EMBO reports

Light-induced spindle assembly through Aurora A clustering

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Referee #1:

Olakkal et al. induce the clustering of Aurora A by utilizing the optogenetic system CRY2/CIB1-MP. The authors demonstrate that this clustering triggers the activation of Aurora A (phosphorylation at T288) and the assembly of spindle pole-like structures that nucleate microtubules. Notably, these clusters promote spindle assembly independently of the Aurora A scaffold CEP192 and centrosomes. These clusters were shown to play a crucial role in mitotic regulation. Their expression rescued the prolonged mitotic duration and the defects in mitotic exit observed under conditions where centrosomes are absent. While the study has successfully reconstructed the minimal unit of centrosome activity in mitosis and the manuscript is well-written with appropriate control experiments, the study in its current form offers limited conceptual advances. Specifically, providing a more mechanistic understanding of the observed phenotypes would significantly strengthen the contribution to the field. Thus, I recommend addressing the following points before publication in EMBO Journal.

Major points

1. Although the clusters of Aurora A nucleate microtubule assembly without recruiting CEP192 (Fig. 3B), it remains unclear whether these clusters recruit other PCM components and the γ-TuRC. If they do not, does the cluster recruit other factors for microtubule assembly such as TPX2? I strongly recommend staining and depleting PCM proteins and MT assembly factors in a nocodazole washout experiment. These data would provide crucial insights into the mechanism of Aurora A-mediated microtubule nucleation in mitosis.
2. The clusters of Aurora A assemble spindle poles and promote spindle assembly in the absence of centrosomes, without recruiting core centrosomal proteins including γ-tubulin (Fig. 4). However, it is important to determine if the cluster recruits other factors, such as TPX2 and the HAUS complexes, to the acentrosomal spindle pole. I recommend staining and depleting MT assembly factors in the cells. Through these experiments, the authors can better demonstrate the essential functions of centrosomes in mitosis-specifically, the regulation of spindle pole factors through Aurora A activity, rather than PCM maturation.
3. Optogenetic activation of Aurora A promoted mitotic progression in acentrosomal cells (Fig. 5). The experiments nicely showed that the optogenetic system restores the functions of centrosomes. However, some cells still exhibit prolonged mitotic progression. A more detailed description of the phenotypes in these cells could reveal the functions of PCM-mediated centrosomal microtubules in mitosis.
4. All experiments were performed with the HeLa Kyoto cell line. It would be preferable to perform the Aurora A clustering experiments in non-transformed human cells, such as

hTERT RPE-1 cells, to validate the physiological relevance of the findings.

Minor points
5. In Fig. 1, the authors employ CIB1-MP to induce the clustering. Have alternative multimerization tags been tested? It is possible that CIB1-MP itself contributes to the integrity of an artificial spindle pole independently of Aurora A function.
6. Fig. 1G shows that Aurora A cluster formation is induced in interphase cells. Are these clusters similarly capable of recruiting microtubules? Furthermore, which endogenous factors are enriched at these sites?
7. In the absence of centrosomes or Cep192, it is unclear how Aurora A clusters are targeted to spindle poles. What factors are proposed to act as recruiters in this context? This could potentially be addressed experimentally, for example by siRNA-mediated depletion of candidate factors.
8. In Fig. 4H-I, spindle length is increased upon light illumination. What mechanism underlies this elongation, and can it be experimentally tested?

Referee #2:

The manuscript is clearly written and well presented. The study introduces an optogenetic system designed to control Aurora A auto phosphorylation by inducing blue light-dependent clustering of Cry2-Aurora A on a CIB1-MP matrix. The authors show that high local concentrations of Aurora A are sufficient to trigger T288 auto phosphorylation, mimicking the physiological centrosomal recruitment by CEP192 that normally generates this auto phosphorylated Aurora A pool. They further report that light induced Aurora A auto-phosphorylation can restore spindle assembly to and partially alleviate mitotic delays in acentrosomal, centrinone treated cells. The tool itself is elegant and valuable for achieving temporal control of Aurora A auto-phosphorylation.

In its current form, beyond the development of this optogenetic system, the manuscript provides limited new insight into Aurora A biology. The central observation that enforced clustering of Aurora A induces auto phosphorylation is not unexpected; similar conclusions were drawn by Joukov et al. (2010) who demonstrated that dimerisation/oligomerisation of the kinase on beads led to auto-phosphorylation and activation. The most interesting finding here concerns the mitotic role of the centrosome. The authors would argue that achieving Aurora-A's auto-phosphorylation is the sole role of the mitotic centrosome, as stated in the abstract. However, the current data are not robust

enough to support this bold claim, in part due to the lack of some important controls.

Key points:

1. The optogenetic tool is the major strength of this work; as such, it is surprising that the authors have not fully characterised the composition of these complexes during interphase and mitosis. With a SNAP-tag present in CIB1-MP, it is straightforward to affinity purify these complexes and identify their components. Incorporating these data would strengthen the manuscript's mechanistic insights while also addressing point 2 below.
2. Normal Aurora A activity requires complex formation with TPX2, and therefore the authors should demonstrate that complex formation occurs as expected with Cry2-AurA.
3. The optogenetic system is expected to massively alter the balance between phosphorylated and unphosphorylated Aurora A, pushing it towards an excess of auto-phosphorylated kinase. The authors should discuss how this may affect Aurora A functions. For instance, light-driven clustering could actually phenocopy Aurora-A overexpression, thus interfering with spindle formation and also the spindle assembly checkpoint. If so, this needs to be taken into consideration when interpreting data in Figure 5. Chromosome mis-segregation frequency should be measured in the various cell lines generated.
4. Throughout the manuscript the authors equate Aurora A activity with its auto phosphorylation. This is not necessarily the case, and it is thus crucial that Aurora-A activity is assessed independently (e.g. phosphorylation and localisation of substrates).
5. In Figures 4 and 5, mitotic phenotypes (e.g., mitotic duration, spindle length) are assessed in parental control cells, with several important controls missing. It would be important to show mitotic duration and spindle length in Cry2-AurA/CIB1-MP cells with and without light in absence of centrinone. These controls are essential, particularly given the differences apparent between centrinone-treated parental and centrinone-treated (no light) Cry2-AurA/CIB1-MP cells. Mitotic duration seems much longer in the latter, indicating that Cry2-AurA/CIB1-MP expression alone sensitises cells to centrinone treatment.
6. Instead of fixed cut offs (100 min, 4 h) in Fig. 5, the authors should consider reporting median mitotic durations for cells that complete mitosis. The selected cells in Fig. 5B-d all show a marked delay with only one undergoing anaphase, so these panels do not seem to be representative of the graph.
7. The T288A non-phosphorylatable Aurora-A construct CRY2-AurA is only mentioned in Figure S6 as a control for the centrinone experiment in Figure 5. It is a potentially interesting observation, but we would need to know more about the behaviour of this mutant under normal conditions to be able to interpret the results.
8. It is difficult to track which experiments endogenous Aurora-A is depleted in.

9. A clear limitation of the manuscript is that all the experiments are conducted in HeLa cells that have a complex, near-triploid genome. In part due to this larger genome HeLa cells take twice as long to complete mitosis than other diploid cells and despite the increased duration they still frequently mis-segregate chromosomes. Unsurprisingly, Aurora-A inhibition/overexpression have particularly profound effects in HeLa cells. While I realise that establishing this system would take considerable effort in another cell type, I question the rationale of choosing HeLa cells, and this should at least be discussed in the manuscript.
10. I would recommend the authors to change the final sentence of their abstract. It is difficult to see how this system would yield a screening platform. If the authors disagree, they should provide a better explanation in the manuscript as they simply repeat the statement as their final sentence of the main text.
11. The recent paper by the Raff group on the mitotic Aurora-A scaffold (Wong et al., 2025) is highly relevant to this study and should be cited.

Referee #3:

In this study, the authors have developed an optogenetic system to induce clustering of Aurora A kinase and tested whether this platform is sufficient to activate Aurora A and centrosome-linked functions in human cells. By combining light-inducible clustering with perturbations such as Cep192 depletion and PLK4 inhibition, the authors propose that Aurora A oligomerization can drive kinase activation, promote microtubule nucleation, and partially restore mitotic defects in cells with experimentally perturbed centrosome formation.
The approach is technically elegant and addresses a relevant question in centrosome and spindle biology: what is the minimal protein composition at mitotic spindle poles required for efficient microtubule nucleation and faithful cell division? Although the manuscript presents some interesting observations, particularly regarding the ability of induced clusters to partially compensate for centrosome loss, several important quantifications are missing, and the most interesting observation is left without any mechanistic insight. Providing the missing quantifications and attempting to understand molecular mechanism underlying the Aurora A clustering-induced microtubule nucleation would significantly improve the manuscript and make it more relevant and attractive to the EMBO Journal readership. Below please find specific comments.

Major concerns:

1) Figure 1D-F: the criteria used to classify monopolar spindles and chromosome instability are not clearly defined. Which markers have been used for these classifications? Chromosome instability is characterized by numerical and structural changes on the karyotype level that is driven by loss or gain of whole or parts of chromosomes. Given that the karyotype has not been analysed, a more precise term would be something like chromosome congression or segregation errors. This should be more precisely defined, and the representative images should be provided.

2) On the page 6, the authors state: "Since centrosome-localized Aurora A rapidly exchanges with the cytoplasmic pool (Kress et al., 2013; Laos et al., 2015; Zheng et al., 2024), we reasoned that Aurora A clusters generated in mitosis are rapidly transported to centrosomes via microtubule-dependent motors." - The authors could confirm their reasoning using live-cell imaging to clarify whether the clusters are transported to centrosomes via minus end-directed motor activity (e.g. using dynein depletion or inhibition).

3) Related to Figure 2: The authors state that clustered Aurora A "exhibits centrosome-like activity by nucleating microtubules", and that "In contrast to the control cells not exposed to blue light, cells expressing CRY2-AurAr/CIB1-MP, when exposed to blue light and released from the nocodazole, were capable of microtubule nucleation from the CRY2-AurAr clusters, which are spread throughout the cells (Fig. 2H; Movies S3 and S4)".
- It is not clear whether the authors propose that Aurora A serves as a microtubule nucleator per se. The presented experiments do not directly test whether Aurora A catalytic activity is required for the observed microtubule assembly (e.g., no kinase inhibition or kinase-dead mutant analysis or direct assay on microtubule nucleation). Microtubule nucleation is superficially analyzed and left at a very observational level. For instance, GFP-EB1 tracking or quantification of tubulin fluorescence intensity at different time points after nocodazole release would strengthen the conclusions.
- Aurora A activation is inferred solely from T288 autophosphorylation. Inclusion of at least one additional substrate readout would strengthen the conclusion that Aurora A is functionally active toward its downstream targets.
- Are active Aurora A clusters able to bind allosteric factors like Tpx2 and Bora? This could strengthen the central claim that clustering drives functional activation.

4) Related to Figure 3: The observation that Aurora A clustering can restore spindle bipolarity in Cep192-depleted cells is important and should be quantified to clarify whether this is a robust population-level effect or limited to a subset of cells.

5) Figure 3D-G, and page 9: The authors state that "Consistent with prior work, siRNA-mediated depletion of Cep192 abolished the accumulation of T288P and produced spindle assembly defects (Fig. 3D-3G; data not shown; Gomez-Ferreria et al., 2007; Holder et al., 2024)". The data should be shown and quantified.

6) Related to Figure 4: The authors report that prolonged centrinone treatment led to loss of several PCM components, including Cep192, PCNT, and Cdk5Rap2. However, previous work has shown that acentriolar HeLa cells retain PCM components at spindle poles. In fact, lack of both centrosomes and these PCM components resulted in mitotic spindle assembly failure in HeLa cells. The discrepancy between these two sets of results should be discussed.

7) Page 10: the authors state that "Strikingly, a brief blue-light pulse induced robust T288P accumulation in centrinone-treated cells (Fig. 4E-4G), despite the absence of Cep192 or other centrosome-localized proteins at CRY2-AurAr/CIB1-MP clusters (Fig. S4A-S4O). Thus, light-induced clustering of CRY2-AurAr is sufficient for Aurora A activation in the absence of centrosomes".
- How could Aurora A clustering initiate microtubule nucleation without g-tubulin and/or other centrosome/PCM components? This remains the biggest unused potential of the study and addressing this would immensely increase the manuscript's impact. Besides acknowledging it in the discussion part, the authors could try to assess which other proteins are within the Aurora A clusters that are required for microtubule nucleation. This could be done by either immunofluorescence with specific markers or by immunoblotting and/or mass spectrometry identification after pull-down of the clustered Aurora A.

Minor points:

1) Page 5: The authors state that CRY2-AurAr fully rescues the phenotypes associated with Aurora A depletion. Given that "chromosome instability" presented in Figure 1F remains at 20%, it would be more precise to state that the construct substantially or partially rescues the phenotype.

2) Figure 4G: the last two labels appear misplaced.

3) Clarify whether quantifications represent biological replicates.

4) Number of experiments are not specified in the figure legends, only number of cells.

5) Consistent terminology when referring to "centrosome-like structures" vs "induced clusters" would be of help to the readers.

6) Figure S5A: All other centrinone-treated cells shown in the manuscript (including quantification of spindle length in Figure 4I) displayed much shorter spindles than the spindles in this figure, which appear of similar length as controls. Could authors comment on this?

7) Figure 4I: The lack of centrosomes reduces astral microtubules and can therefore affect spindle orientation, which could interfere with spindle length measurements if derived from 2D projections. The methods section should specify whether 3D measurements or z-corrections were applied.

8) Discussion: The authors state that "Interestingly, when Aurora A was clustered by light in interphase, it remained inactive, showing no detectable T-loop phosphorylation". The reviewer could not find this data in the manuscript.

Dear Prof. Kotak

Thank you for the transfer of your research manuscript, accompanying referee comments, and proposed revision plan to our journal.

We are interested in your manuscript pending revisions including the inclusion of data demonstrating the functionality of your new tool in additional cell lines, the inclusion of additional controls and analyses to cement the dispensability of Cep192, and the delineation of factors that are recruited to the Aurora A clusters that may contribute to microtubule nucleation.

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (July 22nd). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

I am also happy to discuss the revision further via e-mail or a video call, if you wish.

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- the EXACT p-values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
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relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section describing the methods, ideally using a step-by-step protocol format. The aim is to facilitate adoption of the methodologies across labs. Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guidelines: https://www.embopress.org/page/journal/14693178/authorguide#structuredmethods.
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Kurt Weir
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The point-by-point response to reviewers

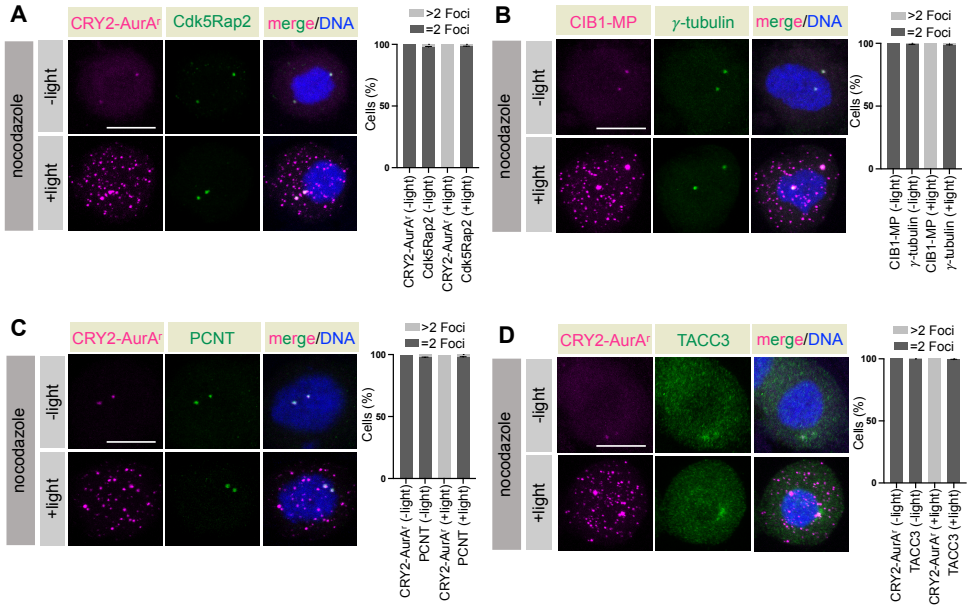
Reviewer #1

We sincerely thank this reviewer for recognizing that "*the study has successfully reconstructed the minimal unit of centrosome activity in mitosis and the manuscript is well-written with appropriate control experiments"*. However, the reviewer noted that, in its current form, the study offers limited conceptual advances. We greatly appreciate the reviewer's insightful remarks and suggestions, which have helped us to improve the manuscript.

Major points:

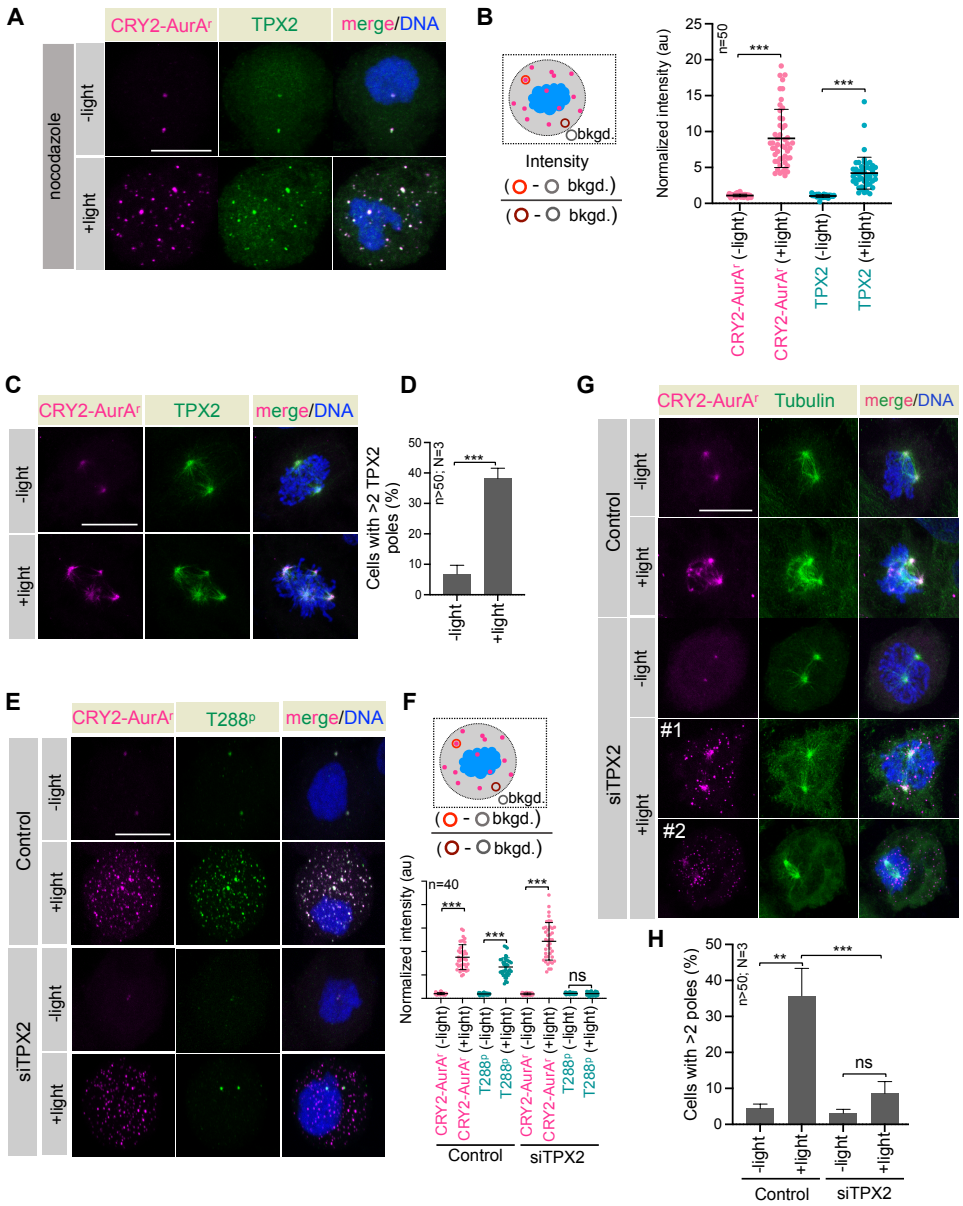
1.Although the clusters of Aurora A nucleate microtubule assembly without recruiting CEP192 (Fig. 3B), it remains unclear whether these clusters recruit other PCM components and the γ-TuRC. If they do not, does the cluster recruit other factors for microtubule assembly, such as TPX2? I strongly recommend staining and depleting PCM proteins and MT assembly factors in a nocodazole washout experiment. These data would provide crucial insights into the mechanism of Aurora A-mediated microtubule nucleation in mitosis.

Response: We thank the reviewer for raising this important point. As suggested, we have now systematically examined the localization of pericentriolar material (PCM) components, including Cdk5Rap2, γ-tubulin, and PCNT, within Aurora A-based clusters. As shown below, none of these canonical PCM proteins were recruited to Aurora A clusters.



CRY2-AurA^r-based clusters do not recruit key centrosomal components when exposed to blue light
(A-D) Immunofluorescence (IF) analysis of HeLa cells stably coexpressing CRY2-AurA^r and CIB1-MP, treated with nocodazole and either kept in the dark or exposed to blue light, as indicated. Cells were stained with anti-mCherry and/or anti-SNAP antibodies (magenta) to detect CRY2-AurA^r and CIB1-MP, together with antibodies against Cdk5Rap2 (A), γ-tubulin (B), PCNT (C), or TACC3 (D) (green). DNA is shown in blue. Representative images show that none of these proteins are detectably recruited to light-induced CRY2-AurA^r/CIB1-MP clusters. Quantification (right) shows the percentage of cells containing either two or more than two CRY2-AurA^r/CIB1-MP clusters that colocalize with Cdk5Rap2, γ-tubulin, PCNT, or TACC3 before and after blue-light illumination. Error bars represent mean ± SD from two independent experiments (N = 2; n > 200 cells per condition).

In addition, we investigated the localization of established Aurora A regulators, including TACC3 (see above) and TPX2. Consistent with the reviewer's suggestion, we found that TPX2, but not TACC3, is robustly recruited to light-induced Aurora A assemblies. Given the well-established roles of TPX2 in Aurora A activation and microtubule nucleation (Brunet et al., 2004, PMID: 15385625; Petry et al., 2013, PMID: 23415226; Roostalu et al., 2015, PMID: 26414402; Liang et al., 2025, PMID: 39821262), we next examined its functional contribution to Aurora A cluster activity. Strikingly, TPX2 depletion significantly reduced Aurora A activation loop phosphorylation ($T288^{P}$), consistent with its established role in stabilizing Aurora A T-loop phosphorylation. Importantly, TPX2 depletion also markedly impaired microtubule nucleation from light-induced Aurora A assemblies. These findings identify TPX2 as a critical component of Aurora A-based clusters and demonstrate that it is required for both Aurora A activation and cluster-dependent microtubule nucleation. These new data are presented below and have been incorporated into Fig. 5 and Fig. S6 of the revised manuscript and discussed on pages 12 and 13.



Aurora A interactor/coactivator TPX2 is recruited to light-induced Aurora A clusters

(A) IF analysis of HeLa cells stably coexpressing CRY2-AurA^r and CIB1-MP treated with nocodazole in the absence and presence of blue light, as indicated. These cells were stained with anti-mCherry (magenta) and anti-TPX2 (green) antibodies. DNA is shown in blue.

(B) Schematic of the method to quantify cluster intensity of CRY2-AurA^r and TPX2 (in au) in the absence or presence of blue light in nocodazole-treated cells, as indicated. The outcome of such analysis is shown to the right. Error bars: mean ± SD (n>50). Exact p values from left to right are ***p<0.001, ***p<0.001.

(C, D) IF analysis of HeLa cells stably coexpressing CRY2-AurA^r and CIB1-MP synchronized in prophase in the absence and presence of blue light, as indicated. These cells were stained with anti-mCherry (magenta) and anti-TPX2 (green) antibodies. DNA is shown in blue. Note the appearance of extra-spindle poles comprising of CRY2-AurA^r and TPX2 upon blue light compared to two poles in cells that were left in the dark (C). Quantification of prophase cells with >2 spindle poles before and after blue light illumination (D). Error bars: mean ± SD (n>50 cells each, N=3). Exact p-value is ***p<0.001.

(E, F) IF analysis of HeLa cells coexpressing CRY2-AurA^r, CIB1-MP in control, or in cells depleted for TPX2 by siRNA. These cells were treated with nocodazole for 12 h, and were either left in dark, or treated with blue light, as indicated. These cells were stained with anti-mCherry (magenta) and anti-T288^p (green) antibodies. DNA is shown in blue. Note the significant loss of T288^p signal in cells depleted for TPX2, in comparison to control cells (E). Schematic of the method to quantify cluster intensity of CRY2-AurA^r and T288^p (in au) in the absence or presence of blue light in nocodazole-treated control or TPX2-depleted cells and the outcome of such analysis (F) Error bars: mean ± SD (n=40). Exact p values from left to right are ***p<0.001, ***p<0.001, ***p<0.001, ns-p=0.3287.

(G, H) IF analysis of control or TPX2-depleted HeLa cells stably coexpressing CRY2-AurA^r and CIB1-MP, synchronized in prophase, and were either kept in dark, or exposed to blue light, as indicated. These cells were stained with anti-mCherry (magenta) and anti-β-tubulin (green) antibodies. DNA is shown in blue. Note the appearance of extra-microtubule emanation centers in control cells, whereas TPX2-depleted cells, despite having clustered CRY2-AurA^r signal, fail to form ectopic microtubule emanation centers; two examples (#1 and #2) are shown (G). Quantification of prophase cells with >2 microtubule emanation centers before and after blue light illumination in control and TPX2-depleted conditions (H). Error bars: mean ± SD (n>50 cells each, N=3). Exact p values from left to right are **p=0.0022, ***p<0.001, ns-p=0.052.

p values are denoted as follows: ns-p≥0.05; **p<0.01; ***p<0.001 as determined by two-tailed unpaired Student's t-test. Scale bars in (A, D, F, and H) represent 10 μm.

2. The clusters of Aurora A assemble spindle poles and promote spindle assembly in the absence of centrosomes, without recruiting core centrosomal proteins, including γ-tubulin (Fig. 4). However, it is important to determine if the cluster recruits other factors, such as TPX2 and the HAUS complexes, to the acentrosomal spindle pole. I recommend staining and depleting MT assembly factors in the cells. Through these experiments, the authors can better demonstrate the essential functions of centrosomes in mitosis, specifically, the regulation of spindle pole factors through Aurora A activity, rather than PCM maturation.

Response: As mentioned in our response to Major Point #1, we have now examined the localization of TPX2 within light-induced Aurora A clusters. In addition, we analyzed TPX2 localization in acentrosomal cells generated by Plk4 inhibitor treatment and found that TPX2 localization is largely unaffected by centrosome loss. Collectively, these experiments reveal a clear distinction between canonical PCM components and TPX2: whereas γ-tubulin, Cdk5Rap2, PCNT, and Cep192 fail to localize to acentrosomal spindle poles in HeLa cells, TPX2 localization remains unperturbed. These new findings have been incorporated into Fig. 6, Fig. S6, and Fig. S8 and are discussed on pages 12, 13, and 15 of the revised manuscript.

Furthermore, in response to a suggestion from Reviewer 3 (see their major point #4), we investigated whether Aurora A-based clusters could support spindle assembly in the absence of the key centrosome component Cep192. To address this, we examined bipolar spindle formation in Cep192-depleted cells, both in the absence and presence of light-induced Aurora A clusters. These experiments demonstrated that Aurora A-based clusters significantly rescue the spindle assembly defects caused by Cep192 depletion, providing additional evidence that these synthetic assemblies can promote spindle formation independently of the canonical PCM scaffold.

These new findings have been incorporated into Fig. 4 and are discussed on pages 12 of the revised manuscript.

3. Optogenetic activation of Aurora A promoted mitotic progression in acentrosomal cells (Fig. 5). The experiments nicely showed that the optogenetic system restores the functions of centrosomes. However, some cells still exhibit prolonged mitotic progression. A more detailed description of the phenotypes in these cells could reveal the functions of PCM-mediated centrosomal microtubules in mitosis.

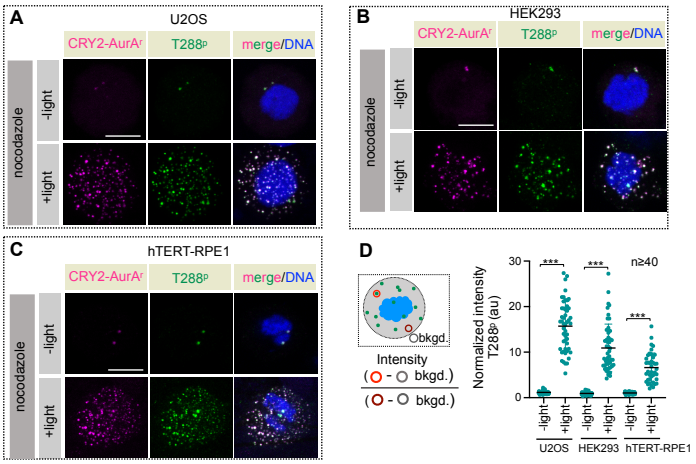
Response: We apologize for the lack of clarity in the original figure panel, which may have led to this misunderstanding. In the initial version of the manuscript, we chose to illustrate the behavior of all analyzed cells in Fig. 5, rather than highlighting only the representative behavior observed in the majority of cells. We believe this presentation may have contributed to the confusion. A similar concern was raised by Reviewer 2 (see their major point #6).

In the revised manuscript, we have now included representative cells that more accurately reflect the predominant behavior observed across the population. For example, in DMSO-treated control cells, 86% of cells exited mitosis within 100 minutes, whereas in Plk4 inhibitor (centrinone)-treated cells, 70% of cells took longer than 100 minutes to exit mitosis. In addition, as suggested by Reviewers 2 and 3, we have now included a more detailed analysis of the phenotypes observed in centrinone-treated cells, including chromosome misalignment, in which Aurora A activity is modulated through clustering. These revised data are now incorporated into Fig. 7 and Fig. S10 and are discussed on pages 16 and 17 of the revised manuscript.

4. All experiments were performed with the HeLa Kyoto cell line. It would be preferable to perform the Aurora A clustering experiments in non-transformed human cells, such as hTERT RPE-1 cells, to validate the physiological relevance of the findings.

Response: We thank the reviewer for this concern, which was also emphasized by the second reviewer (major point #9). As per the reviewer's suggestion, we have now examined Aurora A clustering-based activation across multiple cell lines, including U2OS, HEK293, and hTERT-RPE1 cells. Our analyses revealed that all of these cell lines are competent for Aurora A activation (T-loop phosphorylation) induced by clustering.

These new data are shown below and are now incorporated into Fig. S3 and are discussed on page 9 of the revised manuscript.



Clustered Aurora A molecules can autophosphorylate themselves at the T-loop in various cell lines

(A-C) IF analysis of U2OS (A), HEK293 (B), and hTERT-RPE1 (C) cells that transiently express CRY2-AurA^r and CIB1-MP, treated with nocodazole, and were either exposed or not to the blue light, as indicated. These cells were stained with anti-mCherry (magenta) and anti-T288^p (green) antibodies. DNA is shown in blue.

(D) Schematic of the method to quantify cluster intensity of T288^p (in au) in the absence or presence of blue light in

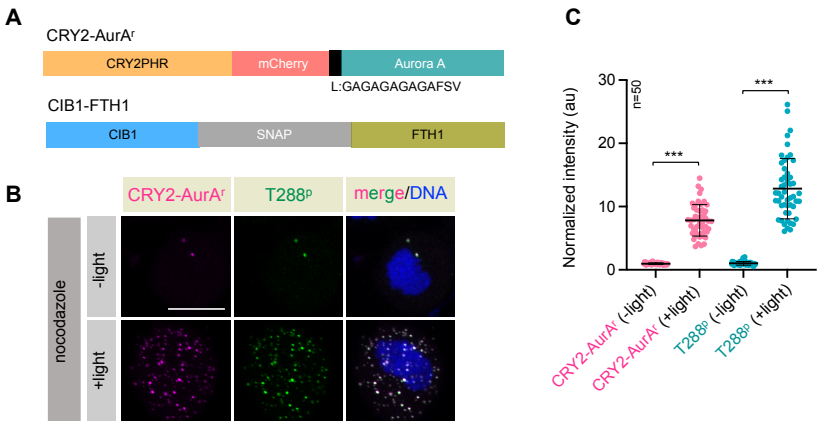
nocodazole-treated diverse cell lines, as indicated. The outcome of such analysis is shown to the right. Error bars: mean ± SD (n=50 for U2OS and HEK293; n=40 for hTERT-RPE1). Exact p values from left to right are ***p<0.001, ***p<0.001 and ***p<0.001.

Minor points:

1. In Fig. 1, the authors employ CIB1-MP to induce the clustering. Have alternative multimerization tags been tested? It is possible that CIB1-MP itself contributes to the integrity of an artificial spindle pole independently of Aurora A function.

-As suggested by the reviewer, we have now employed an additional multimerization scaffold, Ferritin Heavy Chain 1 (FTH1), which self-assembles into a 24-subunit nanocage. We find that CIB1-FTH1 efficiently clusters Aurora A and promotes its activation upon blue-light illumination, analogous to our CIB1-MP-based system. Importantly, neither CIB1-MP nor CIB1-FTH1 exhibits intrinsic directionality or accumulates at centrosomes/spindle poles in the absence of Aurora A, indicating that the localization and activity of the light-induced assemblies are dictated by Aurora A itself rather than by the scaffold.

For completeness, we provide the data below. However, because the CIB1-FTH1 experiments largely recapitulate the findings obtained with CIB1-MP and do not alter the main conclusions of the study, we have chosen not to include them in the revised manuscript to maintain a focused and streamlined presentation.



Engineering Aurora A clustering using a multimerization scaffold of FTH1

(A) Domain organization of the Aurora A clustering constructs. In brief, it contains two modules: 1) light-sensitive photolyase homology region (PHR) domain of CRY2 (CRY2PHR), mCherry-tag, a linker (L) and siRNA-resistant Aurora Ar (CRY2-AurAr), and 2) SNAP-tagged FTH1, which contains CIB1 domain (CIB1-FTH1).

(B) IF analysis of HeLa cells transiently coexpressing CRY2-AurAr and CIB1-FTH1treated with nocodazole in the absence and presence of blue light, as indicated. These cells were stained with anti-mCherry (magenta) and anti-T288P (green) antibodies. DNA is shown in blue. (C) Intensity analysis of clustered CRY2-AurAr molecules and T288P (in au) in the absence or presence of blue light in nocodazole-treated cells, and the outcome of such analysis. Error bars: mean ± SD (n=50). Exact p values from left to right are ***p<0.001, ***p<0.001.

2. Fig. 1G shows that Aurora A cluster formation is induced in interphase cells. Are these clusters similarly capable of recruiting microtubules? Furthermore, which endogenous factors are enriched at these sites?.

-We thank the reviewer for inviting us to discuss this important point. We found that optogenetic clustering of Aurora A during interphase does not induce T-loop phosphorylation, indicating that

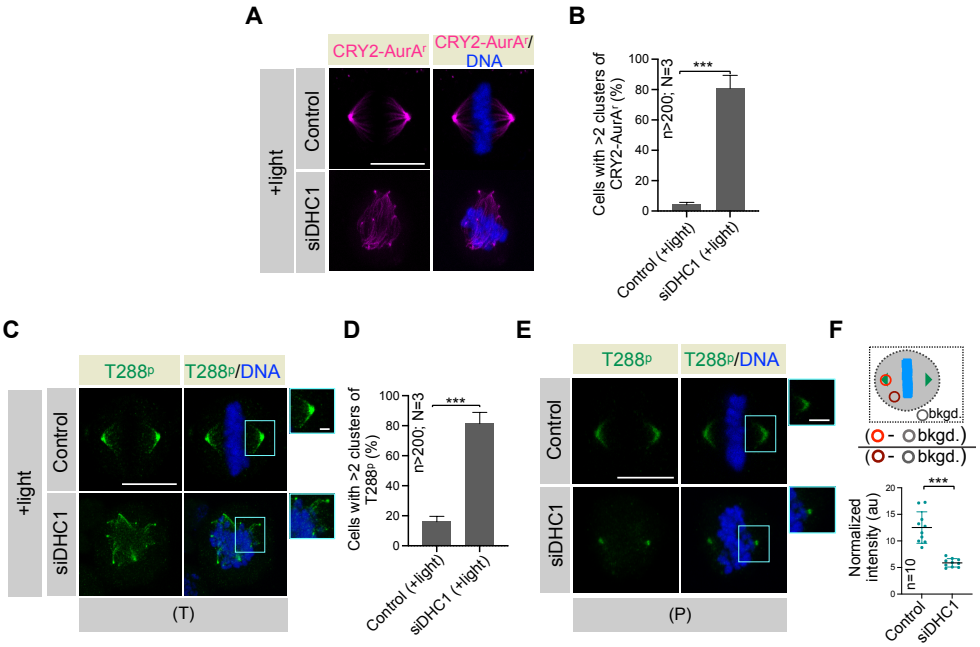
the mitotic molecular environment is required for Aurora A activation and, consequently, for efficient microtubule nucleation. A similar point was also raised by Reviewer 3 (see Minor Point #8). In the interest of maintaining a balanced and focused narrative, we chose not to include these data in the manuscript. However, for the reviewer's evaluation, we have now included these results in our response to Reviewer 3 (Minor Point #8).

Interestingly, one of the key factors that localizes to Aurora A-based clusters is TPX2. TPX2 is known to play a critical role in activation loop stabilization of Aurora A, as well as in microtubule nucleation. However, during interphase, TPX2 predominantly accumulates within the nucleus. Therefore, one intriguing possibility is that the absence of TPX2 from Aurora A clusters during interphase keeps these clusters in an inactive state.

3. In the absence of centrosomes or Cep192, it is unclear how Aurora A clusters are targeted to spindle poles. What factors are proposed to act as recruiters in this context? This could potentially be addressed experimentally, for example, by siRNA-mediated depletion of candidate factors.

-We thank the reviewer for this insightful comment. A related concern was also raised by Reviewer 3 (see Major Point #2). Previous studies have shown that Aurora A accumulation at spindle poles is dependent on microtubules (Silvia and Cassimeris, 2013, PMID: 24152729). Based on this observation, we hypothesized that a minus-end-directed motor protein, most likely dynein, could facilitate the transport of Aurora A clusters toward spindle poles.

To test this possibility, we examined the localization of Aurora A-based clusters following depletion of the dynein heavy chain DHC1. Strikingly, similar to the phenotype observed upon nocodazole treatment, Aurora A clusters failed to efficiently coalesce and accumulate at spindle poles in DHC1-depleted cells. Furthermore, T288-phosphorylated Aurora A (T288^{p}) associated with these clusters was also unable to robustly enrich at spindle poles following dynein depletion. Importantly, we found that endogenous T288^{p} failed to accumulate efficiently at spindle poles in DHC1-depleted cells, indicating that dynein activity is generally required for proper spindle pole localization of Aurora A during mitosis. These findings support a model in which dynein-mediated transport promotes the coalescence and spindle pole accumulation of Aurora A assemblies. The new data are shown below, incorporated into Fig. 2D-2G and Fig. S1G and S1H, and discussed on page 9 of the revised manuscript.



Dynein is crucial for the coalescence of light- induced Aurora A clusters

(A, B) IF analysis of transgenic HeLa cells coexpressing CRY2-AurA^{r} and CIB1-MP (Control) or depleted of dynein heavy chain 1 (DHC1) by siRNA for 60 h. Cells were acutely exposed to blue light and subsequently stained with anti-mCherry (magenta) antibodies. DNA is shown in blue. Quantification on the right represents the percentage of cells containing more than two CRY2-AurA^{r}-positive foci under control and DHC1-depleted conditions (B). Error bars represent mean ± SD from three independent experiments (>200 cells analyzed per condition in each experiment). Exact p values is ***p<0.001.

(C) IF analysis of transgenic (T) HeLa cells coexpressing CRY2-AurA^{r} and CIB1-MP in control and upon transfection with DHC1 siRNA for 60 h. These cells were acutely exposed to blue light and subsequently stained with anti-T288^{P} antibodies (green). DNA is shown in blue. Rectangular regions marked in the image are shown magnified on the top right.

(D) Quantification of the percentage of cells containing more than two T288^{P}-positive foci under control and DHC1-depleted conditions. Error bars represent mean ± SD from three independent experiments (>200 cells analyzed per condition in each experiment). Statistical significance was determined using a two-tailed unpaired t-test; ***p <0.001.

(E) IF analysis of parental (P) HeLa cells in control and upon transfection with DHC1 siRNA for 60 h. These cells were stained with anti-T288^{P} (green) antibodies. DNA is shown in blue. Rectangular regions marked in the image are shown magnified on the top right.

(F) Schematic of the method to quantify spindle pole intensity of T288^{P} in Control and DHC1-depleted cells. The outcome of such analysis is shown below. Error bars: mean ± SD (n=10). Exact p values is ***p<0.001.

4. In Fig. 4H-I, spindle length is increased upon light illumination. What mechanism underlies this elongation, and can it be experimentally tested?

-Since the Aurora A-TPX2 interaction is required for proper spindle length control (Bird and Hyman, 2008, PMID: 18663142; Meaders et al., 2026, PMID: 42090511), and because active Aurora A fails to localize to spindle poles in cells lacking centrosomes, we speculate that one possible explanation is the inability to establish a robust Aurora A-TPX2 interaction under these conditions. Such a defect could account for the shorter spindle phenotype observed in our system.

Notably, as mentioned above, TPX2 protein levels remain unchanged in cells lacking centrosomes, suggesting that it is the functional Aurora A-TPX2 axis, rather than TPX2 abundance itself, that may be responsible for regulating spindle length.

Reviewer #2

We thank the reviewer for acknowledging that "*the manuscript is clearly written and well presented"* and that "*the tool itself is elegant and valuable for achieving temporal control of Aurora A auto-phosphorylation."* However, the reviewer raised the concern that "*in its current form, beyond the development of this optogenetic system, the manuscript provides limited new insight into Aurora A biology."* The reviewer further mentioned that "*enforced clustering of Aurora A induces auto-phosphorylation is not unexpected*," citing the study by Joukov et al., 2010, PMID: 21097701, which demonstrated that antibody-based accumulation of Aurora A on beads could promote Aurora A auto-phosphorylation and activation. We would like to clarify that, although it has long been hypothesized that enforced clustering of Aurora A may induce activation through T-loop phosphorylation, to the best of our knowledge, this has not previously been formally tested in living cells using either chemical or optogenetic approaches.

Moreover, the studies by Joukov et al., 2010, as well as the earlier work by Tsai and Zheng, 2005, PMID: 16332542, proposed that the MTOC-like activity of αAurA-coated beads arises from αAurA-mediated dimerization and subsequent Aurora A activation on the bead surface. Importantly, Aurora A activation in this system still required Cep192 (please see Fig. 2 in Joukov et al., 2010). In contrast, our optogenetic clustering system does not rely on Cep192 for Aurora A activation. We therefore believe that our findings provide mechanistic insight into how higher-order clustering can directly promote Aurora A activation independently of canonical centrosomal scaffolding factors.

We apologize for any misunderstanding arising from our interpretation. We did not intend to imply that promoting Aurora A auto-phosphorylation is the sole function of mitotic centrosomes. Because our clustering-based approach rescues multiple phenotypes occasionally seen in acentrosomal cells, including defects in spindle length, NuMA dynamics, and mitotic duration. We proposed that artificial clustering of Aurora A can partially compensate for the loss of centrosome function, at least for the phenotypes examined in our study. To make this point clearer to our readership, we have slightly revised the abstract accordingly. Furthermore, based on the suggestion of Reviewer 3, we have now included additional data demonstrating that Aurora A clustering-mediated activation can substantially rescue the spindle assembly defects caused by Cep192 depletion. These findings further support the idea that uncoupling Aurora A activation from canonical centrosome/Cep192-dependent regulation is sufficient to rescue the spindle assembly defects examined in our study.

Major points:

1. The optogenetic tool is the major strength of this work; as such, it is surprising that the authors have not fully characterised the composition of these complexes during interphase and mitosis. With a SNAP-tag present in CIB1-MP, it is straightforward to affinity purify these complexes and identify their components. Incorporating these data would strengthen the manuscript's mechanistic insights while also addressing point 2 below.

Response: We thank the reviewer for raising this important point and for their thoughtful suggestions. A comprehensive proteomic characterization of light-induced Aurora A assemblies would indeed be highly informative. However, given the primary objective of this study, to establish an optogenetic strategy for inducing functional centrosome-like Aurora A assemblies, we initially focused on targeted mechanistic analyses rather than large-scale proteomics. Moreover, several proteomic studies have already characterized the Aurora A interactome, including a recent proximity-labeling approach that identified Aurora A-associated proteins (Garrido et al., 2026, PMID: 41475341).

Guided in part by the suggestions of Reviewer 1 (Major Point #1) and by known Aurora A interactors, we therefore examined the localization of TACC3 and TPX2. Notably, we found that

TPX2, but not TACC3, is robustly recruited to light-induced Aurora A assemblies. Furthermore, siRNA-mediated depletion of TPX2 markedly reduced Aurora A T-loop phosphorylation ($T288^P$), consistent with the established role of TPX2 in stabilizing Aurora A activation. Importantly, TPX2 depletion also abolished microtubule nucleation from light-induced Aurora A assemblies. These new findings are now included in Fig. 5, and Fig. S6 and are discussed on pages 12, 13 of the revised manuscript.

We are keen to pursue a comprehensive proteomic analysis of light-induced Aurora A assemblies in future studies to define their complete molecular composition and identify additional factors that may cooperate with TPX2 to promote microtubule nucleation. However, we believe that the new TPX2-related data provide a clear mechanistic framework for understanding how Aurora A assemblies nucleate microtubules, whereas a systematic proteomic dissection of these assemblies would extend beyond the scope of the present study.

2. Normal Aurora A activity requires complex formation with TPX2, and therefore, the authors should demonstrate that complex formation occurs as expected with Cry2-AurA.

Response: We thank the reviewer for raising this important point. Please see our response to Major Point #1, where we discuss the additional experiments performed to address this issue. In brief, we have now examined both the localization and the functional relevance of TPX2 in Aurora A cluster-dependent microtubule nucleation. Notably, we found that TPX2 robustly localizes to the light-induced Aurora A clusters and is required for both efficient T288 phosphorylation and microtubule nucleation. A similar concern was also raised by the reviewer #1 (see response to their major point #1). This new data generated in response to these comments has substantially strengthened the mechanistic basis of our model.

3. The optogenetic system is expected to massively alter the balance between phosphorylated and unphosphorylated Aurora A, pushing it towards an excess of auto-phosphorylated kinase. The authors should discuss how this may affect Aurora A functions. For instance, light-driven clustering could actually phenocopy Aurora-A overexpression, thus interfering with spindle formation and also the spindle assembly checkpoint. If so, this needs to be taken into consideration when interpreting data in Figure 5. Chromosome mis-segregation frequency should be measured in the various cell lines generated.

Response: We thank the reviewer for this insightful comment. We would like to emphasize that, unlike Aurora A overexpression systems, where constitutively elevated Aurora A activity can lead to mitotic abnormalities, our optogenetic approach induces only a transient and reversible increase in the pool of T288-phosphorylated Aurora A. In the original version of the manuscript (Fig. S1D), we showed that Aurora A T-loop phosphorylation rapidly declines following withdrawal of blue light.

To directly address the possibility that transient Aurora A activation might compromise mitotic fidelity, we performed two additional analyses. First, we quantified chromosome congression defects (chromosome misalignment) following optogenetic activation of Aurora A. Second, in the context of the experiments shown in the previous Fig. 5 (now Fig. 7), we measured mitotic duration in cells subjected to repeated rounds of blue-light stimulation. Importantly, neither analysis revealed a significant increase in mitotic errors or delays. Moreover, mitotic timing in CRY2-$AurA^r$/CIB1-MP-expressing cells subjected to repeated blue-light stimulation remained comparable to that of parental control cells. Together, these findings indicate that transient enhancement of Aurora A activity through our optogenetic approach does not measurably impair mitotic progression or chromosome segregation fidelity. These new data have now been incorporated into Fig. S2E and S2F and Fig. S10 of the revised manuscript and are discussed on page 16.

Finally, we would like to point out that the levels of ectopically expressed Aurora A in our system are comparable to those of the endogenous protein (Fig. 1). We believe that this near-

physiological expression level, together with the transient and reversible nature of optogenetic activation, explains why our system differs from previously reported Aurora A overexpression models and avoids the mitotic abnormalities associated with sustained Aurora A hyperactivation.

4. Throughout the manuscript, the authors equate Aurora A activity with its auto-phosphorylation. This is not necessarily the case, and it is thus crucial that Aurora-A activity is assessed independently (e.g. phosphorylation and localisation of substrates).

Response: Prompted by this comment, we obtained and tested the LATS2 pS82 phospho-specific antibody used in Holder et al., 2025, PMID: 39327527, as a target for Aurora A kinase. Unfortunately, despite repeated attempts, we were unable to detect a specific signal with the antibody lot that we received, either by immunofluorescence or by immunoblotting. However, we would like to emphasize that our conclusions regarding Aurora A activation were not based solely on its auto-phosphorylation ($T288^P$ staining). In the previous version of the manuscript, we also examined the localization and dynamics of NuMA, a well-established substrate of Aurora A. Previous studies have demonstrated that Aurora A phosphorylates NuMA at S1969 (Kettenbach et al., 2015, PMID: 21712546), and that Aurora A activity regulates NuMA localization during mitosis (Gillani et al., 2016, PMID: 26832443; Kotak et al., 2016, PMID: 27335426; Rajeevan et al., 2025, PMID: 40940421). More recently, we showed that cells lacking centrosomes fail to accumulate active, T288-phosphorylated species at spindle poles (Rajeevan et al., 2025). Under these circumstances, NuMA accumulates robustly at spindle poles, closely phenocopying cells treated with the Aurora A inhibitor MLN8237 or cells expressing the phospho-dead NuMA S1969 mutant. Importantly, our optogenetic system, which restores Aurora A activity through light-induced clustering, rescues both the aberrant spindle pole localization and the altered dynamics of NuMA. These findings provide functional evidence that the light-induced Aurora A assemblies contain a catalytically active kinase capable of phosphorylating downstream substrates (e.g. NuMA) and modulating their localization and behavior.

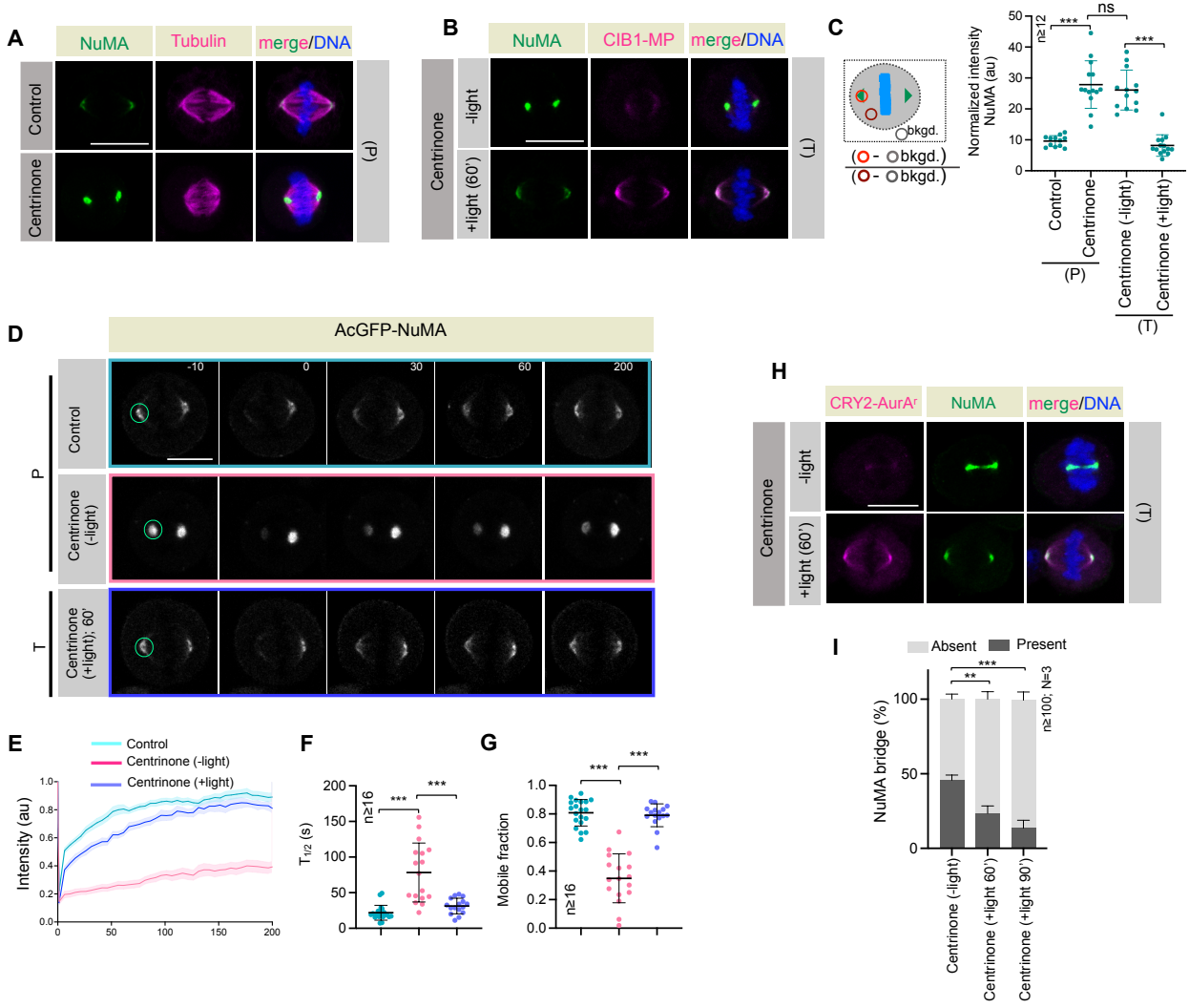
We therefore believe that the combined assessment of Aurora A T-loop phosphorylation ($T288^P$) and the localization and dynamic properties of the bona fide Aurora A substrate NuMA provides strong and complementary evidence for Aurora A activation in our system. Importantly, these analyses were already included in the original submission (original Fig. 4; revised Fig. 6) and have been further expanded and strengthened in the revised manuscript (new Fig. S9). For the reviewer's convenience, we also reproduce the relevant data below.

NuMA abnormal localization and dynamic properties affected in acentrosomal cells are rescued by light-induced Aurora A activation

(A-C) IF analysis of parental (P) (A), or transgenic (T) HeLa cells stably coexpressing CRY2-$AurA^r$ and CIB1-MP that are either left untreated or treated with Plk4 inhibitor-centrinone for 96 h, and also exposed to blue light, as indicated. These cells were then stained with either anti-NuMA (green) and anti-β-tubulin (magenta) antibodies. DNA is shown in blue. The measurement method and the NuMA intensity at the spindle poles in the above-mentioned conditions are shown on the right (C). Error bars: mean ± SD ($n \geq 12$). Exact p values from left to right are ***$p<0.001$, ns;$p>0.05$ ***$p<0.001$.

(D-G) Fluorescence recovery after photobleaching (FRAP) analysis of spindle pole-localized AcGFP-NuMA (gray) in parental metaphase cells (cyan outline), parental metaphase cells treated with centrinone (pink outline), and transgenic cells coexpressing CRY2-AurAr and CIB1-MP, treated with centrinone and exposed to blue light (blue outline) (D). Time is indicated in seconds (s). Bleached regions are marked by green circles. Representative recovery curves are shown below (E). Curves and shaded areas represent mean ± SEM. Consistent with our previous findings (Rajeevan et al., 2025), spindle pole-localized AcGFP-NuMA exhibits markedly slower recovery in centrinone-treated cells. In contrast, optogenetic activation of Aurora A significantly restores NuMA dynamics, resulting in faster fluorescence recovery at spindle poles. Quantification of the half-time of recovery ($T_{1/2}$) (F) and mobile fraction (G)

derived from the FRAP experiments shown in (D) and (E). Error bars represent mean ± SD. Exact p-values: ***p < 0.001 and ***p < 0.001 for both $T_{1/2}$ (F) and mobile fraction (G).
(H, I) IF analysis of HeLa cells stably coexpressing CRY2-AurA^{r} and CIB1-MP treated with centrinone for 96 h in the absence or presence of blue light for 60 min (see methods) stained for T288^{p} (green) and anti-NuMA (grey). Unstained mCherry signal is shown in magenta, and DNA is shown in blue. Note the presence of NuMA bridges in centrinone-treated cells, which disappear upon illumination with blue light (H). The quantification of NuMA bridges, categorized as present and absent, is shown to the right (I). Error bars: mean ± SD (N=3, n>100 cells each). Exact p values from left to right are **p=0.003, ***p<0.001.

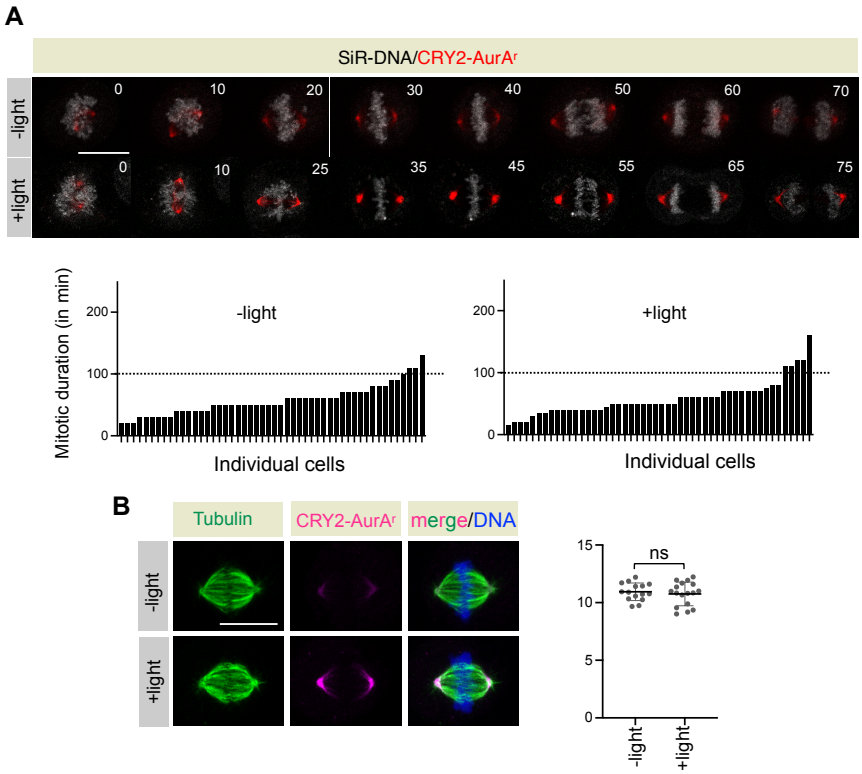


3. In Figures 4 and 5, mitotic phenotypes (e.g., mitotic duration, spindle length) are assessed in parental control cells, with several important controls missing. It would be important to show mitotic duration and spindle length in Cry2-AurA/CIB1-MP cells with and without light in the absence of centrinone. These controls are essential, particularly given the differences apparent between centrinone-treated parental and centrinone-treated (no light) Cry2-AurA/CIB1-MP cells. Mitotic duration seems much longer in the latter, indicating that Cry2-AurA/CIB1-MP expression alone sensitises cells to centrinone treatment.

Response: We thank the reviewer for raising this concern. We would like to respectfully clarify the rationale behind the experiments presented in the previous Fig. 4 and Fig. 5. The primary

objective of these experiments was to determine whether mitotic defects commonly observed in acentrosomal cells generated by centrinone treatment could be rescued by optogenetic activation of Aurora A in CRY2-AurA^{r}/CIB1-MP-expressing cells. Accordingly, our principal comparison was between centrinone-treated CRY2-AurA^{r}/CIB1-MP cells in the absence and presence of blue-light stimulation. Since the focus of these experiments was on rescuing acentrosomal phenotypes, we did not initially consider CRY2-AurA^{r}/CIB1-MP cells, with and without light stimulation, under normal centrosome-containing conditions as a critical control.

Nevertheless, prompted by the reviewer's suggestion, we have now performed these additional analyses and quantified both spindle length and mitotic duration in CRY2-AurA^{r}/CIB1-MP-expressing cells with and without blue-light stimulation in the absence of centrinone. Importantly, we did not detect any significant differences between these conditions. For the reviewer's convenience, we provide the data below. However, because they do not alter the conclusions of the study and because our primary focus is on the ability of optogenetic Aurora A activation to rescue acentrosomal phenotypes, we have only included mitotic duration in the revised manuscript in new Fig. S10 to maintain a focused narrative and avoid being distracted from the central findings.



Light-induced Aurora A clustering does not alter mitotic duration or spindle length
(A) Representative confocal live-cell images of mitotic HeLa cells stably coexpressing CRY2-AurA^{r} and CIB1-MP that were either maintained in the dark (−light) or subjected to repeated blue-light illumination (+light). Mitotic duration was measured from prometaphase to cytokinesis initiation. Quantification is shown below, where each horizontal bar represents an individual cell. Scale bar, 10 μm.
(B) Immunofluorescence (IF) analysis of transgenic HeLa cells coexpressing CRY2-AurA^{r} and CIB1-MP that were either maintained in the dark (−light) or exposed to blue light (+light). Spindle length was quantified as the distance between the two spindle poles. Cells were stained with anti-β-tubulin (green) and anti-mCherry (magenta) antibodies to detect CRY2-AurA^{r}. DNA is shown in blue. Quantification of spindle length is shown on the right. Error bars represent mean ± SD (n ≥ 15 cells). Exact *p* value: ns ($p > 0.05$).

Furthermore, we like to emphasize that we do not observe major differences between parental cells and CRY2-AurA^{r}/CIB1-MP-expressing cells following centrinone treatment with respect to spindle length (Fig 6I), chromosome alignment defects (Fig. S8C-S8E), NuMA localization (Fig. S9A-S9C), or the localization of PCM components analyzed (Fig. S7). Regarding the reviewer's observation that approximately 80% of centrinone-treated parental cells, compared with approximately 70% of centrinone-treated CRY2-AurA^{r}/CIB1-MP cells, failed to exit mitosis within the imaging window in the absence of light stimulation, we believe that this difference falls within the experimental variability typically associated with live-cell imaging assays and likely reflects differences in sample size rather than a biologically meaningful effect.

Taken together, these additional analyses indicate that expression of CRY2-AurA^{r}/CIB1-MP alone does not measurably alter mitotic progression or spindle organization. Rather, the rescue phenotypes described in the manuscript specifically arise from light-induced activation of Aurora A in acentrosomal cells.

6. Instead of fixed cut-offs (100 min, 4 h) in Fig. 5, the authors should consider reporting median mitotic durations for cells that complete mitosis. The selected cells in Fig. 5B-d all show a marked delay, with only one undergoing anaphase, so these panels do not seem to be representative of the graph.

Response: We thank the reviewer for raising this point. We would like to clarify that, in the original Fig. 5 (now Fig. 7), cells were imaged for a total duration of 4 h. Under these conditions, a substantial fraction of centrinone-treated cells failed to complete mitosis within the imaging window. Consequently, it was not possible to accurately determine mitotic duration for all cells. To accommodate this limitation, we categorized cells into two groups: those that completed mitosis within 100 min and those that either required more than 100 min or failed to exit mitosis during the 4 h imaging period. We agree that, when all cells complete mitosis, reporting median mitotic duration is a more conventional and informative metric. However, given the large proportion of centrinone-treated cells that remained arrested throughout the imaging window, we felt that this binning strategy provided a more accurate representation of the population behavior and allowed us to capture both the frequency and severity of the mitotic delay.

Regarding the representative images, our original intention was to illustrate the range of cellular behaviors observed, including cells that progressed normally as well as those that exhibited prolonged mitotic arrest. We agree with the reviewer that this presentation may have been confusing. Therefore, in the revised manuscript, we have replaced these examples with representative cells that better reflect the predominant behavior observed in each experimental condition and are more consistent with the corresponding quantifications.

7. The T288A non-phosphorylatable Aurora-A construct CRY2-AurA is only mentioned in Figure S6 as a control for the centrinone experiment in Figure 5. It is a potentially interesting observation, but we would need to know more about the behaviour of this mutant under normal conditions to be able to interpret the results.

Response: We thank the reviewer for this important suggestion. Prompted by this comment, we have now examined spindle length and NuMA localization at the spindle poles in cells expressing a non-phosphorylatable Aurora A T288A mutant following depletion of endogenous Aurora A. Consistent with our expectations, expression of the T288A mutant resulted in aberrant accumulation of NuMA at the spindle poles, closely resembling the phenotype observed upon Aurora A inhibition with MLN8237 (Rajeevan et al., 2025). In addition, these cells exhibited a significant reduction in spindle length compared with controls. These findings further support the importance of Aurora A T-loop phosphorylation for proper NuMA regulation and spindle assembly. The new data have been incorporated into new Fig. S10 of the revised manuscript.

8. It is difficult to track which experiments endogenous Aurora-A is depleted in.

Response: We thank the reviewer for requesting this clarification. In the revised manuscript, we have now clearly indicated in all relevant figure panels the experimental conditions in which endogenous Aurora A kinase was depleted. We hope that these additions improve the clarity of the data presentation and facilitate interpretation of the results.

9. A clear limitation of the manuscript is that all the experiments are conducted in HeLa cells that have a complex, near-triploid genome. In part due to this larger genome HeLa cells take twice as long to complete mitosis than other diploid cells and despite the increased duration they still frequently mis-segregate chromosomes. Unsurprisingly, Aurora-A inhibition/overexpression has particularly profound effects in HeLa cells. While I realise that establishing this system would take considerable effort in another cell type, I question the rationale of choosing HeLa cells, and this should at least be discussed in the manuscript.

Response: We thank the reviewer for this thoughtful comment. A similar point was also raised by Reviewer 1 (see response to Major Point # 4). There were two main reasons for initially choosing HeLa cells for this study. First, we have established all the necessary experimental tools in this cell line, including stable cell lines expressing siRNA-resistant, near-endogenous levels of our protein of interest, allowing us to rigorously and efficiently test our hypotheses. Second, and more importantly, hTERT-RPE1 TP53$^{-/-}$ cells are relatively resistant to PLK4 inhibitor treatment compared with HeLa cells. Previous studies have shown that these cells can assemble PCM foci and acentrosomal microtubule-organizing centers in the absence of centrosomes (Wong et al., 2015, PMID: 25931445; Watanabe et al., 2020, PMID: 33170211), thereby mitigating many of the mitotic defects associated with centrosome loss. In contrast, HeLa cells are considerably more sensitive to centrosome ablation and exhibit a robust mitotic delay, providing a more suitable system to test whether our Light-Induced Spindle Assembly (LISA) approach can functionally compensate for centrosome loss. We have now incorporated this rationale into the revised Discussion in page 19.

That being said, prompted by the reviewer's suggestion, we have extended our analyses to additional human cell lines, including HEK293, U2OS, and hTERT-RPE1. We are pleased to report that, analogous to HeLa cells, light-induced Aurora A clustering leads to robust accumulation of T288-phosphorylated Aurora A in all three cell lines. These findings demonstrate that our optogenetic strategy is not restricted to a single cellular context and is broadly applicable across diverse human cell types. These new data have been incorporated into Fig. S3 and are discussed on page 9 of the revised manuscript.

10. I would recommend the authors to change the final sentence of their abstract. It is difficult to see how this system would yield a screening platform. If the authors disagree, they should provide a better explanation in the manuscript as they simply repeat the statement as their final sentence of the main text.

Response: In response, we have revised the Abstract and expanded the relevant section of the manuscript to provide a clearer explanation of why we believe our optogenetic system can serve as a versatile screening platform. Specifically, we now better articulate how the reversible and tunable nature of light-induced Aurora A activation can be exploited to interrogate the molecular mechanisms underlying spindle assembly and to identify factors that regulate Aurora A-dependent processes. The revised text can be found on pages 2, 18 and 19 of the revised manuscript.

11. The recent paper by the Raff group on the mitotic Aurora-A scaffold (Wong et al., 2025) is highly relevant to this study and should be cited.

Response: Added, thanks.

Reviewer #3

We genuinely thank the reviewer for stating, "*The approach is technically elegant and addresses a relevant question in centrosome and spindle biology: what is the minimal protein composition at mitotic spindle poles required for efficient microtubule nucleation and faithful cell division?*" However, the reviewer invited us to address a few important concerns that we have attempted, as discussed below.

Major points:

1. Figure 1D-F: The criteria used to classify monopolar spindles and chromosome instability are not clearly defined. Which markers have been used for these classifications? Chromosome instability is characterized by numerical and structural changes on the karyotype level that is driven by loss or gain of whole or parts of chromosomes. Given that the karyotype has not been analysed, a more precise term would be something like chromosome congression or segregation errors. This should be more precisely defined, and the representative images should be provided.
Response: We thank the reviewer for these helpful suggestions. In response, we have clarified our criteria for classifying monopolar spindles and now provide a detailed definition in the corresponding figure legend. We also appreciate the reviewer's recommendation regarding terminology and have replaced "chromosome instability" with the more precise term "chromosome misalignment." These defects were quantified by examining Hoechst 33342-stained chromosomes and assessing their alignment at the metaphase plate.

In addition, to further strengthen our results, we have examined the localization of the spindle assembly checkpoint (SAC) protein Bub1 in cells depleted of Aurora A kinase and in cells in which this phenotype was significantly rescued by CRY2-mCherry-tagged Aurora A. These new data provide an independent readout of mitotic fidelity and have been incorporated into the revised manuscript (See Fig 1D and 1E, and related text on page 6).

2. On page 6, the authors state: "Since centrosome-localized Aurora A rapidly exchanges with the cytoplasmic pool (Kress et al., 2013; Laos et al., 2015; Zheng et al., 2024), we reasoned that Aurora A clusters generated in mitosis are rapidly transported to centrosomes via microtubule-dependent motors." - The authors could confirm their reasoning using live-cell imaging to clarify whether the clusters are transported to centrosomes via minus-end-directed motor activity (e.g. using dynein depletion or inhibition).
Response: We thank the reviewer for raising this important point, which was also highlighted by Reviewer 1 (see our response to their minor Point #3). In response, we investigated the role of dynein in the transport and accumulation of light-induced Aurora A clusters at spindle poles. Notably, siRNA-mediated depletion of DHC1 resulted in the dispersal of CRY2-AurA^{r} clusters, producing a phenotype reminiscent of that observed following nocodazole treatment. These findings suggest that dynein-mediated transport is required for the efficient coalescence of Aurora A clusters at spindle poles.

In addition, we found that the robust accumulation of endogenously active Aurora A at the spindle pole also depends on dynein function, indicating that dynein-mediated transport contributes not only to the behavior of our synthetic Aurora A assemblies but also to the localization of the endogenous kinase during mitosis.

These new findings have been further incorporated into the revised manuscript (page 7) and presented in the new Fig. 2D-2G, and Fig. S1G and S1H.

3. Related to Figure 2: The authors state that clustered Aurora A "exhibits centrosome-like activity by nucleating microtubules", and that "In contrast to the control cells not exposed to blue light, cells expressing CRY2-AurAr/CIB1-MP, when exposed to blue light and released from the

nocodazole, were capable of microtubule nucleation from the CRY2-AurAr clusters, which are spread throughout the cells (Fig. 2H; Movies S3 and S4)".

3a. It is not clear whether the authors propose that Aurora A serves as a microtubule nucleator per se. The presented experiments do not directly test whether Aurora A catalytic activity is required for the observed microtubule assembly (e.g., no kinase inhibition or kinase-dead mutant analysis or direct assay on microtubule nucleation). Microtubule nucleation is superficially analyzed and left at a very observational level. For instance, GFP-EB1 tracking or quantification of tubulin fluorescence intensity at different time points after nocodazole release would strengthen the conclusions.

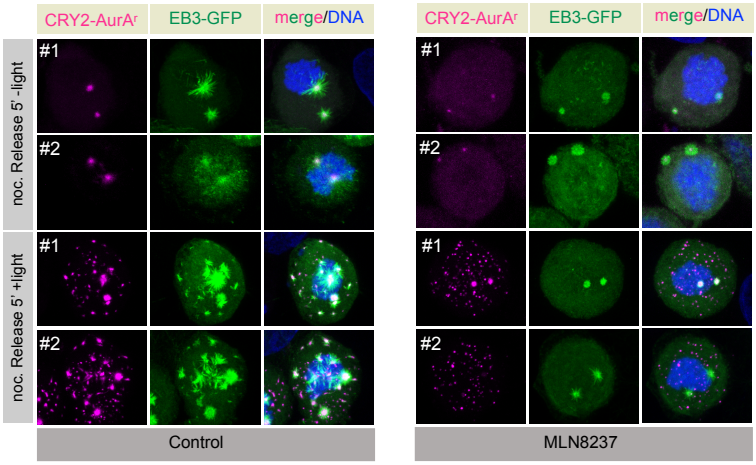
Response: We thank the reviewer for this important comment and apologize for any misunderstanding arising from our original presentation. We did not intend to propose that Aurora A itself functions as a microtubule nucleator. Rather, our working hypothesis is that higher-order Aurora A assemblies recruit microtubule-associated factors that are required for microtubule nucleation.

To address this point, and in response to related suggestions from Reviewers 1 and 2 (see their Major Points #1 and #2, respectively), we have now examined the localization of several established Aurora A interactors, including TPX2 and TACC3. Among these candidates, we found that the evolutionarily conserved microtubule nucleation factor TPX2 robustly accumulates within light-induced Aurora A clusters. Importantly, RNAi-mediated depletion of TPX2 substantially impairs Aurora A cluster-dependent microtubule nucleation, indicating that TPX2 is a critical downstream effector of this process.

As further suggested by the reviewer, we also directly tested the requirement for Aurora A kinase activity by treating cells with the selective Aurora A inhibitor MLN8237. These experiments demonstrate that Aurora A catalytic activity is essential for microtubule nucleation by the light-induced clusters (please see below).

Finally, we appreciate the reviewer's suggestion to employ microtubule plus-end markers to monitor microtubule growth. In response, we have performed nocodazole washout experiments using GFP-EB3 and directly visualized microtubule nucleation and growth from the Aurora A clusters. These experiments provide an independent and dynamic readout of the nucleation process and further support our conclusions.

Collectively, these new data strengthen our model that Aurora A does not act as a microtubule nucleator per se but instead forms signaling assemblies that recruit and activate factors, such as TPX2, required for efficient microtubule nucleation. For the reviewer's convenience, we have shown the data related to kinase inhibition below, and the other results in response to Reviewer 1 point #1. All of these findings have been incorporated into Fig. 3, Fig. 5, Fig. S4 and Fig. S6 of the revised manuscript and are discussed on pages 10-12.



Aurora A activity is vital for the efficient microtubule nucleation capacity of light-induced Aurora A clusters

IF analysis of HeLa cells coexpressing CRY2-AurA^{c}, CIB1-MP, and EB3-GFP. Cells were first treated with nocodazole to depolymerize microtubules and subsequently incubated with either DMSO (control) or the Aurora A-specific inhibitor MLN8237 for 2 h. Cells were either left untreated or exposed to a brief illumination with blue light followed by nocodazole washout to follow microtubule nucleation, as indicated. Cells were stained with anti-mCherry (magenta) and anti-GFP (green). DNA is shown in blue. Note the absence of ectopic EB3-GFP-positive microtubule growth events in two independent examples (#1 and #2) of MLN8237-treated cells exposed to blue light, in contrast to DMSO-treated control cells

3b. Aurora A activation is inferred solely from T288 autophosphorylation. Inclusion of at least one additional substrate readout would strengthen the conclusion that Aurora A is functionally active toward its downstream targets.

Response: A similar point was raised by reviewer 2 (see our response to their major point #4). As suggested by the reviewer, we obtained and tested the LATS2 pS82 phospho-specific antibody utilized in Holder et al., 2025, PMID: 39327527, as a target for Aurora A kinase. Unfortunately, despite repeated attempts, we were unable to detect a specific signal with the antibody lot that we received, either by immunofluorescence or by immunoblotting. However, we would like to emphasize that our conclusions regarding Aurora A activation were not based solely on T288 phosphorylation. In the previous version of the manuscript, we also examined the localization and dynamics of NuMA, a well-established substrate of Aurora A. Previous studies have demonstrated that Aurora A phosphorylates NuMA at S1969 (Kettenbach et al., 2015, PMID: 21712546), and that Aurora A activity regulates NuMA localization at the spindle poles during mitosis (Gillani et al., 2016, PMID: 26832443; Kotak et al., 2016, PMID: 27335426; Rajeevan et al., 2025, PMID: 40940421). More recently, we showed that cells lacking centrosomes fail to accumulate active, T288-phosphorylated Aurora A at spindle poles (Rajeevan et al., 2025). Under these circumstances, NuMA accumulates robustly at spindle poles, closely phenocopying cells treated with the Aurora A inhibitor MLN8237 or cells expressing the phospho-dead NuMA S1969 mutant (Rajeevan et al., 2025). Importantly, our optogenetic system, which restores Aurora A activity through light-induced clustering, rescues both the aberrant spindle pole localization and the altered NuMA dynamics. These findings provide functional evidence that the light-induced Aurora A assemblies contain a catalytically active kinase capable of phosphorylating downstream substrates such as NuMA. We have further strengthened these findings in the new Fig S9.

3c. Are active Aurora A clusters able to bind allosteric factors like Tpx2 and Bora? This could strengthen the central claim that clustering drives functional activation.

Response: We thank the reviewer for this suggestion. As described above, in the revised manuscript, we have now examined the localization of TPX2 within light-induced Aurora A clusters and directly tested its functional relevance for Aurora A cluster-dependent microtubule nucleation. Our results demonstrate that TPX2 robustly accumulates at Aurora A clusters and is required both for efficient stabilization of Aurora A T-loop (T288) phosphorylation and for microtubule nucleation by these assemblies. These findings are consistent with the established role of TPX2 as a key Aurora A co-activator and microtubule nucleation factor and further support our model that Aurora A-dependent microtubule nucleation is mediated through the recruitment and activation of downstream effector proteins rather than by Aurora A itself functioning as a microtubule nucleator.

With regard to Bora, we chose not to pursue its localization or functional analysis in our experimental system. Bora is primarily known for its role in promoting Aurora A activation during the G2/M transition, particularly in the activation of Plk1, rather than in mitotic microtubule nucleation. Given that our new data identify TPX2 as a critical mediator of Aurora A cluster-dependent microtubule nucleation, we decided to focus our mechanistic analyses on this well-established mitotic co-activator. We have therefore not included Bora in the current study, as we believe it falls beyond the primary scope of the present work.

4. Related to Figure 3: The observation that Aurora A clustering can restore spindle bipolarity in Cep192-depleted cells is important and should be quantified to clarify whether this is a robust population-level effect or limited to a subset of cells.

Response: As suggested by the reviewer, we have now examined whether light-induced Aurora A clustering can restore spindle bipolarity in Cep192-depleted cells. We found that Cep192 depletion results in a substantial defect in bipolar spindle assembly, with ~80% of cells failing to form bipolar spindles. Remarkably, light-induced Aurora A clustering significantly rescued this phenotype, reducing the frequency of spindle assembly defects and restoring bipolar spindle formation ~60% of cells. These findings further support our model that Aurora A activation can, at least in part, function independently of the canonical centrosome/Cep192 pathway to promote spindle assembly. These new data have been incorporated into Fig. 4H-4K and are discussed on page 12 of the revised manuscript.

5. Figure 3D-G, and page 9: The authors state that "Consistent with prior work, siRNA-mediated depletion of Cep192 abolished the accumulation of T288P and produced spindle assembly defects (Fig. 3D-3G; data not shown; Gomez-Ferreria et al., 2007; Holder et al., 2024)". The data should be shown and quantified.

Response: As requested by the reviewer, we have now included the Cep192 depletion data in the revised Fig. 4D-4G and mentioned this in the corresponding results section.

6. Related to Figure 4: The authors report that prolonged centrinone treatment led to loss of several PCM components, including Cep192, PCNT, and Cdk5Rap2. However, previous work has shown that acentriolar HeLa cells retain PCM components at spindle poles. In fact, lack of both centrosomes and these PCM components resulted in mitotic spindle assembly failure in HeLa cells. The discrepancy between these two sets of results should be discussed.

Response: We thank the reviewer for raising this important point and for inviting us to discuss the apparent discrepancy in the literature. Previous work by Watanabe et al., 2020 (PMID: 33170211, Fig. 7A) reported weak but detectable accumulation of Cep192, Cdk5Rap2, and PCNT in acentrosomal HeLa cells. In contrast, Chinen et al., 2020 (PMID: 31782546; Fig. S1A and S1B) and Chinen et al., 2021 (PMID: 33443571; Fig. 2F) were unable to detect appreciable levels of these PCM components under similar conditions. Our observations, in which we fail to detect Cep192, PCNT, and Cdk5Rap2 in acentrosomal HeLa cells, are therefore more consistent with the findings of Chinen et al., 2021 in HeLa cells.

One possible explanation for these differing observations is variability in the levels of TRIM37, an E3 ubiquitin ligase that targets Cep192 for degradation, among HeLa cell lines used in different laboratories. Another possibility is that the well-documented genetic heterogeneity of HeLa cell lines, including differences in gene copy number, protein expression levels, and protein turnover rates, could influence the capacity of acentrosomal cells to retain or assemble residual PCM structures after centrosome loss.

7. Page 10: The authors state that "Strikingly, a brief blue-light pulse induced robust T288P accumulation in centrinone-treated cells (Fig. 4E-4G), despite the absence of Cep192 or other centrosome-localized proteins at CRY2-AurAr/CIB1-MP clusters (Fig. S4A-S4O). Thus, light-induced clustering of CRY2-AurAr is sufficient for Aurora A activation in the absence of centrosomes".

- How could Aurora A clustering initiate microtubule nucleation without γ-tubulin and/or other centrosome/PCM components? This remains the biggest unused potential of the study and addressing this would immensely increase the manuscript's impact. Besides acknowledging it in the discussion part, the authors could try to assess which other proteins are within the Aurora A clusters that are required for microtubule nucleation. This could be done by either

immunofluorescence with specific markers or by immunoblotting and/or mass spectrometry identification after pull-down of the clustered Aurora A.

Response: We thank the reviewer for this insightful comment. A similar point was also raised by Reviewers 1 and 2 (see responses to Major Points 1 and 2, respectively). As outlined above, we have now investigated the localization of several Aurora A co-activators and interactors, including TPX2 and TACC3, within the light-induced Aurora A assemblies.

Notably, we found that TPX2 robustly co-localizes with light-induced Aurora A clusters. Given the well-established role of TPX2 in promoting microtubule nucleation (Brunet et al., 2004, PMID: 15385625; Petry et al., 2013, PMID: 23415226; Roostalu et al., 2015, PMID: 26414402; Liang et al., 2025, PMID: 39821262), we next examined its functional relevance in our system. RNAi-mediated depletion of TPX2 resulted in a marked reduction in T288-phosphorylated Aurora A at the light-induced clusters, consistent with the established role of TPX2 in stabilizing the Aurora A activation loop. Importantly, TPX2 depletion also substantially impaired microtubule nucleation from these clusters, demonstrating that TPX2 is a critical mediator of Aurora A cluster-dependent microtubule assembly.

These findings provide mechanistic insight into how light-induced Aurora A assemblies promote microtubule nucleation and support our model that Aurora A functions by recruiting and activating downstream microtubule nucleation factors rather than acting as a nucleator itself. These new data have been incorporated into Fig. 5 and Fig. S6 and are discussed on pages 12 and 13 of the revised manuscript.

Minor points:

1. Page 5: The authors state that CRY2-AurAr fully rescues the phenotypes associated with Aurora A depletion. Given that "chromosome instability" presented in Figure 1F remains at 20%, it would be more precise to state that the construct substantially or partially rescues the phenotype.

-Thanks, it is now corrected.

2. Figure 4G: The last two labels appear misplaced.

-Thanks, corrected.

3. Clarify whether quantifications represent biological replicates.

-We apologize for this omission. All experiments were performed at least twice, and in most cases three independent times. We have now clarified this in the Methods section of the revised manuscript.

4. Number of experiments are not specified in the figure legends, only number of cells.

-We thank the reviewer for this helpful comment. The quantified cell numbers are derived from two to three independent biological experiments. We have now included this information in the Methods section of the revised manuscript.

5. Consistent terminology when referring to "centrosome-like structures" vs "induced clusters" would be of help to the readers.

-Thanks for this point; we have now used consistent terminology throughout the paper.

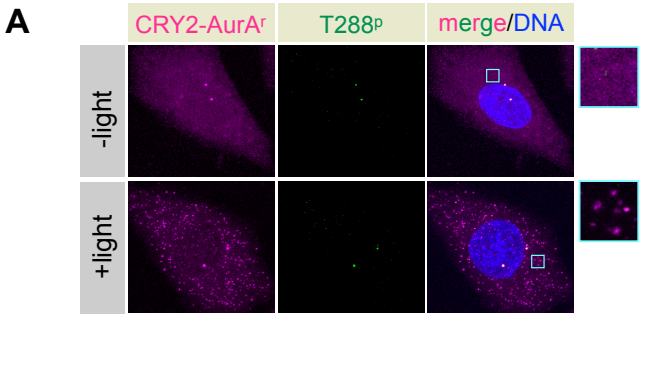
6. Figure S5A: All other centrinone-treated cells shown in the manuscript (including quantification of spindle length in Figure 4I) displayed much shorter spindles than the spindles in this figure, which appear of similar length as controls. Could authors comment on this?

-We thank the reviewer for highlighting this discrepancy. Upon re-examining our dataset, we realized that the representative image included in the original figure was not fully representative

of the quantified phenotype and likely corresponded to an outlier cell with a relatively normal spindle length. In the revised manuscript, we have replaced the representative image from an acentrosomal cell that more accurately reflects the characteristic short-spindle phenotype observed under these conditions and is consistent with our quantitative analyses. We hope that these changes improve the presentation of the data and better align the representative examples with the population-level measurements.

7. Figure 4I: The lack of centrosomes reduces astral microtubules and can therefore affect spindle orientation, which could interfere with spindle length measurements if derived from 2D projections. The methods section should specify whether 3D measurements or z-corrections were applied.
-We thank the reviewer for this important point. We agree that changes in spindle orientation could potentially influence spindle length measurements if these are performed using two-dimensional projections. To avoid this issue, all spindle length measurements in our study were acquired from confocal image stacks encompassing the entire mitotic cell and analyzed in three dimensions using Imaris software (Oxford Instruments). Specifically, we reconstructed the full 3D volume of each spindle and measured the pole-to-pole distance by drawing a straight line between the two spindle poles within the 3D reconstruction, rather than from a 2D projection. This approach minimizes potential artifacts arising from spindle orientation and ensures accurate determination of spindle length irrespective of spindle positioning within the cell. We have now clarified this in the Method section of the revised manuscript.

8. Discussion: The authors state that "Interestingly, when Aurora A was clustered by light in interphase, it remained inactive, showing no detectable T-loop phosphorylation". The reviewer could not find this data in the manuscript.
- We thank the reviewer for raising this point. In the interest of maintaining a balanced and focused narrative, we chose not to include these data in the original version of the manuscript. However, prompted by the reviewer's comment, we have now included these data below for the reviewer's evaluation. We have kept the statement in the discussion section of the revised manuscript indicating that these observations were obtained but are not shown.



Light-induced Aurora A clusters do not accumulate T288 phosphorylated Aurora A in interphase
(A) IF analysis of HeLa cells stably coexpressing CRY2-AurA^r and CIB1-MP in interphase in the absence and presence of blue light, as indicated. These cells were stained with anti-mCherry (magenta) and anti-T288^p (green) antibodies. DNA is shown in blue. Rectangular regions marked in the image are shown magnified on the top right.
(B) Quantification of CRY2-AurA^r and T288^p intensity (in au) within clustered CRY2-AurA^r in the absence or presence of blue light. Error bars: mean ± SD (n≥25). Exact p values from left to right are ***p<0.001, and ns-p>0.05.

Dear Prof. Kotak

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. The numerical source data check has also been completed without any points identified that require clarification.

As you have seen, all referees are very positive about the study and request only minor changes to clarify text and figures.

Before I can accept the manuscript, I need the completion of the editorial requests included in my prior email (recopied here):

- Please adjust the format of the reference list according to EMBO Journal format (up to 10 author names in the reference list before et al). Please refer to our Guide to Authors for additional information on EMBO J reference format.

- Please note that the Methods sections "Transient transfection" and "Small interfering RNA transfection" display high similarity to published works. Please provide a citation for these sections.

- Please incorporate a statement on blinding in data analysis into the Methods section even if no blinding was performed.

- Please remove the Reagents and Tools Table from the manuscript file and provide it as a separate file.

- Please note that the following grants are missing in our system: IE/REDA-23-2061, IE/REDA-24-2061, IE/REDA-25-2061.

- Please provide the main text figures in separate publication-ready files.

- Please merge the two pdf files containing the supplemental figures and their legends into one clean PDF titled Appendix with a title page containing a short table of contents with page numbers. The legend should follow each supplemental figure. The correct nomenclature in all places (Appendix, main text, etc.) should be Appendix Figure S1, etc. Please remove the movie legends from this file.

- Please update the nomenclature for the movies to Movie EV1, etc. in all places (source file names, titles in the system, legend titles, callouts in the manuscript). Movie legends should be provided in a readme file; then each legend should be zipped up with its corresponding movie so that we have 5 movie folders (Movie EV1-Movie EV5).

- Please adjust the in-text callouts for individual figures and figure panels: e.g. callouts for the individual panels of Fig. 8 appear to be missing.

- Please update the Source Data checklist to reflect the nature of the data for each figure panel (such as N/A (schematic) or microscopy image).

- Please provide the image source data along with the numerical source data. We suggest .Tiff format.

- Please note that EMBO Reports does not allow data to not be shown. Please incorporate the evidence that Aurora A clustering fails to induce T-loop phosphorylation into a figure and call this out in the main text.

- Please note that we require plots with N<5 to display individual data points. In particular, please doublecheck figures 2E, 3C, 4K, 5E and I, S1H, S2F, S5B, S8E and S10E.

- Our production/data editors have asked you to clarify several points in the figure legends (see below). Please incorporate these changes in the manuscript and return the revised file with tracked changes with your final manuscript submission.
- 1. Please note that the exact p values are not provided in the legends of figures 1G,I; 2C,E,G; 3C; 4E,G,J,K; 5B,E,G,I; 6B,D,G,I,L,M.

- Please note that EMBO press papers are accompanied online by:
A) a short (2 sentences) summary of the findings and their significance,
B) 2-5 short bullet points highlighting the key results, and
C) a synopsis image in .jpg or .png format that is exactly 550 pixels wide and 300 pixels high. Please note that the text needs to be legible at the final size. Please upload this information along with your revised manuscript (the text for A and B should be provided in one separate Word file uploaded as Cover Art/Synopsis Image).

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Kurt Weir
Editor
EMBO Reports

Referee #1:

The authors have appropriately addressed all of my comments and concerns, including providing the results of feasible experiments. These revisions have significantly improved the manuscript. Therefore, I am pleased to recommend this paper for publication.

Referee #2:

The revised manuscript is substantially improved, and the authors have addressed most of the major concerns raised in the previous round. In particular, the new data strengthen the main conclusion that forced Aurora A clustering is sufficient to trigger Aurora A activation and support microtubule nucleation/spindle assembly independently of canonical centrosomal scaffolds, with an important contribution from TPX2. The additional analyses of Cep192 independence, TPX2 recruitment/function, and Aurora A inhibitor sensitivity considerably reinforce the study. The study provides an original optogenetic framework to probe how Aurora A clustering can organize spindle assembly in the absence of canonical centrosomal scaffolds, and the new revision now gives convincing support for a central role of TPX2 in this process. This reviewer supports publication of the revised manuscript in EMBO Reports.

Minor comment:

- The authors now show convincingly that several canonical PCM components are not detectably recruited to the light-induced clusters, while TPX2 is recruited and functionally required. This is a real strength of the revision. However, the mechanism by which these clusters nucleate microtubules is still only partially resolved. The study would benefit from a more explicit statement that the work identifies a minimal functional module but does not yet define the full molecular composition of the nucleation-competent clusters, which can be addressed by biochemical and/or proteomic characterization in future.

Referee #3:

The authors have responded convincingly to most of the previous reviewer comments. Although not all suggested experiments have been done (e.g. proteomics),they have provided alternative data or robust clarification that adequately address the reviewer concerns. There is one exception, albeit minor:

3. "It would be important to show
mitotic duration and spindle length in Cry2-AurA/CIB1-MP cells with and without light in the
absence of centrinone."

The authors present new data along these lines, some of which is included in Fig. S10. From visual inspection, it appears that the expression of Cry2-AurA/CIB1-MP does not markedly affect mitotic duration or spindle length under control conditions (no centrinone). However, I strongly feel that the data should all be included in the Fig. S10 because it shows a baseline of similarity with the parental cells, and this makes the results with the centrinone even more compelling. Also, the data require quantification as done for the experiments shown in Fig. 7C. While I commend the authors for wanting to maintain the focus on their narrative, just including the control data in the supplementary information with a minimal reference to it in the text won't distract the reader.

One major point about Fig. 7C. I think there is an error in the quantification for panel a (14% seems high when I look at the data), and this number is similar to that in panel d, where the data looks different. Even with different values, I agree that the phenotype is mostly rescued, but its important to get the values correct.

Response to the reviewers

Reviewer #1

We deeply thank the reviewer for their support of our work.

Reviewer #2

We sincerely thank the reviewer for their positive feedback on our work. Below, we address the one minor concern raised by the reviewer.

(1) The authors now show convincingly that several canonical PCM components are not detectably recruited to the light-induced clusters, while TPX2 is recruited and functionally required. This is a real strength of the revision. However, the mechanism by which these clusters nucleate microtubules is still only partially resolved. The study would benefit from a more explicit statement that the work identifies a minimal functional module but does not yet define the full molecular composition of the nucleation-competent clusters, which can be addressed by biochemical and/or proteomic characterization in future.

Response. We thank the reviewer for this important point. As suggested, we have added a sentence to the Discussion section (p. 20) highlighting that future proteomic analyses will be important for identifying the complete repertoire of proteins recruited to light-induced Aurora A clusters. Defining the molecular composition of these assemblies will be crucial for understanding the mechanisms by which they promote microtubule nucleation.

Reviewer #3

We are greatly thankful to the reviewer for their kind words. We have now addressed the remaining minor points raised by the reviewer.

The authors present new data along these lines, some of which is included in Fig. S10. From visual inspection, it appears that the expression of Cry2-AurA/CIB1-MP does not markedly affect mitotic duration or spindle length under control conditions (no centrinone). However, I strongly feel that the data should all be included in the Fig. S10 because it shows a baseline of similarity with the parental cells, and this makes the results with the centrinone even more compelling. Also, the data require quantification as done for the experiments shown in Fig. 7C. While I commend the authors for wanting to maintain the focus on their narrative, just including the control data in the supplementary information with a minimal reference to it in the text won't distract the reader.

One major point about Fig. 7C. I think there is an error in the quantification for panel a (14% seems high when I look at the data), and this number is similar to that in panel d, where the data looks different. Even with different values, I agree that the phenotype is mostly rescued, but its important to get the values correct.

Response. We thank the reviewer for raising this important point. The data showing mitotic duration in cells expressing CRY2-AurA/CIB1-MP in the presence and absence of blue light were already included in the previous Fig. S10. However, in response to the reviewer's concern, we have now additionally included spindle-length measurements in this figure, which is presented as the new Appendix Fig. S5H. As further suggested, we have also added the corresponding quantification directly to the figure panel, consistent with the presentation in the main Fig. 7.

We further thank the reviewer for pointing out the error in the quantification shown in Fig. 7, panel a. Upon re-evaluating the underlying dataset, we identified minor errors in the previously reported values and have corrected them accordingly. We sincerely appreciate the reviewer's careful and astute observation, which enabled us to identify and rectify these errors.

Prof. Sachin Kotak
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