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Novel role of DONSON in CMG helicase assembly during vertebrate DNA replication initiation

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

Dr. Yoshitami Hashimoto
Tokyo University of Pharmacy and Life Sciences
School of Life Sciences
1432-1
Horinouchi
Hachioji, Tokyo 192-0392
Japan

16th Apr 2023

Re: EMBOJ-2023-114131
Novel role of DONSON in CMG helicase assembly during vertebrate DNA replication initiation

Dear Dr. Hashimoto,

Thank you for submitting your study on DONSON as a new vertebrate replication initiation factor to The EMBO Journal. It has now been assessed by three expert referees, whose reports are copied below for your information. I am happy to say that in light of their unanimously supportive comments, we would be interested in pursuing a revised manuscript further for publication, pending adequate revision of a few specific issues raised by the reviewers.

Among the key points to address are potential reasons for the incomplete rescue by recombinant DONSON (refs 1 and 3), where referee 1 proposes a chromatin transfer experiment to check for possible additional DONSON effects on replication initiation; a possible activation of DNA damage responses upon DONSON depletion, which referees 1 and 3 propose to test using checkpoint inhibitors and Chk1 phosphorylation; and the relation of DONSON depletion to CDK/DDK-dependent phosphorylations (major point of referee 2). In addition, all reviewers suggest various presentational modifications and improvements.

Detailed information on preparing, formatting and uploading a revised manuscript can be found below and in our Guide to Authors. Please note that it is our policy to allow only a single round of (major) revision, making it important to carefully respond to all points raised at this stage; therefore, please do not hesitate to contact me in case you would like to discuss any of the issues raised by the reviewers. Also, should revision require more time than our default three-months revision period, we would be open to offering an extension, during which our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) would of course remain valid.

Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to your revision.

Yours sincerely,

Hartmut Vodermaier

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

*** PLEASE NOTE: All revised manuscript are subject to initial checks for completeness and adherence to our formatting guidelines. Revisions may be returned to the authors and delayed in their editorial re-evaluation if they fail to comply to the following requirements (see also our Guide to Authors for further information):

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

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

instead.

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

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

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

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

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9) Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be clearly noted in the figure legend and/or the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure. Finally, we generally encourage uploading of numerical as well as gel/blot image source data; for details see: embopress.org/page/journal/14602075/authorguide#sourcedata

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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (15th Jul 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

Hashimoto et al. present the identification of a new replication origin firing factor, DONSON, that is specific to higher eukaryotes. The requirement of DONSON may be unexpected, because it is not amongst the minimally sufficient set of firing factors in the yeast recombinant replication system. The authors furthermore present a basic characterisation of DONSON function in the form of a structure-function analysis using DONSON deletion mutants. An essential biochemical activity of the DONSON N-terminus, the binding to GINS, is identified and a complex in Xenopus egg extracts with GINS and DNA polymerase epsilon described. Then the authors define the step in the origin firing process, for which DONSON is required. Together, this work advances our model of origin firing in higher eukaryotes, about which little mechanistic detail is known. It presents an important early step towards an understanding of some so far unexplained fundamental differences to the yeast paradigm that can in the future be addressed more directly based on the model developed by this work presented.

The experiments presented are of high quality, and the main point of the paper, that DONSON is a new origin firing factor, is largely convincingly made. The advance in our understanding of origin firing in higher eukaryotes that the paper presents is significant. I can support publication in EMBO J upon addressing a few issues listed below.

Points to be addressed:

1) Changes and control experiments to strengthen that DONSON functions in origin firing
The evidence that DONSON functions in origin firing (it had previously been implicated as a replisome factor) is relatively strong already. I have two issues:

A) The rescue of DNA replication in DONSON-depleted Xenopus extracts by recombinant DONSON is partial, although the statistics presented using replicate experiments make the partial rescue overall convincing.

Please address more clearly what the underlying reasons are.
It seems to me that recombinant DONSON rescues replisome formation (chromatin westerns) better than replication (nucleotide analogue incorporation) in depleted extracts.To better characterise the role of DONSON in replication it could be addressed if DONSON is required for replication elongation in addition of a role in initiation in the extract. If yes partial rescues of DONSON's role in firing and elongation could add up, resulting in a relatively low degree of replication. The authors do chromatin transfer experiments in Fig 6. Such experiments could also address whether DONSON has a role in replication. Replisomes could be formed in undepleted extracts in the presence of aphidicolin to prevent elongation. Chromatin transfer into (initiation-deficient) DSN-depleted and control extracts allows to test if replication is slower if DONSON is not present during elongation.

The functional experiments (Figs 2-5) could be shown in a more coherent way. I suggest to start by presenting the slightly clearer effects on replisome formation (chromatin westerns), including rescue by recombinant DONSON. Show chromatin westerns using the second DSN-antibody too, please. Then continue with the nucleotide incorporation analysis, including a clear addressing of the partial nature of replication rescue. I would put the microscopic images of Figure 5A into the supplement or delete them. The quantification graphs in B carry all the information. Unless the authors have particular reasons, these graphs could be shown in a more concise way, perhaps in one graph not repeating the controls all over again.

B) Formally, DONSON depletion could suppress replication origin firing by activating DNA damage signalling.

Measuring replisome formation upon adding checkpoint signalling inhibitors to DONSON depleted extracts should clarify this issue.

2) DONSON mutant analysis
An overview schematic showing the position of the mutations would be helpful.

3) Manuscript form and language

A) Supplementary figures are often not referenced clearly, which makes following the arguments difficult. Please reference properly including Figure panels referred to.

B) I find a few formulations unclear or slighlty misleading:

- Change' Drosophila humpty dumpty is an essential gene for cell proliferation' into 'Drosophila humpty dumpty, DONSON in vertebrates, is an essential gene for cell proliferation'

- Change 'In contrast, there is no clear vertebrate ortholog of yeast Sld2.' into 'There is nor clear functional equivalent to Sld2 in vertebrates'. Ortholog implicates homology. RecQ4-N is homologous.

- Change 'in vertebrates, it has not been yet achieved partly due to the lack of a complete set of initiator proteins.' into 'in vertebrates, origin-dependent CMG formation has not been yet achieved partly due to the lack of a complete set of initiator proteins.
The structure of human CMG is known (Yeeles lab), but this was made not by origin firing.

- Change 'In the control condition, the amount of associated DONSON gradually increased,...' into 'Upon adding sperm DNA to untreated interphase extracts, the amount of associated DONSON gradually increased, ...'

- Change '..., and found that both the antibodies caused a severe reduction in Cy3 intensities (Fig. 2A).' into '..., and found that both anti-DONSON antibodies caused a severe reduction in Cy3 intensities (Fig. 2A).'

- Add , without affecting origin licensing to '..., these results suggest that DONSON is required for replisome assembly during DNA replication initiation.'

- 'The concentration of endogenous DONSON was estimated as 50-100 nM in comparison': Add a sentence how it was estimated.

- Change 'These results suggest that the entire DONSON protein is necessary for replisome assembly.' into 'These results suggest that the entire DONSON protein contains essential parts for replisome assembly.'

- Change 'The full-length and N-terminal 1-157 amino acids fragments co-precipitated strongly with Polε, Cdc45, and GINS (Sld5/Psf2) and slightly with TopBP1, but not with RecQL4, Treslin, Mcm7, and Claspin.' into 'The full-length and N-terminal 1-157 amino acids fragments co-precipitated strongly Polε, Cdc45, and GINS (Sld5/Psf2) and slightly TopBP1, but not RecQL4, Treslin, Mcm7, and Claspin.

Is TopBP1 really detectable over backgound in the IPs?

- 'These results show that DONSON forms a sub-complex with GINS, Polε, and Cdc45 in solution prior to replication initiation,...'
Too strong, because an order of events is not shown here

- '...suggesting that the interaction with Polε and the integrity of the C-terminal regionare required for stably maintaining DONSON as part of the replisome.'
Too strong, change into '...may be required...'

- I do not understand the sentence 'To examine whether CDK and DDK without DONSON were sufficient for replisome assembly...'. These 3 factors are clearly not sufficient for replisome assembly.

- Add '...when DONSON is not present at the same time as the kinases' to 'These results suggest that both CDK and DDK require DONSON for initiating DNA replication and that CDK and DDK without DONSON are not sufficient for replisome assembly.'

- An 'l' is mssing from 'Meier-Gorlin syndrome'.

Referee #2:

In the manuscript "Novel role of DONSON in CMG helicase assembly during vertebrate DNA replication initiation", Hashimoto and colleagues use Xenopus egg extracts to provide evidence that DONSON promotes dormant MCM2-7 helicase activation by enabling recruitment of CDC45 and GINS and formation of the CMG complex. Depletion of DONSON from egg extract largely abolished replication of sperm chromatin and prevented stable association of CDC45, GINS, Pol , and CLASPIN with chromatin. These effects could be partially rescued by addition of recombinant DONSON protein. Although the failure to observe a full rescue even with 10-fold excess of rDONSON is somewhat unsatisfying, the partial rescue nonetheless indicates that the replication defects caused by DONSON depletion or addition of DONSON antibody are specific. The authors additionally show that DONSON is retained on chromatin when replication is blocked with the polymerase inhibitor aphidicolin or when replisome disassembly is blocked upon p97 or Cullin inhibition, indicating that DONSON is a constitutive component of elongating replisomes. The function of DONSON is further characterized and an N-terminal DONSON fragment encompassing PGY and NPF motifs shown to mediate interactions with CDC45, GINS, and Pol , providing a physical basis for DONSON's function in CMG activation. Finally, the authors attempt to show that DONSON is required for CDK and DDK function by incubating nuclei first in DONSON-inhibited (but CDK- and DDK-proficient) extract and then transferring the nuclei to DONSON-proficient, CDK- or DDK-inhibited extract. Failure to observe CMG assembly in this experimental setup is taken as evidence that DONSON is required for proper CDK and DDK function. However, this seems like an over interpretation of the data that could be resolved with modifications to the experimental design (as described below). Overall, though, the data are of high quality and support the authors' conclusion that DONSON is an important new player in replication initiation. This manuscript is therefore likely to be of high interest to the DNA replication and cell cycle fields.

Major points -

1. In the extract transfer experiment described in Figure 6, MCM4 phosphorylation is lost upon transfer from DONSON-inhibited extract to DONSON proficient, DDK-inhibited extract, indicating that MCM4 (and likely other CDK- or DDK-dependent phosphorylations are rapidly removed in extract). It may be then that CDK and DDK are fully capable of phosphorylating all the necessary target proteins in the absence of DONSON, but that these modifications are simply lost before DONSON can activate CMG when the kinases are inactivated. Have the authors performed this experiment in the presence of phosphatase inhibitors that spreserve MCM phosphorylation after kinase inhibition? Alternatively, do the authors have data to suggest that DONSON or a factor that interacts with DONSON is phosphorylated in a CDK- or DDK-dependent manner?

Minor points -

1. SybrGold staining of replicated DNA in mock and DONSON-depleted extract (e.g. Figure 3C) seems to indicate that replication of sperm chromatin is very inefficient. One would expect the amount of DNA present to roughly double upon incubation in mock depleted extract. Is the apparent replication inefficiency typical for these reactions?

2. It might be worthwhile to show the Alphafold prediction for DONSON as a figure rather than simply describing it in the results section.

3. The manuscript is somewhat difficult to read in certain passages and should be edited for clarity throughout.

Referee #3:

- general summary and opinion about the principal significance of the study, its questions and findings

DNA replication is essential for genome integrity and defects in replication initiation lead to cancer and rare diseases such as Meier-Gorlin syndrome. DONSON is a protein that associates with the replisome and has previously been shown to be important for replication fork stability. Here the authors use Xenopus egg extracts to identify a new function for DONSON in being critical for helicase (CMG) assembly during replication initiation. They show that CMG assembly and DNA replication are severely impaired in the absence of DONSON and this is likely due to a direct interaction between DONSON and the GINS component of the CMG complex. They show similar functions for the human DONSON protein, suggesting that these functions are conserved across vertebrates. They further show that DONSON function is unlikely to be important during the recovery of stalled forks after treatment with aphidicolin. They also show that several replication factors are required for DONSON interaction with chromatin and that the critical functions of CDK and DDK in replication initiation cannot occur in the absence of DONSON. Together this places DONSON at the heart of the critical steps in replication initiation control, which will be of significance for the replication, cell cycle and genome integrity fields.

- specific major concerns essential to be addressed to support the conclusions

I have none. This is a clear and well-executed study that identifies a new and critical function for a protein in the essential process of DNA replication initiation control.
Although there are loose ends, which I describe next, this should not preclude publication of this interesting study.
The loose ends include...
How does DONSON regulate CMG? - This is a big question that is beyond the scope of this initial study.
How is DONSON's function in replication fork stability related to its role described here in initiation? Why is Donson always in the replisome if it's not required for elongation? - Again, these are big question that are beyond the scope of this initial study.
What is the role of DONSON binding to pol epsilon? - An exciting new avenue to explore in future studies.
Why is Donson depletion not fully rescued by the add back of recombinant protein? - This is likely to be due to the recombinant protein not behaving well, but it may be that another factor is partially depleted in this case.

- minor concerns that should be addressed

Is it possible (perhaps very unlikely) that DONSON depletion from egg extracts is affecting CMG assembly and replication initiation by activating ATR/Chk1? As ATR/Chk1 are known inhibitors of replication initiation via phosphorylation of Treslin, I think it is worth doing a simple western blot for Chk1 activation after depletion of Donson to rule out that this is having an effect.

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

Meier-Gorlin syndrome misspelled.
Misspelling of DONSON on p.8 and p.15 (DOSNON) and on p.22 (DNONSON)

Unnecessary sentence in the discussion "DONSON could be the last initiation factor to be identified, this may mean completion of the full list of vertebrate replication initiators." I would remove it. I don't think it's very scientific to describe anything as the "last". Who knows what will be discovered next!

27 May 2023

Dear Dr Hartmut Vodermaier,
Senior Editor
The *EMBO Journal*

Re: "Novel role of DONSON in CMG helicase assembly during vertebrate DNA replication initiation" by Yoshitami Hashimoto, Kota Sadano, Nene Miyata, Haruka Ito and Hirofumi Tanaka to the *EMBO Journal*; the tracking number EMBOJ-2023-114131.

Through the utilization of a *Xenopus* cell-free system, we discovered that a microcephaly gene product, DONSON, was essential for CMG helicase assembly, which is a pivotal event for the initiation of eukaryotic DNA replication. Almost all replication proteins are highly conserved among eukaryotes; however, some known replication initiators have divergent primary structures. Sld2/Drc1 is the final initiator protein whose authentic functional counterpart has not yet been identified in higher eukaryotes. In this study, we propose that DONSON from vertebrates is a functional counterpart of yeast Sld2/Drc1, based on the similarity of their roles in CMG helicase assembly. We are confident that we have identified a novel, and likely the final, initiator of vertebrate DNA replication; this work will greatly help in the establishment of an *in vitro* reconstitution system for vertebrate DNA replication.

We are grateful to you and the reviewers for your careful consideration of our manuscript for publication in the *EMBO Journal*. We have taken all the comments into careful consideration and made the suggested changes, and we believe that our manuscript has benefited greatly from this.

We have addressed the concerns raised by the reviewers in a point-by-point response provided below, including the key points: potential reasons for the incomplete rescue by recombinant DONSON, and the relation of DONSON-depletion to CDK/DDK-dependent phosphorylation.

We hope that you will consider the revised version of our manuscript and find it suitable for publication in the *EMBO Journal*.

Yours Sincerely,

Yoshitami HASHIMOTO, Ph.D.

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Point-by-point response

Referee #1:

Hashimoto et al. present the identification of a new replication origin firing factor, DONSON, that is specific to higher eukaryotes. The requirement of DONSON may be unexpected, because it is not amongst the minimally sufficient set of firing factors in the yeast recombinant replication system. The authors furthermore present a basic characterisation of DONSON function in the form of a structure-function analysis using DONSON deletion mutants. An essential biochemical activity of the DONSON N-terminus, the binding to GINS, is identified and a complex in Xenopus egg extracts with GINS and DNA polymerase epsilon described. Then the authors define the step in the origin firing process, for which DONSON is required. Together, this work advances our model of origin firing in higher eukaryotes, about which little mechanistic detail is known. It presents an important early step towards an understanding of some so far unexplained fundamental differences to the yeast paradigm that can in the future be addressed more directly based on the model developed by this work presented.

The experiments presented are of high quality, and the main point of the paper, that DONSON is a new origin firing factor, is largely convincingly made. The advance in

our understanding of origin firing in higher eukaryotes that the paper presents is significant. I can support publication in EMBO J upon addressing a few issues listed below.

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The evidence that DONSON functions in origin firing (it had previously been implicated as a replisome factor) is relatively strong already. I have two issues:

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Please address more clearly what the underlying reasons are.
It seems to me that recombinant DONSON rescues replisome formation (chromatin westerns) better than replication (nucleotide analogue incorporation) in depleted extracts. To better characterise the role of DONSON in replication it could be addressed if DONSON is required for replication elongation in addition of a role in initiation in the extract. If yes partial rescues of DONSON's role in firing and elongation could add up, resulting in a relatively low degree of replication. The authors do chromatin transfer experiments in Fig 6. Such experiments could also address whether DONSON has a role in replication. Replisomes could be formed in undepleted extracts in the presence of aphidicolin to prevent elongation. Chromatin transfer into (initiation-deficient) DSN-depleted and control extracts allows to test if replication is slower if DONSON is not present during elongation.

Response

We thank the reviewer for their comment. In the original manuscript, we examined the requirement of DONSON for restart and elongation (progression) using the suggested chromatin transfer protocol and it was revealed that DONSON was not necessary for restart and elongation after fork stalling. We presented these results in Supplementary

Figure 4 in the original manuscript, but mentioned it only briefly in the Discussion section without sufficient explanation. In the revised manuscript, we have moved this data to the main Figure 4 and explained it fully in the Results section.

The functional experiments (Figs 2-5) could be shown in a more coherent way. I suggest to start by presenting the slightly clearer effects on replisome formation (chromatin westerns), including rescue by recombinant DONSON. Show chromatin westerns using the second DSN-antibody too, please. Then continue with the nucleotide incorporation analysis, including a clear addressing of the partial nature of replication rescue. I would put the microscopic images of Figure 5A into the supplement or delete them. The quantification graphs in B carry all the information. Unless the authors have particular reasons, these graphs could be shown in a more concise way, perhaps in one graph not repeating the controls all over again.

Response

We thank the reviewer for their comment. As suggested, we have rearranged the main figures in the revised manuscript as detailed below. We have also deleted the microscopic images from the original Figure 5A and redundant graphs of the original Figure 5B.

New Figure 1: Unchanged from the original Figure 1

New Figure 2: (DONSON requirement for replisome formation); Data from the original Figures 2B, 3A, and 3B

New Figure 3: (DONSON requirement for nucleotide incorporation); Data from the original Figures 2A and 3C

New Figure 4: (DONSON requirement for elongation); Data from the original Supplementary Figure 4

New Figure 5: (DONSON interaction with GINS and Polε); Data from the original Figures 4A, 4B, 4C, 4D, and 4E

New Figure 6: (Requirement of DONSON-GINS interaction for replication); Data from the original Figures 4F and 5B

New Figure 7: Minor changes from the original Figure 6

B) Formally, DONSON depletion could suppress replication origin firing by activating

DNA damage signalling.

Measuring replisome formation upon adding checkpoint signalling inhibitors to DONSON depleted extracts should clarify this issue.

Response

We thank the reviewer for their comment. As suggested, we have examined the activation of the checkpoint signaling and effect of a checkpoint inhibitor on the replisome formation as well as the nucleotide incorporation in the DONSON-depleted extract. We found that the ATR/Chk1 pathway was not abnormally activated in the DONSON-depleted extract and that treatment with caffeine, an ATM/ATR inhibitor, did not restore the replisome formation and replication activity to that of the control level in the DONSON-depleted extract with recombinant DONSON. We have presented this data in Expanded View Figure 3 in the revised manuscript.

2) DONSON mutant analysis

An overview schematic showing the position of the mutations would be helpful.

Response

We thank the reviewer for their comment. We have added a schematic and 3D structure model indicating the position of the mutations in Figure 5C and Expanded View Figure 1 in the revised manuscript, respectively.

3) Manuscript form and language

A) Supplementary figures are often not referenced clearly, which makes following the arguments difficult. Please reference properly including Figure panels referred to.

Response

We thank the reviewer for their comment. In the revised manuscript, we have prepared five Expanded View Figures (Figure EV1–5) and referenced the EV Figures by each of their Figure panels (e.g., Fig. EV1A, Fig. EV1B, etc.).

B) I find a few formulations unclear or slighlty misleading:

- Change' Drosophila humpty dumpty is an essential gene for cell proliferation' into 'Drosophila humpty dumpty, DONSON in vertebrates, is an essential gene for cell proliferation'

Response

We thank the reviewer for their comment. We have changed the text as suggested in the revised manuscript.

- Change 'In contrast, there is no clear vertebrate ortholog of yeast Sld2.' into 'There is nor clear functional equivalent to Sld2 in vertebrates'. Ortholog implicates homology. RecQ4-N is homologous.

Response

We thank the reviewer for their comment. We have changed the text as suggested in the revised manuscript.

- Change 'in vertebrates, it has not been yet achieved partly due to the lack of a complete set of
initiator proteins.' into 'in vertebrates, origin-dependent CMG formation has not been yet achieved partly due to the lack of a complete set of initiator proteins.
The structure of human CMG is known (Yeeles lab), but this was made not by origin firing.

Response

We thank the reviewer for their comment. We have changed the text as suggested in the revised manuscript.

- Change 'In the control condition, the amount of associated DONSON gradually increased,...' into 'Upon adding sperm DNA to untreated interphase extracts, the

amount of associated DONSON gradually increased, ...'

Response

We thank the reviewer for their comment. We have changed the text as suggested in the revised manuscript.

- Change '..., and found that both the antibodies caused a severe reduction in Cy3 intensities (Fig. 2A).' into '..., and found that both anti-DONSON antibodies caused a severe reduction in Cy3 intensities (Fig. 2A).'

Response

We thank the reviewer for their comment. Since the order of data was rearranged, the chromatin western is presented first in the revised manuscript and we used the following expression to explain the effect of anti-DONSON antibodies on the replisome assembly: 'Both anti-DONSON antibodies almost completely inhibited the chromatin binding of Claspin, Cdc45, and Psf2 at 20–30 min, which is the timing of replication initiation (Fig. 2C, lanes 10–15).'

The original section (a severe reduction in Cy3 intensities) was combined with the Cy5 data in DONSON depletion and changed to: 'We found that both the addition of anti-DONSON antibodies and DONSON depletion caused a severe reduction in Cy3 and Cy5 intensities, ---.'

- Add , without affecting origin licensing to '..., these results suggest that DONSON is required for replisome assembly during DNA replication initiation.'

Response

We thank the reviewer for their comment. We have changed the text as suggested in the revised manuscript.

- 'The concentration of endogenous DONSON was estimated as 50-100 nM in comparison': Add a sentence how it was estimated.

Response

We thank the reviewer for their comment. In the revised manuscript, we have changed the text to: ‘The concentration of endogenous DONSON was estimated as 50–100 nM through the comparison of the signal intensities of the endogenous and recombinant DONSON immunoblots (Fig. 2A, lanes 1–8; Fig. EV1C).’

- Change 'These results suggest that the entire DONSON protein is necessary for replisome assembly.' into 'These results suggest that the entire DONSON protein contains essential parts for replisome assembly.'

Response

We thank the reviewer for their comment. We have changed the text as suggested in the revised manuscript.

- Change 'The full-length and N-terminal 1-157 amino acids fragments co-precipitated strongly with Polε, Cdc45, and GINS (Sld5/Psf2) and slightly with TopBP1, but not with RecQL4, Treslin, Mcm7, and Claspin.' into 'The full-length and N-terminal 1-157 amino acids fragments co-precipitated strongly Polε, Cdc45, and GINS (Sld5/Psf2) and slightly TopBP1, but not RecQL4, Treslin, Mcm7, and Claspin.

Is TopBP1 really detectable over backgound in the IPs?

Response

We thank the reviewer for their comment. We agree that the TopBP1 signals were too weak to definitively indicate an interaction with DONSON. In the revised manuscript we have changed the text to: ‘The full-length and N-terminal 1–157 amino acid fragments co-precipitated strongly with Polε, Cdc45, and GINS (Sld5/Psf2), but not with TopBP1, RecQL4, Treslin, Mcm7, and Claspin.’

- 'These results show that DONSON forms a sub-complex with GINS, Polε, and Cdc45 in solution prior to replication initiation,...'
Too strong, because an order of events is not shown here

Response

We thank the reviewer for their comment. In the original manuscript we used the expression “prior to replication initiation” in four places. In the revised manuscript, we have deleted the “prior to replication initiation” expressions and replaced them with “independent of replication initiation” in three places.

- '...suggesting that the interaction with Polε and the integrity of the C-terminal regionare required for stably maintaining DONSON as part of the replisome.'
Too strong, change into '...may be required...'

Response
We thank the reviewer for their comment. We have changed the text as suggested in the revised manuscript.

- I do not understand the sentence 'To examine whether CDK and DDK without DONSON were sufficient for replisome assembly...'. These 3 factors are clearly not sufficient for replisome assembly.

Response
We thank the reviewer for their comment. In the revised manuscript we have changed the text to: “To examine whether the actions of CDK and DDK, before the loading of DONSON onto the chromatin, were sufficient for replisome assembly, we performed a nuclear transfer experiment.”

- Add '...when DONSON is not present at the same time as the kinases' to 'These results suggest that both CDK and DDK require DONSON for initiating DNA replication and that CDK and DDK without DONSON are not sufficient for replisome assembly.'

Response
We thank the reviewer for their comment. We have changed the text as suggested in the revised manuscript.

- An 'l' is mssing from 'Meier-Gorlin syndrome'.

Response

We thank the reviewer for their comment. We have corrected the spelling errors ("Gorin" to "Gorlin") in the revised manuscript.

Referee #2:

In the manuscript "Novel role of DONSON in CMG helicase assembly during vertebrate DNA replication initiation", Hashimoto and colleagues use Xenopus egg extracts to provide evidence that DONSON promotes dormant MCM2-7 helicase activation by enabling recruitment of CDC45 and GINS and formation of the CMG complex. Depletion of DONSON from egg extract largely abolished replication of sperm chromatin and prevented stable association of CDC45, GINS, Pol□, and CLASPIN with chromatin. These effects could be partially rescued by addition of recombinant DONSON protein. Although the failure to observe a full rescue even with 10-fold excess of rDONSON is somewhat unsatisfying, the partial rescue nonetheless indicates that the replication defects caused by DONSON depletion or addition of DONSON antibody are specific. The authors additionally show that DONSON is retained on chromatin when replication is blocked with the polymerase inhibitor aphidicolin or when replisome disassembly is blocked upon p97 or Cullin inhibition, indicating that DONSON is a constitutive component of elongating replisomes. The function of DONSON is further characterized and an N-terminal DONSON fragment encompassing PGY and NPF motifs shown to mediate interactions with CDC45, GINS, and Pol□, providing a physical basis for DONSON's function in CMG activation. Finally, the authors attempt to show that DONSON is required for CDK and DDK function by incubating nuclei first in DONSON-inhibited (but CDK- and DDK-proficient) extract and then transferring the nuclei to DONSON-proficient, CDK- or DDK-inhibited extract. Failure to observe CMG assembly in this experimental setup is taken as evidence that DONSON is required for proper CDK and DDK function. However, this seems like an over interpretation of the data that could be resolved with modifications to the experimental design (as described below). Overall, though, the data are of high quality and support the authors' conclusion that DONSON is an important new player in replication initiation. This manuscript is therefore likely to be

of high interest to the DNA replication and cell cycle fields.

Major points -

1. In the extract transfer experiment described in Figure 6, MCM4 phosphorylation is lost upon transfer from DONSON-inhibited extract to DONSON proficient, DDK-inhibited extract, indicating that MCM4 (and likely other CDK- or DDK-dependent phosphorylations are rapidly removed in extract). It may be then that CDK and DDK are fully capable of phosphorylating all the necessary target proteins in the absence of DONSON, but that these modifications are simply lost before DONSON can activate CMG when the kinases are inactivated. Have the authors performed this experiment in the presence of phosphatase inhibitors that spreserve MCM phosphorylation after kinase inhibition? Alternatively, do the authors have data to suggest that DONSON or a factor that interacts with DONSON is phosphorylated in a CDK- or DDK-dependent manner?

Response

We thank the reviewer for their comment. We performed the same experiment in the presence of phosphatase inhibitors and observing the results we came to the same conclusion that DONSON is required for proper CDK and DDK functioning. We have presented this data in Expanded View Figure 5 in the revised manuscript.

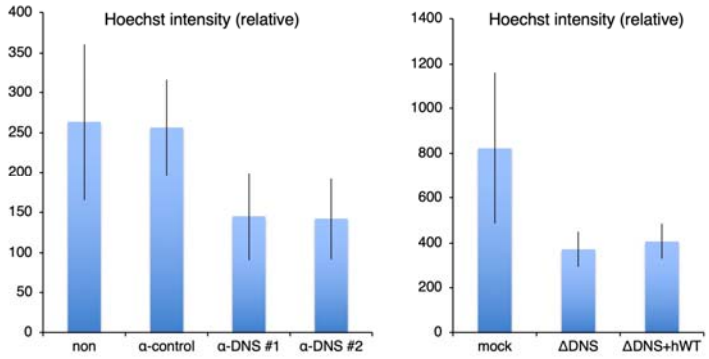
Minor points -

1. SybrGold staining of replicated DNA in mock and DONSON-depleted extract (e.g. Figure 3C) seems to indicate that replication of sperm chromatin is very inefficient. One would expect the amount of DNA present to roughly double upon incubation in mock depleted extract. Is the apparent replication inefficiency typical for these reactions?

Response

We thank the reviewer for their comment. Although we did not measure the absolute amount of synthesized DNA in this study, it is generally accepted that template sperm

DNA is almost fully duplicated in *Xenopus* egg extract (Gillespie *et al*, *Methods Mol Biol* 2016; Gillespie *et al*, *Method*s 2012). However, it is also well known that the depletion procedure (even in the control depletion) sometimes delays the timing of replication initiation by several tens of minutes. Since we measured the relative replication activity at 60 min in many cases, it may be possible that the template DNA was not fully duplicated at that timepoints. However, we noticed that Hoechst intensities in the microscopic images were nearly doubled as indicated in the graph below (this data was not included in the revised Figures): Left (New Figure 3A) non, α-control conditions vs. α-DONSON antibodies (#1, #2); Right (New Figure 6B) mock vs. ΔDNS.



Therefore, we believe that the sperm DNA was efficiently replicated in our extract, but SybrGold staining did not detect the DNA quantitatively.

2. It might be worthwhile to show the Alphafold prediction for DONSON as a figure rather than simply describing it in the results section.

Response

We thank the reviewer for their comment. As suggested, we have added the AlphaFold prediction in the revised Figure 5A and Expanded View Figure 1A.

3. The manuscript is somewhat difficult to read in certain passages and should be edited for clarity throughout.

Response

We thank the reviewer for their comment. We have rearranged the main Figures and Expanded View Figures so that the manuscript would be easier to understand in the revised version. We have had the manuscript professionally edited by an editor whose native language is English.

Referee #3:

- general summary and opinion about the principal significance of the study, its questions and findings

DNA replication is essential for genome integrity and defects in replication initiation lead to cancer and rare diseases such as Meier-Gorlin syndrome. DONSON is a protein that associates with the replisome and hsas previously been shown to be important for replication fork stability. Here the authors use Xenopus egg extracts to identify a new function for DONSON in being critical for helicase (CMG) assembly during replication initiation. They show that CMG assembly and DNA replication are severely impaired in the absence of DONSON and this is likely due to a direct interaction between DONSON and the GINS component of the CMG complex. They show similar functions for the human DONSON protein, suggesting that these functions are conserved across vertebrates. They further show that DONSON function is unlikely to be important during the recovery of stalled forks after treatment with aphidicolin. They also show that several replication factors are required for DONSON interaction with chromatin and that the critical functions of CDK and DDK in replication initiation cannot occur in the absence of DONSON. Together this places DONSON at the heart of the critical steps in replication initiation control, which will be of significance for the replication, cell cycle and genome integrity fields.

- specific major concerns essential to be addressed to support the conclusions

I have none. This is a clear and well-executed study that identifies a new and critical function for a protein in the essential process of DNA replication initiation control.
Although there are loose ends, which I describe next, this should not preclude

publication of this interesting study.

The loose ends include...

How does DONSON regulate CMG? - This is a big question that is beyond the scope of this initial study.

How is DONSON's function in replication fork stability related to its role described here in initiation? Why is Donson always in the replisome if it's not required for elongation? - Again, these are big question that are beyond the scope of this initial study.

What is the role of DONSON binding to pol epsilon? - An exciting new avenue to explore in future studies.

Why is Donson depletion not fully rescued by the add back of recombinant protein? - This is likely to be due to the recombinant protein not behaving well, but it may be that another factor is partially depleted in this case.

Response

We thank the reviewer for their comment. We have deleted this section of the original second paragraph (regarding the partial rescue), and the latter half of the original fourth paragraph (regarding the constitutive association of DONSON with chromatin and CMG regulation) from the Discussion section in the revised manuscript.

- minor concerns that should be addressed

Is it possible (perhaps very unlikely) that DONSON depletion from egg extracts is affecting CMG assembly and replication initiation by activating ATR/Chk1? As ATR/Chk1 are known inhibitors of replication initiation via phosphorylation of Treslin, I think it is worth doing a simple western blot for Chk1 activation after depletion of Donson to rule out that this is having an effect.

Response

We thank the reviewer for their comment. As suggested, we have examined the Chk1 activation (as well as the effect of a checkpoint inhibitor) and found that this was not the reason for the inefficient replication in the DONSON depleted extract plus recombinant

DONSON. We have presented this data in Expanded View Figure 3 in the revised manuscript.

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

Meier-Gorlin syndrome misspelled.
Misspelling of DONSON on p.8 and p.15 (DOSNON) and on p.22 (DNONSON)

Response

We thank the reviewer for their comment. We have corrected all these spelling errors in the revised manuscript.

Unnecessary sentence in the discussion "DONSON could be the last initiation factor to be identified, this may mean completion of the full list of vertebrate replication initiators." I would remove it. I don't think it's very scientific to describe anything as the "last". Who knows what will be discovered next!

Response

We thank the reviewer for their comment. As suggested, we have deleted this sentence in the revised manuscript.

Dr. Yoshitami Hashimoto
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Hachioji, Tokyo 192-0392
Japan

29th Jun 2023

Re: EMBOJ-2023-114131R
Novel role of DONSON in CMG helicase assembly during vertebrate DNA replication initiation

Dear Dr. Hashimoto,

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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

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

Yours sincerely,

Hartmut Vodermaier

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

Referee #1:

Very good revisions

Referee #2:

In this revised manuscript, that authors provide compelling evidence that vertebrate DONSON functions analogously to yeast Sld2 to promote recruitment of CDC45, GINS, and Pol e during helicase activation. All of my criticisms have been addressed. The authors' conclusions are well-supported by the data presented and appropriately stated. The inclusion of new data demonstrating that DONSON-depletion does not activate ATR-dependent checkpoint signaling and that addition of phosphatase inhibitors does not permit temporal separation of CDK/DDK and DONSON activities strengthens the manuscript. Overall, this work will be of high significance to the field.

EMBO Press Author Checklist

Corresponding Author Name: Yoshitami Hashimoto
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2023-114131

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The data shown in figures should satisfy the following conditions:
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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
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- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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Each figure caption should contain the following information, for each panel where they are relevant:
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- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
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  - are tests one-sided or two-sided?
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  - definition of 'center values' as median or average;
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Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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