour growth is reduced in cells expressing a mutated version of CPAP (Fig 1F)
- CCB02 alters the interaction of CPAP with free tubulin (Fig S4C, Fig S6D-E). It could also have other side effects but it is impossible to test them all and cells without CPAP do not display the main phenotype in response to CCB02, which is extra-centrosome declustering (Fig S10E, F). This key control should be moved to the main figures (Fig 3 for example).
- The amount of cells with extra-centrosome is reduced when cultured for two days in the presence of CCB02 (Fig S7B). This results is key and should be move to the main figures (Fig 3 for example). Longer exposure would be even more convincing.
- Cells treated with CCB02 showed prolonged mitosis (Fig S8C-D) and activation of the cell cycle checkpoints (Bub1, Mad1) (Fig S9).
- CCB02 does not seem to impact microtubule growth and dynamics, neither in vitro nor in cells (Fig 5E-F).
- CCB02 impacts cell invasion (in addition to cell survival) (Fig 6A)

I was less enthusiastic about the following points
- CCB02 increases the amount of PCM around declustered extra-centrosomes. First the effect on the images (Figure 4A) is small and the quantification showing a 8-fold increase (Fig AB) sounds not in adequation with those images. Second the impact of PCM accumulation and microtubule nucleation on extra-centrosomes declustering is not demonstrated. So the relevance of this observation to the understanding of the corresponding cell death is questionable. It can be involved or it can be a side effect. I must say that I was first enoyed by the writing of the introduction posing this mechanism as the potential cause of the observed phenotype. But authors conclusion is clear and the rationale which led them to their discovery is not claimed to be the actual mechanism accounting for extra-centrosome declustering and cell death. Authors acknowledge they don't know the actual mechanism. So conclusions are solid. But I found that the introduction was a bit misleading. However, one should be free to explain freely the rationale behind and experiment as long as the conclusion is clearly independent of it.
- Again, regarding the underlying mechanism, even if we are only talking about the introduction, I was not convinced that the nucleation of more microtubule should lead to declustering. Indeed, current understanding of the clustering mechanism is based on microtubules. Kinesins, such as HSET, are believed to slide anti-parallel microtubules and promote extra-centrosome clustering. So additional microtubules from extra-centrosome should help rather than prevent clustering. I think this discrepancy with current dogma should be stated and discussed. This would draw people attention to the progress that still need to be done to understand the observed phenotype. I personally tend to think that CCB02, by binding to microtubules, compete with other MAPs or motors, such as HSET, and thereby prevent centrosome clustering. The discussion should be more open to all potential mechanisms.
- the effect of CCB02 on tumor growth is not very significant ($P<0.01$ only, Fig 6C). At least it is less significant than the effect of the mutated CPAP. Error bars showing SEM and not SD are misleading since they show highly distinct averages although the statistical test show the effect is not that strong. This could be due to high variability between tumour responses, which is somehow a bit tempered by this representation. I suggest to show standard deviations to be more transparent.

 

However, it is important to state that this not-so-strong but significant effect on tumour growth, and the absence of clear underlying mechanism to account for observations on cells, should not prevent the publication of this study in a high standard journal such EMBO Journal. Indeed, this work is a great demonstration of the importance of good biochemistry and good cell biology on the identification of new drugs against cancer. It could be that the not-so-strong effect on tumours is only a problem of drug delivery to the tumours, which could be improved later. In addition, this work will definitely encourage further works to explain the cellular phenotypes (which are very strong) and increase the impact on tumor growth in mice.

Referee #2:

Centrosome amplification is frequently observed in multiple human tumors. In order to survive, those cancer cells acquire their intrinsic ability to efficiently cluster their extra centrosomes to circumvent mitotic catastrophe. Therefore, the induction of centrosome declustering has been proposed as a prime target for cancer therapeutics. In this manuscript, the authors identified a selective inhibitor (CCB02) against the CPAP-Tubulin interaction that prevents efficient clustering in tumor cells. Mechanistically, this chemical inhibition activates interphase centrosomes to increase microtubule nucleation prior to mitosis, thus causing mitotic cancer cells to undergo centrosome declustering and cell death. This study could have important consequences to the development of selective therapies targeting to supercentrosomal cancer cells. Overall, the paper is very well written and data presented here are well validated. I therefore in principle support the publication of this interesting story in EMBO, when the following issues are addressed.

1. I have a major concern with the results described in Figure 1E-F. Here, the authors report that MDA-MB-231 cells expressing CPAPΔT can cause around 20% of multipolar mitotic cells in Figure 1E, while implantation of those cells shows an over 3-fold decrease of in vivo growth of breast cancer xenografts when compared to control in Figure 1F. I just wonder how a small portion of supercentrosomal cells contributes to such a significant difference in tumor growth? Therefore, it is essential to see whether CPAPΔT expression will affect the cell cycle progression of MDA-MB-231 cells without extra centrosomes.

2. To state that CPAPΔT expression or CCB02 treatment indeed increased microtubule nucleation in interphase cells, the authors may want to perform canonical microtubule regrowth assay to provide quantitative assessment of this phenotype. It is also necessary for authors to provide appropriate statistic data to back up the MT nucleation statement in their movie S1C-D and sFigure 7C-G, since this is a key conclusion of the paper.

3. It has been shown that CPAP determines the centriole length, and abnormalities in centriole length may lead to centrosome fragmentation. To this end, the author should at least test the impact of CCB02 treatment on centrosome fragmentation in addition to centrosome declustering. The common assay for centrosome fragmentation is staining for gamma-tubulin.

4. It will be of interest to test whether chemical perturbation of CPAP-Tubulin interaction will also affect the cancer cell migration using wound healing assay.

5. A schematic illustrating the mouse xenograft experimental setup will be helpful to understand the impact of CCB02 on tumor growth .

6. The authors should explain in the manuscript why they used different concentrations of the CCB02 drug in different cancer cell lines and how they determined it.

Referee #3:

Here, the authors report that inhibiting the interaction between CPAP (the human Sas-4 homologue) and tubulin, through either genetic or chemical means, leads to increased microtubule nucleation from cells with amplified centrosomes. This in turn interferes with centrosome clustering and

 

apparently leads to selective elimination of cells with amplified centrosomes when cultured in 2D and 3D, as well as in mouse xenografts.

While the data look potentially interesting, I struggled to follow this manuscript with many sections poorly explained. For example, it wasn't indicated how constructs were 'introduced' into cells or what was meant by the 'frequent hitters' that were excluded from the screen. There was also insufficient detail in the legends to the six main figures with, for example, it not being clear what was being imaged in the live cell experiments shown in Figure 1A or how the cells shown on the left in Figure 3B were identified as being in G2. In addition, it was quite frustrating that the bulk of the data was put into eleven Supplementary Figures, including some potentially interesting results such as that depletion of CPAP abrogated the effect of the compound. Finally, there are some plainly inaccurate statements, such as that in interphase the centrosome is incompetent to nucleate robust microtubules (p14); this might be true in flies but not in human cells. Hence, overall I do not feel that this manuscript reaches the standards expected for this prestigious journal and cannot recommend publication. I have highlighted some additional concerns below.

In the genetic-based experiments shown in Figure 1, I'm unclear as to whether the authors think that blocking the CPAP-tubulin interaction leads to enhanced microtubule nucleation at all times or only in mitosis, and only in cells with amplified centrosomes or all cells. In several places, the authors talk about enhancing microtubule nucleation "prior to mitosis" without clarifying whether they mean throughout the cell cycle or just in G2. Crucially, microtubule regrowth experiments are required to answer this point and should be done on interphase and mitotic cells with and without doxycycline-induction of Plk4. Quantified microtubule regrowth experiments would also be useful in other parts of the manuscript, such as in Figure 4 when cells are treated with CCB02. Figure 4C apparently shows microtubule nucleation but again it isn't clear from the legend how this experiment has been done. Figure 4D shows an immunoprecipitation experiment but without controls.

While I am not an NMR expert, I wasn't convinced that the data shown in Figure 2 unambiguously confirms that CCB02 directly binds to the same site on tubulin to which CPAP binds. Indeed, the text hints at this lack of definitive proof stating that "this observation suggests that the CPAP peptide and CCB02.1 have the same binding site on tubulin". When combined just with in silico docking and not a crystal structure then I don't feel that the authors can draw the conclusion that "these results indicate that CCB02 is a novel tubulin binder whose binding site overlaps with the CPAP peptide". Biochemical competition experiments are also required here. Likewise, the CCB02-biotin pull-down assay identified interacting proteins beyond tubulin (Fig. S6E), and so the authors are overinterpreting the results to claim that CCB02 is a specific tubulin binder in cells.

Figure 3 presents the main data in support of the title of the manuscript that inhibiting CPAP-tubulin interaction selectively acts on cells with amplified centrosomes. However, the correlation of CCB02 IC50 with centrosome number isn't sufficient to suggest that the effect of this drug is selective to cells with amplified centrosomes, as these cancer cells are likely to share many other genetic defects. Indeed, in the Discussion the authors admit that CCB02 is only "likely to be selective for extra centrosomes-containing cells".

In Figure 5, it is claimed that taxol and docetaxel do not induce multipolar mitoses when it is well know from the literature that they do and indeed the figures suggest that they do. The analysis of the consequences of CCB02 on microtubule dynamics is very superficially described.

The abstract highlights the use of 3D-organotypic invasive assays but only a limited set of data are shown using this approach in Fig. 6, and the graph in Fig. 6B lacks errors bars.

Many of the main and supplementary figures are only shown as merged images in which it is not always possible to see individual protein signals, e.g. CPAP and Cep152 in Figure 3B or S2.

1st Revision - authors' response 17 September 2018

(Please see next page)

 

**Referee #1:**

In this manuscript Jay Gopalakrishnan and colleagues identified and characterized a drug, CCB02, which prevents extra-centrosome clustering and selectively alters the proliferation and invasion of the corresponding cells. The drug was found to inhibit the interaction between free tubulin and CPAP and thus impact the nucleation of microtubules from the extra-centrosome. How this impacts extra-centrosome clustering and cell cycle arrest is not shown but the final effect on cancer cell survival is clear and most important. The results are clearly presented and data are convincing. Considering the importance of finding a drug that selectively impact cancer cells with extra-centrosome, I strongly support the publication of this work.

We highly appreciate this reviewer's view on looking at the final effects of CPAP-Tubulin inhibition on cancer cells. We are encouraged and happy to address this reviewer's concerns with valid experiments.

I was mostly convinced by the following points:

- cells with extra-centrosomes are delayed in mitosis when they express a mutated version of CPAP (Fig 1A)

- extra-centrosomes can't cluster in cells expressing a mutated version of CPAP (Fig 1B-E)

- tumour growth is reduced in cells expressing a mutated version of CPAP (Fig 1F)

- CCB02 alters the interaction of CPAP with free tubulin (Fig S4C, Fig S6D-E). It could also have other side effects but it is impossible to test them all and cells without CPAP do not display the main phenotype in response to CCB02, which is extra-centrosome declustering (Fig S10E, F). This key control should be moved to the main figures (Fig 3 for example).

Agreeing to this reviewer, we now have moved this important control experiment to the main figure as **Fig. 4**

- The amount of cells with extra-centrosome is reduced when cultured for two days in the presence of CCB02 (Fig S7B). This results is key and should be move to the main figures (Fig 3 for example). Longer exposure would be even more convincing.

We indeed exposed cells to CCB02 for 14 days (longer treatment). We now have moved this figure to the main figure as **Fig. 3A**

- Cells treated with CCB02 showed prolonged mitosis (Fig S8C-D) and activation of the cell cycle checkpoints (Bub1, Mad1) (Fig S9).

- CCB02 does not seem to impact microtubule growth and dynamics, neither in vitro nor in cells (Fig 5E-F).

- CCB02 impacts cell invasion (in addition to cell survival) (Fig 6A)

I was less enthusiastic about the following points:

- CCB02 increases the amount of PCM around declustered extra-centrosomes. First the effect on the images (Figure 4A) is small and the quantification showing a 8-fold increase (Fig AB) sounds not in adequation with those images.

This experiment is to show CCB02 treatment increases PCM recruitment to interphase centrosomes. In this experiment, we analyzed two centrosome-containing cells **(Fig. 5A).** For clarity, we now have highlighted this aspect in the revised version.

We appreciate this reviewer for bringing up this point. We realized that there was an inconsistency between the values given in the graph and the intensities of interphase centrosomes at the representative images (**Fig. 5A-B**). We reanalyzed them and here are the reasons for the inconsistency:

- ***i)*** We chose to present nearly similar representative figures in each panel.
- ***ii)*** While we re-quantified the intensities, we noticed that there are few outliers and images that were not looking perfect interphase-like cells. We have now excluded them from our calculation estimating intensities.
- ***iii)*** Revised quantification is now provided **(Fig. 5B).**
- ***iv)*** We would like to emphasize that this experiment i.e increased PCM recruitment is also supported by our biochemical experiments (such as IP and sucrose gradient fractionation of centrosomes, **Fig. 5C, S11** in the manuscript).
- ***v)*** Finally, the newly added microtubule regrowth assay again confirms an enhanced recruitment of PCM (γ-Tubulin) (**Fig. R2** see below)

-Second the impact of PCM accumulation and microtubule nucleation on extra-centrosomes declustering is not demonstrated. So the relevance of this observation to the understanding of the corresponding cell death is questionable. It can be involved or it can be a side effect. I must say that I was first enoyed by the writing of the introduction posing this mechanism as the potential cause of the observed phenotype. But authors conclusion is clear and the rationale which led them to their discovery is not claimed to be the actual mechanism accounting for extra-centrosome declustering and cell death. Authors acknowledge they don't know the actual mechanism. So conclusions are solid. But I found that the introduction was a bit misleading. However, one should be free to explain freely the rationale behind and experiment as long as the conclusion is clearly independent of it.

We apologize that our upfront rationale has annoyed this reviewer. We now realized it and toned down the statement. We agree that we should have been flexible about our working hypothesis and open for more options. To make the introduction more appropriate, we now have changed our statement as follows (which appears at the introduction part). We are happy to adapt any more changes if this reviewer suggests.

***Page 4 in the manuscript***: "*Thus, we wondered that activating extra centrosomes to nucleate an enhanced level of microtubules before they cluster in mitosis could potentially lead to centrosome declustering. Although, this rationale may differ from current view of centrosome declustering mechanisms*[1-4]*, it may represent as one of the alternative mechanisms linking microtubule-nucleating activity and centrosome declustering*"

- Again, regarding the underlying mechanism, even if we are only talking about the introduction, I was not convinced that the nucleation of more microtubule should lead to declustering. Indeed, current understanding of the clustering mechanism is based on microtubules. kinesins, such as HSET, are believed to slide anti-parallel microtubules and promote extra-centrosome clustering. So additional microtubules from extra-centrosome should help rather than prevent clustering. I think this discrepancy with current dogma should be stated and discussed. This would draw people attention to the progress that still need to be done to understand the observed phenotype.

We are thankful for this reviewer's points, which are well taken. We have now discussed various possibilities at the introduction (as stated above) as well as at the discussion as below.

***Page 15 in the manuscript***: *"Given the inherent nature of small molecules such as off-target effects and cross reactivity, it is plausible that CCB02 can compete with microtubule binding proteins including kinesins such as HSET which has been shown to promote clustering of extra centrosomes*[3, 4]*."*

We hope that the revised version is no more carrying our biased opinion. We are happy to adapt any more changes if this reviewer suggests.

I personnaly tend to think that CCB02, by binding to microtubules, compete with other MAPs or motors, such as HSET, and thereby prevent centrosome clustering. The discussion should be more open to all potential mechanisms.

We agree. We were curious to test the possibility of CCB02 preventing HSET from binding to MTs. This is because; CCB02 at high concentration could potentially also bind to polymerized tubulins and stabilize them.

We wish we could have shared this data to this reviewer. Thus, we did experiment by staining HSET on CCB02 treated cells. However, we could not find a conclusive result as, we require optimizing the conditions with the anti-HSET (Bethyl laboratory). We will continue testing this in the future.

- the effect of CCB02 on tumor growth is not very significant ($P<0.01$ only, Fig 6C). At least it is less significant than the effect of the mutated CPAP. Error bars showing SEM and not SD are misleading since they show highly distinct averages although the statistical test show the effect is not that strong. This could be due to high variability between tumour responses, which is somehow a bit tempered by this representation. I suggest to show standard deviations to be more transparent.

Reviewer's point is well taken. We do now consider variability between tumor volumes and present the statistics as SD **(Fig. 7D).**

However, it is important to state that this not-so-strong but significant effect on tumour growth, and the absence of clear underlying mechanism to account for observations on cells, should not prevent the publication of this study in a high standard journal such EMBO Journal. Indeed, this work is a great demonstration of the importance of good biochemistry and good cell biology on the identification of new drugs against cancer. It could be that the not-so-strong effect on tumours is only a problem of drug delivery to the tumours, which could be improved later. In addition, this work will definitely encourage further works to explain the cellular phenotypes (which are very strong) and increase the impact on tumor growth in mice.

We highly appreciate this reviewer's view on the significance of our work. The revised version addresses the concerns of this reviewer.

**Referee #2:**

Centrosome amplification is frequently observed in multiple human tumors. In order to survive, those cancer cells acquire their intrinsic ability to efficiently cluster their extra centrosomes to circumvent mitotic catastrophe. Therefore, the induction of centrosome declustering has been proposed as a prime target for cancer therapeutics. In this manuscript, the authors identified a selective inhibitor (CCB02) against the CPAP-Tubulin interaction that prevents efficient clustering in tumor cells. Mechanistically, this chemical inhibition activates interphase centrosomes to increase microtubule nucleation prior to mitosis, thus causing mitotic cancer cells to undergo centrosome declustering and cell death. This study could have important consequences to the development of selective therapies targeting to supercentrosomal cancer cells. Overall, the paper is very well written and data presented here are well validated. I therefore in principle support the publication of this interesting story in EMBO, when the following issues are addressed.

We are highly encouraged by this reviewer's opinion on our work. We are very happy to address the concerns with valid experiments.

1. I have a major concern with the results described in Figure 1E-F. Here, the authors report that MDA-MB-231 cells expressing CPAPΔT can cause around 20% of multipolar mitotic cells in Figure 1E, while implantation of those cells shows an over 3-fold decrease of in vivo growth of breast cancer xenografts when compared to control in Figure 1F. I just wonder how a small portion of supercentrosomal cells contributes to such a significant difference in tumor growth? Therefore, it is essential to see whether CPAPΔT expression will affect the cell cycle progression of MDA-MB-231 cells without extra centrosomes.

We are thankful to this reviewer for this very intriguing point. Here are our possible answers. Unlike in vitro experiments (where CPAP-tubulin inhibition does not affect normal cells), the situation might be complex in in vivo (in tissues), which we could not monitor in the time scale. There are number of possibilities for the tumor reduction which we would like to share here:

***i)*** It could be possible that in 3D tissue environment, the residual number of cells, which did not harbor extra centrosomes, could start amplifying them. This is difficult to visualize /analyze.

***ii)*** Indeed, it has been shown that tumors appear to maintain extra centrosomes in tissues[5, 6].

**To answer the effect of CPAPΔT on cell cycle progression of two centrosome containing cells:**

***i)*** We have already shown CPAPΔT expression does not delay the cell cycle progression of MCF10A cells that harbor two centrosomes **(Fig. 1Aii).** The effect is specific only for extra centrosome-containing cells **(Fig. 1Aiv)**.

***ii)*** To precisely address this reviewer's concern, we now performed a similar live imaging experiment with two-centrosome-containing MDA-MB-231 cells. The results are similar to that of CPAPΔT expressing MCF10A cells that harbor two centrosomes. Meaning that CPAPΔT expression does not cause mitotic delay in two-centrosome-containing MDA-MB-231 cells **(Fig. R1 and Movies R1A-B).** If required, we are happy to add this data in the revised version.

***iii)*** Finally, we would like to share that mutant flies expressing Sas-4ΔT (fly version of CPAP) did not show any observable delay in development. This has been verified in two of our independent papers[7, 8]

Taken together CPAPΔT has no effect in cell cycle progression of two centrosome-containing cells.

**Fig. R1**

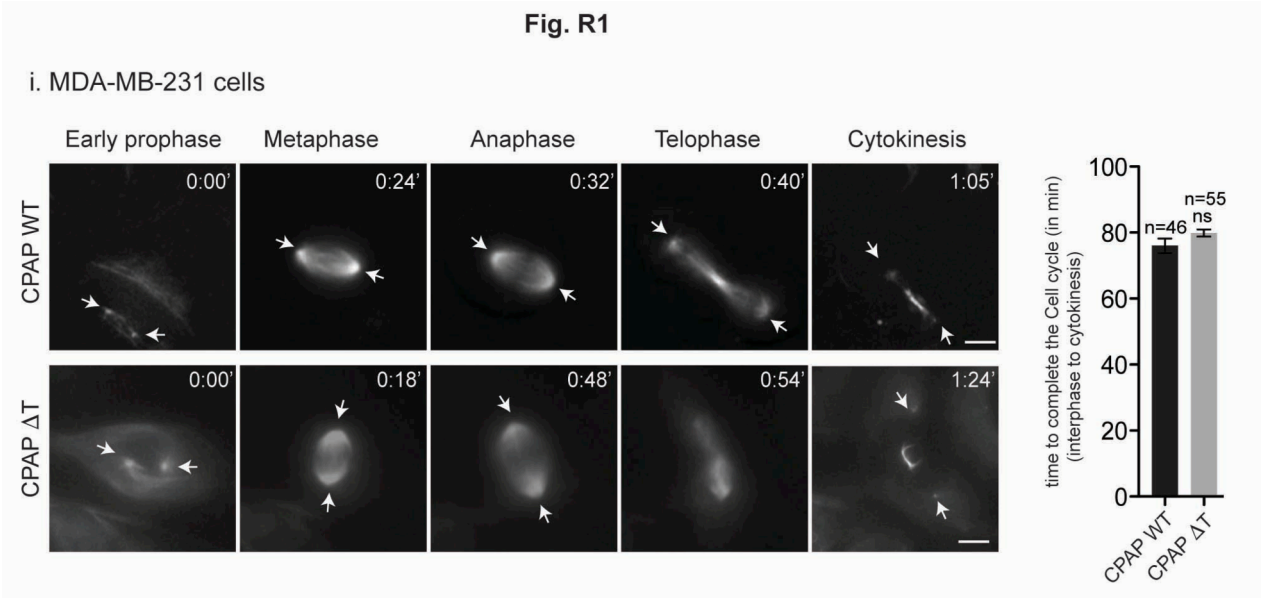

**Fig.R1 (A)** Snap shot images of MDA-MB-231 cells expressing CPAP WT and CPAPΔT in two centrosome-containing cells. Note that CPAPΔT expression does not alter cell cycle progression significantly. SiR-tubulin was used to visualize both centrosomes and microtubules. Bar graph at right quantifies cell cycle duration. Number of cells (n) analyzed in each condition is indicated at the top of each bar. Scale bar, 5µm. (*N*)=3. Error bars, mean ± SEM. Unpaired *t*-test.

2. To state that CPAPΔT expression or CCB02 treatment indeed increased microtubule nucleation in interphase cells, the authors may want to perform canonical microtubule regrowth assay to provide quantitative assessment of this phenotype. It is also necessary for authors to provide appropriate statistic data to back up the MT nucleation statement in their movie S1C-D and sFigure 7C-G, since this is a key conclusion of the paper.

We are thankful to this reviewer for asking us to do a key experiment "microtubule regrowth assay". This experiment strengthens two key aspects of the paper namely,

CCB02 treatment ***i)*** Enhances MT nucleation prior to mitosis ***ii)*** Enhances PCM recruitment

We preformed a three-time point microtubule regrowth assay with MCF10A (-Dox, two centrosomes), MCF10A (+Dox, extra centrosomes) and MDA-MB-231 cells[9, 10]. CCB02 treatment caused centrosomes to nucleate an enhanced level of microtubules already at 1.5 min after induction of regrowth. Importantly, these centrosomes indeed recruited significantly higher levels of γ-tubulin **(Fig.R2)**.

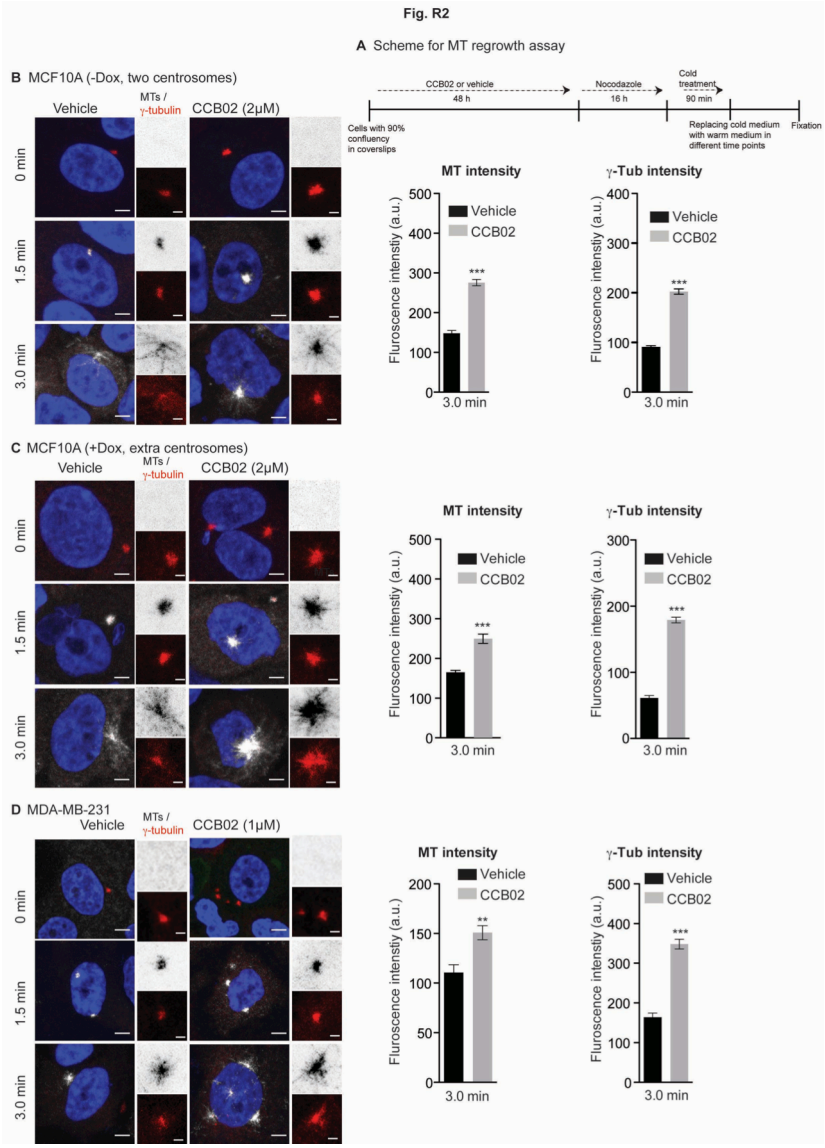

**Fig.R2 (A)** Experimental scheme of microtubule (MT) regrowth assay.
**(B-D)** MT-regrowth assays at 0, 1.5 and 3.0 min using MCF10A (-Dox, two centrosomes), MCF10A (+Dox, extra centrosomes) and MDA-MB-231 cells. MT nucleation panel is shown in grey scale (inset images are inverted) and γ-tubulin is shown in red. Note that in contrast to vehicle treatment, CCB02 treatment caused centrosomes to nucleate an enhanced level of microtubules already at 1.5 min after induction of regrowth with simultaneous increase in γ-tubulin recruitment. All these cells were stained with γ-tubulin (red), microtubules (α-tubulin, grey) and DNA (DAPI blue). Scale bar, 2μm and insets, 0.5 μm. Bar graphs show MT and γ-tubulin intensities at 3 min after induction of MT regrowth. (*N*)=3. At least 80 centrosomes were considered to calculate intensities from each cell line. Error bars, mean ± SEM. Unpaired *t*-test. $^{**}P < 0.001$, $^{***}P < 0.0001$.

3. It has been shown that CPAP determines the centriole length, and abnormalities in centriole length may lead to centrosome fragmentation. To this end, the author should at least test the impact of CCB02 treatment on centrosome fragmentation in addition to centrosome declustering. The common assay for centrosome fragmentation is staining for gamma-tubulin.

Here, the reviewer asks us to test if CCB02 has centrosome fragmentation effect. To test this aspect, we performed two experiments (**Fig. R3A-C**).

**First experiment:** We analyzed fragmented centrosome in MDA-MB231 and MCF10A cells (Dox +, extra centrosomes) before and after CCB02 treatment (**Fig. R3A-B**).

*Before CCB02-treatment:* We did observe centrosome fragmentation in these cells as determined by PCNT-negative centrin dots (centrin-3)[11-13]. Indeed, these cells also contained intact centrosomes as revealed by centrin co-localized with PCNT. Kindly note that we used PCNT instead of gamma-tubulin for the following reasons:

i) Antibodies compatibility
ii) Centrin and PCNT combination has been used to determine centrosome fragmentation[11]
iii) Centrin has been used to label centrioles[11, 12, 14] and PCNT has been used to label pericentriolar material[15, 16]

*After CCB02-treatment:* We did not observe any increase in fragmented centrosomes. From this, we think CCB02 does not induce centrosome fragmentation.

**Second experiment:** To further substantiate the above finding, we performed experiments in two centrosome-containing cells. This is because, in centrosome-amplified cells, extra centrosomes might mislead us in distinguishing them from fragmented centrosomes.

To do this, we analyzed MCF10A cells before and after CCB02 treatment. We did not observe any centrosome fragmentation before and after CCB02 treatment (**Fig. R3C**).

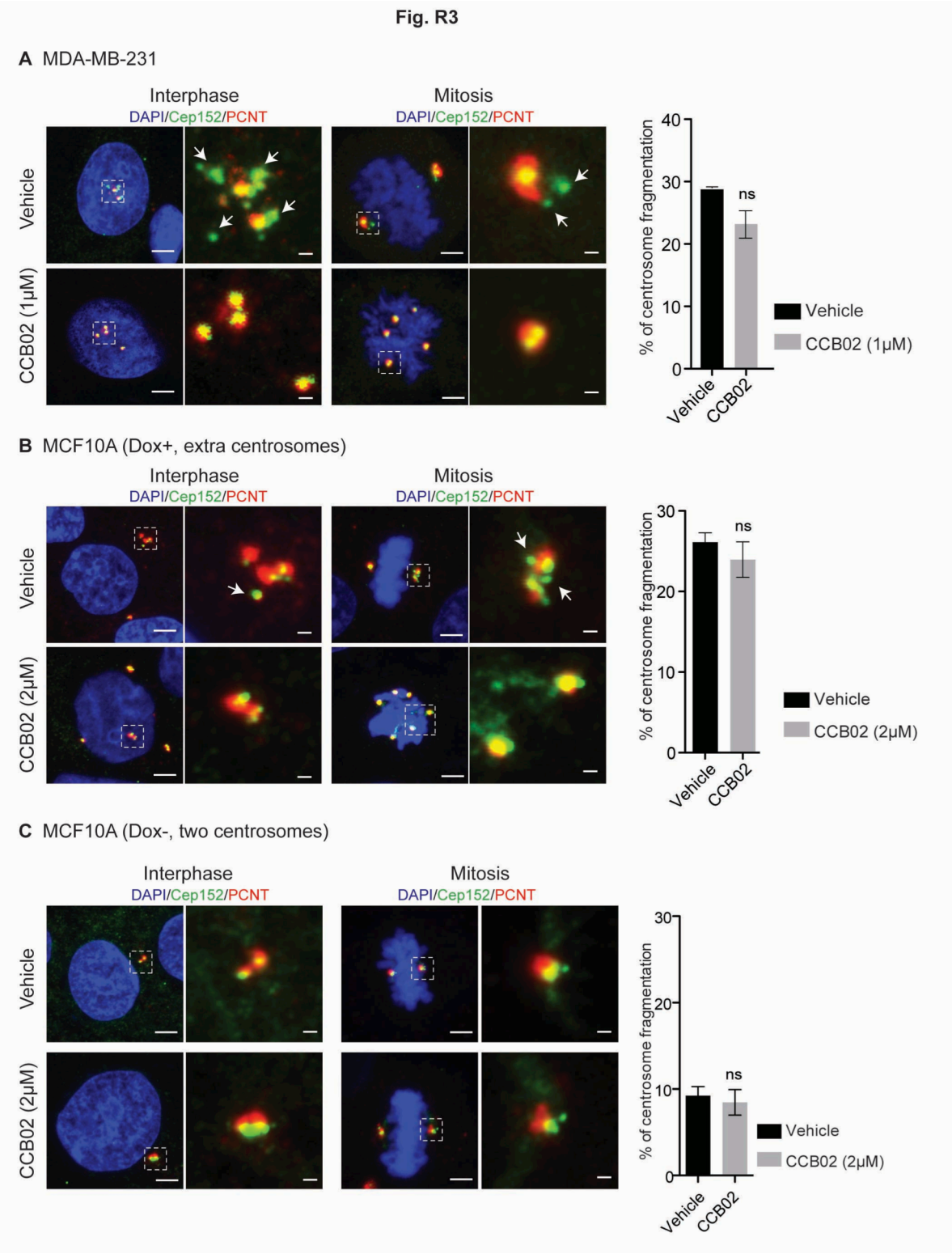


**Fig.R3 (A-B)** Fragmented centrosomes in extra centrosomes-containing MDA-MB231 and MCF10A cells (+Dox, extra centrosomes) before and after CCB02 treatment. Fragmented centrosomes in these cells are determined by PCNT-negative centrin dots (centrin-3). No increase in centrosome fragmentation is observed after CCB02 treatment. **(C)** Similar experiment was performed in two centrosome-containing MCF10A cells. This was because, in centrosome-amplified cells, extra centrosomes might mislead us in distinguishing them from fragmented centrosomes. No centrosome fragmentation is identified before and after CCB02 treatment. All these cells were stained for centrin3 (green) and PCNT (red), and DNA (DAPI, blue). Scale bar, 2μm and insets, 0.5μm. Bar diagrams at right quantify the percentage of fragmented centrosomes. (*N*)=3. At least 200 cells were used for quantifications from each cell line. Error bars, mean ± SEM. Unpaired *t*-test.

4. It will be of interest to test whether chemical perturbation of CPAP-Tubulin interaction will also affect the cancer cell migration using wound-healing assay.

We completely agree as it nicely complements our 3D spheroid invasion assay (**Fig. 7A-B**).

We pretreated MDA-MB-231 cells with 1µM CCB02 for 12 hrs. This is to ensure that the effect of CCB02 is already present while we perform experiments.

We noticed that CCB02 significantly delayed the wound closure possibly perturbing cell migration. We measured the delay at least in three-time points (**Fig. R4**).

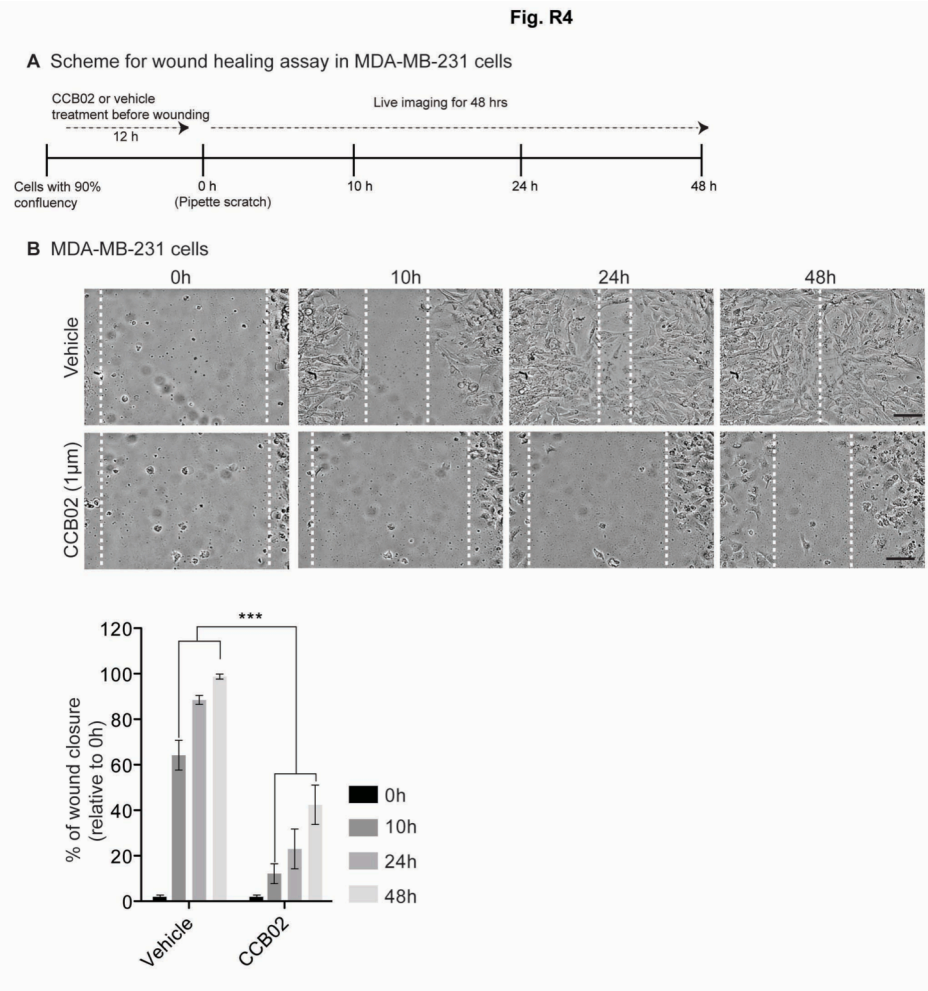


**Fig.R4 (A)** Experimental scheme of wound healing assay using MDA-MB-231 cells.
**(B)** Snap shot of live cell images show wound closure at various time points (0, 10, 24 and 48 hours). Relative to vehicle treatment, CCB02 delays wound closure. Dashed lines mark cell free empty space. Scale bar, 100µm. Bar diagrams below quantifies the percentage of relative wound closure. (*N*)=3. At Error bars, mean ± SEM. *p* values were obtained using Two-way ANOVA. ****P* < 0.0001.

5. A schematic illustrating the mouse xenograft experimental setup will be helpful to understand the impact of CCB02 on tumor growth.

As noted by this reviewer, we have now provided the scheme of mouse xenograft experiment in the main **Fig. 7C**.

6. The authors should explain in the manuscript why they used different concentrations of the CCB02 drug in different cancer cell lines and how they determined it.

We thank the reviewer to point out this important missing information. The concentration of the CCB02 used to treat different cancer cell lines were determined based on estimated $IC_{50}$ values of respective cell lines. For several unknown reasons, $IC_{50}$ values differed across various cell lines that we used. However, for uniformity, we wanted to stick to use the same concentration of CCB02 throughout the manuscript.

For example, MDA-MB-231 and MCF10A (+Dox, extra centrosomes) cells were treated with 1μM and 2μM, respectively. These cell lines were widely used in the manuscript. This information has been provided now in the figure legend (**Fig.S7C-G**) of the revised manuscript.

**Referee #3:**

Here, the authors report that inhibiting the interaction between CPAP (the human Sas-4 homologue) and tubulin, through either genetic or chemical means, leads to increased microtubule nucleation from cells with amplified centrosomes. This in turn interferes with centrosome clustering and apparently leads to selective elimination of cells with amplified centrosomes when cultured in 2D and 3D, as well as in mouse xenografts.

While the data look potentially interesting, I struggled to follow this manuscript with many sections poorly explained.

We apologize that some aspects of the manuscript is not clear to this reviewer. We have improved the clarity of the revised version.

For example, it wasn't indicated how constructs were 'introduced' into cells or what was meant by the 'frequent hitters' that were excluded from the screen.

These are indeed methodological aspects, which are described in the method section. We have now elaborated these aspects in method section.

"Frequent hitters" – Frequent hitters mean that some of the compounds have inherent ability to bind proteins non-specifically. There are number of ways to eliminate these frequent hitters from the final list of compounds. To do it efficiently, we generated an algorithm[17]. This aspect has been elaborated in our earlier work Schorpp, K. et al 2013 that has been referred in the methods section (Reference 1, Page.16).

There was also insufficient detail in the legends to the six main figures with, for example, it not being clear what was being imaged in the live cell experiments shown in Figure 1A or how the cells shown on the left in Figure 3B were identified as being in G2.

It turned out that some information were not evident. For example, in Figure 1A, we mentioned the cell cycle stage that was being imaged. For clarity, we now mentioned it also in the figure legend **(Fig.1A)**.

In addition, it was quite frustrating that the bulk of the data was put into eleven Supplementary Figures, including some potentially interesting results such as that depletion of CPAP abrogated the effect of the compound.

As suggested, we now have rearranged the figures brining key findings into main figures. For example, we moved the **Fig.S10** to Main **Fig. 4** and **Fig.S7B to Fig. 3A**.

Finally, there are some plainly inaccurate statements, such as that in interphase the centrosome is incompetent to nucleate robust microtubules (p14); this might be true in flies but not in human cells.

Our apologies for the lack of clarity. We clarified these issues. To better improve the accuracy of our statements, we add relevant literatures concerning *Drosophila* and human centrosomes.

Hence, overall I do not feel that this manuscript reaches the standards expected for this prestigious journal and cannot recommend publication.

I have highlighted some additional concerns below.

In the genetic-based experiments shown in Figure 1, I'm unclear as to whether the authors think that blocking the CPAP-tubulin interaction leads to enhanced microtubule nucleation at all times or only in mitosis, and only in cells with amplified centrosomes or all cells. In several places, the authors talk about enhancing microtubule nucleation "prior to mitosis" without clarifying whether they mean throughout the cell cycle or just in G2. Crucially, microtubule regrowth experiments are required to answer this point and should be done on interphase and mitotic cells with and without doxycycline-induction of Plk4. Quantified microtubule regrowth experiments would also be useful in other parts of the manuscript, such as in Figure 4 when cells are treated with CCB02. Figure 4C apparently shows microtubule nucleation but again it isn't clear from the legend how this experiment has been done.

We appreciate these comments, as they are crucial to the manuscript. By the term “microtubule nucleation before mitosis” means from interphase until mitosis. This was because, inherent difficulty in clearly distinguishing cell cycle stages of cancer cells. Cancer cells are difficult to synchronize. However, we used Cyclin-A staining to profile interphase cells that nucleate enhanced level of microtubules **(Fig. S8A).**

Microtubule regrowth assay suggested by this reviewer is crucial to solve this ambiguity. Thus, we eliminated Figure 4C and have performed a detailed microtubule regrowth assay (**Fig.R2**). This experiment strengthens two key aspects of the paper namely,

CCB02 treatment
***i)*** Enhances MT nucleation prior to mitosis
***ii)*** Enhances PCM recruitment

We preformed a three-time point microtubule regrowth assay with MCF10A (-Dox, two centrosomes), MCF10A (+Dox, extra centrosomes) and MDA-MB-231 cells[9, 10]. CCB02 treatment caused centrosomes to nucleate an enhanced level of microtubules already at 1.5 min after induction of regrowth. Importantly, these centrosomes indeed recruited significantly higher levels of γ-tubulin **(Fig. R2)**.

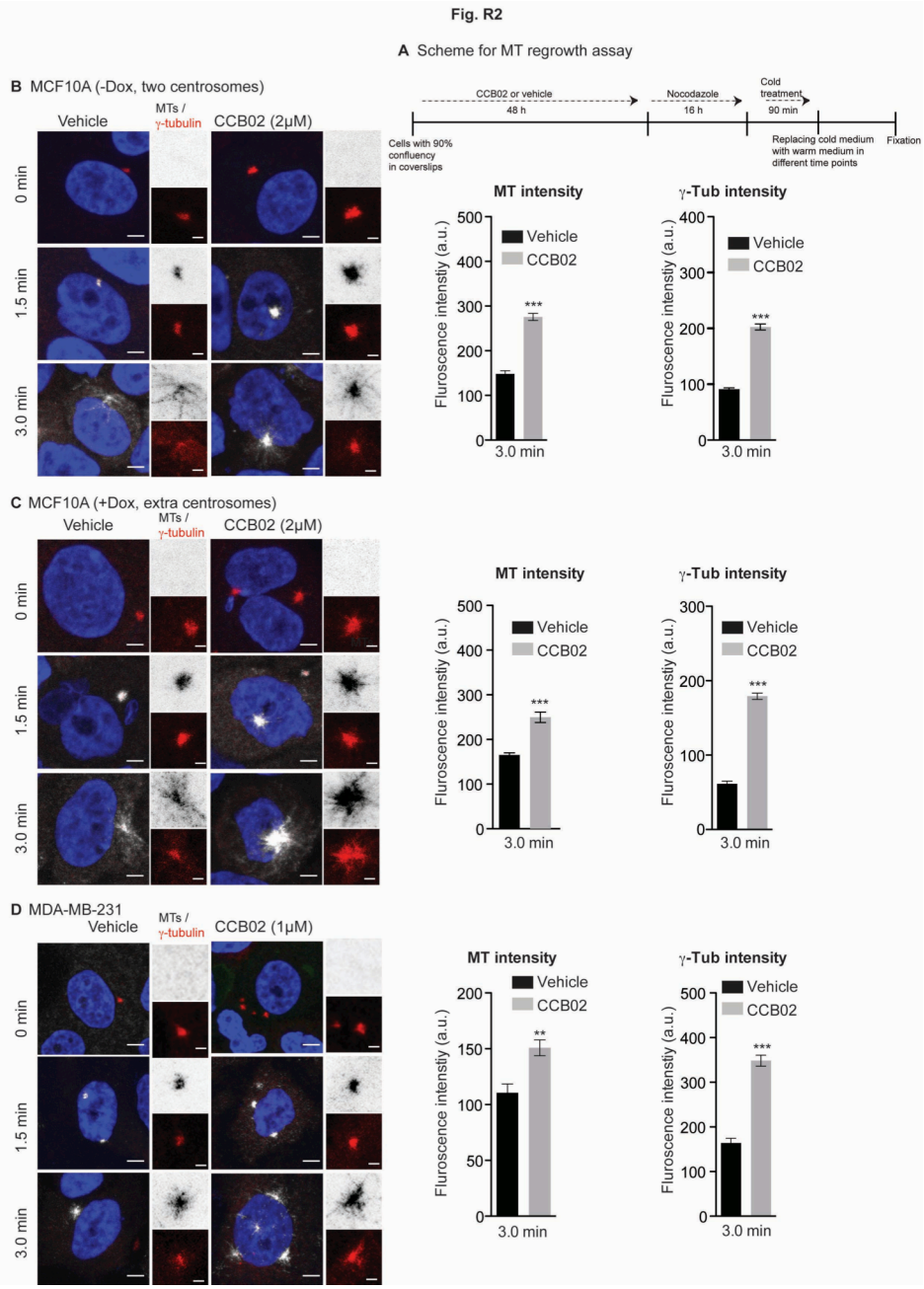


**Fig.R2 (A)** Experimental scheme of microtubule (MT) regrowth assay.
**(B-D)** MT-regrowth assays at 0, 1.5 and 3.0 min using MCF10A (-Dox, two centrosomes), MCF10A (+Dox, extra centrosomes) and MDA-MB-231 cells. MT nucleation panel is shown in grey scale (inset images are inverted) and γ-tubulin is shown in red. Note that in contrast to vehicle treatment, CCB02 treatment caused centrosomes to nucleate an enhanced level of microtubules already at 1.5 min after induction of regrowth with simultaneous increase in γ-tubulin recruitment. All these cells were stained with γ-tubulin (red), microtubules (α-tubulin, grey) and DNA (DAPI blue). Scale bar, 2μm and insets, 0.5 μm. Bar graphs show MT and γ-tubulin intensities at 3 min after induction of MT regrowth. (*N*)=3. At least 80 centrosomes were considered to calculate intensities from each cell line. Error bars, mean ± SEM. Unpaired *t*-test. ***P* < 0.001, ***P < 0.0001.

Figure 4D shows an immunoprecipitation experiment but without controls.

This experiment is to show CCB02 prevents CPAP-tubulin interaction in dose dependent manner. The control is actually the lane without CCB02 (where CPAP-tubulin interacting is intact and CPAP is able to co-purify with Ce152 and gamma-tubulin). However, as this reviewer asked, we now have given an IgG control in **Fig. 5C** and **Fig. S11C**.

While I am not an NMR expert, I wasn't convinced that the data shown in Figure 2 unambiguously confirms that CCB02 directly binds to the same site on tubulin to which CPAP binds. Indeed, the text hints at this lack of definitive proof stating that "this observation suggests that the CPAP peptide and CCB02.1 have the same binding site on tubulin". When combined just with in silico docking and not a crystal structure then I don't feel that the authors can draw the conclusion that "these results indicate that CCB02 is a novel tubulin binder whose binding site overlaps with the CPAP peptide". Biochemical competition experiments are also required here. Likewise, the CCB02-biotin pull-down assay identified interacting proteins beyond tubulin (Fig. S6E), and so the authors are overinterpreting the results to claim that CCB02 is a specific tubulin binder in cells.

We notice that the #3 is not a structural biologist. The structural biology studies have been performed at the Sattler lab (TU-Munich), a well-regarded structural biologist who paid careful attention in interpreting the results. Importantly, we used the state-of-the-art methods to pin point CCB02 binding to tubulin and modeled it's binding mode. These findings were further strongly backed up by number of orthogonal experiments such as

-ITC (**Fig.S6**),
-ALPHA screen (**Fig.S3**),
-INPHARMA assays (**Fig.2 and S5**),
-protein-protein interactions (**Fig.4 and S4**),
-M/S (**Fig.S6**)
-IP experiments (**Fig.S11**).

We indeed tried to co-crystal CCB02-tubulin complex. This is extremely challenging task. Kindly refer our earlier work where CPAP-tubulin complex was determined[18]. Tubulin is very dynamic protein that could be crystalized only in the presence of CPAP domain that somehow stabilizes the complex for crystallization.

Besides all of these aspects, in our opinion, crystallizing CCB02-Tubulin is beyond the scope of the current paper.

Figure 3 presents the main data in support of the title of the manuscript that inhibiting CPAP-tubulin interaction selectively acts on cells with amplified centrosomes. However, the correlation of CCB02 IC50 with centrosome number isn't sufficient to suggest that the effect of this drug is selective to cells with amplified centrosomes, as these cancer cells are likely to share many other genetic defects. Indeed, in the Discussion the authors admit that CCB02 is only "likely to be selective for extra centrosomes-containing cells".

We think it requires clarifications. We toned down our statements in the discussion section as agreed to this reviewer's concern that correlation of CCB02 IC50 with centrosome number is not sufficient to concretely claim the selectivity of CCB02 against cells with extra centrosomes. However, to strengthen our statement, we have also performed an additional experiment. **(Fig. 3A of the revised manuscript)**. For example, our long-term CCB02 treatment selectively eliminated cells with extra centrosomes. This data additionally supports our statement that CCB02 is selective for extra centrosome containing cells.

In Figure 5, it is claimed that taxol and docetaxel do not induce multipolar mitoses when it is well know from the literature that they do and indeed the figures suggest that they do.

Our experiment was to determine whether extra centrosomes are de-clustered upon Taxol

addition. What we reported is centrosomes are not de-clustered. There are few acentrosomal spindles emerge due to the effect of Taxol-mediated microtubule stability.

For clarification, we have provided the same image of the taxol panel of **Fig. 6B** below showing the individual channels. The yellow arrowheads indicate centrosomes and white arrows indicate the acentrosomal microtubules.

**Fig. R5**

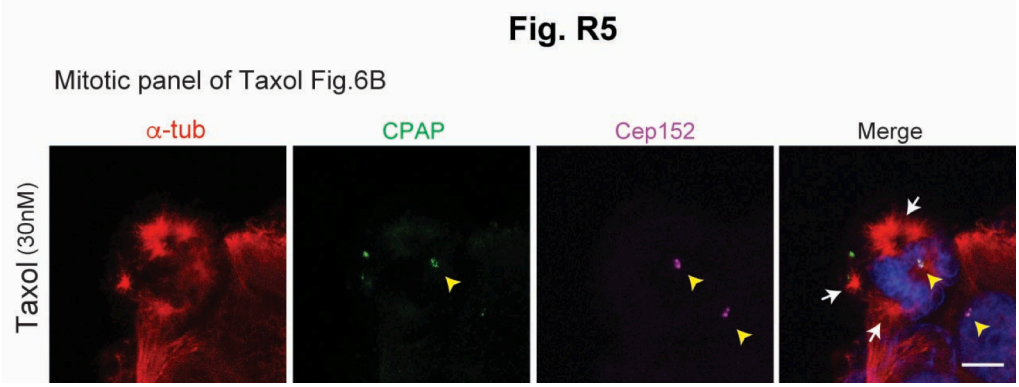


**Fig.R5** The individual channels of taxol mitotic panel of Fig.6B. The white arrow indicates the acentrosomal microtubules and yellow arrow indicates the centrosomes. Cells were stained with CPAP (green), cep152 (magenta), microtubules (α-tubulin, red) and DNA (DAPI blue). Scale bar, 2μm.

The analysis of the consequences of CCB02 on microtubule dynamics is very superficially described.

It is unfortunate to hear this comment, which surprises us. We have adapted our assays both in vitro and in cells. These are the well-accepted and standard assays (MT-tracking and EB1 /EB3 live imaging)[19-22].

The abstract highlights the use of 3D-organotypic invasive assays but only a limited set of data are shown using this approach in Fig. 6, and the graph in Fig. 6B lacks errors bars.

We how have provided error bars in **Fig. 7B**.

Many of the main and supplementary figures are only shown as merged images in which it is not always possible to see individual protein signals, e.g. CPAP and Cep152 in Figure 3B or S2.

This was because of space constraint. Splitting the individual channels will consume larger space and could still be redundant (For an example, **Fig. S7C-G**. Splitting channels will cause at least 40 panels). However, as asked, we have split channels for upper panel of **Fig. 3B**.

**References:**


1. Kramer, A., Maier, B. & Bartek, J. Centrosome clustering and chromosomal (in)stability: a matter of life and death. *Molecular oncology* **5**, 324-335 (2011).
2. Watts, C.A. *et al.* Design, synthesis, and biological evaluation of an allosteric inhibitor of HSET that targets cancer cells with supernumerary centrosomes. *Chemistry & biology* **20**, 1399-1410 (2013).
3. Fielding, A.B., Lim, S., Montgomery, K., Dobreva, I. & Dedhar, S. A critical role of integrin-linked kinase, ch-TOG and TACC3 in centrosome clustering in cancer cells. *Oncogene* **30**, 521-534 (2011).
4. Kwon, M. *et al.* Mechanisms to suppress multipolar divisions in cancer cells with extra centrosomes. *Genes & development* **22**, 2189-2203 (2008).

5. Raab, M.S. *et al.* GF-15, a novel inhibitor of centrosomal clustering, suppresses tumor cell growth in vitro and in vivo. *Cancer research* **72**, 5374-5385 (2012).
6. Wong, Y.L. *et al.* Cell biology. Reversible centriole depletion with an inhibitor of Polo-like kinase 4. *Science* **348**, 1155-1160 (2015).
7. Gopalakrishnan, J. *et al.* Sas-4 provides a scaffold for cytoplasmic complexes and tethers them in a centrosome. *Nature communications* **2**, 359 (2011).
8. Gopalakrishnan, J. *et al.* Tubulin nucleotide status controls Sas-4-dependent pericentriolar material recruitment. *Nature cell biology* **14**, 865-873 (2012).
9. Choi, Y.K., Liu, P., Sze, S.K., Dai, C. & Qi, R.Z. CDK5RAP2 stimulates microtubule nucleation by the gamma-tubulin ring complex. *The Journal of cell biology* **191**, 1089-1095 (2010).
10. Sankaran, S., Starita, L.M., Groen, A.C., Ko, M.J. & Parvin, J.D. Centrosomal microtubule nucleation activity is inhibited by BRCA1-dependent ubiquitination. *Molecular and cellular biology* **25**, 8656-8668 (2005).
11. Karki, M., Keyhaninejad, N. & Shuster, C.B. Precocious centriole disengagement and centrosome fragmentation induced by mitotic delay. *Nature communications* **8**, 15803 (2017).
12. Godinho, S.A. & Pellman, D. Causes and consequences of centrosome abnormalities in cancer. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **369** (2014).
13. Kohlmaier, G. *et al.* Overly long centrioles and defective cell division upon excess of the SAS-4-related protein CPAP. *Current biology : CB* **19**, 1012-1018 (2009).
14. Marteil, G. *et al.* Over-elongation of centrioles in cancer promotes centriole amplification and chromosome missegregation. *Nature communications* **9**, 1258 (2018).
15. Lawo, S., Hasegan, M., Gupta, G.D. & Pelletier, L. Subdiffraction imaging of centrosomes reveals higher-order organizational features of pericentriolar material. *Nature cell biology* **14**, 1148-1158 (2012).
16. Chen, C.T. *et al.* A unique set of centrosome proteins requires pericentrin for spindle-pole localization and spindle orientation. *Current biology : CB* **24**, 2327-2334 (2014).
17. Schorpp, K. *et al.* Identification of Small-Molecule Frequent Hitters from AlphaScreen High-Throughput Screens. *J Biomol Screen* **19**, 715-726 (2013).
18. Zheng, X. *et al.* Molecular basis for CPAP-tubulin interaction in controlling centriolar and ciliary length. *Nature communications* **7**, 11874 (2016).
19. Straube, A. & Merdes, A. EB3 regulates microtubule dynamics at the cell cortex and is required for myoblast elongation and fusion. *Current biology : CB* **17**, 1318-1325 (2007).
20. Mimori-Kiyosue, Y. *et al.* CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex. *The Journal of cell biology* **168**, 141-153 (2005).
21. Komarova, Y. *et al.* EB1 and EB3 control CLIP dissociation from the ends of growing microtubules. *Molecular biology of the cell* **16**, 5334-5345 (2005).
22. Tirnauer, J.S. & Bierer, B.E. EB1 proteins regulate microtubule dynamics, cell polarity, and chromosome stability. *The Journal of cell biology* **149**, 761-766 (2000).

2nd Editorial Decision 4 October 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your revised study was sent back to the three referees for re-evaluation, and we have received comments from all of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues regarding material and methods, formatting, data representation and wording, as outlined below, which need to be adjusted at re-submission.

REFEREE REPORTS

Referee #1:

Most of my comments have been addressed and I consider that the paper is now acceptable for publication.

Referee #2:

In their revised manuscript, Mariappan et al have performed an extensive round of revision to address the issues I raised. Overall, the authors have done an excellent job. In particular the additional microtubule regrowth assay and wound-healing assay give much more weight to their conclusions. The manuscript is quite complete in its current version and therefore I fully support its publication in EMBO.

Referee #3:

This revised manuscript describes a new approach to selective targeting of cancer cells with amplified centrosomes. It exploits a previous finding from this group that in flies centrosomal nucleation of microtubules in interphase is suppressed by the interaction of tubulin with Sas-4. Here, they use a mutant of the human Sas-4 homologue, CPAP, to demonstrate that microtubule nucleation in human cells is suppressed by a similar mechanism and that this is necessary to allow clustering of amplified centrosomes in mitosis. They also describe identification of a novel chemical inhibitor of the tubulin-CPAP interaction that not only blocks centrosome clustering but promotes death of cancer cells with amplified centrosomes in vitro and in vivo.

The authors have made significant efforts in this new version to add valuable controls to what is already a substantial amount of data and ensure that appropriate conclusions are drawn. I was also pleased to see some of the key data brought from the supplementary section into the main figures; this certainly made the manuscript easier to follow. One of the key experiments requested by myself and another referee was to analyse microtubule regrowth rates. This has been well done but has been placed in the supplementary material as Figure S10. Again, I would strongly advise that this is incorporated in the main figures. With these changes, the authors have gone a long way to address the concerns of myself and the other two referees.

I do though remain concerned about the writing with sentences that I find rather poorly constructed and potentially confusing or misleading. For example, on p14 it states that "At interphase of the cell cycle, the centrosome contains a basal level of PCM and is incompetent to nucleate robust microtubules." It is correct to state that there is a basal level of PCM, but human interphase cells are perfectly competent to nucleate a robust microtubule network that contributes to cell shape, migration, vesicular transport and organelle positioning. Similarly, the sentence on p15 that states that "we identified that CCB02 binds beta-tubulin's microtubule outer surface" would benefit from revising. Finally, there are also several references on p13 to Fig. 14 that are not correct.

 

Overall though, I am persuaded that this is an interesting study that presents important new findings worthy of publication and so if, perhaps with support from the journal editorial team, the writing can be sufficiently improved then I would have no further objections to publication.

2nd Revision - authors' response 11 October 2018

Authors made requested editorial changes.

 

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| Manuscript Number: EMBOJ-2018-99876 |

**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? | To have enough sample size and to avoid random variability, each experiment was preformed in triplicate or multiplicate to collect the data. In each experiment, the amount of cell numbers and appropriate statistical methods used are described under each figure legend in the manuscript. Also, given under the methods section on page #4 and page #9. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | Sample size for animal studies were determined based on the literatures and as described in the methods on page #7 and page #8.. |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No samples or animals were excluded from the analysis. We performed experiments as described in the methods on page #7, #page4 (end of paragraph 1) and page #8. |
| 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. | Randomization of animals was used in mouse xenograft assays as described in the methods on page #7 and page #8. |
| For animal studies, include a statement about randomization even if no randomization was used. | Randomization of animals was used in mouse xenograft assays as described in the methods on page #7 and page #8. |
| 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. | Investigator was not blinded during any experiment. Blinding was not relevant to our study. Please refer to first paragraph of #page 9 of methods section. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | Blinding was not relevant to our study. |
| 5. For every figure, are statistical tests justified as appropriate? | Statistical justifications for each experiment, the amount of cell numbers and appropriate statistical methods used are described under in figure legend in the manuscript. Also, given under the methods section on page #4 and page #9. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | |
| Is there an estimate of variation within each group of data? | |
| Is the variance similar between the groups that are being statistically compared? | |

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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). | Antibodies used in this study are reported with catalogue number or reference on page #4, #5, #6and #8 of methods section. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | Cell lines were purchased from ATCC and DSMZ. It is mentioned in the page#2 of methods section. Yes, cell lines have been tested for mycoplasma. It is mentioned in the page#3 (first paragraph) of methods section. |

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

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | For mouse xenograft, NMRI-nu (RjOrl:NMRI-Foxn1nu/Foxn1nu) female mice with a age of four to six weeks were used for experiments after at least one week of quarantine. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | NA |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | NA |

## E- Human Subjects

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

## F- Data Accessibility

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

## G- Dual use research of concern

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