

Coordination of chromosome segregation and cell division in the archaeon *Sulfolobus acidocaldarius*.

Corresponding Author: Professor Stephen Bell

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)

In this study, Samson et al., describe an investigation on the coordination of chromosome segregation and cell division in a Crenarchaeon *Sulfolobus acidocaldarius*. They performed Hi-C/3C-seq analysis on wild type cells after synchronization to G1, S and G2 phases and revealed a local conformation change of the chromosome near the segAB locus. Phenotypic and transcriptomic analysis, ChIP-seq and Hi-C contact matrices of the strain without SegAB, two proteins involved in chromosome segregation, were systematically conducted to reveal the impacts of the segAB deletion on the cell and chromosome architecture. They found segAB deletion influenced cell viability with elevated DNA-less (<1C) cells, anucleate cells and altered nucleoid appearance, but no arrest of cell cycle was observed. In addition, the deletion did not affect chromosome compartmentation or the occupancy of the SMC-superfamily protein, CIsN. Instead, SegB only mainly bound to the region adjacent to the segAB locus possibly by which SegB affected the local contact of the locus. Further, the absence of SegA/B resulted in a transcriptomic change partially akin to the DNA damage response in Sulfolobales. Interestingly, they observed a significant increase number (66%) of cells contained either partial or complete ring-structures of CdvA in asynchronous cultures of the Δ segAB strain compared to only 1% in the wild type strain and there is an interaction between SegA and CdvA, indicating a direct interplay between the SegAB system and the ESCRTIII system. The authors provide novel findings how SegAB involves in chromosome architecture and its segregation, and a potential role of SegA-CdvA interaction in the coordination of chromosome segregation and cell division in this archaeon. Several specific points for improving the manuscript are as follows.

Major points:

1. The authors observed a region of B compartment (~160 kb-500 kb, encompasses the segAB locus) in G1-enriched cells gradually disappeared from G1 to G2 phase. In addition, a plume-like feature in the segAB-proximal region was observed as cells undergo from G1 to S phase, indicating a conformational change in which it transitions from a locally insulated feature to making broader contacts with its encompassing domain. Since the transcription of segAB peaks at 150-180 min after synchronization during which SegAB mediates chromosome segregation, Hi-C analysis should also be conducted at this stage to examine whether the region of B compartment appeared again and a plume-like feature disappeared. This will confirm the cell cycle-dependent working pattern of SegAB in chromosome conformational dynamics.
2. Based on the re-analysis of the previous 3C data, the authors observed the presence of loop structures emanating from the vicinity of the segAB locus. The loop structures are dependent on SegAB since these structures were absent in Δ segAB. However, it is unclear how SegAB modulates the chromosome structure. It would greatly improve the manuscript if the authors provide some data to mechanistically show how SegAB may mediate the formation of loop structures. For example, whether is this dependent on the binding motif of SegB across the chromosome? It will be highly attractive to delete the main binding site adjacent to the segAB locus on the chromosome to see whether other SegB binding site(s) would mediate formation of new loop structures and be used subsequently for chromosome segregation.
3. It has been reported recently that the binding motif consensus of SegB is highly conserved in various species of Sulfolobales (Kabli et al. 2025. bioRxiv preprint doi: <https://doi.org/10.1101/2025.02.07.637068>), and the author conducted Hi-C and 3C analysis on both *S. acidocaldarius* and *Saccharolobus islandicus* frequently. Therefore, the Hi-C analysis in this manuscript should be performed in *Saccharolobus islandicus* as well to reveal whether it is a common mechanism for SegAB to modulate conformation of chromosomes in Sulfolobales.
4. The observed interaction between SegA and CdvA is quite interesting. However, the data seem a bit preliminary for explanation of a role of this interaction in the cell. Further experiments are suggested to be conducted such as using truncated mutants of SegA and CdvA to reveal potential interaction domains, molecular dynamic simulation to detect

putative inhibition on the CdvA polymerization, or expression of truncated mutants of SegA in *S. acidocaldarius* to observe its effect on the CdvA ring structure, etc. These would provide more specific evidences showing how the SegA-CdvA interaction may module the coordination of chromosome segregation and cell division.

Minor points:

1. Line 221-222, In addition to in vitro studies of SegB binding sites, recent ChIP-seq analysis had been already performed in *S. acidocaldarius* and *Saccharolobus solfataricus* (Kabli et al. 2025. bioRxiv preprint doi: <https://doi.org/10.1101/2025.02.07.637068>) which is consistent with this manuscript. I would suggest to involve the citation here.
2. As the sequencing data shown in Supplementary Figure 2, the segA/B genes have been deletion. However, the transcriptomic data in Figure 3e showed that the two genes were only decreased less than about log2 (2.5)-fold, which suggests that there might be still the presence of the two genes in partial cells of the Δ segAB culture.
3. Supplementary Figure 4A can be removed to the main text in Figure 4b for better comparison of ChIP-seq data in the wild type and the Δ segAB strains.
4. Has the raw transcriptomic data been deposited in SRA of NCBI? I did not find the submission number.
5. Supplementary dataset is not consistent with that in Figure 3e. The Log2 Ratio fold change of segAB/Wild-type is from -1.5 to 2.5 in Figure 3e, whereas the Log2 Ratio in Supplementary dataset is from | 2.79e-05 | to | 5.19 | . The authors should unify the presentation of the data so that readers can find differentially expressed genes more easily.
6. The interaction between SegA and CdvA should be included in the proposed model in Figure 6d.

Reviewer #2

(Remarks to the Author)

The manuscript by Samson and colleagues describes the role of the SegAB system in chromosome segregation and its interplay with the cell division machinery in the archaeon *Sulfolobus acidocaldarius*. This elegant study integrates chromosome segregation, chromosome conformation and cell division. It describes the changes in the conformation of loci close to the SegAB locus throughout the cell cycle, the binding of SegB and the interplay between SegA and CdvA. By using a wide range of genetic, genomics, and imaging approaches, the authors performed experiments that support their conclusions, unveiling important insights into the link between chromosome organization, segregation and cell division in archaea. These results, will certainly be important for the scientific community. Overall, I have a few comments that may need further clarification before publication.

1. Concerning the changes in the segAB locus along the cell cycle:

a. In lines 143-144 the authors wrote: '...expression of the segAB locus drops concomitant with the reabsorption of this locus into a broader architectural domain...'

3C maps show that after 40 min of restarting growth, the insulation of the segAB locus is decreased but expression is not increased compared with time 0 min. At 100 min, 3C maps show a poorly insulated region, and the level of expression starts to increase at 120 min, being maximal at 180 min in M phase. The drop in segA expression is only observed 5 min after restarting growth... could the authors please clarify this point?

b. Would it be possible to compare contact maps at timepoints 100 and 0 min to show that the decrease of insulation when cells are in G2 is qualitatively higher than at 40min (S)?

a. Would it be possible to perform a 3C-seq at 180 min in which the expression of segA is maximal, and compare this map with that obtained at time 0 (G1)? This 3C may help to understand the folding dynamics of this region.

b. In G2, the two copies of the chromosome are replicated but not segregated yet: Would the decrease in the insulation of this locus be caused by the presence of the two sister chromosomes? This possibility is indeed compatible with the fact that the conformation of the segAB locus is dynamic and changes with the replication cycle.

2. Concerning the loops observed in the 3C maps:

a. In figure 1, the G1 matrix seems to show better defined loops than in S or G2. Could the authors confirm this? If this is the case, can the authors provide more information about these loops? Are they conserved in S and G2?

b. Figure 5b shows that in the absence of segAB, loop anchors are reduced, although there is no specific binding of segB in these regions. Do the authors have any idea about what could be the cause of this decrease? Could this be due to a global change in the 3D folding? Is the expression of these loci affected in the absence of segAB?

3. Discussion: I found the discussion very interesting and I agree the authors' model, although I have a few questions that may be worth discussing:

a. How would the insulation of the segAB locus be coordinated with chromosome replication and segregation in this model?

b. Do the authors have any hypotheses about the binding of SegB in other regions of the chromosome?

c. Why are loop anchors decreased in the segAB mutant?

4. Minor points:

In the pdf, Fig 4 and Fig5 are inverted, please correct this.

Fig4a: could the authors include the gene names below each box? It is interesting to see the genetic region around the locus segAB.

Could the authors show the contact matrices of the WT and segAB mutant in supplementary figure? This would help readers understand the matrices shown in figure 5a.

Could the authors include the position of the ori (fig 5d) in their circular schemes. Could they also indicate the genes that are no longer anchors in the 3C maps in the absence of segAB?

Section 239-251. It seems clear that the authors do not observe any change in compartments in the absence of segAB. They discuss an unpublished article and I understand their points. I agree that citing the preprint is necessary, but I would advise the authors to maintain a friendly and constructive tone.

Line 265: Figure 5c instead of Figure 5d

I am curious: why segSX and segSR? What do X and R mean? Finally, these motifs are mainly in intragenic regions: do these genes belong to any specific category?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all questions and comments I raised. I have no further questions.

Reviewer #2

(Remarks to the Author)

The authors have done a great job addressing my concerns. The new experiments strengthen the manuscript, and the points addressed improve the clarity of the article. I have only one issue regarding the response to point 3a:

Although the authors addressed my comment and mentioned that the explanation would be expanded in the discussion, I could not find this addition in the revised version, which appears largely unchanged compared to the original manuscript. It seems this might have been unintentionally omitted. Is that correct?

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We are indebted to the referees for their helpful and constructive comments. We address their individual points below.

Reviewer #1 (Remarks to the Author):

In this study, Samson et al., describe an investigation on the coordination of chromosome segregation and cell division in a Crenarchaeon *Sulfolobus acidocaldarius*. They performed Hi-C/3C-seq analysis on wild type cells after synchronization to G1, S and G2 phases and revealed a local conformation change of the chromosome near the *segAB* locus. Phenotypic and transcriptomic analysis, ChIP-seq and Hi-C contact matrices of the strain without SegAB, two proteins involved in chromosome segregation, were systematically conducted to reveal the impacts of the *segAB* deletion on the cell and chromosome architecture. They found *segAB* deletion influenced cell viability with elevated DNA-less (<1C) cells, anucleate cells and altered nucleoid appearance, but no arrest of cell cycle was observed. In addition, the deletion did not affect chromosome compartmentation or the occupancy of the SMC-superfamily protein, CIsN. Instead, SegB only mainly bound to the region adjacent to the *segAB* locus possibly by which SegB affected the local contact of the locus. Further, the absence of SegA/B resulted in a transcriptomic change partially akin to the DNA damage response in *Sulfolobales*. Interestingly, they observed a significant increase number (66%) of cells contained either partial or complete ring-structures of CdvA in asynchronous cultures of the Δ *segAB* strain compared to only 1% in the wild type strain and there is an interaction between SegA and CdvA, indicating a direct interplay between the SegAB system and the ESCRTIII system. The authors provide novel findings how SegAB involves in chromosome architecture and its segregation, and a potential role of SegA-CdvA interaction in the coordination of chromosome segregation and cell division in this archaeon. Several specific points for improving the manuscript are as follows.

Major points:

1. The authors observed a region of B compartment (~160 kb-500 kb, encompasses the *segAB* locus) in G1-enriched cells gradually disappeared from G1 to G2 phase. In addition, a plume-like feature in the *segAB*-proximal region was observed as cells undergo from G1 to S phase, indicating a conformational change in which it transitions from a locally insulated feature to making broader contacts with its encompassing domain. Since the transcription of *segAB* peaks at 150-180 min after synchronization during which SegAB mediates chromosome segregation, Hi-C analysis should also be conducted at this stage to examine whether the region of B compartment appeared again and a plume-like feature disappeared. This will confirm the cell cycle-dependent working pattern of SegAB in chromosome conformational dynamics.

We have added the requested experiment to Figure 1 and also a new set of experiments using acetic acid arrested and released cells. The latter data are a new Figure 2.

2. Based on the re-analysis of the previous 3C data, the authors observed the presence of loop structures emanating from the vicinity of the segAB locus. The loop structures are dependent on SegAB since these structures were absent in Δ segAB. However, it is unclear how SegAB modulates the chromosome structure. It would greatly improve the manuscript if the authors provide some data to mechanistically show how SegAB may mediate the formation of loop structures. For example, whether is this dependent on the binding motif of SegB across the chromosome? It will be highly attractive to delete the main binding site adjacent to the segAB locus on the chromosome to see whether other SegB binding site(s) would mediate formation of new loop structures and be used subsequently for chromosome segregation.

The loop anchor points are not SegB binding sites, but deleting the SegB binding sites would still be a great experiment, however, we respectfully suggest that it is beyond the scope of the current paper. Because many of the SegB binding sites are intragenic, we cannot simply delete them without risking confounding effects of altering expression of the host gene product. In principle we could try to introduce synonymous codon substitutions at the various sites, in parallel with biochemical experiments to test the effect of the substitutions on SegB binding in vitro. With some luck we might be able to identify substitutions might disrupt binding while hopefully not affecting translation (although we note that the codon bias is quite marked in *Sulfolobus* and since we do not have antisera against the various gene products we would have to perform full proteome mass spec analyses to confirm that the gene product has not been quantitatively altered). However, this approach is likely to take months if not years to perform.

3. It has been reported recently that the binding motif consensus of SegB is highly conserved in various species of Sulfolobales (Kabli et al. 2025. bioRxiv preprint doi: <https://doi.org/10.1101/2025.02.07.637068>), and the author conducted Hi-C and 3C analysis on both *S. acidocaldarius* and *Saccharolobus islandicus* frequently. Therefore, the Hi-C analysis in this manuscript should be performed in *Saccharolobus islandicus* as well to reveal whether it is a common mechanism for SegAB to modulate conformation of chromosomes in Sulfolobales.

Sadly, the baby machine does not work well with *S. islandicus* (we believe this due to the inherent propensity of this strain to form aggregates, resulting in inefficient separation of new born cells from the membrane). However, to satisfy the referee's request we have performed 3C-seq on acetic acid arrested and released *S. acidocaldarius* and *S. islandicus*. As we discuss in the paper, the acetic acid approach has been employed by several labs to modulate cell cycle distribution of the population in conjunction with genome wide expression profiling. As we discuss in the paper, it's a potentially pleiotropic effects – the arrest is believed to work by respiration uncoupling – essentially causing a precocious stationary phase like starvation response, resulting in a G2 arrest. Indeed, we observe a general weakening of compartmentalization in the arrested cells, similar to what we have previously reported for stationary phase cells.

Nevertheless, when comparing contact matrices from arrested and dividing cell populations we do observe increased insulation at the seg locus in the dividing cell-enriched population in both *S. acidocaldarius* and *S. islandicus* species.

4. The observed interaction between SegA and CdvA is quite interesting. However, the data seem a bit preliminary for explanation of a role of this interaction in the cell. Further experiments are suggested to be conducted such as using truncated mutants of SegA and CdvA to reveal potential interaction domains, molecular dynamic simulation to detect putative inhibition on the CdvA polymerization, or expression of truncated mutants of SegA in *S. acidocaldarius* to observe its effect on the CdvA ring structure, etc. These would provide more specific evidences showing how the SegA-CdvA interaction may module the coordination of chromosome segregation and cell division.

We are currently performing detailed structure /function analyses of the interaction between SegA and CdvA, however, we believe that will constitute an entire manuscript in its own right, particularly now that we have expanded the current manuscript to extend our analyses to *S. islandicus* in response to the referee's request above.

Minor points:

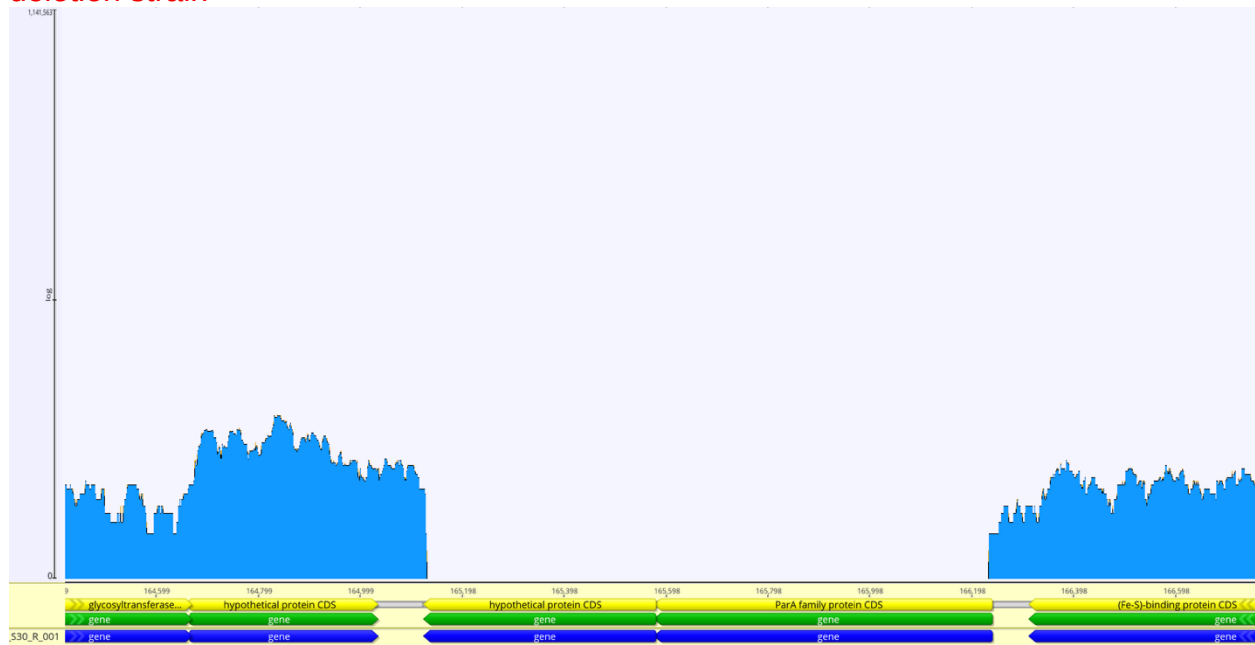
1. Line 221-222, In addition to in vitro studies of SegB binding sites, recent ChIP-seq analysis had been already performed in *S. acidocaldarius* and *Saccharolobus solfataricus* (Kabli et al. 2025. bioRxiv preprint doi: <https://doi.org/10.1101/2025.02.07.637068>) which is consistent with this manuscript. I would suggest to involve the citation here.

We have added this reference.

2. As the sequencing data shown in Supplementary Figure 2, the segA/B genes have been deletion. However, the transcriptomic data in Figure 3e showed that the two genes were only decreased less than about log2 (2.5)-fold, which suggests that there might be still the presence of the two genes in partial cells of the Δ segAB culture.

Thanks for catching this - we made an error when performing the initial alignment and neglected to filter very low (spurious) alignments. Because of this, in the original table we saw values for the four genes that are deleted in the strain (argD, the SmaI restriction enzyme, and segA and B. We have repeated the alignment filtering out low mapped reads (<15) and have included the resultant table as Supplementary material. For the referee, here is a graph of mapped reads in the vicinity of the locus in the segAB

deletion strain



3. Supplementary Figure 4A can be removed to the main text in Figure 4b for better comparison of ChIP-seq data in the wild type and the Δ segAB strains.

We hope the referee won't mind if we leave this figure in the Supplementary data – we appreciate the importance of showing it as control data but it will be readily accessible to readers.

4. Has the raw transcriptomic data been deposited in SRA of NCBI? I did not find the submission number.

Yes, it is present

5. Supplementary dataset is not consistent with that in Figure 3e. The Log2 Ratio fold change of segAB/Wild-type is from -1.5 to 2.5 in Figure 3e, whereas the Log2 Ratio in Supplementary dataset is from $|2.79e-05|$ to $|5.19|$. The authors should unify the presentation of the data so that readers can find differentially expressed genes more easily.

Thanks for catching this too – as described above in response to point 2, we made an error when performing the initial alignment and neglected to filter very low (spurious) alignments. Because of this, in the original table we saw values for the four genes that are deleted in the strain (argD, the Sual restriction enzyme, and segA and B. We have repeated the alignment filtering out low mapped reads (<15) and have included the resultant table as Supplementary material. For the referee, here is a graph of mapped reads in the vicinity of the locus in the segAB deletion strain. The figure was generated using the filtered dataset.

6. The interaction between SegA and CdvA should be included in the proposed model in Figure 6d.

Thank you. We have included this in the discussion beginning on line 390.

Reviewer #2 (Remarks to the Author):

The manuscript by Samson and colleagues describes the role of the SegAB system in chromosome segregation and its interplay with the cell division machinery in the archaeon *Sulfolobus acidocaldarius*. This elegant study integrates chromosome segregation, chromosome conformation and cell division. It describes the changes in the conformation of loci close to the SegAB locus throughout the cell cycle, the binding of SegB and the interplay between SegA and CdvA. By using a wide range of genetic, genomics, and imaging approaches, the authors performed experiments that support their conclusions, unveiling important insights into the link between chromosome organization, segregation and cell division in archaea. These results, will certainly be important for the scientific community. Overall, I have a few comments that may need further clarification before publication.

1. Concerning the changes in the segAB locus along the cell cycle:

a. In lines 143-144 the authors wrote: ‘...expression of the segAB locus drops concomitant with the reabsorption of this locus into a broader architectural domain...’

3C maps show that after 40 min of restarting growth, the insulation of the segAB locus is decreased but expression is not increased compared with time 0 min. At 100 min, 3C maps show a poorly insulated region, and the level of expression starts to increase at 120 min, being maximal at 180 min in M phase. The drop in segA expression is only observed 5 min after restarting growth... could the authors please clarify this point?

We believe the extensive SegB binding to the multiple sites in the vicinity of the seg locus is what causes the insulation/individualization of the locus. As expression levels of the operon drop and presumably protein levels of SegB correspondingly drop, the degree of individualization will also drop

b. Would it be possible to compare contact maps at timepoints 100 and 0 min to show that the decrease of insulation when cells are in G2 is qualitatively higher than at 40min (S)?

We have done this analysis and created a new Supplementary Figure 1 with the heat maps comparing the 100 and 0 time points.

a. Would it be possible to perform a 3C-seq at 180 min in which the expression of *segA* is maximal, and compare this map with that obtained at time 0 (G1)? This 3C may help to understand the folding dynamics of this region.

We have added the requested data to Figure 1. We have also performed a separate set of experiments in both *S. acidocaldarius* and *S. islandicus* using acetic acid arrested and released populations, these data are a new Figure 2.

b. In G2, the two copies of the chromosome are replicated but not segregated yet: Would the decrease in the insulation of this locus be caused by the presence of the two sister chromosomes? This possibility is indeed compatible with the fact that the conformation of the *segAB* locus is dynamic and changes with the replication cycle.

This is an excellent idea. Based on our synchronization experiments, we see the feature at the *segAB* locus emerging in G2 cells when there are two copies of the chromosome and persisting in newborn G1 cells. We believe the locus is reabsorbed into the surrounding chromosome during S phase via intrachromosomal contacts, but we cannot rule out the contribution of interchromosomal contacts.

2. Concerning the loops observed in the 3C maps:

a. In figure 1, the G1 matrix seems to show better defined loops than in S or G2. Could the authors confirm this? If this is the case, can the authors provide more information about these loops? Are they conserved in S and G2?

The Hi-C data used to generate Figure 1 is not high enough resolution to accurately predict loops using Chromosight.

b. Figure 5b shows that in the absence of *segAB*, loop anchors are reduced, although there is no specific binding of *segB* in these regions. Do the authors have any idea about what could be the cause of this decrease? Could this be due to a global change in the 3D folding? Is the expression of these loci affected in the absence of *segAB*?

Five of the loops correlate with the individualization of the *seg* locus from the rest of the chromosome. The loop anchor points are not *SegB* binding sites, so we hypothesize that the loops are mediated by protein-protein interactions between *SegB* and yet-to-be-identified protein factors. We have added Supplementary Figure 7 to show that the expression of the genes at these five anchor points is unaffected by the absence of *SegAB*.

3. Discussion: I found the discussion very interesting and I agree the authors' model,

although I have a few questions that may be worth discussing:

a. How would the insulation of the *segAB* locus be coordinated with chromosome replication and segregation in this model?

We suggest that the extensive binding of SegB at the *seg* locus during and following segregation is what causes the local insulation. Loss of SegB, potentially concomitant with replication fork passage likely resets the locus and allows its reabsorption into the broader domain of the chromosome. We have expanded the explanation of this in the discussion.

b. Do the authors have any hypotheses about the binding of SegB in other regions of the chromosome?

Great question. From our expression analyses SegB binding is not involved in modulating transcription at those sites. Maybe they serve as redundant/ancillary segregation hubs?

c. Why are loop anchors decreased in the *segAB* mutant?

Another interesting point – the distal anchor points are not associated with SegB binding sites so presumably there is/are additional unidentified factor(s) that mediate the bridging events. It/they could be contacted by SegB or its/their expression is dependent on SegB.

4. Minor points:

In the pdf, Fig 4 and Fig5 are inverted, please correct this.

Done

Fig4a: could the authors include the gene names below each box? It is interesting to see the genetic region around the locus *segAB*.

We attempted to draw this but the figure became hopelessly cluttered, instead, we have included Extended Data Table 1 with the genomic coordinates and gene identities of the SegB binding sites.

Could the authors show the contact matrices of the WT and *segAB* mutant in supplementary figure? This would help readers understand the matrices shown in figure 5a.

Sure, we've added that – it's now Supplementary Figure 8.

Could the authors include the position of the *ori* (fig 5d) in their circular schemes. Could

they also indicate the genes that are no longer anchors in the 3C maps in the absence of segAB?

Done

Section 239-251. It seems clear that the authors do not observe any change in compartments in the absence of segAB. They discuss an unpublished article and I understand their points. I agree that citing the preprint is necessary, but I would advise the authors to maintain a friendly and constructive tone.

Our apologies if the phraseology was too aggressive, we've modified that sentence, also taking into account that the pre-print is now published in Nature Comms.

Line 265: Figure 5c instead of Figure 5d

Thank you.

I am curious: why segSX and segSR? What do X and R mean? Finally, these motifs are mainly in intragenic regions: do these genes belong to any specific category?

X is for expanded (compared to the previously proposed site) and R is for purine-rich. We have a supplementary table showing the identities of the genes in which the binding sites reside.

Our thanks again to the referees for their helpful and positive comments.

To address the issue raised by referee 2, we did indeed accidentally omit this revision – sorry about that. We have corrected our error by addition of the following explanation to the end of the first paragraph of the discussion

“In light of our transcription, ChIP-Seq and 3C data, we suggest that as SegB is expressed in late G2 phase (Figure 1f), it binds to the 25 kb region encompassing the *segAB* genes (Figure 5a) and that this extensive nucleoprotein structure acts to sequester this region. Following cell division, as expression of the *segAB* genes decreases, protein turnover will gradually remove SegB and consequently the 25 kb region will be able to reform contacts with its surrounding region.”

Reviewer #1 (Remarks to the Author):

The authors have addressed all questions and comments I raised. I have no further questions.

Reviewer #2 (Remarks to the Author):

The authors have done a great job addressing my concerns. The new experiments strengthen the manuscript, and the points addressed improve the clarity of the article. I have only one issue regarding the response to point 3a:

Although the authors addressed my comment and mentioned that the explanation would be expanded in the discussion, I could not find this addition in the revised version, which appears largely unchanged compared to the original manuscript. It seems this might have been unintentionally omitted. Is that correct?