nature portfolio

Peer Review File

The DNA replication machinery transmits dual signals to prevent unscheduled licensing and execution of centrosome duplication

Corresponding Author: Professor Daiju Kitagawa

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
While the authors added some data to strengthen their conclusion that DONSON controls centrosome biogenesis independently of DNA replication, caveats remain. Also, they did not adequately address many of my original points. In particular, they continue to ignore the very strong data that DONSON is required for replication initiation. This is based on rigorous analysis with degron alleles by two independent groups in two different cell types, not to mention information from 1000 cell lines in Depmap. In my estimation the paper is still not suitable for publication.

Remaining issues (no particular order):

The logic at the start of the paper is still flawed. Cdc6, CDC45 etc. are known to be essential replication factors yet the statement “not all these genes were characterized as essential…” suggests otherwise. The lack of an S phase defect in Figure 1C simply shows they were insufficiently knocked down by the siRNA treatment. That is the only reasonable interpretation, and it should be stated clearly as such. This clearly incomplete knock-down in itself does not suggest these proteins could have roles in centrosome biogenesis.

Why is the nucleus shown in S1e green? This high fluorescence signal might obscure the presence of extra censtrosomes.

No clear conclusions can be drawn based on images such as the one shown in 1g or S1h. In both cases, the Cep192 focus on the top left is simply not visible. There is no explanation anywhere of how the authors reliably counted foci using such images. Nature allows unlimited supplementary figures, so space limitations are not an excuse to not show the separate channels.

The conclusion that DONSON is not required for initiation of replication in mammalian cells remains unfounded. It’s based on an siRNA experiment that shows that cells move through S phase very slowly when DONSON is knocked down, as expected if origin firing is compromised. The absence of a complete block is almost certainly due to incomplete knock-down. Indeed, more rigorous degron-based experiments showed that DONSON is required for replication initiation in humans, where DONSON destruction compromises CMG assembly during S phase entry (Lim et al 2023; Evrin et al., 2023). Making the claim that DONSON is not required for initiation on the basis of the presented evidence is unwarranted and will cause confusion in the field. As an aside, the authors may be correct that DONSON also promotes elongation (Reynolds et al., 2017; Lim et al. 2023), but this does not justify ignoring its clearly established role in initiation.

Thank you for adding the diagram of centrosome biogenesis in Figure S2A. It would be helpful to also add key landmarks in spindle assembly and chromosome segregation. The accompanying description in the results is redundant with the almost identical description in the introduction. Therefore, the diagram should be referenced with the original description in the introduction.

The CDC7-i experiment is consistent with their conclusion, but it’s not definitive. CDC7 itself could play a role in centriole disengagement, independently of its role in replication initiation. The EMI1 experiment (whose rationale was not explained), was uncontrolled (no demonstration that knock-down was successful), so provided no support for their conclusion.

On page 17, they again ignore Lim et al., which showed that CMG assembly is compromised in the absence of DONSON in mammalian cells. The data presented that DONSON protects CMG from disassembly is unconvincing. In particular, the CDC45 staining in Figure 5g looks like it's some kind of background, lacking the punctate staining shown in other panels. Indeed, why would the signal go away when cells are released into S phase?

The recovery of CDC45 signal in Fig 5k could be because the low level of CMGs assembled in siDONSON conditions fail to be unloaded (rather than an increased level of termination in the absence of DONSON).

The structural hypothesis that DONSON competes with LRR1 for binding to MCM3 is not compelling. As noted in my original review, there is no difficulty in folding the three proteins together.

The failure of MCM3D354-375 to rescue precocious PLK1 activation could be due to defective replication initiation.

The analogy that the authors make between replication and centrosome biogenesis is tortured. Strand separation is NOT the licensing step in replication. Licensing refers to helicase recruitment in G1. This event, not strand separation, is strictly prohibited in S phase to limit replication to a single round per cell cycle. The parallel with CDC6 is not compelling because CDC6 is normally released from pre-RCs immediately upon MCM recruitment. It does not stay bound until the origin fires. The effect of CDC6 ATPase mutants that are not released is therefore an artifact that does not reflect the normal function of then protein.

They did not adequately address original point 13, because from their description, it appears samples were denatured after IP, not during or before the IP.

Point 16 was not addressed: they did not show that their p97 signal reflects p97 bound to CMGs.

Regarding point 19, MCMs form a stable complex, so where they sit does not matter.

Reviewer #4

(Remarks to the Author)
I found this paper to be exciting and appropriate for publication in Nature Communications. In this manuscript by Matsuhashi et al., the authors present a previously unidentified role for DONSON in centrosome duplication and the stability of replicasome machinery. Overall, the work is novel and exciting, representing a significant connection between replication and centrosome duplication. The authors demonstrate that the loss of DONSON leads to the premature degradation of ETAA-1, an activating partner of ATR, resulting in a failure of the S-phase checkpoint and inappropriate activation of PLK1 at the centrioles. Overall, I found the paper well-written and the experiments robust.

The authors indeed addressed all of the reviewers' concerns, and I found this paper to be scientifically robust and exciting for our field. There are a few general questions that I have that may be important to answer before publication and could be addressed. These experiments are not necessary for an exciting publication but would help clarify some aspects of the work.

Fig 1:
- The authors conclude that some genes characterized are essential factors for S phase entry. However, a pulse of EdU does not account for that as cells could remain stuck in G2 or have decreased replication speed, which would affect EdU incorporation. The authors should use their images and quantify EdU and DAPI signals to identify if decreased EdU incorporation occurs due to arrests in G1 or G2 or just affects replication itself. Alternatively, the authors could conduct FACS.
- The CEP192 signal in Fig 1d is very difficult to see. The authors should revise the representative images to make the signal more clear.
- Are there changes in CEP192 protein abundance in CEdc6 and DONSON knockdown cells? It visually looks like there are even in G1 from Fig 1i. The authors should quantify this and assess whether decreased CEP192 expression could be a cause of centrosome dysfunction observed in DONSON knockdown

Fig 2:
- The authors suggest that mNG-DONSON localizes to the nucleus and that it localizes to some centrosomes. However, this is a very weak finding as there is just a generalized staining across the nucleus, and the signal is highly oversaturated for mNG. The authors should 1) measure the abundance of mNG DONSON compared to endogenous DONSON/Cdc6. 2) Use PLA or other more specific methods to determine if DONSON/Cdc6 is really localizing to centrioles or use pre-extraction or other methods to remove unbound DONSON/Cdc6 and use 3D imaging to assess localization in a more precise manner. 3) Measure the percent of centrosomes with DONSON/Cdc6 localization as in its current form it is very vague.
- For overexpression (2e,f) the authors should clearly state how much overexpression. Is centrosome disengagement linearly correlated Cdc6 levels or localization to centrosomes? Or is there a threshold or some sort of binary amount?

Fig 3:
- The authors should test whether the p-Plk1 (T210) antibody is indeed accurate and specific, as this antibody is notorious for having off-target binding. The authors should also measure PLK1 at centrioles.
- The authors demonstrate that Chk1 activity is elevated and cite work demonstrating that this is indeed caused by replication stress. The authors should demonstrate how much DNA damage is occurring in siDONSON cells.
- The authors should blot for ATR and Chk1 activity at those inhibitor concentrations to determine if complete inhibition is acquired. The authors should also assess if this is indeed ATR/Chk1 specific or just due to a general activation of the DNA damage response pathway by inhibiting ATM and determining if there is an increase in p-PLK1 at centrioles.

Fig 5:
- Chromatin-bound cdc45 looks like it exclusively localizes to nucleoli in the presence of aphidicolin and it is unclear if the signal is actually just an off-target issue. The authors should quantify chromatin-bound Cdc45 using fractionation western blotting.

Fig 6:
- ETAA1 also plays a function in mitotic ATR signaling. Therefore, it is possible that the effect observed with ETAA1 rescue on the rate of lagging chromosomes and mitotic defects is not direct but solely due to hyperactivation of the mitotic ATR-Chk1 pathway. The authors should assess if ETAA1 is overexpressed specifically in S-phase alone or if S-phase-specific inhibition of plk1 would rescue the multipolarity defects in the subsequent mitosis.

Reviewer #5

(Remarks to the Author)
This work describes how the microcephaly protein DONSON couples the DNA and centrosome cycle via the relocalization of Cdc6 to centrosomes. I was not an original reviewer, but I was asked to comment specifically on the points raised by reviewer 3, which concerned the relationship of this manuscript to the work described in Dwivedi et al., 2023, which was not discussed in the original mansucript.

First, for full disclosure, there is a certain conflict of interest, since my laboratory generated the Dwivedi study.

Second, with regard to the concerns of reviewer 3, the good (and always re-assuring) point is that this study reproduces our original study (mild replication stress induced by aphidicolin leads to a loss of orthogonal orientation of the centriole pairs) and it shows that DONSON depletion results in a more pronounced phenotype. The conclusion of the authors that this represents two different mechanism is sound and robust, and I feel that the authors have thus addressed this point. More generally, I feel that this study adds an important new perspective on the relationship between the DNA and centrosome cycle, and would thus be a nice manuscript for the present journal.

Nevertheless, I differ with the interpretation of the authors in the rebuttal letter that the sole loss of orthogonal centriole orientation does not represent centriole disengagement. Some of the earliest electron-microscopy studies in cells called such a loss of orthogonal arrangement centriole disengagement, showing centriole pairs that were close but without an orthogonal arrangement in G1 cells (see Kuriyama and Borisy JCB, 1981, or the earliest review article on the subject, Tsou and Stears, Curr. Opin in Cell Biol, 2006). Moreover, we have shown that this type of non-orthogonal centrioles will, as soon as they enter mitosis and bind to microtubules, move apart by several microns, recruit microtubule-nucleating pericentriolar material, and thus form a functional spindle pole with single disengaged centrioles (Wilhelm et al, Nat Comm, 2019), indicating that loss of orthogonal orientation in G2 is sufficient to give raise four functional mitotic centrosomes with single centrioles. One possibility to reconcile this different interpretations, would be to discuss that this study points to the fact that different extents of centriole disengagement might exist. In particular, one could speculate that while loss of orthogonal orientation might allow single centrioles to become mitotic MTOC, a higher physical separation might be necessary to allow the next round of centriole duplication. This is, however, just a suggestion, and the authors should feel free to discuss this point or not.

Patrick Meraldi

Version 1:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The revised manuscript, by Matsuhashi and colleagues, is significantly improved, and they have sufficiently addressed my initial concerns.

My major concern is with the representative images. Many images are out of focus, which could affect their quantification (e.g. Fig 4b, 4e, Fig 5e, Fig 5m). The images are often merged only (e.g., Fig 2a, 2h,Fig 4b), which makes it almost impossible to compare or even see in any significant detail what the authors are trying to demonstrate. The authors should show all channels separately and then a merged image. At other times, the single channel image is hard to see at all; for

example, in fig 5e and 5g, the authors should show a nuclear boundary. Another example is Fig 5i, where the chromatin-bound p97 image is more magnified than the merge, a line demarcating the nuclear boundary would help remove this doubt.

Authors need to detail how they are processing the representative images in the figure legends, as it looks like there are differences in processing between single channels and merged channels (e.g. Fig 4i, Fig 5g) and between the original image and the inset (e.g. Fig 5m)

These issues are throughout the manuscript and not just exclusive of the few figures marked. This should be fixed for all representative images.

Reviewer #5

(Remarks to the Author)
The authors have addressed my comments, and I support publication

Reviewer #6

(Remarks to the Author)
I have been asked to assess the authors' responses to the issues raised by Reviewer #1, who is currently unavailable. Having carefully read the revised manuscript, I can say that all the concerns raised by this reviewer have been satisfactorily addressed. I think that this study provides important insights into the molecular mechanisms underlying the coordination between DNA replication and chromosome segregation in human cells.

We thank all the reviewers (#1, #4 and #5) of our manuscript for their critical reading and for their useful and constructive comments (typed in black). In response to all the reviewer comments, we have made significant revisions again to our manuscript with new data (our responses are typed in blue) as we detail below. We now believe it meets the high-quality standards of Nature Communications.

Reviewer #1 (Remarks to the Author)

While the authors added some data to strengthen their conclusion that DONSON controls centrosome biogenesis independently of DNA replication, caveats remain. Also, they did not adequately address many of my original points. In particular, they continue to ignore the very strong data that DONSON is required for replication initiation. This is based on rigorous analysis with degron alleles by two independent groups in two different cell types, not to mention information from 1000 cell lines in Depmap. In my estimation the paper is still not suitable for publication.

> We thank this reviewer for the comment. We fully agree with this point and have explicitly stated in all relevant sections of the manuscript that DONSON is essential for the initiation of DNA replication (page 8, 17-18). Additionally, as detailed below, we have addressed all other points raised by this reviewer, providing the new data from additional experiments.

Remaining issues (no particular order):

The logic at the start of the paper is still flawed. Cdc6, CDC45 etc. are known to be essential replication factors yet the statement “not all these genes were characterized as essential…” suggests otherwise. The lack of an S phase defect in Figure 1C simply shows they were insufficiently knocked down by the siRNA treatment. That is the only reasonable interpretation, and it should be stated clearly as such. This clearly incomplete knock-down in itself does not suggest these proteins could have roles in centrosome biogenesis.

> We thank the reviewer for raising this issue. As this reviewer pointed out, under this siRNA experimental condition, it is possible that the gene knockdown efficiency was insufficient. Given this, it would not be appropriate to discuss whether each gene is essential for DNA replication based on these results. Therefore, we have removed this data from the revised version of the manuscript and modified the main text. However, we believe an important implication of these results is that siRNA targeting either DONSON or Cdc6 specifically induces the phenotype of centrosome amplification, irrespective of the extent to which DNA replication is inhibited.

Why is the nucleus shown in S1e green? This high fluorescence signal might obscure the presence of extra centrosomes.

> In the right panel of the original Supplementary Figure 1e (Supplementary Fig. 1f in the revised manuscript), the nucleus appears green due to the staining of exogenously expressed Flag-DONSON within the nuclei. The channels were observed separately, and this signal does not influence the centrosome count. Quantitative data are also provided (Supplementary Fig. 1g).

No clear conclusions can be drawn based on images such as the one shown in 1g or S1h. In both cases, the Cep192 focus on the top left is simply not visible. There is no explanation anywhere of how the authors reliably counted foci using such images. Nature allows unlimited supplementary figures, so space limitations are not an excuse to not show the separate channels.

>We thank the reviewer for pointing this out and apologize for the difficulty in visualizing the Cep192 foci. In response, we have adjusted the images to make the Cep192 foci more easily recognizable (Fig. 1g and Supplementary Fig. 1i). Additionally, to avoid any confusion, we have included enlarged insets of all Cep192 foci.

The conclusion that DONSON is not required for initiation of replication in mammalian cells remains unfounded. It's based on an siRNA experiment that shows that cells move through S phase very slowly when DONSON is knocked down, as expected if origin firing is compromised. The absence of a complete block is almost certainly due to incomplete knock-down. Indeed, more rigorous degron-based experiments showed that DONSON is required for replication initiation in humans, where DONSON destruction compromises CMG assembly during S phase entry (Lim et al 2023; Evrin et al., 2023). Making the claim that DONSON is not required for initiation on the basis of the presented evidence is unwarranted and will cause confusion in the field. As an aside, the authors may be correct that DONSON also promotes elongation (Reynolds et al., 2017; Lim et al. 2023), but this does not justify ignoring its clearly established role in initiation.

> We fully agree with the reviewer's opinion on this point. Accordingly, we have revised all relevant claims in the manuscript. As shown in previous studies (Lim et al 2023; Evrin et al., 2023), the significant delay in progression to S phase caused by siRNA targeting DONSON is most likely due to the inhibition of DNA replication initiation. While DNA replication is not completely blocked under our experimental conditions, this is likely due to the incomplete effect of siRNA. To avoid any confusion in the field, as the reviewer suggested, we have clearly stated in the revised manuscript that DONSON is an essential factor for DNA replication initiation (please see the modified texts below). Furthermore, we clarified that while origin firing is significantly affected under our experimental conditions, DNA replication progresses at a reduced

rate. This allowed us to uncover a novel function of DONSON in contributing to CMG helicase stability in DNA elongation (Reynolds et al., 2017; Lim et al. 2023), in addition to its essential role in DNA replication initiation.

(page 8)

Recent studies reported that DONSON is required for DNA replication initiation in *Caenorhabditis elegans* and *Xenopus laevis*[13–16]. Consistent with this, our flow cytometric analyses with synchronous human cells, revealed that depletion of DONSON efficiently suppressed the entry into S phase. However, at least under this experimental condition, a very gradual entry of the cells into S phase was detectable, albeit with a significant delay (Supplementary Fig. 1k).

(page 17-18)

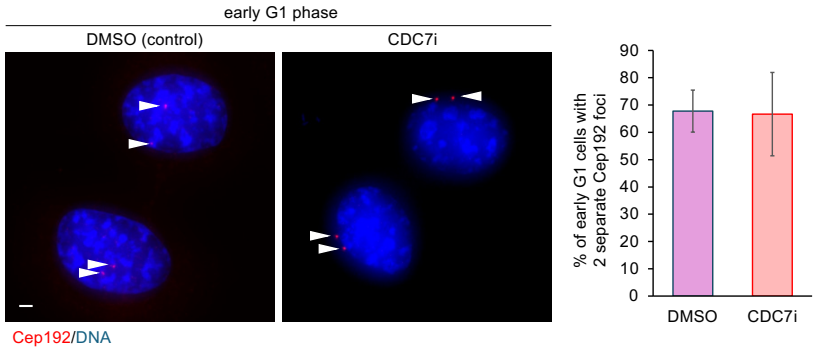
Recent studies demonstrated that DONSON is required for the initial assembly of the CMG helicase in *Caenorhabditis elegans* and *Xenopus laevis*[13–16]. Consistently, in our experimental condition, in which DONSON expression was partially suppressed with siRNA in human cells, the initiation of DNA replication was significantly inhibited but DNA replication still proceeded very gradually (Supplementary Fig. 1k). This might be the reason why the precocious disassembly of the CMG helicase was detectable in DONSON-depleted cells released into S phase (Fig. 5g,h).

Thank you for adding the diagram of centrosome biogenesis in Figure S2A. It would be helpful to also add key landmarks in spindle assembly and chromosome segregation. The accompanying description in the results is redundant with the almost identical description in the introduction. Therefore, the diagram should be referenced with the original description in the introduction.

> We thank the reviewer for this suggestion. We have added key landmarks for spindle assembly and chromosome segregation to the original Supplementary Fig. 2a. Additionally, we have cited this diagram in the relevant section of the introduction. Accordingly, we have moved this diagram to Supplementary Fig. 1a in the current version of the manuscript.

The CDC7-i experiment is consistent with their conclusion, but it's not definitive. CDC7 itself could play a role in centriole disengagement, independently of its role in replication initiation. The EMI1 experiment (whose rationale was not explained), was uncontrolled (no demonstration that knock-down was successful), so provided no support for their conclusion.

>We thank the reviewer for raising this point. In response to this comment, we performed additional experiments to confirm that CDC7 does not directly participate in centriole disengagement. As shown in the attached data, the addition of a CDC7 inhibitor did not suppress normal centriole disengagement during the transition from telophase to G1 phase.



CDC7 inhibitor does not affect centriole disengagement in early G1 phase. Representative images of HeLa cells treated with DMSO or CDC7 inhibitor (10 μM PHA767491, 3 hours) and immunostained with antibodies against Cep192. Arrowheads indicate centrosomes. The histogram represents the frequency of early G1 cells with successful centriole disengagement. Values are mean percentages ± s.d. from three independent experiments (n = 30 for each experiment).

>Regarding the EMI1 experiment, we conducted Western blotting to confirm the knockdown efficiency of siEMI1 and observed a significant reduction in protein levels (new data in Supplementary Fig. 1p). The purpose of this experiment was to ensure that even when cells are arrested in the S phase by siEMI1, centrosome amplification does not occur (page 8 in the revised manuscript).

On page 17, they again ignore Lim et al., which showed that CMG assembly is compromised in the absence of DONSON in mammalian cells. The data presented that DONSON protects CMG from disassembly is unconvincing. In particular, the CDC45 staining in Figure 5g looks like it's some kind of background, lacking the punctate staining shown in other panels. Indeed, why would the signal go away when cells are released into S phase?

> As mentioned above, we have fully accepted the reviewer's comment on this point and revised the text accordingly (pages 17–18 in the revised manuscript). Specifically, we now state that DONSON is essential for the initiation of DNA replication and further clarify that, under our experimental conditions, we address the contribution of DONSON to the stability of the CMG helicase after the initiation of DNA replication.

Regarding the CDC45 staining in Fig. 5g, we replaced it with a representative image showing a punctate-like nuclear pattern, which is more appropriate. This data is quantified, as shown in Fig. 5h. We assume that the disappearance of the CDC45 signal from chromatin during the S phase upon DONSON depletion is due to DONSON's role in contributing to the stability of the CMG helicase during DNA replication progression.

The recovery of CDC45 signal in Fig 5k could be because the low level of CMGs assembled in siDONSON conditions fail to be unloaded (rather than an increased level of termination in the absence of DONSON).

> We thank the reviewer for this comment. Our interpretation of these experimental results is that, under the siDONSON + aphidicolin conditions, CMG assembly does occur, albeit at lower levels than normal. However, CMG disassembly is likely being accelerated via the ubiquitin-proteasome system, including p97, in S phase progression, because the treatment with a p97 inhibitor suppresses CMG disassembly, leading to an increase in the amount of CMGs remaining on chromatin.

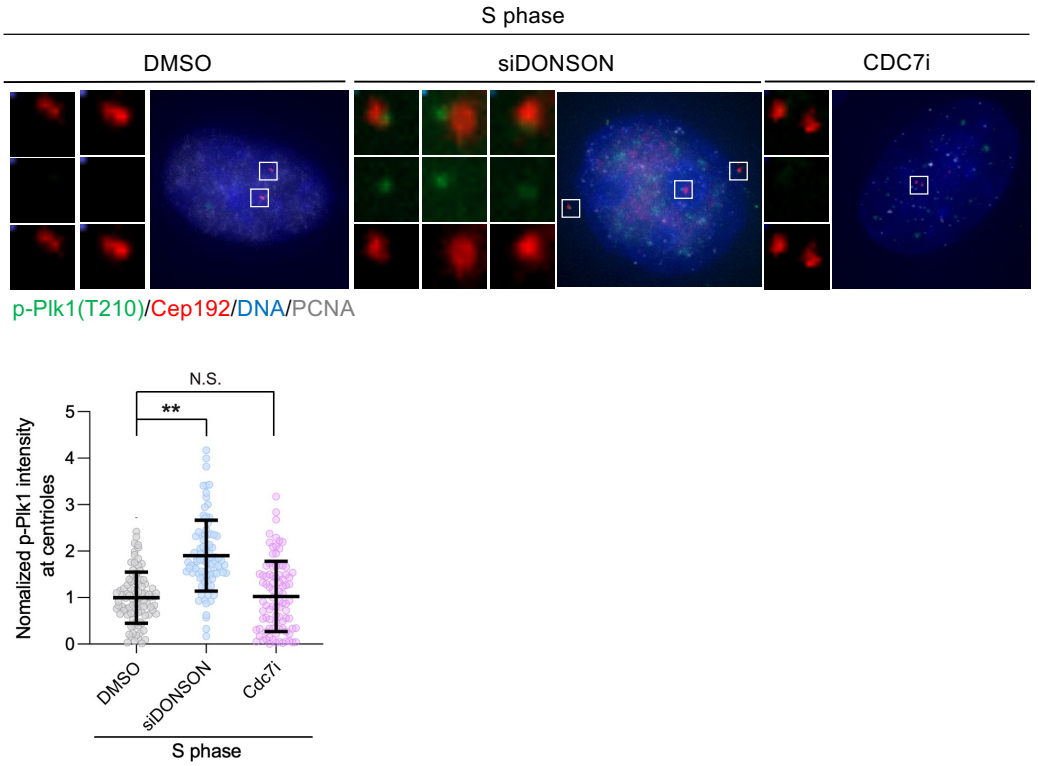
The structural hypothesis that DONSON competes with LRR1 for binding to MCM3 is not compelling. As noted in my original review, there is no difficulty in folding the three proteins together.

> We thank the reviewer for the constructive advice. As mentioned in our rebuttal letter from the previous revision, we have confirmed using AlphaFold3 that the formation of a ternary complex consisting of DONSON, MCM3, and LRR1 is indeed possible. However, in order to better mimic the state of MCM3 during DNA replication, we investigated the effect of DONSON on the interaction between the MCM3/5/7 complex and the LRR1/ELOB/ELOC/Cul2/RBX1 ubiquitin ligase complex. This ubiquitin ligase complex is predicted to bind to the MCM3/5/7 complex mainly via LRR1, as shown in previous studies (new data in Fig. 6e, f and Supplementary Fig. 8b, d). Importantly, when DONSON is introduced, it is predicted that DONSON strongly interacts with the MCM3/5/7 complex (new data in Supplementary Fig. 8a), making it difficult for LRR1 to bind to the MCM3/5/7 complex at its original binding site, ultimately causing the displacement of LRR1 from the MCM3/5/7 complex (new data in Fig. 6e, f and Supplementary Fig. 8c, e). In other words, we anticipate that DONSON would block the interaction between the ubiquitin ligase complex containing LRR1 and the MCM complex. This trend is clearly demonstrated by the high reproducibility of the pattern of the PAE values between LRR1 and the MCM3/5/7 complex in the presence or absence of DONSON (new data in Fig. 6e). Notably, in the AlphaFold structural predictions, in the absence of DONSON, the Cul2-LRR1 ubiquitin ligase is able to approach the primary ubiquitination sites on MCM7 as shown in the previous study (Jenkyn-Bedford M. et al. (2021) Nature). However, in the presence of DONSON, the positions of LRR1 and Cul2 become random, preventing the ubiquitin ligase from approaching these ubiquitination sites at all (new data in Fig. 6f). A possible explanation for these outcomes is the relatively close proximity of the binding sites of DONSON and LRR1 on MCM3. The bulky size of the ubiquitin ligase complex involving LRR1 could lead to competitive interactions between DONSON and the ubiquitin ligase complex for binding to the MCM complex. Furthermore, in support of these structural predictions, our biochemical experiments show that DONSON and LRR1 compete for binding to the MCM complex. Specifically, overexpression of DONSON decreases the endogenous MCM3-LRR1 binding, and this effect is not observed with DONSON mutants lacking the MCM3 binding

site (Supplementary Fig. 7e). In contrast, suppression of DONSON expression increases the endogenous MCM3-LRR1 binding (Fig. 6g). As shown above, the results of this new structural prediction have been clearly presented in the revised manuscript, with corresponding figures and explanations in the main text (new data in Fig. 6 and Supplementary Fig. 8, page 19-21).

The failure of MCM3D354-375 to rescue precocious PLK1 activation could be due to defective replication initiation.

>The mutant used in our experiments was not MCM3 but rather DONSONΔ354-375. Precocious PLK1 activation was not observed when DNA replication initiation was inhibited using a CDC7 inhibitor (new data attached below). Therefore, we believe that the phenotype associated with the DONSONΔ354-375 mutant is not attributable to defective DNA replication initiation.



Phosphorylation of Plk1 at T210 residue in S phase upon CDC7 inhibition.
Control and DONSON-depleted HeLa cells were treated with DMSO or 10 μM PHA767491 (CDC7 inhibitor, 24hr). Quantification of the normalized signal intensity of phosphorylated-Plk1 (T210) at centrioles. Values are mean percentages ± s.d. from three independent experiments (n = 30 for each experiment).

The analogy that the authors make between replication and centrosome biogenesis is tortured. Strand separation is NOT the licensing step in replication. Licensing refers to helicase recruitment in G1. This event, not strand separation, is strictly prohibited in S phase to limit replication to a single round per cell cycle. The parallel with CDC6 is not compelling because CDC6 is normally released from pre-RCs immediately upon MCM recruitment. It does not stay bound until the

origin fires. The effect of CDC6 ATPase mutants that are not released is therefore an artifact that does not reflect the normal function of then protein.

> We apologize for any confusion regarding this discussion. We understand and appreciate the reviewer's feedback and, as a result, have substantially modified the section in the current manuscript (please see page 24-25). Specifically, the statement regarding licensing was misleading, so it was revised to avoid any discrepancies. Additionally, the reference to the CDC6 mutant mentioned by this reviewer was removed, and further discussion based on it was refrained from.

They did not adequately address original point 13, because from their description, it appears samples were denatured after IP, not during or before the IP.

> We apologize for not explaining this point more explicitly. In this experiment, we performed the IP in the denaturing conditions and addressed in the Methods section of the revised manuscript (page 36).

Point 16 was not addressed: they did not show that their p97 signal reflects p97 bound to CMGs.

> We thank the reviewer for this suggestion. In response to this comment, we validated the specificity of the chromatin-bound p97 signal through siRNA knockdown experiments, which confirmed its specificity (new data in Supplementary Fig. 6 d,e).

Regarding point 19, MCMs form a stable complex, so where they sit does not matter.

>We apologize for the insufficient explanation regarding this experiment. This experiment involved the overexpression of DONSON along with MCM3 or MCM6 in cells to investigate their interaction, primarily detecting one-to-one binding. We regret any confusion caused by the lack of clarity in our previous description.

Reviewer #4 (Remarks to the Author):

I found this paper to be exciting and appropriate for publication in Nature Communications. In this manuscript by Matsuhashi et al., the authors present a previously unidentified role for DONSON in centrosome duplication and the stability of replisome machinery. Overall, the work is novel and exciting, representing a significant connection between replication and centrosome duplication. The authors demonstrate that the loss of DONSON leads to the premature degradation of ETAA-1, an activating partner of ATR, resulting in a failure of the S-phase checkpoint and inappropriate activation of PLK1 at the centrioles. Overall, I found the paper well-

written and the experiments robust.

The authors indeed addressed all of the reviewers' concerns, and I found this paper to be scientifically robust and exciting for our field. There are a few general questions that I have that may be important to answer before publication and could be addressed. These experiments are not necessary for an exciting publication but would help clarify some aspects of the work.

> We thank this reviewer for the encouraging feedback. We also appreciate the various constructive comments provided. Following these suggestions, we performed a series of experiments that were feasible and incorporated the resulting new data into our revised manuscript. Our detailed responses to each comment are provided below.

Fig 1:

- The authors conclude that some genes characterized are essential factors for S phase entry. However, a pulse of EdU does not account for that as cells could remain stuck in G2 or have decreased replication speed, which would affect EdU incorporation. The authors should use their images and quantify EdU and DAPI signals to identify if decreased EdU incorporation occurs due to arrests in G1 or G2 or just affects replication itself. Alternatively, the authors could conduct FACS.

> We thank this reviewer for raising this issue. The point highlighted by the reviewer is well-taken; to accurately assess the efficiency of DNA replication, it is crucial to also consider the influence on the cell cycle. One significant implication of our results is that siRNA targeting either DONSON or Cdc6 specifically induces the phenotype of centrosome amplification, irrespective of the extent to which DNA replication is inhibited. However, the EdU incorporation data may not be sufficient for a rigorous discussion of DNA replication efficiency under each condition. Furthermore, since the primary objective of this experiment is to identify MPD causative genes newly implicated in centrosome number regulation, we have opted to exclude this result from the revised version of the manuscript.

- The CEP192 signal in Fig 1d is very difficult to see. The authors should revise the representative images to make the signal more clear.

> We thank this reviewer for pointing this out. To improve visibility, we adjusted the intensity of the CEP192 signal in Fig. 1b of the revised manuscript.

- Are there changes in CEP192 protein abundance in Cdc6 and DONSON knockdown cells? It visually looks like there are even in G1 from Fig 1i. The authors should quantify this and assess whether decreased CEP192 expression could be a cause of centrosome dysfunction observed in

DONSON knockdown

> Under siDONSON and siCdc6 conditions, we confirmed that there is no significant change in the signal intensity of Cep192 at centrosomes in multiple samples. For example, in Fig. 1g, where the Cep192 signal appears slightly weaker under siDONSON or siCdc6 conditions during the S/G2 phase, it is possible that Cep192 recruitment to the disengaged daughter centriole is less than that to the mother centriole. However, this is unlikely to be the cause of the centriole disengagement phenotype observed in DONSON or Cdc6 knockdown.

Fig 2:

- The authors suggest that mNG-DONSON localizes to the nucleus and that it localizes to some centrosomes. However, this is a very weak finding as there is just a generalized staining across the nucleus, and the signal is highly oversaturated for mNG. The authors should 1) measure the abundance of mNG DONSON compared to endogenous DONSON/Cdc6. 2) Use PLA or other more specific methods to determine if DONSON/Cdc6 is really localizing to centrioles or use pre-extraction or other methods to remove unbound DONSON/Cdc6 and use 3D imaging to assess localization in a more precise manner. 3) Measure the percent of centrosomes with DONSON/Cdc6 localization as in its current form it is very vague.
- For overexpression (2e,f) the authors should clearly state how much overexpression. Is centrosome disengagement linearly correlated Cdc6 levels or localization to centrosomes? Or is there a threshold or some sort of binary amount?

> Thank you for pointing out these critical aspects. First, it should be noted that Cdc6 localizes to centrosomes, whereas DONSON is exclusively localized within the nucleus. Due to the difficulty of detecting endogenous DONSON via immunostaining, it is challenging to make accurate comparisons with the intracellular levels of mNG-DONSON in each cell.

We agree that confirming the specificity of Cdc6 localization to centrosomes is essential. The centrosomal Cdc6 signal disappears following siCdc6 treatment, supporting its specificity (new data in Supplementary Fig. 3a,b). Furthermore, this Cdc6 signal persists even after cytoplasmic extraction, suggesting that it represents a centrosome-bound fraction.

We quantified the distribution of Cdc6 between the nucleus and centrosomes (Supplementary Fig. 3c). In cells positive for EdU incorporation, Cdc6 localization to centrosomes was prominently observed, suggesting that this event occurs around the entry into the S phase.

In response to the reviewer's comment, we further examined the relationship between mNG-Cdc6 localization to centrosomes and the suppression of the centriole disengagement phenotype induced by siDONSON. Quantitative analysis revealed a clear positive correlation between these two events (new data in Supplementary Fig. 3f).

Fig 3:

- The authors should test whether the p-Plk1 (T210) antibody is indeed accurate and specific, as this antibody is notorious for having off-target binding. The authors should also measure PLK1 at centrioles.

> We thank this reviewer for pointing this out. The specificity of the p-Plk1 antibody for centrosomal signals used in this study has been validated in our previous research (Supplementary Figure 7 in Takeda Y. et al. (2019), *Journal of Cell Science*). In siDONSON-treated cells, no changes were observed in the overall protein expression levels of Plk1 (Supplementary Fig. 4g), nor were there any significant differences in the Plk1 signal intensity at centrosomes.

- The authors demonstrate that Chk1 activity is elevated and cite work demonstrating that this is indeed caused by replication stress. The authors should demonstrate how much DNA damage is occurring in Simonson cells.

> In this study, we detected Chk1 phosphorylation at S296 within the context of the intrinsic S/G2 checkpoint, which reflects the basal activity of Chk1 during DNA replication, distinct from its phosphorylation and activation in response to DNA damage (Michelena J. et al. (2019), *Journal of Cell Biology*). Under siDONSON conditions, this phosphorylation is reduced (Fig. 3J), indicating that DONSON is essential for maintaining Chk1's basal activity during DNA replication, a mechanism entirely separate from its role in the context of DNA damage.

- The authors should blot for ATR and Chk1 activity at those inhibitor concentrations to determine if complete inhibition is acquired. The authors should also assess if this is indeed ATR/Chk1 specific or just due to a general activation of the DNA damage response pathway by inhibiting ATM and determining if there is an increase in p-PLK1 at centrioles.

> Western blot analysis confirmed that Chk1 S296 phosphorylation within the intrinsic S/G2 checkpoint is suppressed by the Chk1 inhibitor (new data in Supplementary Fig. 4k). While the specific ATR phosphorylation sites relevant to this context have not been identified and therefore cannot be monitored, we have used ATR inhibitors at concentrations previously demonstrated in various studies to be sufficiently effective and specific. The activation of ATR and Chk1 discussed in this study is observed as part of the intrinsic S/G2 checkpoint pathway during DNA replication and does not reflect a response to DNA damage.

Fig 5:

- Chromatin-bound cdc45 looks like it exclusively localizes to nucleoli in the presence of aphidicolin and it is unclear if the signal is actually just an off-target issue. The authors should quantify chromatin-bound Cdc45 using fractionation western blotting.

> As the reviewer correctly pointed out, the appropriate representative localization pattern for chromatin-bound Cdc45 observed here is punctate-like in the nucleus, and we have replaced the image accordingly. As shown in the fractionation experiment in Supplementary Fig. 6c, Cdc45 is present in the chromatin fraction. Since the same antibody was used in the immunostaining experiments, we consider the nuclear signal of Cdc45 shown here to be specific.

Fig 6:

- ETAA1 also plays a function in mitotic ATR signaling. Therefore, it is possible that the effect observed with ETAA1 rescue on the rate of lagging chromosomes and mitotic defects is not direct but solely due to hyperactivation of the mitotic ATR-Chk1 pathway. The authors should assess if ETAA1 is overexpressed specifically in S-phase alone or if S-phase-specific inhibition of plk1 would rescue the multipolarity defects in the subsequent mitosis.

> We thank the reviewer for this valuable suggestion. However, previous studies have shown that the phenotypes resulting from ETAA1 inactivation and inhibition of the mitotic ATR pathway are associated with errors in chromosome alignment and spindle checkpoint response (Bass T.E. et al., 2019, *Journal of Cell Biology*). These phenotypes differ significantly from those we observed under siDONSON conditions. Based on these considerations, it can be inferred that the effect of ETAA1 overexpression in rescuing the multiple spindle formation phenotype differs from the function of ETAA1 previously demonstrated in the context of mitotic ATR signaling. Additionally, establishing an experimental system for temporally controlled overexpression of ETAA1 presents significant technical challenges.

Reviewer #5 (Remarks to the Author):

This work describes how the microcephaly protein DONSON couples the DNA and centrosome cycle via the relocalization of Cdc6 to centrosomes. I was not an original reviewer, but I was asked to comment specifically on the points raised by reviewer 3, which concerned the relationship of this manuscript to the work described in Dwivedi et al., 2023, which was not discussed in the original mansucript.

First, for full disclosure, there is a certain conflict of interest, since my laboratory generated the Dwivedi study.

Second, with regard to the concerns of reviewer 3, the good (and always re-assuring) point is that this study reproduces our original study (mild replication stress induced by aphidicolin leads to a

loss of orthogonal orientation of the centriole pairs) and it shows that DONSON depletion results in a more pronounced phenotype. The conclusion of the authors that this represents two different mechanism is sound and robust, and I feel that the authors have thus addressed this point. More generally, I feel that this study adds an important new perspective on the relationship between the DNA and centrosome cycle, and would thus be a nice manuscript for the present journal.

Nevertheless, I differ with the interpretation of the authors in the rebuttal letter that the sole loss of orthogonal centriole orientation does not represent centriole disengagement. Some of the earliest electron-microscopy studies in cells called such a loss of orthogonal arrangement centriole disengagement, showing centriole pairs that were close but without an orthogonal arrangement in G1 cells (see Kuriyama and Borisy JCB, 1981, or the earliest review article on the subject, Tsou and Stears, Curr. Opin in Cell Biol, 2006). Moreover, we have shown that this type of non-orthogonal centrioles will, as soon as they enter mitosis and bind to microtubules, move apart by several microns, recruit microtubule-nucleating pericentriolar material, and thus form a functional spindle pole with single disengaged centrioles (Wilhelm et al, Nat Comm, 2019), indicating that loss of orthogonal orientation in G2 is sufficient to give raise four functional mitotic centrosomes with single centrioles. One possibility to reconcile this different interpretations, would be to discuss that this study points to the fact that different extents of centriole disengagement might exist. In particular, one could speculate that while loss of orthogonal orientation might allow single centrioles to become mitotic MTOC, a higher physical separation might be necessary to allow the next round of centriole duplication. This is, however, just a suggestion, and the authors should feel free to discuss this point or not.

Patrick Meraldi

> We thank this reviewer for the thoughtful comments. We propose that centriole disengagement primarily occurs in late mitosis (Tsou M.B. et al., 2006, *Nature*) through a two-step process. The first critical step is centriole disorientation during the G2/M phase, where the mother and daughter centrioles remain closely associated through the expanded PCM surrounding them. Subsequently, the activation of separase leads to PCNT cleavage, causing PCM disassembly and enabling the physical separation of mother and daughter centrioles during telophase-early G1 phase (centriole disengagement) (please also see a recently published paper from our lab (Ito K et al. (2025) EMBO J, now cited in the revised manuscript)).
Indeed, when separase is inactivated, the mother and daughter centrioles become disoriented during mitosis, but the PCM maintains their close proximity, and neither centriole converts into a centrosome (Figure 6 in Ito K et al. (2025) EMBO J)). Under conditions of replication stress,

premature separase activation leads to compromised PCM integrity and partial disassembly in early mitosis (Mullee L.I. et al., 2016, *Chromosome Res*). Therefore, in such conditions, once the mother and daughter centrioles reach a disoriented state during the G2/M phase, they can immediately separate (centriole disengagement).

We thank all the reviewers (#4, #5 and #6) of our manuscript for their critical reading and for their useful and constructive comments (typed in black). In response to the comments from Reviewer 4, we have improved the quality of all representative panels, including those specifically pointed out, to enhance their clarity and make them easier for readers to interpret as we detail below (our responses are typed in blue). We now believe it meets the high-quality standards of Nature Communications.

Reviewer #4 (Remarks to the Author):

The revised manuscript, by Matsuhashi and colleagues, is significantly improved, and they have sufficiently addressed my initial concerns.

> We thank this reviewer for the encouraging comment.

My major concern is with the representative images. Many images are out of focus, which could affect their quantification (e.g. Fig 4b, 4e, Fig 5e, Fig 5m). The images are often merged only (e.g., Fig 2a, 2h,Fig 4b), which makes it almost impossible to compare or even see in any significant detail what the authors are trying to demonstrate. The authors should show all channels separately and then a merged image. At other times, the single channel image is hard to see at all; for example, in fig 5e and 5g, the authors should show a nuclear boundary. Another example is Fig 5i, where the chromatin-bound p97 image is more magnified than the merge, a line demarcating the nuclear boundary would help remove this doubt.

>We appreciate the reviewer's constructive comment regarding the quality of the representative panels. In response, we have revised the panels that were noted as potentially unfocused (Fig 4b, 4e, Fig 5e, Fig 5m). For images where only merged channels were initially presented, we have now separated and displayed individual channels (Fig 2a, 2h, Fig 4b). In cases where the boundaries between the nucleus and cytoplasm were unclear, we have added dashed lines along the nuclear periphery to enhance visibility (Fig 5e, 5g, 5i and 5k). These improvements to the representative panels have made the results easier to interpret and have increased the clarity and credibility of our findings.

Authors need to detail how they are processing the representative images in the figure legends, as it looks like there are differences in processing between single channels and merged channels (e.g. Fig 4l, Fig 5g) and between the original image and the inset (e.g. Fig 5m)

>We thank the reviewer for pointing out this important issue. In response, we carefully re-examined all figures to ensure consistency in intensity and contrast between merged and single-channel images (e.g. Fig 4l, Fig 5g), as well as between the original images and their

corresponding insets (e.g. Fig 5m). Furthermore, we have revised the legend of Figure 1 to provide a detailed description concerning the single channel and the inset (page 49).

These issues are throughout the manuscript and not just exclusive of the few figures marked. This should be fixed for all representative images.
>In response to the reviewer's comment, we conducted a thorough re-evaluation of the quality of all representative panels in the manuscript.

Reviewer #5 (Remarks to the Author):

The authors have addressed my comments, and I support publication
>We are grateful to this reviewer for the positive feedback on our manuscript.

Reviewer #6 (Remarks to the Author):

I have been asked to assess the authors' responses to the issues raised by Reviewer #1, who is currently unavailable. Having carefully read the revised manuscript, I can say that all the concerns raised by this reviewer have been satisfactorily addressed. I think that this study provides important insights into the molecular mechanisms underlying the coordination between DNA replication and chromosome segregation in human cells.
>We appreciate that this reviewer took the time to critically evaluate our manuscript in place of Reviewer #1 and are grateful for the positive and constructive feedback.