

# Distinct roles of MCM2-7 subunits in replication licensing in human cells

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript studies the roles of MCM C-terminal extensions (CTE) during MCM loading in human cells. The authors determine a 3.5 angstrom resolution structure of single hexamers (SH) isolated from human cells which form an expected hexameric ring with an open Mcm2/5 gate. The authors observe 5 of the CTEs positioned at the C-terminal side of the MCM ring. Overall, the organization and structure of the human SH resembles that of the yeast SH, the main difference being that yeast does not have a Mcm2 CTE and that human Mcm3 CTE docks onto Mcm2. Similarly to what is known for yeast, several CTEs need to rearrange during OCCM formation for allowing interactions with ORC and Cdc6. For the Mcm3 CTE the authors propose that its interactions with Mcm2 locks the gate and prevents premature MCM loading. Although this hypothesis is interesting it has not been tested by the authors and remains highly speculative. Finally the authors express CTE deletion mutants in cells and show that some CTEs are more important for preRC formation than others. Here an interesting finding is that human Mcm3 CTE is not required for DH formation, one of the few differences to yeast. In summary, although it is important to study human MCM loading, many of the current findings and conclusions parallel those from earlier studies in yeast (autoinhibitory CTEs, different contributions of CTEs to loading, CTE rearrangement during OCCM formation) and new insights into licensing mechanisms are somewhat limited.

Major concerns:

1. The authors identify five stable CTEs in their structure and note that only Mcm7 CTE is flexible. What percentage of total SH particles show visible CTEs? Or in other words, how stable are these CTEs really? In fig S1, only a quarter of MCM particles went into the 3.47 angstrom SH structure. In previous structures some of these CTEs were mostly flexible. Even in the SH map provided the Mcm4 CTE is flexible because the EM density is weak.
2. Related to the previous point, the authors spend substantial time describing CTE interactions but provide no evidence that CTE-CTE interactions and a stable organization is functionally important. The authors claim CTE autoinhibition serves as “a failsafe mechanisms to ensure precise MCM loading across the genome” but no evidence is provided that this is the case or that there is any functional relevance of stable positioning of CTEs in the SH. CTE point mutations (not deletions) that support efficient OCCM assembly but destabilize CTEs in the SH could be used to test this.
3. The authors further suggest that Mcm3 CTE that docks onto Mcm2 prevents ring closure. If this is true one would expect to see closed MCM rings when this domain is deleted. Do the authors see closed rings in their deletion dataset to support this conclusion? Again functional relevance of the Mcm3 CTE interaction with Mcm2 could be tested using point mutations (not CTE deletion) that specifically destabilize this interface but not the OCCM interface.
4. In fig 1 the authors should include a direct comparison with the yeast SH structure to show the expected similarities between human and yeast structures. The SH structure also deserves to be compared to the structure of human MCM dimer with open MCM rings that seems very similar to the authors structure (for example the Mcm5 CTE is also in the central channel there).
5. Fig 1 j,k: Based on the provided SH map, the Mcm6-4 site likely contains ATP and not ADP. For the DH, which structure is used for the comparison?
6. For the SH structure without Mcm3 CTE, the authors follow a different processing pipeline than for the wt structure and do not include a 3D classification step within a Mcm3 CTE mask (figs S1 and S2). In order to confirm that the map region assigned as Mcm3 CTE is really missing, the authors should redo this processing.
7. The authors propose inhibitory roles for Mcm4, Mcm5 and Mcm6 CTEs during loading based on comparison of their SH structure and the OCCM. These conformational changes and roles are expected and similar to what has been seen in yeast SH and OCCM structures (Zhai NSMB 2017, Yuan NSMB 2017, Yuan PNAS 2000). This should at least be acknowledged

by the authors but it limits new insights this comparison provides.

8. In fig 2 the authors show side chain contacts for different CTEs but many of these side chains are not seen in the cryoEM map. For Mcm2 R850 the authors even built an alanine instead of an arginine presumably because there is no side chain density. If the authors want to claim these specific contacts they should be tested and validated by mutating these residues (not CTE deletion).

9. In fig 4 there is some inconsistency in the data. The authors show that Mcm5 CTE deletion reduces SH formation in cells by 90% but this truncation does not affect chromatin association and MCM loading. One would expect that a 90% reduction in SH levels would also cause a reduction in loading but this is not observed.

Other comments:

1. The authors refer to their SH structure as a “closed spiral”. This is misleading because the Mcm2/5 gate is open and not closed.
2. The methods sections is missing residue numbers for the Mcm truncations.
3. The authors report three states of the SH structure but deposit only one into databases. All maps and models included in the manuscript should be deposited.
4. In figures or legends, please include PDB codes for all published structures shown.
5. Page 3, lines 51-53: sliding of intermediates could also account for licensing at origins with extended ACS-B2 spacing and should be acknowledged (see Zhang PNAS 2023).

References:

1. Page 3, lines 47-48: single molecule work from the Bell lab should be cited when correlating ATP hydrolysis with Cdt1 release and Mcm ring closure.
2. Page 4, line 73: reference 36 does not report a human MO
3. Page 4, line 92: reference 37 is missing
4. The work from Hongtao Yu reporting the same interaction between Mcm3 CTE and Mcm2 as the authors should be acknowledged (Liu bioRxiv 2025).
5. Page 7, line 176: The OCCM structure from the Speck lab should be referenced for the interactions of the Mcm3 CTE with Orc2 and Cdc6.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

Mcm2-7 is a molecular motor containing 6 homologous but distinct subunits that together form the catalytic core of the replicative helicase. Although it has become clear that the subunits make different contributions to DNA replication, a detailed delineation of these differences has proved challenging. This study by Fan et. al., uses a largely structural approach to determine the conformational differences between the complex immediately prior to (single hexamer) and immediately after (the double hexamer) stable DNA binding. Relative to these 2 different Mcm2-7 conformations, they find that in the single hexamer form, the C-terminal extension of Mcm5 in combination with a similar region in Mcm3, block closure of the Mcm2/5 gate and occlude the DNA binding domain of Mcm2-7. These data imply that pre-RC formation converts Mcm2-7 into a DNA binding form by relieving the inhibition caused by the Mcm3 and 5 C-terminal extensions, thus preventing Mcm2-7 from productively binding DNA earlier. These data, in combination with others, suggests the specific and differential mechanistic involvement of various Mcm subunits during initial DNA binding.

I find this manuscript to be well-written, and the data of high quality. However, I have some concerns regarding the biological relevance of the conclusions. As mentioned, this study is mostly based upon structural analysis. In summary, the authors propose that C-termini of several subunits (e.g. Mcm5 and Mcm3) inhibit inappropriate DNA binding until ultimately this inhibition is lifted during pre-RC formation. This is an elegant conjecture, as this simple mechanism would maintain Mcm2-7 in an inactive form until the correct point in the cell cycle.

However, an alternative and less interesting hypothesis remains – perhaps spontaneous interactions of the single hexamer with DNA (that is without ORC and its friends) can spontaneously remodel the single hexamer into a DNA binding form. Biochemical evidence is needed to distinguish between these hypotheses. It should be noted in budding yeast that single hexamers of Mcm2-7 also have an open Mcm2/5 gate, but in this case bind they ssDNA well but lack helicase activity. Although to their credit the authors examined the effects of appropriate C-terminal truncations on each of the 6 subunits for both chromatin loading and Mcm2-7 complex stability. However, contrary to simple expectations, both the Mcm5 and Mcm3 C-terminal truncations reduce rather than stimulate chromatin binding, presumably because these domains have additional functions within the Mcm2-7 complex (e.g., promote Mcm2-7 complex stability). Simple biochemical experiments with informative mutant Mcm2-7 complexes (e.g., possibly removal of the appropriate hydrogen binding acceptors/donors between Mcm3 and 5 as suggested in Fig. 2) would test the author's hypothesis and greatly strengthen their paper.

Smaller issues

Possible typo line 107 - Mcm2-6 rather than Mcm2-7?

Typo legend Fig 5 panels ACEGIK “containing” rather than “contianing”

Sup fig 3 - I'm having a very difficult time seeing the critical difference  $\pm$  Mcm3 WHD. Make more clear.

(Remarks on code availability)

### Reviewer #3

#### (Remarks to the Author)

In the present work, Fan et al. present a structural and functional studies of the human MCM2-7 replicative helicase isolated natively from G1 cells and the role of the C-terminal extensions (CTEs) of each of the MCM subunits (which included a winged-helix domain (WHD)), in supporting hexamer assembly and helicase loading. The structure obtained from native material is consistent with a deposited, lower-resolution structure of human MCM2-7 (also natively purified) from the Zhu lab and a comparable resolution model of the over-expressed and reconstituted complex from the Bleichert lab, revealing a view of the hexamer in a pre-loaded, 'ground state' which shows that MCM2-7 on its own adopts a cracked, left-handed spiral conformation wherein access to the helicase pore is blocked by the WHD of MCM5. However, the structure also captures new features, such as showing that the MCM3 WHD can reach across and bridge the crack between the MCM2 and MCM5 subunits, and that WHDs of MCM2, MCM6, and (to a lesser extent) MCM4 also interact with each other. Deletion analyses are used to show that: 1) the CTEs of MCM2 and MCM5 are important for stable interaction of the subunits with other MCM partners by co-IP, 2) the CTEs of MCM2, MCM6, and MCM7 play a role in stable chromatin association in cells, and 3) the CTEs of MCM6 and MCM7 are important for double hexamer formation in vitro.

The data appear of good technical quality and generally support the stated conclusions. The findings of the paper are well organized and the writing accessible to non-specialists. The observations will be of interest to the community for highlighting new roles for the relatively enigmatic and multifunctional MCM WHDs. Some questions requiring additional clarification are noted below.

#### Specific comments:

The work compares the human MCM2-7 structure reported here with that of a CDT1/MCM2-7 complex from budding yeast from the team of Gao and Tye and a low-resolution human MCM2-7 model from Zhu and colleagues, but it does not mention nor analyze a higher resolution model of human MCM2-7 from by Bleichert and coworkers. How does the new structure presented here compare to the Bleichert model and what new features/concepts does it advance? Some notation and discussion of this is important to both properly acknowledge prior findings and help distinguish what is new in the structural versus what is already known.

Although the deletion studies are important and useful, it would have been satisfying to see a few more precise substitutions in some of the functional studies. For example, given that the CTEs of MCM2 and MCM6 seem important for stable subunit interactions and double hexamer formation, respectively, does mutating the interface between these two subunits' WHDs give rise to the same effects? Similarly, do point mutations on the MCM5 WHD (or even a deletion of the CTE) affect the efficiency of and timing of MCM2-7 loading (as per the nocodazole arrest assay presented in Fig. 6j)?

In previous work (Ref.# 40), a 3×FLAG tag was placed on C-terminal of MCM7, whereas in the current study it is placed on C-terminal of MCM2, and the tag is not cleaved prior to structure determination. Given that the 3×FLAG tag is highly negatively charged, and that the intrinsically disordered region (IDR) of the MCM3 C-terminal extension (CTE) contains two highly positively charged patches (each six amino acids in length), the spatial proximity of MCM3 CTE to the MCM2 C-terminal domain (CTD) might be influenced by electrostatic interactions with the 3×FLAG tag. It would be helpful to do a control experiment to show that 3×FLAG tag does not influence position of the MCM3 WHD.

Lines 145-156. Why should the MCM6 CTE have a less significant effect on stabilizing MCM2-7 interactions than MCM4 or MCM2 by co-IP, when it seems critical for bridging the WHD interactions between MCM4 and MCM2 in the structure?

Conversely, why does removal of the MCM2 (or MCM5) CTE destabilize inter-MCM interactions by pull-down, but have little effect on double hexamer formation or chromatin association?

Fig. 4e. Why are the effects of the MCM5 CTE so variable compared to the other deletions? MCM3 still co-associates with the deletion mutant to some extent, as might be expected from the direct subunit contact (and is noted in the text); however, so does MCM6, which is not discussed and is more puzzling.

Given the removal of the MCM3 and MCM5 CTEs appears to have no effect on either chromatin association or double hexamer formation, is there any reason to believe that the ability of these elements to block the MCM2/5 gap is functionally important?

#### Minor points:

It would be helpful to include a table that lists/compares the effects of the CTE deletions on supporting MCM2-7 interactions vs. chromatin association in cells vs. double hexamer formation.

If the MCM5 WHD binds to the pore to blocks ORC and DNA binding, what might release it to enable OCCM formation? Please comment in the Discussion.

In Fig. 2e, the label "MCM2-NTD" should be corrected to "MCM2-CTD."

Figs. 3q and 3r do not appear to be called out in the text with the other Fig. 3 panels.

Fig. 4. Panel e is called out in the text before panels c and d. Suggest swapping order in the figure to match the text.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this revision the authors have addressed some but not all previous concerns. The following issues remain:

1. Stability of CTEs: the authors use crosslinker to trap transient interactions and even with crosslinker only a subset of particles have CTEs resolved. Therefore the CTEs are quite dynamic and not "stable" as stated in the manuscript (page 5), which is even acknowledged by the authors in the reviewer responses. Referring to these transient CTE interactions as "conformational lock" is misleading (page 6, line 142).
2. ATPase sites: page 5 line 112: nucleotide exchanges could also occur after DH assembly.
3. The authors state in the revision that MCM5-CTE positioning in the DNA binding channel contributes to MCM ring stability. What is the evidence for this? The role of MCM5-CTE in MCM hexamer stability could be independent of its position in the channel.
4. Role of MCM-CTE autoinhibition in MCM loading: the authors include new data showing that an MCM3 mutant in Meier Gorlin syndrome (Q761L) stabilizes interactions with MCM2 and reduces MCM loading to support the functional relevance of autoinhibition. Although this is a nice finding, the loading defect could also be a result of inefficient OCCM assembly and not because of autoinhibition.
5. MCM5-CTE deletion and role in SH assembly and MCM loading: the authors have not sufficiently explained the discrepancy between the strong reduction of MCM SH formation upon MCM5-CTE deletion to 10% of WT and the robust MCM loading observed by this mutant. Even if WT MCM5 is coexpressed and MCM5-CTE MCM can be loaded as stated by the authors, one would expect that only 10% of loaded MCM is mutant and the other 90% loaded MCM is WT. Both hexamers would compete for loading. Equal amounts of MCM5-CTE and WT MCM on chromatin as in the manuscript would mean the mutant MCM is 10 times more efficient in being loaded than the WT hexamer. Is this plausible?

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

My concern for this manuscript largely focused on the author's main hypothesis that the CTE of Mcm3 and 5 clog the central channel of Mcm2-7 to prevent aberrant DNA binding during G1. To test this hypothesis, I suggested some good-ol' fashion biochemistry to measure DNA binding affinity of their complex. The authors attempted such measurements in the rebuttal using an electrophoretic mobility shift assay (EMSA) in combination with a double-stranded DNA substrate. They concluded that their "wild type" Mcm complex does not bind DNA, which is consistent with their hypothesis. Moreover, they repeated their assay using a Mcm complex lacking the CTE of Mcm3, which also did not bind DNA in their assay (contrary to their hypothesis). They concluded from this experiment that life is complicated and somehow that this result still supported their hypothesis.

It would have been helpful to read the previously published biochemical literature on Mcm2-7 prior to assaying DNA binding. Rarely has an EMSA been successfully used with the Mcm complex, possibly because 1) the Mcm complex has very poor affinity for double-strand DNA as opposed to single-stranded DNA, 2) the function of the Mcm complex is to move along the DNA rather than to tightly bind to the DNA (such as the ORC positive control that they used for their experiment), which likely reduces the stability of protein/DNA during a long electrophoretic run, and 3) DNA binding by the complex is heavily ATP (or better yet ATP-gamma S) dependent, and it is unclear if the authors added (sufficient) ATP to either the binding assay or the gel. Generally, filter binding has proven to be a much better way to assay protein/DNA interactions with Mcm2-7.

In summary, the experiment and positive control provided in the rebuttal are completely uninformative.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily addressed all comments raised during the prior review. No further revisions are suggested.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have now addressed all concerns. I have no additional comments.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

OK, my concerns are satisfied.

(Remarks on code availability)

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

## REVIEWER COMMENTS

### Reviewer #1 (Remarks to the Author):

This manuscript studies the roles of MCM C-terminal extensions (CTE) during MCM loading in human cells. The authors determine a 3.5 angstrom resolution structure of single hexamers (SH) isolated from human cells which form an expected hexameric ring with an open Mcm2/5 gate. The authors observe 5 of the CTEs positioned at the C-terminal side of the MCM ring. Overall, the organization and structure of the human SH resembles that of the yeast SH, the main difference being that yeast does not have a Mcm2 CTE and that human Mcm3 CTE docks onto Mcm2. Similarly to what is known for yeast, several CTEs need to rearrange during OCCM formation for allowing interactions with ORC and Cdc6. For the Mcm3 CTE the authors propose that its interactions with Mcm2 locks the gate and prevents premature MCM loading. Although this hypothesis is interesting it has not been tested by the authors and remains highly speculative. Finally the authors express CTE deletion mutants in cells and show that some CTEs are more important for preRC formation than others. Here an interesting finding is that human Mcm3 CTE is not required for DH formation, one of the few differences to yeast.

In summary, although it is important to study human MCM loading, many of the current findings and conclusions parallel those from earlier studies in yeast (autoinhibitory CTEs, different contributions of CTEs to loading, CTE rearrangement during OCCM formation) and new insights into licensing mechanisms are somewhat limited.

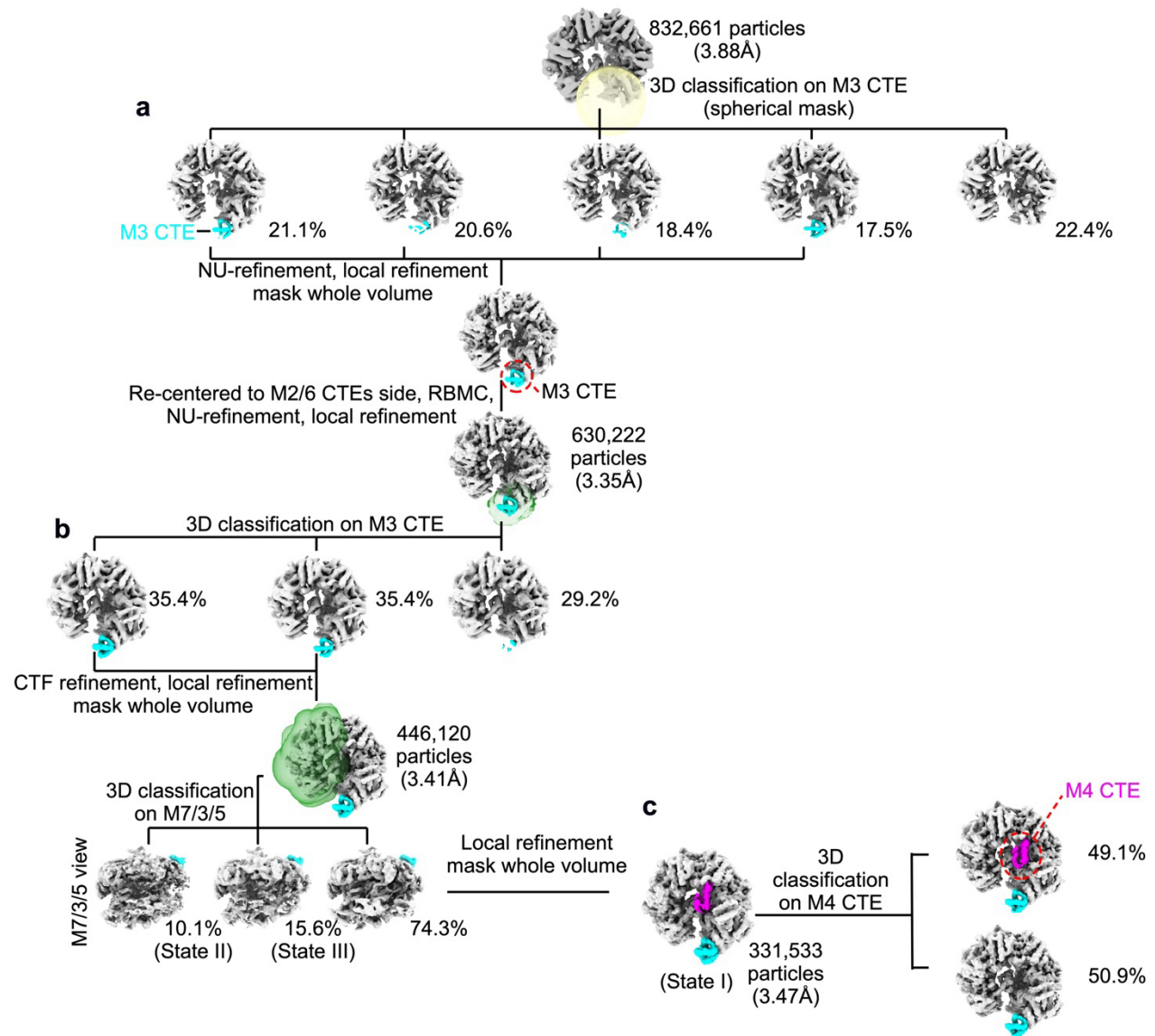
We thank the reviewer for the valuable comments. The relevant concerns have been addressed as listed below.

### Major concerns:

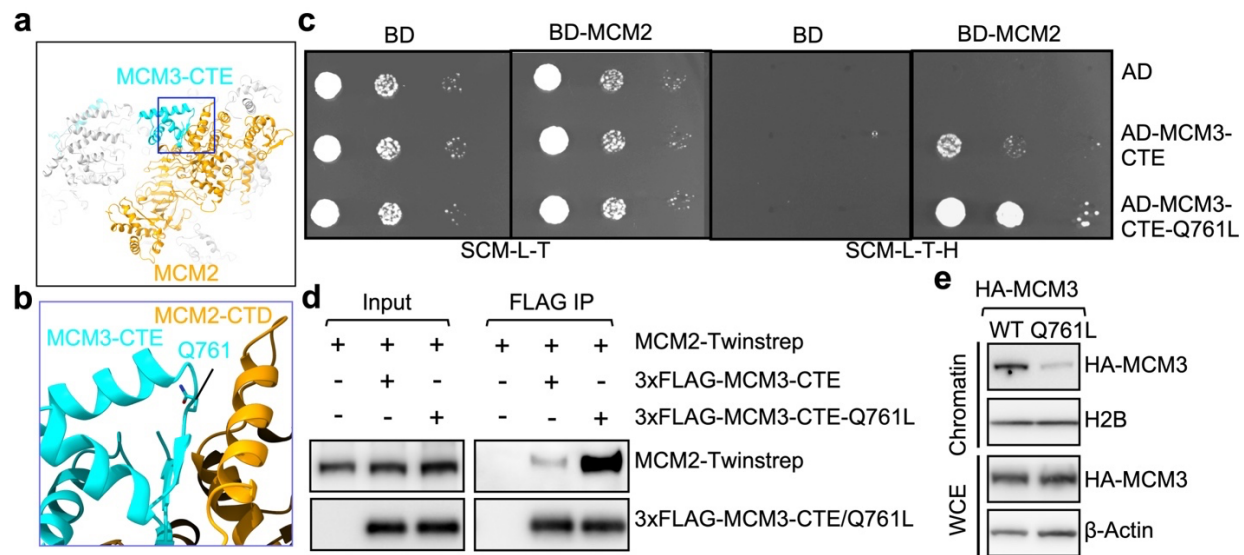
1. The authors identify five stable CTEs in their structure and note that only Mcm7 CTE is flexible. What percentage of total SH particles show visible CTEs? Or in other words, how stable are these CTEs really? In fig S1, only a quarter of MCM particles went into the 3.47 angstrom SH structure. In previous structures some of these CTEs were mostly flexible. Even in the SH map provided the Mcm4 CTE is flexible because the EM density is weak.  
Response: We sincerely thank the reviewer for raising this important point regarding the stability and visibility of the C-terminal extensions (CTEs) in our MCM-SH structure.

Unlike previous structural studies, our analysis was performed on chemically crosslinked samples, which better preserve transient conformations and allow us to capture distinct dynamic behaviors among the CTEs of individual MCM subunits. In our cryo-EM reconstructions, the CTEs of MCM2, MCM5, MCM6 are consistently well-resolved and

visible across all SH particles. In contrast, MCM3-CTE is detectable in 77.6% of SH particles; within this subset, 29.2% exhibit unstable interactions with MCM2-CTD (Fig. R1a, b; Supplementary Fig. 1h, i), suggesting conformational flexibility at the MCM2-MCM3 interface. This structural observation is further supported by functional data: both yeast two-hybrid assays and *in vivo* affinity pull-down experiments demonstrate a weak interaction between MCM3-CTE and MCM2 (Fig. R2c, d; Fig. 2d-f). We propose that the crosslinking used in our sample preparation helps stabilize dynamic CTE conformations, thereby enabling their visualization in our cryo-EM reconstitutions.



**Figure R1.** Distinct conformations of MCM3/4-CTEs in the MCM-SH structure. **a, b.** 3D classification focused on MCM3-CTE with the SH particles. **c.** 3D classification focused on MCM4-CTE with the SH particles from State I showed a bimodal distribution of MCM4-CTE.



**Figure R2.** Meier-Gorlin syndrome-associated mutation MCM3-Q761L affects MCM loading. **a.** The atomic model highlighting the MCM3-CTE/MCM2 interface. **b.** The magnified view of the boxed region in **a**, illustrating the location of MCM3-Q761 precisely at the MCM3/MCM2 interface. **c.** Physical interaction between MCM3-CTE and MCM2 detected by yeast two-hybrid analysis. Growth of the AH109 yeast cells transformed with DNA-binding domain (BD)-MCM2, together with the activation domain (AD) vector or AD-MCM3-CTE plasmids was assessed by spotting equal number of cells onto synthetic complete medium (SCM) drop-out plates as indicated. T, tryptophan; L, leucine; H, histidine. **d.** FLAG immunoprecipitation (IP) using soluble cell extracts from HeLa S3 cells ectopically expressing MCM2-Twinstrep and 3xFLAG-MCM3-CTE constructs. Immunoblotting was performed to detect the relevant subunits is indicated. **e.** Crude chromatin was isolated to assess the chromatin association of HA-tagged MCM3 or MCM3-Q761L.

We fully agree with the reviewer's observation that MCM4-CTE density appears weak in the consensus map. Further 3D subclassification revealed a bimodal distribution: in State I, 49.1% of the SH particles display a well-resolved MCM4-CTE, whereas the remaining 50.9% exhibit substantially weaker or disordered densities. This result suggests that MCM4-CTE exist in a conformational equilibrium between an ordered state and a more flexible or dynamic state (Figure R1c, Figure S1j).

2. Related to the previous point, the authors spend substantial time describing CTE interactions but provide no evidence that CTE-CTE interactions and a stable organization is functionally important. The authors claim CTE autoinhibition serves as "a failsafe mechanisms to ensure precise MCM loading across the genome" but no evidence is provided that this is the case or that there is any functional relevance of stable positioning of CTEs in the SH. CTE point mutations (not deletions) that support efficient OCCM assembly but destabilize CTEs in the SH could be used to test this.

**Response:**

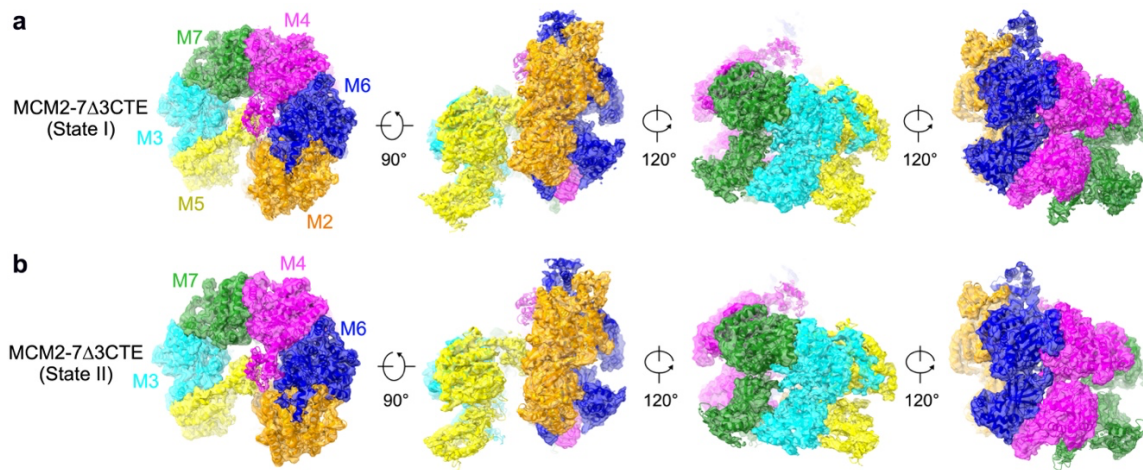
We appreciate the reviewer's comments. Following the suggestion, we have incorporated an analysis of the Meier-Gorlin syndrome-associated MCM3-Q761L mutant (Fig. R2, Fig. 2). Our structural and functional data showed that this mutation, which locates precisely at the MCM3-CTE/MCM2-CTD interface (Fig. R2a-b), enhances the docking affinity of MCM3-CTE for MCM2-CTD (Fig. R2c-d). However, this tighter interaction concurrently inhibits chromatin loading of the MCM complex containing MCM3-Q761L (Fig. R2e).

These results suggest that efficient MCM loading requires a precise regulation of the MCM3-CTE/MCM2-CTD interaction. Specifically, it also implies that a failure to disrupt this interface in a timely manner could impair replication licensing, likely preventing the proper conformational transition necessary for replication licensing. This mechanistic defect also provides a direct molecular link between dysregulated CTE-CTD dynamics and the pathogenesis of Meier-Gorlin syndrome.

3. The authors further suggest that Mcm3 CTE that docks onto Mcm2 prevents ring closure. If this is true one would expect to see closed MCM rings when this domain is deleted. Do the authors see closed rings in their deletion dataset to support this conclusion? Again functional relevance of the Mcm3 CTE interaction with Mcm2 could be tested using point mutations (not CTE deletion) that specifically destabilize this interface but not the OCCM interface.

**Response:**

We thank the reviewer for these insightful suggestions. Our structural analysis of the MCM3- $\Delta$ CTE mutant showed that it does not form planar or closed MCM rings (Supplementary Figs.3 and 4). Notably, in both of its major conformations (Fig. R3; Supplementary Fig.3e, f), MCM5-CTE remains positioned within the central channel, similar to its conformation in the wild-type SH.



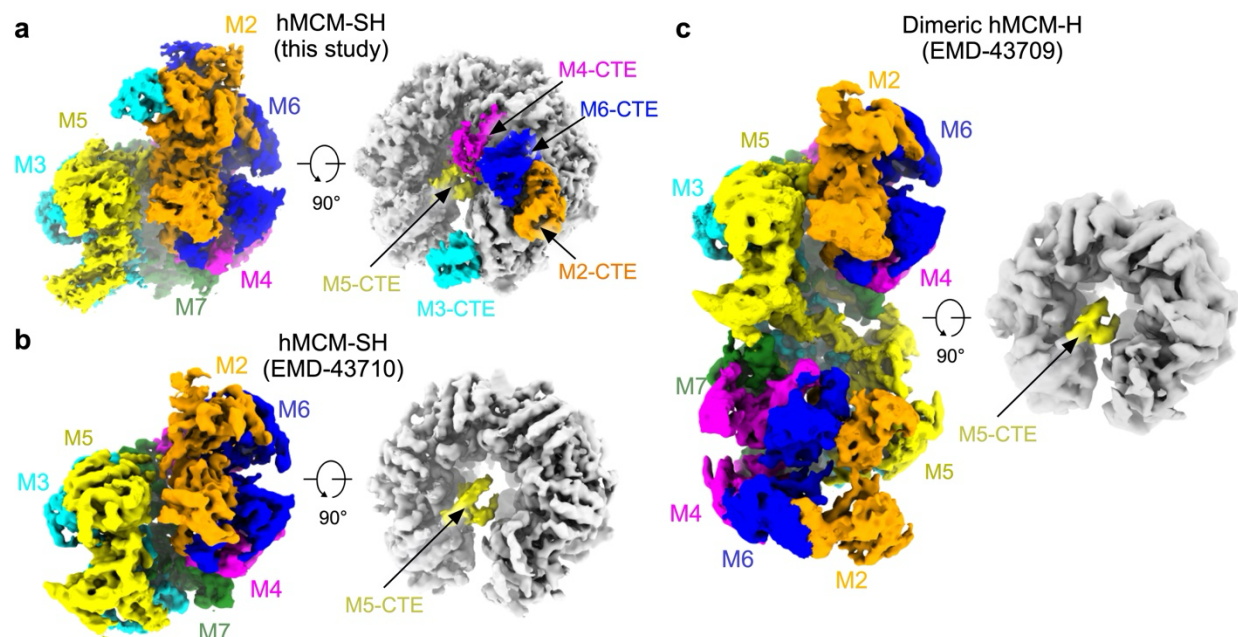
**Figure R3.** Conformational states of the MCM2-7 $\Delta$ 3CTE complex. The overall architecture of the MCM2-7 $\Delta$ 3CTE complex (MCM2-7-SH with a CTE truncation in MCM3) is shown. The two major conformational states are presented as State I (a) and State II (b).

Following the reviewer's suggestion, we attempted to identify point mutations that selectively destabilize the MCM3-MCM2 interface without perturbing the OCCM interface. Despite extensive efforts, we were unable to isolate such mutations. As an alternative approach, we incorporated the Meier-Gorlin syndrome-associated mutation MCM3-Q761L, which, as described above, stabilizes the MCM3-MCM2 interface and impairs MCM loading on chromatin (Fig. R2, Fig. 2).

4. In fig 1 the authors should include a direct comparison with the yeast SH structure to show the expected similarities between human and yeast structures. The SH structure also deserves to be compared to the structure of human MCM dimer with open MCM rings that seems very similar to the authors structure (for example the Mcm5 CTE is also in the central channel there).

Response:

We appreciate the reviewer's suggestion. A direct comparison of the human SH structure with its yeast counterpart is now provided in Figure 1. In addition, we also compared our structure with previously reported mono- and dimeric-hMCM-SHs (Fig. R4, Supplementary Fig. 2a-c).



**Figure R4.** Comparison of the hMCM-SH structures. The structure of hMCM-SH determined in this study (a) is shown alongside previously reported structures of the monomeric (b, EMD-43710) and dimeric (c, EMD-43709) forms.

5. Fig 1 j,k: Based on the provided SH map, the Mcm6-4 site likely contains ATP and not ADP. For the DH, which structure is used for the comparison?

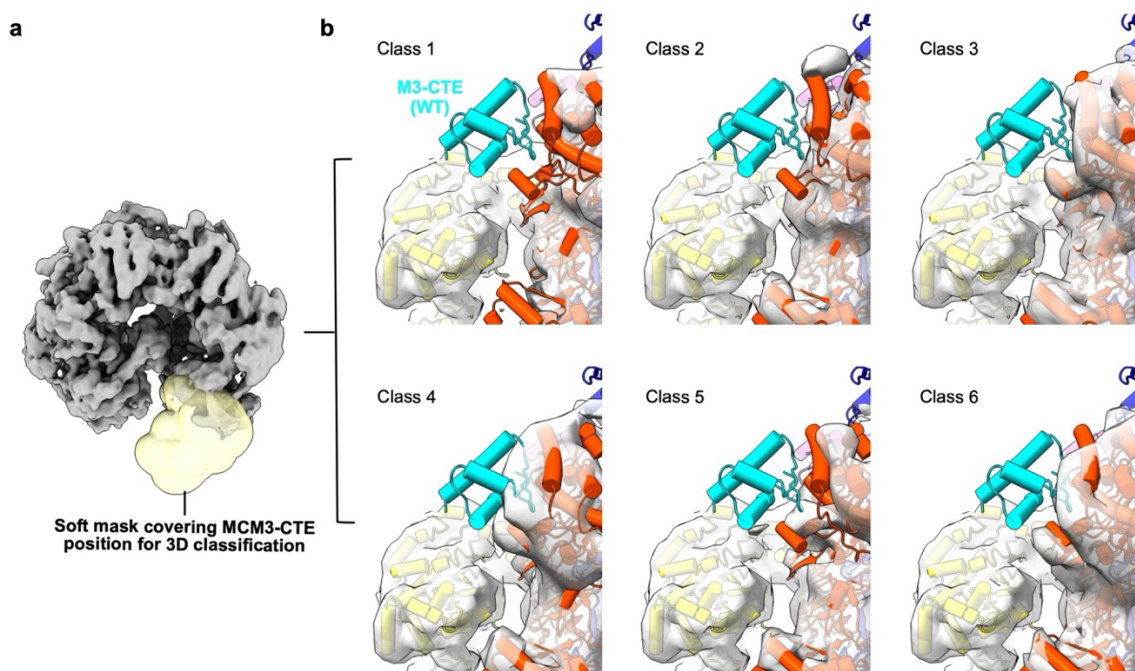
**Response:**

We thank the reviewer for pointing out this issue. We have corrected the relevant nucleotide assignment to ATP in the atomic model and updated the corresponding panel in **Figure 1** accordingly. The information of the DH (PDB: 7W1Y) used for the comparison has been added to the figure legend.

6. For the SH structure without Mcm3 CTE, the authors follow a different processing pipeline than for the wt structure and do not include a 3D classification step within a Mcm3 CTE mask (figs S1 and S2). In order to confirm that the map region assigned as Mcm3 CTE is really missing, the authors should redo this processing.

**Response:**

We thank the reviewer for this helpful suggestion. As recommended, we performed focused 3D classification on the MCM3 $\Delta$ CTE dataset using a soft mask centered on the MCM3-CTE/MCM2-CTD interface. This analysis resolved six distinct conformational classes; however, none of them exhibited additional density corresponding to MCM3-CTE (**Figure R5**). The absence of such density is consistent with the expected outcome of MCM3-CTE deletion.



**Figure R5.** Focused 3D classification of the MCM3 $\Delta$ CTE dataset. (a) The cryo-EM map of MCM3 $\Delta$ CTE SH, indicating the masked region (yellow) used for focused 3D classification. (b) The resulting six classes (transparent surfaces) superimposed with the atomic model the WT SH (cartoon).

7. The authors propose inhibitory roles for Mcm4, Mcm5 and Mcm6 CTEs during loading based on comparison of their SH structure and the OCCM. These conformational changes and roles are expected and similar to what has been seen in yeast SH and OCCM structures (Zhai NSMB 2017, Yuan NSMB 2017, Yuan PNAS 2000). This should at least be acknowledged by the authors but it limits new insights this comparison provides.

**Response:**

We thank the reviewer for this comment. We have now included the relevant references in the revised manuscript. This comparison highlights a significant conformational change in MCM3-CTE during the transition from SH to OCCM, suggesting that MCM3-CTE plays a distinct or species-specific role in the MCM loading.

8. In fig 2 the authors show side chain contacts for different CTEs but many of these side chains are not seen in the cryoEM map. For Mcm2 R850 the authors even built an alanine instead of an arginine presumably because there is no side chain density. If the authors want to claim these specific contacts they should be tested and validated by mutating these residues (not CTE deletion).

**Response:**

We thank the reviewer for pointing out this issue and apologize for the oversight. To better resolve the CTE-related interfaces, we performed focused local refinement using State I particles from the WT SH, which enabled more accurate model building. The updated interactions are now depicted in **Supplementary Fig. 2d-h**.

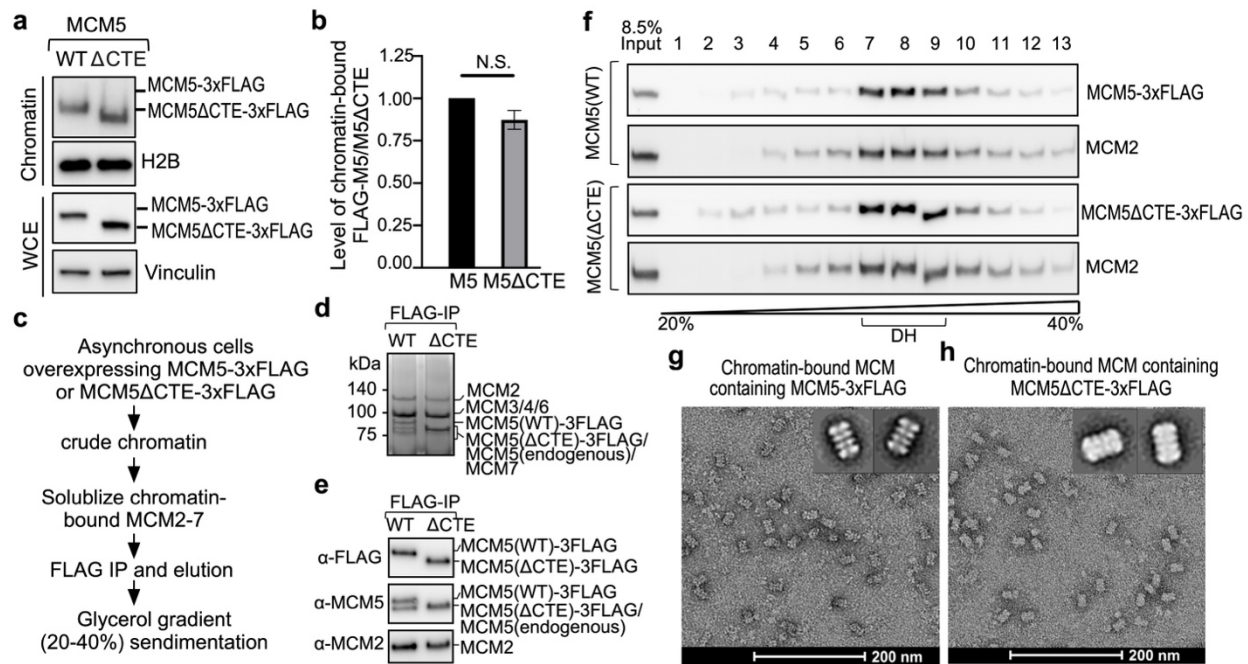
Given that the CTEs of MCM2, MCM3, and MCM4 are dispensable for proliferation and MCM loading, we have not pursued further characterization of these interactions in the current study.

9. In fig 4 there is some inconsistency in the data. The authors show that Mcm5 CTE deletion reduces SH formation in cells by 90% but this truncation does not affect chromatin association and MCM loading. One would expect that a 90% reduction in SH levels would also cause a reduction in loading but this is not observed.

**Response:**

We thank the reviewer for highlighting this important point. To clarify the experimental context: in our HeLa S3 cell system, both endogenous MCM5 and overexpressed constructs (MCM5-WT or MCM5 $\Delta$ CTE) are present. Our result showed that the overexpressed MCM5 $\Delta$ CTE mutant is less efficient at stably assembling into the MCM ring than its overexpressed wild-type counterpart (**Supplementary Fig. 6g, h**). However, once

incorporated, the MCM5 $\Delta$ CTE-containing SHs can be efficiently loaded onto chromatin to form pre-RCs (Fig. R6; Fig. 4e, k, t, u). This is further supported by our observation that the levels of chromatin-bound DHs containing the mutant are not significantly reduced under overexpression conditions in HeLa S3 cells.



**Figure R6.** The effect of MCM5-CTE deletion on MCM loading. **a.** Crude chromatin was isolated to assess the chromatin association of MCM5-3xFLAG or MCM5 $\Delta$ CTE-3xFLAG. **b.** Quantification of chromatin-bound MCM5 $\Delta$ CTE-3xFLAG level normalized to WT MCM5-3xFLAG. **c.** Schematic workflow for affinity purification of the chromatin-bound MCM5/MCM5 $\Delta$ CTE-3xFLAG containing MCM complexes. **d-e.** The isolated MCM complexes were analyzed by silver staining (**d**) and western blotting (**e**). **f.** The Eluates from (**d**) were fractionated on 20-40% glycerol gradient. Immunoblot analysis shows the distribution of WT MCM5 (**c**) or MCM5 $\Delta$ CTE-containing complexes (**d**) across the gradient fractions. **g-h.** Representative negative-staining EM images of MCM-DHs from the isolated MCM complex in (**d**). Insets show representative 2D class averages of the particles. Scale bar is 200nm.

Other comments:

1. The authors refer to their SH structure as a “closed spiral”. This is misleading because the Mcm2/5 gate is open and not closed.

**Response:**

We thank the reviewer for this comment. We have changed it to a “latched spiral” to describe the role of MCM3-CTE as a latch securing the MCM2/5 gate.

2. The methods sections is missing residue numbers for the Mcm truncations.

**Response:**

We thank the reviewer for identifying this omission. As suggested, we have now added the relevant information for each truncation mutant used in this study to the Methods section.

3. The authors report three states of the SH structure but deposit only one into databases. All maps and models included in the manuscript should be deposited.

**Response:**

We appreciate the reviewer's suggestion. The EM maps for States II and III of the MCM-SH are now deposited to EMDB (EMD-68350 and EMD-68363). As these states share the same core architecture with State I and vary only in the flexibility of the NTDs of MCM3 and MCM5, we did not generate specific models for them.

4. In figures or legends, please include PDB codes for all published structures shown.

**Response:**

We thank the reviewer for pointing out this issue. As suggested, we have now added the relevant PDB codes for the published structures.

5. Page 3, lines 51-53: sliding of intermediates could also account for licensing at origins with extended ACS-B2 spacing and should be acknowledged (see Zhang PNAS 2023).

**Response:**

We thank the reviewer for the suggestion. We have incorporated this point into the revised manuscript.

**References:**

1. Page 3, lines 47-48: single molecule work from the Bell lab should be cited when correlating ATP hydrolysis with Cdt1 release and Mcm ring closure.

**Response:**

We appreciate the reviewer's suggestion. The relevant references have been cited.

2. Page 4, line 73: reference 36 does not report a human MO

**Response:**

We thank the review for pointing out this issue. The relevant references have been updated accordingly.

3. Page 4, line 92: reference 37 is missing

**Response:**

We thank the review for the suggestion. The reference has been added.

4. The work from Hongtao Yu reporting the same interaction between Mcm3 CTE and Mcm2 as the authors should be acknowledged (Liu bioRxiv 2025).

**Response:**

We thank the review for the suggestion. The work from Hongtao Yu has been acknowledged accordingly.

5. Page 7, line 176: The OCCM structure from the Speck lab should be referenced for the interactions of the Mcm3 CTE with Orc2 and Cdc6.

**Response:**

We appreciate the reviewer's suggestion. The relevant reference has been added.

## **Reviewer #2 (Remarks to the Author):**

Mcm2-7 is a molecular motor containing 6 homologous but distinct subunits that together form the catalytic core of the replicative helicase. Although it has become clear that the subunits make different contributions to DNA replication, a detailed delineation of these differences has proved challenging. This study by Fan et. al., uses a largely structural approach to determine the conformational differences between the complex immediately prior to (single hexamer) and immediately after (the double hexamer) stable DNA binding. Relative to these 2 different Mcm2-7 conformations, they find that in the single hexamer form, the C-terminal extension of Mcm5 in combination with a similar region in Mcm3, block closure of the Mcm2/5 gate and occlude the DNA binding domain of Mcm2-7. These data imply that pre-RC formation converts Mcm2-7 into a DNA binding form by relieving the inhibition caused by the Mcm3 and 5 C-terminal extensions, thus preventing Mcm2-7 from productively binding DNA earlier. These data, in combination with others, suggests the specific and differential mechanistic involvement of various Mcm subunits during initial DNA binding.

I find this manuscript to be well-written, and the data of high quality. However, I have some concerns regarding the biological relevance of the conclusions. As mentioned, this study is mostly based upon structural analysis. In summary, the authors propose that C-termini of several subunits (e.g. Mcm5 and Mcm3) inhibit inappropriate DNA binding until ultimately this inhibition is lifted during pre-RC formation. This is an elegant conjecture, as this simple mechanism would maintain Mcm2-7 in an inactive form until the correct point in the cell cycle.

[We thank the reviewer for these valuable comments.](#)

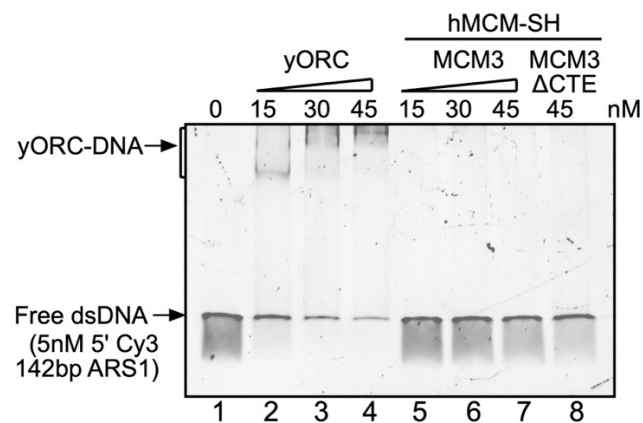
However, an alternative and less interesting hypothesis remains – perhaps spontaneous interactions of the single hexamer with DNA (that is without ORC and its friends) can spontaneously remodel the single hexamer into a DNA binding form. Biochemical evidence is needed to distinguish between these hypotheses. It should be noted in budding yeast that single hexamers of Mcm2-7 also have an open Mcm2/5 gate, but in this case bind they ssDNA well but lack helicase activity. Although to their credit the authors examined the effects of appropriate C-terminal truncations on each of the 6 subunits for both chromatin loading and Mcm2-7 complex stability. However, contrary to simple expectations, both the Mcm5 and Mcm3 C-terminal truncations reduce rather than stimulate chromatin binding, presumably because these domains have additional functions within the Mcm2-7 complex (e.g., promote Mcm2-7 complex stability). Simple biochemical experiments with

informative mutant Mcm2-7 complexes (e.g., possibly removal of the appropriate hydrogen binding acceptors/donors between Mcm3 and 5 as suggested in Fig. 2) would test the author's hypothesis and greatly strengthen their paper.

Response:

We thank the reviewer for these constructive suggestions. To test whether hMCM-SH can spontaneously remodel itself into a DNA-binding competent conformation, we performed *in vitro* DNA binding assays using purified hMCM-SH. Given that hMCM-SH is normally loaded onto double-stranded origin DNA during replication licensing, we incubated hMCM-SH with 142-bp double-stranded ARS1 DNA. Our results show that unlike the yeast ORC which can retard DNA mobility in gel-shift assays (Fig. R7, lane 2-4), hMCM-SH did not produce discernible slower-migrating DNA-protein complexes (Fig. R7, lane 5-7), indicating that it lacks DNA-binding activity under these conditions. Moreover, even deletion of MCM3-CTE failed to induce a conformational change sufficient to enable DNA binding (Fig. R7, lane 8). These findings support the model that hMCM-SH exists in an auto-inhibited state, which intrinsically blocks premature association of the MCM ring with origin DNA.

Notably, recent *in vitro* pre-RC assembly studies have demonstrated that hMCM-SH can be efficiently recruited onto dsDNA only in the presence of ORC1-5 and CDT1, suggesting that ORC, together with CDT1, actively remodels the hMCM-SH into a conformation competent for stable DNA binding during replication licensing.



**Figure R7.** hMCM-SH alone does not bind DNA *in vitro*. Electrophoretic mobility shift assay using 5'-Cy3-labeled 142bp ARS1 dsDNA incubated with hMCM-SH containing either MCM3 or MCM3ΔCTE. Yeast ORC was included as a positive control for DNA binding. Reactions were resolved on a 5% native polyacrylamide gel to distinguish protein-bound from free DNA. Protein concentrations are as indicated above each lane.

Smaller issues

Possible typo line 107 - Mcm2-6 rather than Mcm2-7?

Response:

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

Typo legend Fig 5 panels ACEGIK “containing” rather than “contianing”

Response:

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

Sup fig 3 - I’m having a very difficult time seeing the critical difference  $\pm$  Mcm3 WHD. Make more clear.

Response:

We thank the reviewer for raising this point. The overall architecture of the MCM complex remains largely unchanged in the absence of MCM3-CTE. The primary structural difference is the absence of MCM3-CTE interaction with MCM2-CTD in the mutant, a point we now highlighted in the revised **Supplementary Fig. 3a-c**.

**Reviewer #3 (Remarks to the Author):**

In the present work, Fan et al. present a structural and functional studies of the human MCM2-7 replicative helicase isolated natively from G1 cells and the role of the C-terminal extensions (CTEs) of each of the MCM subunits (which included a winged-helix domain (WHD)), in supporting hexamer assembly and helicase loading. The structure obtained from native material is consistent with a deposited, lower-resolution structure of human MCM2-7 (also natively purified) from the Zhu lab and a comparable resolution model of the over-expressed and reconstituted complex from the Bleichert lab, revealing a view of the hexamer in a pre-loaded, 'ground state' which shows that MCM2-7 on its own adopts a cracked, left-handed spiral conformation wherein access to the helicase pore is blocked by the WHD of MCM5. However, the structure also captures new features, such as showing that the MCM3 WHD can reach across and bridge the crack between the MCM2 and MCM5 subunits, and that WHDs of MCM2, MCM6, and (to a lesser extent) MCM4 also interact with each other. Deletion analyses are used to show that: 1) the CTEs of MCM2 and MCM5 are important for stable interaction of the subunits with other MCM partners by co-IP, 2) the CTEs of MCM2, MCM6, and MCM7 play a role in stable chromatin association in cells, and 3) the CTEs of MCM6 and MCM7 are important for double hexamer formation in vitro.

The data appear of good technical quality and generally support the stated conclusions. The findings of the paper are well organized and the writing accessible to non-specialists. The observations will be of interest to the community for highlighting new roles for the relatively enigmatic and multifunctional MCM WHDs. Some questions requiring additional clarification are noted below.

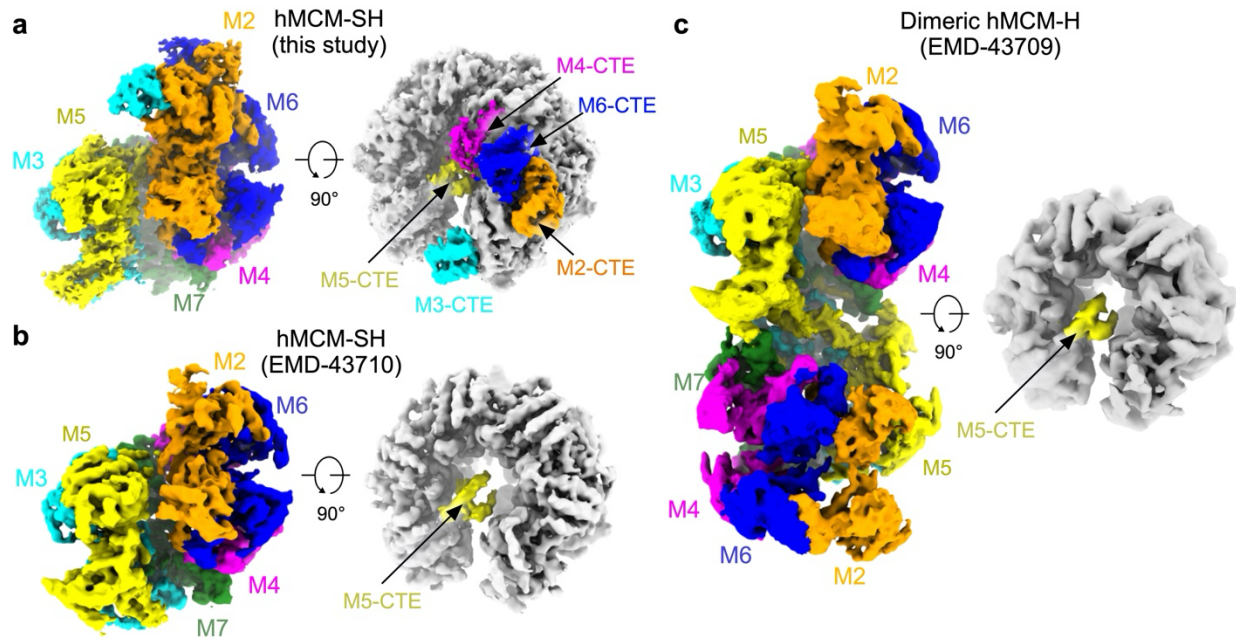
[We thank the reviewer for these valuable comments. The relevant concerns have been addressed as listed below.](#)

**Specific comments:**

The work compares the human MCM2-7 structure reported here with that of a CDT1/MCM2-7 complex from budding yeast from the team of Gao and Tye and a low-resolution human MCM2-7 model from Zhu and colleagues, but it does not mention nor analyze a higher resolution model of human MCM2-7 from by Bleichert and coworkers. How does the new structure presented here compare to the Bleichert model and what new features/concepts does it advance? Some notation and discussion of this is important to both properly acknowledge prior findings and help distinguish what is new in the structural versus what is already known.

[Response:](#)

We thank the reviewer for this insightful suggestion. We have compared our structure with the hMCM-SH structure reported in a recent elegant study from the Bleichert lab. While both structures adopt a similar coiled conformation with MCM5-CTE positioned within the central DNA binding channel, the CTEs of MCM2, MCM3, MCM4, and MCM6 are not resolved in the prior structure (Fig. R8; Supplementary Fig. 2a-c).



**Figure R8.** Comparison of the hMCM-SH structures. The structure of hMCM-SH determined in this study (a) is shown alongside previously reported structures of the monomeric (b, EMD-43710) and dimeric (c, EMD-43709) forms.

We attribute this discrepancy to two key methodological differences:

(1) Source of hMCM-SH complexes: The Bleichert's study used recombinant complexes expressed and purified from insect cells using recombinant baculovirus system. In contrast, our complexes were isolated directly from human cells, a condition that may better preserve native post-translational modifications that may be critical for stabilizing dynamic regions of the hMCM-SH such as those CTEs.

(2) Use of chemical crosslinking: Our samples were stabilized via mild chemical crosslinking prior to cryo-EM grid preparation, a step not employed in the prior study. whereas no crosslinking was applied in the Bleichert Study. Flexible and loosely associated regions such as the CTEs are highly susceptible to denaturation or displacement at the air-water interface during vitrification. The crosslinking in our protocol likely helped preserve these labile elements, allowing their visualization in our high-resolution reconstructions.

Together, these methodological distinctions likely explain the enhanced visibility of CTE densities in our cryo-EM map.

Although the deletion studies are important and useful, it would have been satisfying to see a few more precise substitutions in some of the functional studies. For example, given that the CTEs of MCM2 and MCM6 seem important for stable subunit interactions and double hexamer formation, respectively, does mutating the interface between these two subunits' WHDs give rise to the same effects? Similarly, do point mutations on the MCM5 WHD (or even a deletion of the CTE) affect the efficiency of and timing of MCM2-7 loading (as per the nocodazole arrest assay presented in Fig. 6j)?

Response:

We thank the reviewer for this valuable suggestion. To evaluate the functional significance of the CTEs of MCM2, MCM3, MCM4, and MCM5 in human cells, we employed CRISPR/Cas9 genome editing to generate homozygous CTE-knockout cell lines. We successfully established three viable homozygous mutants: MCM2 $\Delta$ CTE (MCM2CTE $^{-/-}$ ), MCM3 $\Delta$ CTE (MCM3CTE $^{-/-}$ ), and MCM4 $\Delta$ CTE (MCM4CTE $^{-/-}$ ) (Figs. 5h; 6a, b). Notably, while these human mutants remain viable, deletion of Mcm3-CTE or Mcm4-CTE in yeast causes severe growth defects (Figs. 5i; 6c). This suggests that the roles of these two CTEs in MCM loading may be species-specific, and that the human licensing machinery exhibits greater functional redundancy or flexibility compared to its yeast counterpart. Consistent with this notion, MCM loading still occurred in MCM2CTE $^{-/-}$ , MCM3CTE $^{-/-}$ , and MCM4CTE $^{-/-}$  cells, albeit with reduced efficiency (Figs. 5l; 6d, e).

To investigate the function of human MCM5-CTE, we generated MCM5-mAID cell lines expressing either wild-type MCM5 or MCM5 $\Delta$ CTE. Under restrictive conditions, wild-type MCM5 but not MCM5 $\Delta$ CTE rescued cell proliferation, confirming that MCM5-CTE is essential for viability. Subsequent analysis showed that removal of MCM5-CTE causes an obvious reduction in chromatin MCM loading after endogenous MCM5-mAID depletion (Fig. 6i). Thus, human MCM5-CTE, like its yeast ortholog, plays a conserved role in replication licensing.

In previous work (Ref.# 40), a 3 $\times$ FLAG tag was placed on C-terminal of MCM7, whereas in the current study it is placed on C-terminal of MCM2, and the tag is not cleaved prior to structure determination. Given that the 3 $\times$ FLAG tag is highly negatively charged, and that the intrinsically disordered region (IDR) of the Mcm3 C-terminal extension (CTE) contains two highly positively charged patches (each six amino acids in length), the spatial proximity of Mcm3 CTE to the Mcm2 C-terminal domain (CTD) might be influenced by electrostatic

interactions with the 3×FLAG tag. It would be helpful to do a control experiment to show that 3×FLAG tag does not influence position of the MCM3 WHD.

Response:

We thank the reviewer for this comment. Notably, a recent paper (Liu et al, 2025, bioRxiv) reports a human MCM structure in which MCM3-CTE is similarly engaged with MCM2-CTD. In that study, a 3XFLAG tag was fused to MCM7, suggesting that the presence of the 3xFLAG tag does not perturb the positioning of MCM3-CTE.

Lines 145-156. Why should the MCM6 CTE have a less significant effect on stabilizing MCM2-7 interactions than MCM4 or MCM2 by co-IP, when it seems critical for bridging the WHD interactions between MCM4 and MCM2 in the structure?

Response:

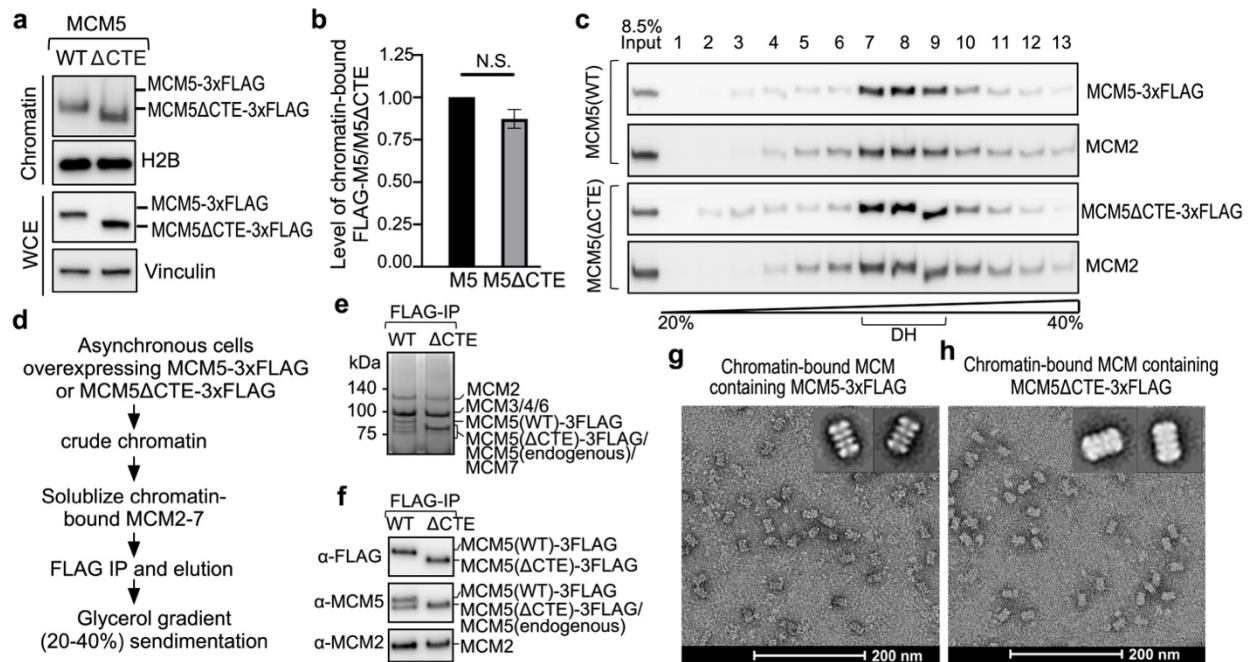
We thank the reviewer for raising this point. While MCM4-CTE is positioned near the CTE of MCM6 in our WT SH structure, we observe no direct interaction between them. In addition, MCM2-CTE engages both the CTE and CTD of MCM6. Even in the absence of MCM6-CTE, MCM2-CTE could still maintain physical contact with MCM6-CTD, thereby preserving a key interaction interface (Supplementary Fig. 2d, e). These features may explain why MCM6-CTE have a less significant impact on overall MCM ring integrity.

Conversely, why does removal of the MCM2 (or MCM5) CTE destabilize inter-MCM interactions by pull-down, but have little effect on double hexamer formation or chromatin association?

Response:

We thank the reviewer for this question. To clarify the experimental context: in our HeLa S3 cell system, both endogenous MCM2 (or MCM5) and overexpressed constructs (MCM2-WT or MCM2ΔCTE) are present. Our result showed that the overexpressed MCM2ΔCTE mutant is less efficient at stably assembling into the MCM ring than its overexpressed wild-type counterpart (Supplementary Fig. 6g, h). However, once incorporated, the MCM2ΔCTE-containing SHs can be efficiently loaded onto chromatin to form pre-RCs (Fig. R9; Fig. 4e, k, t, u). This is further supported by our observation that the levels of chromatin-bound DHs containing the mutant are not significantly reduced under overexpression conditions.

However, with homozygous MCM2CTE<sup>-/-</sup> or MCM3CTE<sup>-/-</sup> mutant cell lines, we indeed observed an obvious reduction in MCM loading. These results suggest that while not essential, these CTEs facilitate an efficient pre-RC assembly on chromatin. The human licensing machinery exhibits greater functional redundancy or flexibility compared to its yeast counterpart.



**Figure R9.** Impact of MCM5-CTE deletion on MCM loading. **a.** Crude chromatin was isolated to assess the chromatin association of MCM5-3xFLAG or MCM5ΔCTE-3xFLAG. **b.** Quantification of chromatin-bound MCM5ΔCTE-3xFLAG level normalized to WT MCM5-3xFLAG. **c.** The solubilized chromatin extracts were fractionated on a 20-40% glycerol gradient. Migration of WT MCM5 or MCM5ΔCTE-containing complexes was detected by western blotting. **d.** Schematic workflow for affinity purification of the chromatin-bound MCM5/MCM5-ΔCTE-3xFLAG containing MCM complexes. **e-f.** The isolated MCM complexes were analyzed by silver staining (**e**) and western blotting (**f**). **g-h.** Representative negative-staining EM images of MCM-DHs from the isolated MCM complex in (**e**). Insets show representative 2D class averages of the particles. Scale bar is 200nm.

Fig. 4e. Why are the effects of the MCM5 CTE so variable compared to the other deletions? MCM3 still co-associates with the deletion mutant to some extent, as might be expected from the direct subunit contact (and is noted in the text); however, so does MCM6, which is not discussed and is more puzzling.

**Response:**

We thank the reviewer for this comment. We have included a section to the Discussion (page 10) addressing the distinct contributions of the CTEs to MCM ring integrity.

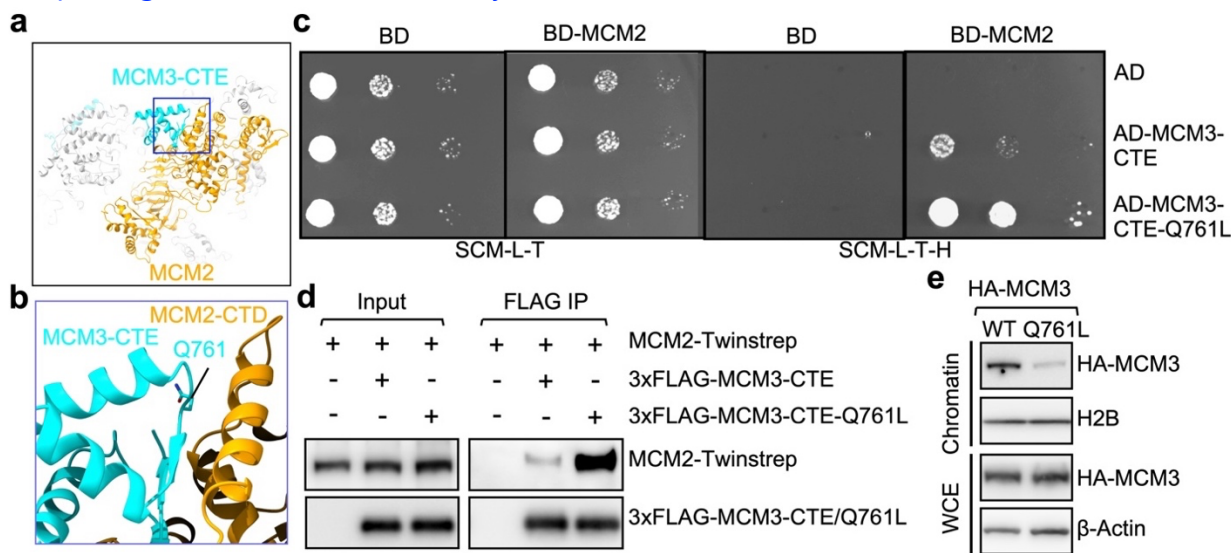
Given the removal of the MCM3 and MCM5 CTEs appears to have no effect on either chromatin association or double hexamer formation, is there any reason to believe that the ability of these elements to block the MCM2/5 gap is functionally important?

**Response:**

We thank the reviewer for this question. In the revised manuscript, we have incorporated an analysis of the Meier-Gorlin syndrome-associated MCM3-Q761L mutant (Fig. R10, Fig. 2).

Our structural and functional data showed that this mutation, which locates precisely at the MCM3-CTE/MCM2-CTD interface (Fig. R10a-b), enhances the docking affinity of MCM3-CTE for MCM2-CTD (Fig. R10c-d). However, this tighter interaction concurrently inhibits chromatin loading of the MCM complex containing MCM3-Q761L (Fig. R10e).

These results suggest that efficient MCM loading requires a precise regulation of the MCM3-CTE/MCM2-CTD interaction. Specifically, it also implies that a failure to disrupt this interface in a timely manner could impair replication licensing, likely preventing the proper conformational transition necessary for replication licensing. This mechanistic defect also provides a direct molecular link between dysregulated CTE-CTD dynamics and the pathogenesis of Meier-Gorlin syndrome.



**Figure R10.** Meier-Gorlin syndrome-associated mutation MCM3-Q761L affects MCM loading. **a.** The atomic model highlighting the MCM3-CTE/MCM2 interface. **b.** The magnified view of the boxed region in **a**, illustrating the location of MCM3-Q761 precisely at the MCM3/MCM2 interface. **c.** Physical interaction between MCM3-CTE and MCM2 detected by yeast two-hybrid analysis. Growth of the AH109 yeast cells transformed with DNA-binding domain (BD)-MCM2, together with the activation domain (AD) vector or AD-MCM3-CTE plasmids was assessed by spotting equal number of cells onto synthetic complete medium (SCM) drop-out plates as indicated. T, tryptophan; L, leucine; H, histidine. **d.** FLAG immunoprecipitation (IP) using soluble cell extracts from HeLa S3 cells ectopically expressing MCM2-Twinstrep and 3xFLAG-MCM3-CTE constructs. Immunoblotting was performed to detect the relevant subunits is indicated. **e.** Crude chromatin was isolated to assess the chromatin association of HA-tagged MCM3 or MCM3-Q761L.

#### Minor points:

It would be helpful to include a table that lists/compares the effects of the CTE deletions on supporting MCM2-7 interactions vs. chromatin association in cells vs. double hexamer

formation.

Response:

We thank the reviewer for this suggestion. A summary table detailing the functional consequences of each CTE deletion has been included as [Supplementary Table 2](#).

If the MCM5 WHD binds to the pore to blocks ORC and DNA binding, what might release it to enable OCCM formation? Please comment in the Discussion.

Response:

We thank the reviewer for this insightful suggestion. Our structure shows that MCM5-WHD occupies the central channel, sterically blocking DNA entry and likely preventing premature engagement with ORC. The release of this WHD is therefore a critical regulatory step in MCM loading. Recent *in vitro* pre-RC assembly studies have shown that hMCM-SH can be efficiently recruited onto dsDNA only in the presence of ORC1-5 and CDT1 (Yang et al 2024 Nature; Weissmann et al 2024 Nature; Wells et al 2025 NC), indicating that ORC and CDT1 actively remodel the hMCM-SH into a conformation competent for stable DNA binding. Notably, previous works in yeast demonstrated that ORC-Cdc6 binding allosterically induces a conformational rearrangement in MCM ring, which is coupled to the displacement of the Mcm5-WHD from the central pore, thereby clearing the channel for DNA loading (Yuan et al 2020 PNAS; Faull et al 2025 NC). Given the high structural conservation of the MCM and ORC-Cdc6 complexes across eukaryotes, it is likely that a similar mechanism repositions MCM5-CTE during MCM loading in human cells. We have incorporated this discussion on [page 11](#).

In Fig. 2e, the label “MCM2-NTD” should be corrected to “Mcm2-CTD.”

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

Figs. 3q and 3r do not appear to be called out in the text with the other Fig. 3 panels.

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

Fig. 4. Panel e is called out in the text before panels c and d. Suggest swapping order in the figure to match the text.

Response: We thank the reviewer’s suggestion. The relevant adjustment has been made accordingly.

We thank the referees once again for their valuable comments and suggestions. We have addressed each point below.

Reviewer #1 (Remarks to the Author):

In this revision the authors have addressed some but not all previous concerns. The following issues remain:

1. Stability of CTEs: the authors use crosslinker to trap transient interactions and even with crosslinker only a subset of particles have CTEs resolved. Therefore the CTEs are quite dynamic and not "stable" as stated in the manuscript (page 5), which is even acknowledged by the authors in the reviewer responses. Referring to these transient CTE interactions as "conformational lock" is misleading (page 6, line 142).

**Response:** We thank the reviewer for this valuable comment. We agree that the CTEs are dynamic and that referring to them as a "conformational lock" was misleading. Accordingly, we have revised the relevant text on page 6.

2. ATPase sites: page 5 line 112: nucleotide exchanges could also occur after DH assembly.

**Response:** We appreciate the reviewer's comment. The relevant description has been revised as the follows: *"No nucleotide binds the MCM2-5 interface due to the ring breach. A comparison with hMCM-DH suggests that nucleotides exchange might occur at some of the ATPase interfaces, including MCM2-5, MCM5-3, MCM3-7, and MCM4-6 sites, during the SH-to-DH transition."*

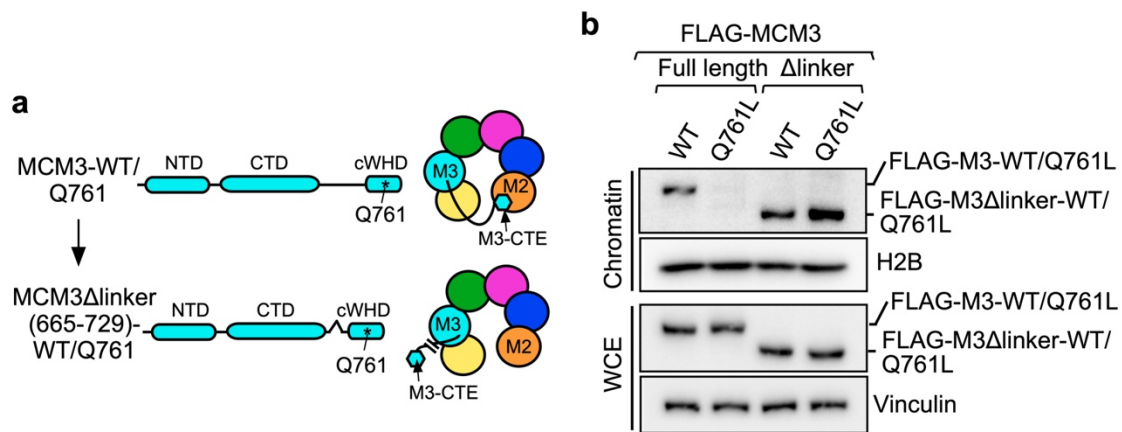
3. The authors state in the revision that MCM5-CTE positioning in the DNA binding channel contributes to MCM ring stability. What is the evidence for this? The role of MCM5-CTE in MCM hexamer stability could be independent of its position in the channel.

**Response:** We thank the reviewer for the valuable comment. The relevant statement has been removed.

4. Role of MCM-CTE autoinhibition in MCM loading: the authors include new data showing that an MCM3 mutant in Meier Gorlin syndrome (Q761L) stabilizes interactions with MCM2 and reduces MCM loading to support the functional relevance of autoinhibition. Although this is a nice finding, the loading defect could also be a result of inefficient OCCM assembly and not because of autoinhibition.

**Response:** We thank the reviewer for bringing up this important point. We agree that the loading defect caused by the MCM3-Q761L mutation could also result from impaired OCCM assembly rather than enhanced autoinhibition. To distinguish between these possibilities, we generated an MCM linker deletion mutant ( $\Delta 665-729$ ) designed to disrupt the interaction between MCM3-CTE and MCM2-CTD (Rebuttal Fig. R1a). Our analysis indicated that this deletion mutation alone had no effect on wild-type MCM loading (Rebuttal Fig. R1b), indicating that it does not comprise OCCM assembly or general loading competence of MCM complex. Importantly, this same linker deletion largely rescued the loading defect of the MCM3-Q761L mutant (Rebuttal

Fig. R1b). These results suggest that the Q761L mutation impairs loading efficiency mainly through stabilizing the MCM3-CTE-MCM2-CTD interaction. It is likely that a precise temporal regulation of the MCM3-CTE/MCM2-CTD interaction is required for efficient MCM loading. Failure to disrupt this interface in a timely manner may impair replication licensing.



**Rebuttal Fig. R1** (added as Fig. 2g, h in the revised manuscript). Meier-Gorlin syndrome-associated mutation MCM3-Q761L impairs MCM loading.

**a** Domain organization of MCM3 and MCM3-linker (665-729) truncation mutant (left panels). Schematic illustration of the potential impact of MCM3-Δlinker mutation on MCM3-cWHD binding to MCM2-CTD (right panels)

**b** Crude chromatin fractions were isolated and probed for the chromatin-bound 3xFLAG-tagged full-length MCM3, MCM3-Q761L, and their corresponding linker truncation mutants.

5. MCM5-CTE deletion and role in SH assembly and MCM loading: the authors have not sufficiently explained the discrepancy between the strong reduction of MCM SH formation upon MCM5-CTE deletion to 10% of WT and the robust MCM loading observed by this mutant. Even if WT MCM5 is coexpressed and MCM5-CTE MCM can be loaded as stated by the authors, one would expect that only 10% of loaded MCM is mutant and the other 90% loaded MCM is WT. Both hexamers would compete for loading. Equal amounts of MCM5-CTE and WT MCM on chromatin as in the manuscript would mean the mutant MCM is 10 times more efficient in being loaded than the WT hexamer. Is this plausible?

**Response:** We really appreciate the reviewer's insightful comment. We agree that the apparent efficiency of MCM5-ΔCTE loading relative to wild-type warrants careful consideration. In our structure, MCM5-CTE occludes the central DNA-binding channel of the MCM ring, suggesting that its removal may relieve an autoinhibitory block and enhance loading efficiency. Thus, it is plausible that the MCM5-ΔCTE mutant loads more efficiently than the wild-type complex, compensating for its reduced stability during SH assembly. Further studies using in vitro pre-RC assembly assays are needed to test this possibility.

We have included this discussion to the revised manuscript on page 11.

Reviewer #2 (Remarks to the Author):

My concern for this manuscript largely focused on the author's main hypothesis that the CTE of Mcm3 and 5 clog the central channel of Mcm2-7 to prevent aberrant DNA binding during G1. To test this hypothesis, I suggested some good-ol' fashion biochemistry to measure DNA binding affinity of their complex. The authors attempted such measurements in the rebuttal using an electrophoretic mobility shift assay (EMSA) in combination with a double-stranded DNA substrate. They concluded that their "wild type" Mcm complex does not bind DNA, which is consistent with their hypothesis. Moreover, they repeated their assay using a Mcm complex lacking the CTE of Mcm3, which also did not bind DNA in their assay (contrary to their hypothesis). They concluded from this experiment that life is complicated and somehow that this result still supported their hypothesis.

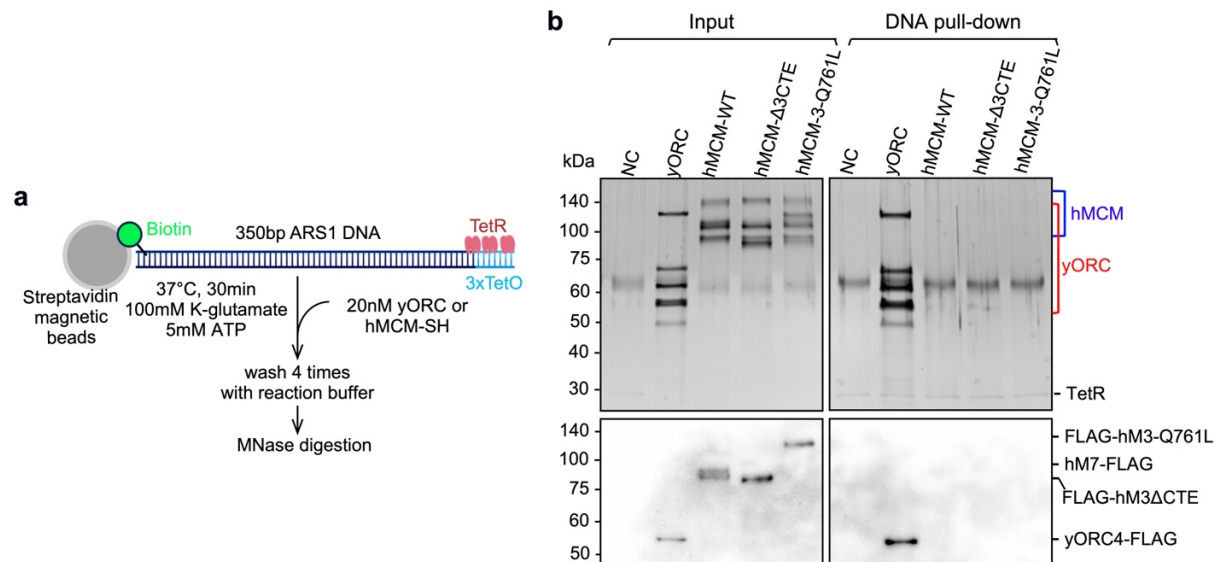
It would have been helpful to read the previously published biochemical literature on Mcm2-7 prior to assaying DNA binding. Rarely has an EMSA been successfully used with the Mcm complex, possibly because 1) the Mcm complex has very poor affinity for double-strand DNA as opposed to single-stranded DNA, 2) the function of the Mcm complex is to move along the DNA rather than to tightly bind to the DNA (such as the ORC positive control that they used for their experiment), which likely reduces the stability of protein/DNA during a long electrophoretic run, and 3) DNA binding by the complex is heavily ATP (or better yet ATP- $\gamma$ S) dependent, and it is unclear if the authors added (sufficient) ATP to either the binding assay or the gel. Generally, filter binding has proven to be a much better way to assay protein/DNA interactions with Mcm2-7.

In summary, the experiment and positive control provided in the rebuttal are completely uninformative.

**Response:** We thank the reviewer for the invaluable comment and constructive suggestions. We agree that our initial EMSA-based approach was inadequate for assessing MCM-DNA binding, and we appreciate the reviewer's guidance regarding the technical challenges associated with this method. To address this concern more rigorously, we have developed an improved *in vitro* binding assay designed to overcome the limitations noted by the reviewer.

In this assay, a biotinylated ARS1 DNA fragment (350 bp) was immobilized on streptavidin magnetic beads via one end. The opposite end harbors a 3×TetO sequence, which was blocked by TetR binding to prevent protein complexes from sliding off the DNA (Fig. R2a). Purified yORC (as a positive control), hMCM-WT, hMCM- $\Delta$ 3CTE, or hMCM-3-Q761L was incubated with the immobilized DNA in the presence of 5mM of ATP. Following extensive washing, DNA-bound proteins were eluted by MNase digestion and analyzed. As expected, yORC showed robust binding to ARS1 DNA. In contrast, none of the MCM complexes tested, including WT,  $\Delta$ 3CTE, or Q761L mutant, exhibited detectable DNA binding under these conditions (Fig. R2b). These results indicate that the hMCM SH resides in an autoinhibitory state that prevents autonomous DNA binding. Furthermore, disrupting the MCM3-CTE-MCM2-CTD interaction by deletion of MCM3-CTE ( $\Delta$ 3CTE) is not sufficient to relieve this autoinhibition and enable DNA binding. This finding is consistent with a model in which multiple autoinhibitory elements, including the CTEs of MCM3, MCM5, and MCM6, cooperatively block DNA engagement, and relief of inhibition likely

requires the coordinated action of licensing factors such as ORC and CDC6 during pre-RC assembly.



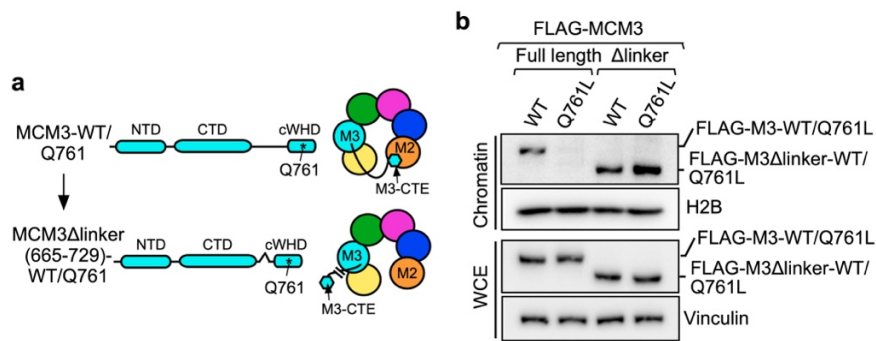
**Rebuttal Fig. R2.** (added as **Supplementary Fig. 4h, i** in the revised manuscript) *In vitro* DNA binding assay to examine the affinity of hMCM complexes for DNA.

**a** Schematic illustration of the *in vitro* experimental setup for analyzing protein-DNA interaction and the workflow of the binding assay. Biotinylated ARS1 DNA (350 bp) containing a 3×TetO sequence at one end was immobilized on streptavidin-coated magnetic beads. The free end was blocked by TetR binding to prevent protein complexes from sliding off the DNA.

**b** Purified yORC (positive control), hMCM-WT, hMCM-Δ3CTE, and hMCM-Q761L were incubated with the immobilized DNA in the presence of 5 mM ATP. Following extensive washing, bound proteins were eluted by MNase digestion, analyzed by SDS-PAGE, and visualized by silver staining (upper panels) and immunoblotting (lower panels) as indicated.

In parallel, we also investigated the functional consequences of the MCM3-CTE-MCM2-CTD interaction *in vivo*. We found that a Meier-Gorlin syndrome-associated mutation, MCM3-Q761L, stabilizes the MCM2-MCM3 interface, and chromatin loading of the MCM complex harboring the Q761L mutation is significantly impaired (**Fig. R1b**). This finding suggests that a persistent MCM3-CTE-MCM2-CTD interaction may hinder subsequent steps required for productive loading onto chromatin. To test this hypothesis, we examined the role of the MCM3 linker region, which connects its CTD to cWHD (**Fig. R1a**). Deletion of the linker region (residues 665-729; MCM3Δlinker) does not affect chromatin loading of wild-type MCM3 (**Fig. R1b**), indicating that the linker is dispensable for MCM loading under normal conditions. However, this same linker truncation largely rescues the chromatin loading defect of the MCM3-Q761L mutant (**Fig. R1b**). We propose that the linker deletion restricts the ability of MCM3-CTE to engage MCM2-CTD, thereby restoring the loading of the Q761L mutant.

Together these results suggest that efficient MCM loading requires precise temporal regulation of the MCM3-CTE/MCM2-CTD interaction. Failure to disrupt this interface in a timely manner may impair replication licensing.



*Rebuttal Fig. R1. (added as Fig. 2g, h in the revised manuscript) Meier-Gorlin syndrome-associated mutation MCM3-Q761L impairs MCM loading.*

***a** Domain organization of MCM3 and MCM3-linker (665-729) truncation mutant (left panels). Schematic illustration of the potential impact of MCM3-Δlinker mutation on MCM3-cWHD binding to MCM2-CTD (right panels)*

***b** Crude chromatin fractions were isolated and probed for the chromatin-bound 3xFLAG-tagged full-length MCM3, MCM3-Q761L, and their corresponding linker truncation mutants.*