nature portfolio

Peer Review File

Replication licensing regulated by a short linear motif within an intrinsically disordered region of origin recognition complex

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Review for Nature Communications submission, "*Replication licensing regulated by an intrinsically disordered region of origin recognition complex*."

Here, *Wu et al.* investigate the functional significance of the yeast Orc2 N-terminal intrinsically disordered region (IDR). Notably, there is increasing appreciation for the role of protein disordered regions – and the sequence motifs embedded therein – across biology and specifically in DNA replication with recent reports demonstrating that licensing factor IDRs have essential roles in both human and *Drosophila* replication initiation (Parker Lab, Bleichert Lab, Stillman Lab amongst others). Here the authors show for the first time that a subsequence (amino acids 175-200) within the yeast Orc2 IDR is critically required for replication licensing. Using genetic and cell biological experiments the authors convincingly show that this peptide is necessary for viability and proliferation *in vivo* and that loss of this sequence results in defects in DNA replication initiation across the genome. Mechanistic work connects this strong phenotypic data to a defect in the licensing reaction with elegant reconstitution/structural studies where they find that the Orc2 IDR is necessary in Pre-RC assembly with a specific requirement between the loading of single (OCCM) and double hexamers (MO). Finally, biochemical studies show that this peptide stimulates Mcm2-7 ATPase activity (even when provided in trans) and that this may occur through an interaction with Mcm2 (identified by pull down). Importantly, this region of the Orc2 IDR contains regulatory phosphorylation sites, and they show that when phosphorylated this region no longer binds Mcm2-7, thus providing new mechanistic insight into the role of regulatory phosphorylation in replication licensing.

Overall, this is a well-written, rigorous study that provides new mechanistic insight into the fundamental process of DNA replication licensing. The data are clearly presented and support the primary conclusions of the paper. I have a single experimental critique concerning the data presented to support Mcm2 as the relevant Orc2-IDR interaction partner (Figure 5B). In my view, the experimental approach is deficient and additional support is needed. Alternatively, these data could altogether be removed without detracting from the impact of the work. Once this point is addressed it is my opinion the manuscript will be suitable for publication. These points are discussed in detail below where I have divided my suggestions to the authors into a MAJOR COMMENTS section (which may require additional experimentation) and a MINOR COMMENTS section that includes additional, non-essential points aimed at improving the clarity and/or readability of the manuscript.

MAJOR COMMENTS

1) This comment concerns the experiment(s) leading to the identification of Mcm2 as the binding partner for the Orc2 IDR. I unfortunately do not find these data convincing as there appear to be multiple problems with the assay. However, the importance of this manuscript does not rest on validating a specific interaction between Mcm2 and Orc2-IDR. Thus, I would suggest simply removing these more tenuous experiments (Figure 5B and 5D) and publishing the work essentially as is. However, if the authors wish to include these data, then the authors should:

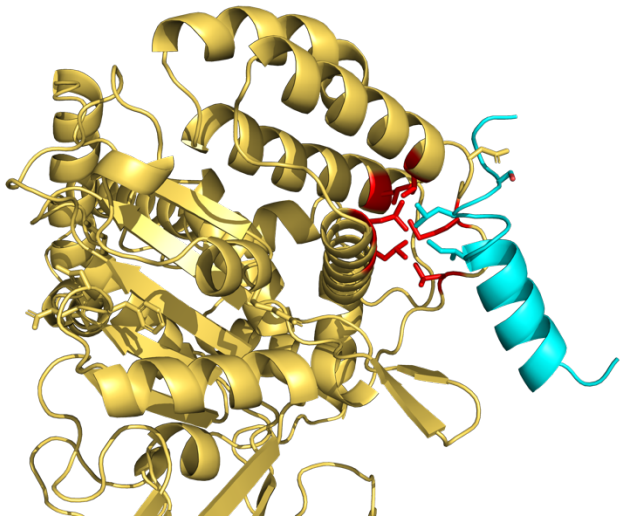
    a. <u>Cite relevant literature on disassembling Mcm2-7.</u>

    b. <u>Demonstrate that Mcm2-7 is, in fact, disassembled</u> – one accessible experiment would be to analyze the disassembled complex by size exclusion. If disassembled, perfect co-elution of the similarly sized Mcm subunits should be lost and the Cdt1 elution profile should be fully distinct.

    c. <u>Provide an unambiguous statement about how the complex was disassembled</u> – the authors suggest high salt is used to disassemble the complex (line 210: "a high salt buffer to disrupt interactions"). This method lacks empirical support. Indeed, even higher salt concentrations are used in their loading reaction and the large macromolecular interfaces that keep Cdt1-Mcm2-7 in a stable complex are predominantly hydrophobic in nature. On

closer inspection of their methods, it becomes clear that salt is likely not the primary component leading to disassembly. Instead, they use 0.5% NP40S, which is 25x the concentration present in their other assay buffers (e.g., BF2 contains 0.02%). The authors should state in the main text that detergent was used to disrupt the complex. However, this raises another concern, and that is that these relatively high concentrations of detergent may partially unfold Mcm subunits. Indeed, if 0.5% NP40S is sufficient to disrupt inter-molecular hydrophobic interactions (leading to disassembly of Mcm subunits), then it is logical that it may also disrupt intra-molecular interactions responsible for folding. This is a more problematic issue to address. Thus, in addition to acknowledging this, the authors should also:

d. Use an orthogonal approach to identify the Mcm subunit bound by Orc2 IDR – for example, AlphaFold multimer could be used to "assay" all pairwise interactions between the Orc2 peptide and each Mcm subunit to demonstrate which Mcm subunit scores highest and to identify the interaction site which could be experimentally validated by mutagenesis. For example, the screenshot at right shows the Alphafold multimer prediction for yeast Mcm2 (gold)-Orc2^{IDR} (cyan), revealing I194 (red) completely buried in a solvent-exposed surface on Mcm2.



MINOR COMMENTS

1) I suggest the title be changed to reflect the findings more accurately. The title emphasizes intrinsic disorder, but the region identified in Orc2 seems instead to be structured. The authors allude to this in their peptide experiments where they name the peptide "short a-helix region (residues 187-205)." In fact, calling this region "IDR2" seems somewhat misleading since it is predicted to be ordered (based on Figure1b), a finding supported by the Alphafold model which predicts a helical element. The field of protein intrinsic disorder refers to sub-sequences embedded within an IDR as Short Linear Motifs (or "SLiMs"). This may be a more apt name for this Orc2 element and the title could be revised to reflect this.

2) Similarly, it is confusing that the authors refer to this region both as "SH" and as a "IDR2". I believe their idea is that SH resides within IDR2, but in my view, these are the same. I suggest naming this region SH from the beginning of the manuscript, consistent with the disorder predictions shown in Figure 1B (they could also show AlphaFold prediction as supplemental support for naming it so).

3) Introduction, paragraph 3: in the context of "how ORC accomplishes its flipping" (line 64) it would be useful to state the known role for Orc6 in this process. This would provide context for how they are thinking about Orc2 but also contrast, as Orc6 does not to my knowledge impact Mcm2-7 enzymatic activity like they show for Orc2.

4) Introduction, paragraph 4: the final paragraph of the introduction reads more like a results section. I suggest moving the majority of this material into the first paragraph of the results and rewriting the last introductory paragraph to summarize the major findings of the paper and how they impact the field.

5) Figure 2A – the differences in Figure 2A are somewhat difficult to see, although believable given the very convincing data in the next panel (Figure 2B). To improve Figure 2A, the authors could quantify differences based on fraction of cells in S-phase (or S-phase length) and additionally include these quantified data in graph format.

6) Line 190: “similar to the level induced by Mcm2-7 alone (Fig. 4a)” – I don’t see the data for Mcm2-7 alone, they should add this.

7) Line 219: “It is likely that S-CDK phosphorylation at this site hinders the binding” – why is this likely? Evidence is lacking for this statement. I suggest either changing the wording to make it more suggestive or provide additional evidence, such as the position of the phosphorylation site in an Alphafold model of the interaction.

8) The authors should note in the text the observed shift in Orc2-IDR gel migration upon phosphorylation as support that phosphorylation is happening.

9) Can the authors determine, based on the published structures of the OCCM, whether the Orc2-IDR (helical region) can extend far enough to engage Mcm2? This would provide additional support for their model in Figure 6.

10) Within the results section, the authors should comment on the inefficiency of the pull down in Figure 5A. Is it sub-stoichiometric because other regions of ORC are required for full tethering efficiency (such as Orc6)?

11) Line 53: “establishing ~~as~~ a platform”

12) Line 161: “however, ~~these~~ reactions”

13) Line 168: “we conducted ~~a~~ time-resolved”

14) Figure 3B figure legend: “WEC” should be “WCE”

15) Figure 3d: in reaction (3) schematic: was ATP also added in first step?

16) Figure 3h-j and Figure 4c: please indicate scale for EM reconstructions

17) Figure 5a-b: Cdt1 band is barely visible (problem with printed version?) and appears sub-stoichiometric to Mcm subunits. Why is this?

I appreciate the opportunity to review your work and the visual and written clarity with which it was presented in this manuscript. I was impressed by the quality of your mechanistic work which in my opinion represents an important contribution to the field.

Matthew Parker, Ph.D.

Department of Biophysics
University of Texas Southwestern Medical Center

Reviewer #2 (Remarks to the Author):

In this manuscript Wu et al. investigate the role of the intrinsically disordered region of Orc2 in facilitating the DNA replication initiation process and show that the IDR of Orc2 is essential for the formation of the fully licensed double hexamer Mcm (DH-Mcm). The genetic data do show that a specific motif within the IDR is essential for viability and that specific stages of the licensing reaction are defective or slower upon deletion or mutation within the IDR. This is important new data. The biochemistry leads them to present a model that is not firmly proven, yet nevertheless interesting. The key steps where the IDR is proposed to play it's roles occurs is in the conversion of the intermediate ORC/Cdc6/Cdt1Mcm2-7 (OCCM) complex through to the DH-Mcm. The data show that the IDR of Orc2 interacts with Mcm2 in pull down assays. They set up a licensing reaction where the Orc 2 subunit in the complex is mutated within the IDR and the ATPase of the OCCM is diminished and remarkably the fragment of ORC 2 containing the IDR can partially rescue by itself the ATPase. Below are comments that we believe need to be addressed either with clarifications or new experiments before publication.

Major-

1. Authors identified that Orc2-IDR (1-200) interacts with Mcm2 by using pulldown assay claiming that in the MO (Mcm-ORC) complex Orc2 IDR interacts with Mcm2. However, the pulldown assays were done with only Orc2-IDR and Mcm/cdt1 complex, and not under Mcm loading conditions. We point out that the data do not show that within the OCCM complex other subunits may have conformations that allow for this interaction not detectable with single subunits alone. Authors might show this interaction they propose under loading conditions and /or determine the Mcm 2 site required that have similar phenotypes in vitro. Further, the data provided do show the specific region of the IDR (175-200) or I194 that is important for this interaction. However, it would be reasonable to ask if Orc2-IDR (1-200) with similar mutation (I194E) or deletion (Δ175-200) interacts with Mcm.

2. Is there a specific reason for using Orc2ΔN175 construct for in-vitro phosphorylation and phospho-mimic (S188D), in the loading experiments (Figure 5e)? Would not the Wt-Orc2 represent the proper scenario rather than an Orc2ΔN175 construct. The authors might consider performing loading experiments with phospho-mimic mutant S188D and in-vitro phosphorylation using Wt-Orc.

3. The key novel aspects of their model the Orc2-IDR would play it's critical role in converting the OCCM to MO (figure 6). To do this job, Orc2IDR interacts with Mcm after OCCM formation which increases Mcm ATPase activity leading to removal of Cdc6 and Cdt1, and flipping of Orc on to DNA, forming an MO complex. This is proposed through the quantifying percentages of total Mcm at different time points during Mcm recruitment and loading process and identification of the intermediates. The authors must describe how they quantify the percentage of total Mcm in Figure 3h, I, and J.

4. More importantly, If Orc2IDR is important for MO formation but not OCCM formation as claimed the authors should have seen equal amounts of Mcm being converted to OCCM in Figure 3i and 3j upon deletion of Orc2IDR (Orc2ΔN200) in the presence of ATP and ATPγS, since OCCM can not be converted to MO upon deletion of Orc2IDR (shown in Figure 4c) even in the presence of ATP. However, this does not seem to be observed. About 5% of the total Mcms form OCCM in the presence of ATP in 10mins (Figure3i) and around 70% total Mcm form OCCM in the presence of ATP in 10mins (Figure3j), upon deletion of Orc2IDR. Please explain.

5. In Figure 4C, the authors do not describe how MO formation was quantified and at what time point during the loading process MO formation was quantified. One presumes it is based upon quantifying the number of particles visualized with a negative stain. However, quantification of MO should to be supported by another biochemical assay as negative staining quantification can lead to biases. Also, it is not mentioned at which step Orc2-IDR2 peptide was added.

Minor-

1. For some of the experiment's authors need to provide more details to get a better Idea what does the data mean. An example: ATPase assay is not described well, it is not clear whether authors get the rate from the initial slope from many different time points or just from 2 time points.
2. The authors do not provide the source of S-Cdk.
3. Figure 1d, there is a typo saying "orc2-all HF to A" which should be "orc2-all HF to E".
4. It is very minor, in Figure 1c, in the representation of constructs, ΔN175-215 looks smaller than ΔN175-200.
5. It seems Orc2/I194E subunit runs slower than Orc2, see panel c and g in Figure3. Is there

any reason for Orc2/I194E subunit runs slower than Orc2?
6. In Figure 5e, there is Orc6/Clb5. What is Clb5, We could not see any mention of Clb5 in the text.
7. Error bars in Figure 3h, I and j, are shown however, it is not detailed how they derive these error bars or from how many experiments. Same for panel C in Figure 4.
8. Statistical significance needs to be done for the ATPase assay in Figure 4 panel a and b to substantiate the findings

Reviewer #3 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):
The first step of DNA replication initiation in eukaryotes involves the loading of a double hexamer of the Mcm2-7 replicative helicase onto DNA at replication origins. Mcm2-7 loading has been extensively studied in the budding yeast model system, but certain aspects of the mechanism and regulation of this critical process remain incompletely understood. In this manuscript, the authors investigate the function of the N-terminal Intrinsically Disordered Region (IDR) of Orc2 in Mcm2-7 loading. This is an excellent piece of work, that uses a compelling mixture of yeast genetics, in vivo and in vitro functional assays and structural biology, to convincingly demonstrate that the Orc2 IDR is required for the conversion of OCCM to MO, a key step in double hexamer formation. Additionally, the authors reveal that the Orc2 IDR plays a key regulatory role in Mcm2-7 loading, as CDK phosphorylation of this region blocks its interaction with Mcm2, and, consequently, MO formation. I have some suggestions for where the authors should better explain their experimental strategy and / or expand their discussion, but would otherwise recommend that this work is published without delay.

Specific points:

• Why are the complementation assays in Fig. 1-2 all performed in the context of the Orc2-1 missense mutant? This is not explained in the text, and seems a little strange, as the same experiments could presumably have been performed in the same fashion, but with wildtype Orc2 under the control of the gal promoter?

• The data revealing that ARS1614 and 1621 can replicate in the absence of the Orc2 IDR is very interesting, particularly in the context of the recent BioRxiv preprint (Lim et al., 2024) from the Diffley lab, which is mentioned in the discussion. From the methods section, it sounds like these analyses were performed genome-wide, but data is only provided for specific genomic regions (Fig. 2c-e). If the authors have the data for the whole genome, it should be included in this manuscript. For example, it would be a very interesting and valuable addition to this paper if there are other origins that replicate independently of the Orc2IDR, and if their identity correlates with their sequence / structure.

• The AlphaFold-predicted structure of budding yeast Orc2, including the fact that part of the Orc2-IDR is predicted to be α-helical (https://alphafold.ebi.ac.uk/entry/P32833), should be discussed alongside the DISOPRED predictions. Related to this, it also seems relevant to mention the evolutionary conservation of this region of Orc2 (e.g. are the residues that are mutated in Orc2 conserved, even within fungi?)

• In places, the text is a little colloquial. Words / phrases such as ‘floppy’ and ‘acrobatic gymnastics’ should be replaced with more precise scientific language.

We thank all four referees for their constructive comments, which we have addressed one by one as listed below. As a result, the paper is much improved in accessibility and quality.

Reviewer #1 (Remarks to the Author):

Review for Nature Communications submission, "*Replication licensing regulated by an intrinsically disordered region of origin recognition complex.*"

Here, *Wu et al.* investigate the functional significance of the yeast Orc2 N-terminal intrinsically disordered region (IDR). Notably, there is increasing appreciation for the role of protein disordered regions – and the sequence motifs embedded therein – across biology and specifically in DNA replication with recent reports demonstrating that licensing factor IDRs have essential roles in both human and *Drosophila* replication initiation (Parker Lab, Bleichert Lab, Stillman Lab amongst others). Here the authors show for the first time that a subsequence (amino acids 175-200) within the yeast Orc2 IDR is critically required for replication licensing. Using genetic and cell biological experiments the authors convincingly show that this peptide is necessary for viability and proliferation *in vivo* and that loss of this sequence results in defects in DNA replication initiation across the genome. Mechanistic work connects this strong phenotypic data to a defect in the licensing reaction with elegant reconstitution/structural studies where they find that the Orc2 IDR is necessary in Pre-RC assembly with a specific requirement between the loading of single (OCCM) and double hexamers (MO). Finally, biochemical studies show that this peptide stimulates Mcm2-7 ATPase activity (even when provided in trans) and that this may occur through an interaction with Mcm2 (identified by pull down). Importantly, this region of the Orc2 IDR contains regulatory phosphorylation sites, and they show that when phosphorylated this region no longer binds Mcm2-7, thus providing new mechanistic insight into the role of regulatory phosphorylation in replication licensing.

Response: We thank the referee for these valuable comments.

Overall, this is a well-written, rigorous study that provides new mechanistic insight into the fundamental process of DNA replication licensing. The data are clearly presented and support the primary conclusions of the paper. I have a single experimental critique concerning the data presented to support Mcm2 as the relevant Orc2-IDR interaction partner (Figure 5B). In my view, the experimental approach is deficient and additional support is needed. Alternatively, these data could altogether be removed without detracting from the impact of the work. Once this point is addressed it is my opinion the manuscript will be suitable for publication. These points are discussed in detail below where I have divided my suggestions to the authors into a MAJOR COMMENTS section (which may require additional experimentation) and a MINOR COMMENTS section that includes additional, non-essential points aimed at improving the clarity and/or readability of the manuscript.

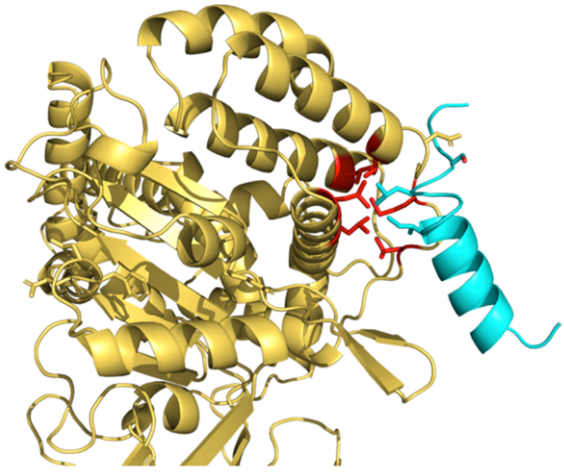
Response: The relevant concerns have been addressed as listed below.

MAJOR COMMENTS

1) This comment concerns the experiment(s) leading to the identification of Mcm2 as the binding partner for the Orc2 IDR. I unfortunately do not find these data convincing as there appear to be multiple problems with the assay. However, the importance of this manuscript does not rest on validating a specific interaction between Mcm2 and Orc2-IDR. Thus, I would suggest simply removing these more tenuous experiments (Figure 5B and 5D) and publishing the work essentially as is. However, if the authors wish to include these data, then the authors

should:

a. Cite relevant literature on disassembling Mcm2-7.
b. Demonstrate that Mcm2-7 is, in fact, disassembled – one accessible experiment would be to analyze the disassembled complex by size exclusion. If disassembled, perfect co-elution of the similarly sized Mcm subunits should be lost and the Cdt1 elution profile should be fully distinct.
c. Provide an unambiguous statement about how the complex was disassembled – the authors suggest high salt is used to disassemble the complex (line 210: "a high salt buffer to disrupt interactions"). This method lacks empirical support. Indeed, even higher salt concentrations are used in their loading reaction and the large macromolecular interfaces that keep Cdt1-Mcm2-7 in a stable complex are predominantly hydrophobic in nature. On closer inspection of their methods, it becomes clear that salt is likely not the primary component leading to disassembly. Instead, they use 0.5% NP40S, which is 25x the concentration present in their other assay buffers (e.g., BF2 contains 0.02%). The authors should state in the main text that detergent was used to disrupt the complex. However, this raises another concern, and that is that these relatively high concentrations of detergent may partially unfold Mcm subunits. Indeed, if 0.5% NP40S is sufficient to disrupt inter-molecular hydrophobic interactions (leading to disassembly of Mcm subunits), then it is logical that it may also disrupt intra-molecular interactions responsible for folding. This is a more problematic issue to address. Thus, in addition to acknowledging this, the authors should also:
d. Use an orthogonal approach to identify the Mcm subunit bound by Orc2 IDR – for example, AlphaFold multimer could be used to "assay" all pairwise interactions between the Orc2 peptide and each Mcm subunit to demonstrate which Mcm subunit scores highest and to identify the interaction site which could be experimentally validated by mutagenesis. For example, the screenshot at right shows the Alphafold multimer prediction for yeast Mcm2 (gold)-Orc2^{IDR} (cyan), revealing I194 (red) completely buried in a solvent- exposed surface on Mcm2.



Response: We thank the referee for the suggestion. In the revision, the data related to Cdt1-Mcm2-7 disassembly has been removed.

MINOR COMMENTS

1) I suggest the title be changed to reflect the findings more accurately. The title emphasizes intrinsic disorder, but the region identified in Orc2 seems instead to be structured. The authors allude to this in their peptide experiments where they name the peptide "short a-helix region (residues 187-205)." In fact, calling this region "IDR2" seems somewhat misleading since it is predicted to be ordered (based on Figure1b), a finding supported by the Alphafold model which predicts a helical element. The field of protein intrinsic disorder refers to sub-sequences

embedded within an IDR as Short Linear Motifs (or "SLiMs"). This may be a more apt name for this Orc2 element and the title could be revised to reflect this.

Response: We appreciate the referee's suggestion. The title has been revised accordingly.

2) Similarly, it is confusing that the authors refer to this region both as "SH" and as a "IDR2". I believe their idea is that SH resides within IDR2, but in my view, these are the same. I suggest naming this region SH from the beginning of the manuscript, consistent with the disorder predictions shown in Figure 1B (they could also show AlphaFold prediction as supplemental support for naming it so).

Response: The suggestion has been well taken. The IDR2 region has been named as SH.

3) Introduction, paragraph 3: in the context of "how ORC accomplishes its flipping" (line64) it would be useful to state the known role for Orc6 in this process. This would provide context for how they are thinking about Orc2 but also contrast, as Orc6 does not to my knowledge impact Mcm2- 7 enzymatic activity like they show for Orc2.

Response: The suggestion has been well taken. We have included a sentence to state the role of Orc6 in this process on page 3.

4) Introduction, paragraph 4: the final paragraph of the introduction reads more like a results section. I suggest moving the majority of this material into the first paragraph of the results and rewriting the last introductory paragraph to summarize the major findings of the paper and how they impact the field.

Response: We thank the referee for the suggestion. The text has been revised accordingly.

5) Figure2A– the differences in Figure2A are some what difficult to see, although believable given the very convincing data in the next panel (Figure 2B). To improve Figure 2A, the authors could quantify differences based on fraction of cells in S-phase (or S-phase length) and additionally include these quantified data in graph format.

Response: We agree with the referee regarding the issue. In the revised version, the original Figure 2a has been removed to avoid any confusion.

6) Line 190: "similar to the level induced by Mcm2-7 alone (Fig. 4a)" – I don't see the data for Mcm2-7 alone, they should add this.

Response: We thank the referee for pointing out this issue. We have included the data for Cdt1-Mcm2-7 alone in Fig. 4a.

7) Line 219: "It is likely that S-CDK phosphorylation at this site hinders the binding" – why is this likely? Evidence is lacking for this statement. I suggest either changing the wording to make it more suggestive or provide additional evidence, such as the position of the phosphorylation site in an Alphafold model of the interaction.

Response: We thank the referee for the suggestion. We have changed the wording on page 8 accordingly.

8) The authors should note in the text the observed shift in Orc2-IDR gel migration upon phosphorylation as support that phosphorylation is happening.

Response: The suggestion has been well taken. The observed band shift of Orc2-IDR has been noted on page 8.

9) Can the authors determine, based on the published structures of the OCCM, whether the Orc2- IDR (helical region) can extend far enough to engage Mcm2? This would provide additional support for their model in Figure 6.

Response: We appreciate the reviewer's valuable suggestion. In the OCCM structure, Orc2-NTD is positioned adjacent to the CTDs of Mcm2 and Mcm5. As a result, the helical region of Orc2-SH can be easily extended to interact with Mcm2 and other neighbouring MCM subunits (Supplementary Fig. 4).

10) Within the results section, the authors should comment on the inefficiency of the pull down in Figure 5A. Is it sub-stoichiometric because other regions of ORC are required for full tethering efficiency (such as Orc6)?

Response: We have added a comment on page 8 acknowledging that the low pull-down efficiency observed in our study may indicate a weak or transient interaction between Orc2N200 and Mcm2-7 containing complexes.

11) Line 53: "establishing as a platform"

Response: The typo has been corrected.

12) Line 161: "however, these reactions"

Response: The typo has been corrected.

13) Line 168: "we conducted a time-resolved"

Response: We have revised the sentence.

14) Figure 3B figure legend: "WEC" should be "WCE"

Response: The typo has been corrected.

15) Figure 3d: in reaction (3) schematic: was ATP also added in first step?

Response: We apologize for this mistake. ATPγS was added in the first step of reaction (3).

16) Figure 3h-j and Figure 4c: please indicate scale for EM reconstructions

Response: The EM reconstructions were utilized to provide an overall 2D shape of the relevant complexes. A box size of 210 Å was applied for particle picking.

17) Figure 5a-b: Cdt1 band is barely visible (problem with printed version?) and appears sub-stoichiometric to Mcm subunits. Why is this?

Response: It could be due to a poor staining efficiency with Cdt1 in the silver staining reaction.

I appreciate the opportunity to review your work and the visual and written clarity with which it was presented in this manuscript. I was impressed by the quality of your mechanistic work which in my opinion represents an important contribution to the field.

We thank the reviewer for recognizing the quality and impact of our study.

Reviewer #2 (Remarks to the Author):

In this manuscript Wu et al. investigate the role of the intrinsically disordered region of Orc2 in facilitating the DNA replication initiation process and show that the IDR of Orc2 is essential for the formation of the fully licensed double hexamer Mcm (DH-Mcm). The genetic data do show that a specific motif within the IDR is essential for viability and that specific stages of the licensing reaction are defective or slower upon deletion or mutation within the IDR. This is important new data. The biochemistry leads them to present a model that is not firmly proven, yet nevertheless interesting. The key steps where the IDR is proposed to play it's roles occurs is in the conversion of the intermediate ORC/Cdc6/Cdt1Mcm2-7 (OCCM) complex through to the DH-Mcm. The data show that the IDR of Orc2 interacts with Mcm2 in pull down assays. They set up a licensing reaction where the Orc2 subunit in the complex is mutated within the IDR and the ATPase of the OCCM is diminished and remarkably the fragment of ORC2 containing the IDR can partially rescue by itself the ATPase. Below are comments that we believe need to be addressed either with clarifications or new experiments before publication.

Response: We appreciate the referee's valuable comments.

Major-

1. Authors identified that Orc2-IDR (1-200) interacts with Mcm2 by using pulldown assay claiming that in the MO (Mcm-ORC) complex Orc2 IDR interacts with Mcm2. However, the pulldown assays were done with only Orc2-IDR and Mcm/cdt1 complex, and not under Mcm loading conditions. We point out that the data do not show that within the OCCM complex other subunits may have conformations that allow for this interaction not detectable with single subunits alone. Authors might show this interaction they propose under loading conditions and /or determine the Mcm2 site required that have similar phenotypes in vitro. Further, the data provided do show the specific region of the IDR (175-200) or I194 that is important for this interaction. However, it would be reasonable to ask if Orc2-IDR (1-200) with similar mutation (I194E) or deletion (Δ175-200) interacts with Mcm.

Response: We thank the referee for the suggestions. We have performed the pull-down assays under a loading condition. Our results demonstrate that Orc2-IDR(1-200) can interact with both Cdt1-Mcm2-7 and OCCM (Fig. 5a, b). We observed that the removal of the special SH region (176-200) from Orc2-IDR blocks its binding to Mcm2-7 containing complexes. However, I194E mutation does not affect this interaction. These results suggest that the binding of Orc2-IDR to Mcm2-7 is mediated by Orc2-SH(175-200). We speculate that the residue I194 of Orc2-SH plays a crucial role in reconfiguring Mcm2-7 ring into a state that is competent for ATP hydrolysis to drive the OCCM-MO transition.

2. Is there a specific reason for using Orc2ΔN175 construct for in-vitro phosphorylation and phospho-mimic (S188D), in the loading experiments (Figure 5e)? Would not the Wt-Orc2 represent the proper scenario rather than an Orc2ΔN175 construct. The authors might consider performing loading experiments with phospho-mimic mutant S188D and in-vitro phosphorylation using Wt-Orc.

Response: We thank the reviewer for the suggestion. Notably, Orc2ΔN175 can be considered equivalent to Orc2-WT. Our results have demonstrated that Orc2-S1(1-175), which contains four S-CDK phosphorylation sites, is dispensable for cell viability (Fig. 1c) as well as pre-RC assembly (Fig. 3c-f). To specifically examine the effect of S-CDK phosphorylation upon Orc2-SH(176-200), we utilized Orc2ΔN175-containing ORC in our *in vitro* assays. As shown in Fig 5d, both the phosphorylated Orc2ΔN175 and Orc2ΔN175-S188D exhibit a similar defect in pre-RC assembly. This emphasizes the unique role of S-CDK in regulating replication re-licensing through targeting Orc2-SH(176-200).

3. The key novel aspects of their model the Orc2-IDR would play it's critical role in converting the OCCM to MO (figure 6). To do this job, Orc2IDR interacts with Mcm after OCCM formation which increases Mcm ATPase activity leading to removal of Cdc6 and Cdt1, and flipping of Orc on to DNA, forming an MO complex. This is proposed through the quantifying percentages of total Mcm at different time points during Mcm recruitment and loading process and identification of the intermediates. The authors must describe how they quantify the percentage of total Mcm in Figure 3h, I, and J.

Response: The relevant information has been provided in the method part on page 17.

4. More importantly, If Orc2IDR is important for MO formation but not OCCM formation as claimed the authors should have seen equal amounts of Mcm being converted to OCCM in Figure 3i and 3j upon deletion of Orc2IDR (Orc2ΔN200) in the presence of ATP and ATPγS, since OCCM can not be converted to MO upon deletion of Orc2IDR (shown in Figure 4c) even in the presence of ATP. However, this does not seem to be observed. About 5% of the total Mcms form OCCM in the presence of ATP in 10mins (Figure3i) and around 70% total Mcm form OCCM in the presence of ATP in 10mins (Figure3j), upon deletion of Orc2IDR. Please explain.

Response: We thank the reviewer for pointing out this issue. It is possible that ATPγS synchronizes all the factors into a static state that is suitable for OCCM formation. However, in the presence of ATP, the relevant complexes may exist in highly dynamic states, which could result in MCM loading and subsequent OCCM formation with relatively low efficiency. Alternatively, it is plausible the OCCM assembled with ATPγS might exhibit greater stability on DNA compared to those bound with ATP.

5. In Figure 4C, the authors do not describe how MO formation was quantified and at what time point during the loading process MO formation was quantified. One presumes it is based upon quantifying the number of particles visualized with a negative stain. However, quantification of MO should to be supported by another biochemical assay as negative staining quantification can lead to biases. Also, it is not mentioned at which step Orc2-IDR2 peptide was added.

Response: We thank the referee for the suggestions. The quantification of MO formation was performed following a previous study (Miller et al 2019 Nature). The typical shape of the MO particles consists of one ORC coupled to a single MCM ring, which is tilted at an angle of about 90° offset from the central channel of ORC. We collected EM data for MO formation after the pre-RC reactions for 4 minutes. Due to the fast and transient nature of MO formation, it is challenging to directly capture this process using biochemical assays. Therefore, EM is currently the only approach available to visualize and quantify MO formation. Moreover, we premixed Orc2-SH peptide with Cdt1-Mcm2-7 heptamer before adding them to the reactions for MO formation. The relevant information has been included in the method part on page 16.

Minor-

1. For some of the experiment's authors need to provide more details to get a better Idea what does the data mean. An example: ATPase assay is not described well, it is not clear whether authors get the rate from the initial slope from many different time points or just from 2 time points.

Response: We thank the referee for the suggestions. More details have been provided in the relevant Figure legends and methods.

2. The authors do not provide the source of S-Cdk.

Response: The source of the yeast strain for S-CDK purification has been provided in the Supplementary Table 1.

3. Figure 1d, there is a typo saying "orc2-all HF to A" which should be "orc2-all HF to E".

Response: Typo corrected.

4. It is very minor, in Figure 1c, in the representation of constructs, ΔN175-215 looks smaller than ΔN175-200.

Response: We thank the reviewer for pointing out this mistake. It has been fixed.

5. It seems Orc2/I194E subunit runs slower than Orc2, see panel c and g in Figure3. Is there any reason for Orc2/I194E subunit runs slower than Orc2?

Response: Orc2-I194E was fused with one FLAG tag at its N-terminus.

6. In Figure 5e, there is Orc6/Clb5. What is Clb5, We could not see any mention of Clb5 in the text.

Response: Clb5 refers to the regulatory subunit of S-CDK. This information has been included in the figure legend (Fig. 5d).

7. Error bars in Figure 3h, I and j, are shown however, it is not detailed how they derive these error bars or from how many experiments. Same for panel C in Figure 4.

Response: The information has been provided in the relevant figure legends (Figs. 3 and 4).

8. Statistical significance needs to be done for the ATPase assay in Figure 4 panel a and b to substantiate the findings

Response: The suggestion was well taken. Analysis of statistical significance has been conducted with the ATPase assays.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We thank the reviewer for the valuable comments and suggestions. Please refer to the relevant point-to-point responses provided in this rebuttal letter for the details.

Reviewer #4 (Remarks to the Author):

The first step of DNA replication initiation in eukaryotes involves the loading of a double hexamer of the Mcm2-7 replicative helicase onto DNA at replication origins. Mcm2-7 loading has been extensively studied in the budding yeast model system, but certain aspects of the mechanism and regulation of this critical process remain incompletely understood. In this manuscript, the authors investigate the function of the N-terminal Intrinsically Disordered Region (IDR) of Orc2 in Mcm2-7 loading. This is an excellent piece of work, that uses a compelling mixture of yeast genetics, in vivo and in vitro functional assays and structural biology, to convincingly demonstrate that the Orc2 IDR is required for the conversion of OCCM to MO, a key step in double hexamer formation. Additionally, the authors reveal that the Orc2 IDR plays a key regulatory role in Mcm2-7 loading, as CDK phosphorylation of this region blocks its interaction with Mcm2, and, consequently, MO formation. I have some suggestions for where the authors should better explain their experimental strategy and / or expand their discussion, but would otherwise recommend that this work is published without delay.

Response: We thank the referee for these valuable comments. The relevant concerns have been addressed as listed below.

Specific points:

• Why are the complementation assays in Fig. 1-2 all performed in the context of the Orc2-1 missense mutant? This is not explained in the text, and seems a little strange, as the same experiments could presumably have been performed in the same fashion, but with wildtype Orc2 under the control of the gal promoter?

Response: We appreciate the referee's suggestions. This *GAL-orc2-1* strain was adopted from Susan Gasser's lab (Shimada and Gasser 2007 Cell). While Orc2-1 mutant proteins have a relatively short half-life (~8 min) at 30°C, GAL-orc2-1 cells express Orc2-1 at high levels in galactose-containing medium and grow with wild-type kinetics. However, this strain allows

efficient depletion of Orc2 proteins. Therefore, we used this strain to examine the impact of the Orc2-IDR mutants upon cell viability and replication initiation after Orc2-1 depletion.

• The data revealing that ARS1614 and 1621 can replicate in the absence of the Orc2 IDR is very interesting, particularly in the context of the recent BioRxiv preprint (Lim et al., 2024) from the Diffley lab, which is mentioned in the discussion. From the methods section, it sounds like these analyses were performed genome-wide, but data is only provided for specific genomic regions (Fig. 2c-e). If the authors have the data for the whole genome, it should be included in this manuscript. For example, it would be a very interesting and valuable addition to this paper if there are other origins that replicate independently of the Orc2IDR, and if their identity correlates with their sequence / structure.

Response: We thank the referee for the suggestion. The syn-seq data for the whole genome has been included in Supplementary Figure 2. We have observed a few additional origins that exhibit origin firing independently of Orc2-IDR. We are currently characterizing these origins systematically to obtain a comprehensive view of their regulation during the cell division cycle. We plan to report the relevant results in a future manuscript.

• The AlphaFold-predicted structure of budding yeast Orc2, including the fact that part of the Orc2-IDR is predicted to be α-helical (https://alphafold.ebi.ac.uk/entry/P32833), should be discussed alongside the DISOPRED predictions. Related to this, it also seems relevant to mention the evolutionary conservation of this region of Orc2 (e.g. are the residues that are mutated in Orc2 conserved, even within fungi?)

Response: We appreciate the referee's suggestions. In the revised version, we have included a multiple sequence alignment of Orc2-NTD (Supplementary Figure 6). Our analysis indicates that Orc2-SH is conserved only among fungi, suggesting that Orc2-SH dependent pre-RC assembly might be specific to yeast species.

• In places, the text is a little colloquial. Words / phrases such as 'floppy' and 'acrobatic gymnastics' should be replaced with more precise scientific language.

Response: The suggestion was well taken. The relevant words/phrases have been revised accordingly.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily responded to all of my previous comments. However, in the revised manuscript they have now included additional data (Figure 5A-B) that to my understanding runs contrary to the model put forward in the original paper. In Figures 1-4 the authors present convincing evidence that a short helix in Orc2 is critical for replication licensing. Relatedly, they find a conserved isoleucine residue (I194) within this helix that when mutated to glutamate (I194E) results in a severe growth defect in vivo and reduced Mcm2-7 loading in vitro. The paper builds to show that the short helix in Orc2 stimulates Pre-RC ATPase activity and, consistently, that the I194E mutation abolishes this effect. Thus, all the data point to a direct interaction between the Orc2 short helix and Mcm2-7, and with I194 playing a critical role in this interaction. It is no surprise then when in Figure 5 they observe a direct interaction between the Orc2 short helix and Mcm2-7. However, what was unexpected was that the I194E mutant interacts with Mcm2-7 normally (same as wild-type Orc2 protein). The authors provide no discussion of this contradictory and unanticipated result, but say only that "In addition, the Orc2N200-I194E mutant maintained its binding affinity to Mcm2-7"... how do we understand these data with respect to the other in vitro and in vivo data that demonstrate an essential role for I194 and a loss of function when this residue is mutated (I194E). How do these new data impact the model?

Reviewer #2 (Remarks to the Author):

The authors have revised their manuscript and we find the revisions adequate for publication. We believe that the SLIM within the region of the ORC2 IDR, reported here is critical for a pivotal step in the loading process and calls attention to a new and novel activity. The name change for the title of the manuscript also highlights an interesting feature of structures within IDR's that can be overlooked without careful structural analysis.Some point for this might be made in the abstract or introduction. On this point we suggest that In some of the responses to our initial review and those of the others it was not clear when a few of the issues and response were actually addressed in the revisions.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

The authors have addressed all the points raised by this reviewer. I recommend publication of this excellent piece of work.

Reviewer #1 (Remarks to the Author)

The authors have satisfactorily responded to all of my previous comments. However, in the revised manuscript they have now included additional data (Figure 5A-B) that to my understanding runs contrary to the model put forward in the original paper. In Figures 1-4 the authors present convincing evidence that a short helix in Orc2 is critical for replication licensing. Relatedly, they find a conserved isoleucine residue (I194) within this helix that when mutated to glutamate (I194E) results in a severe growth defect in vivo and reduced Mcm2-7 loading in vitro. The paper builds to show that the short helix in Orc2 stimulates Pre-RC ATPase activity and, consistently, that the I194E mutation abolishes this effect. Thus, all the data point to a direct interaction between the Orc2 short helix and Mcm2-7, and with I194 playing a critical role in this interaction. It is no surprise then when in Figure 5 they observe a direct interaction between the Orc2 short helix and Mcm2-7. However, what was unexpected was that the I194E mutant interacts with Mcm2-7 normally (same as wild-type Orc2 protein). The authors provide no discussion of this contradictory and unanticipated result, but say only that "In addition, the Orc2N200-I194E mutant maintained its binding affinity to Mcm2-7"... how do we understand these data with respect to the other in vitro and in vivo data that demonstrate an essential role for I194 and a loss of function when this residue is mutated (I194E). How do these new data impact the model?

Response: We appreciate the reviewer's valuable comments. We believe that our additional data (Fig. 5a-b) does not conflict with our model presented in Fig. 6 for the reasons stated below.

(1) All our *in vivo* and *in vitro* results indicate that the SLIM within Orc2-IDR plays a crucial role in driving pre-RC assembly at replication origins.

(2) While I194E mutation has similar effects as the removal of the entire SLIM, it does not necessarily mean that the I194E mutation will disrupt the interaction between Orc2-SLIM and Mcm2-7.

(3) Our results suggest that the binding of the SLIM of Orc2-IDR to Mcm2-7 is not sufficient to transform OCCM into MO. After Orc2 binding, it is likely that the residue I194 is needed to reconfigure Mcm2-7 ring into a state that can stimulate its ATPase activity. The exact mechanism of how this process is accomplished remains unclear. Further study is necessary to elucidate the structural basis of how Orc2-SLIM impacts Mcm2-7, leading to conformational changes that initiate ATP hydrolysis by OCCM.

We have incorporated the following section in our discussion on page 10 (please refer to the revised manuscript attached).

"Notably, Orc2-I194E mutation does not affect the interaction between Orc2-SH and Mcm2-7, suggesting that the residue I194 plays a crucial role in remodeling Mcm2-7 ring following Orc2-SH binding. The exact mechanism of how this process is accomplished remains unclear. Further structural study is needed to determine how Orc2-SH influences Mcm2-7, inducing conformational changes that trigger ATP hydrolysis by OCCM, and to clarify the precise function of the residue I194 in this process."