nature portfolio

Peer Review File

Chromosomal domain formation by archaeal SMC, a roadblock protein, and DNA structure

Corresponding Author: Professor Naomichi Takemata

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)
The manuscript by Yamaura, Takemata et al. investigates the role of Smc and nucleoid-associated proteins in 3D chromosome architecture in archaea. The authors find Smc-dependent long-range DNA contacts throughout the T. kodakarensis chromosome, except across two boundary sites where such contacts are reduced. The major boundary is located at the dif site, with similar observations noted in other archaea. However, the boundary is largely unaffected by the removal of dif or XerA in T. kodakarensis. Instead, a NAP (TrmBL2) is shown to be crucial for Smc accumulation at the boundary site and for boundary formation. In addition to TrmBL2, DNA sequence features appear to contribute to boundary formation, likely due to altering DNA mechanics. Altogether, the work suggests that TrmBL2 bound to rigid/stretched DNA acts as a roadblock for Smc loop extrusion.
The study goes into impressive depth in its examination of T. kodakarensis chromosome organization (using various experimental approaches and simulation) and extrapolates some of the findings through additional experiments conducted in other archaea. The findings are striking and supported by convincing data. A minor drawback is the absence of an obvious phenotype and the lack of clarity on the physiological relevance of roadblocking/domain formation. Regardless, this is a strong study that appears essentially ready for publication. The following comments may be taken into consideration to further improve the manuscript:

Comments:

Is the dif-surrounding sequence sufficient for Smc roadblocking? Can the authors insert a second copy of this region at an ectopic location to test for roadblocking and to provide further support for the suggestion of bidirectional DNA motoring by Smc-ScpAB (by Hi-C)?

The main figures are highly accessible, but critical information might be lost to some readers by zooming in on selected genomic regions. Showing whole-genome Chip-seq and Hi-C profiles for selected experiments in some of the main figures might be useful. For example, the differential contact maps for smc, scpA… mutants to give the reader an indication of the overall impact of Smc activity.

In the manuscript, the authors prominently contrast the activity of Smc in domain formation and in sister chromosome resolution, while the two activities/functions may actually be identical. In T. kodakarensis, a chromosome domain is indeed visible in Hi-C maps, but the function of Smc might still be limited to supporting chromosome segregation. The appearance of a domain boundary might be an indirect consequence of removing Smc from the chromosome dimer resolution sites (to support chromosome segregation). With the currently available information, this option cannot be disregarded.

The fact that Smc is excluded from chr dimer resolution sites appears strikingly conserved in diverse prokaryotes. This is not well represented in the manuscript. The potentially related instance of MukBEF and MatP/matS appears entirely omitted from the manuscript. Additionally, suggestions/speculation on the putative function of Smc exclusion from these sites should be added. This is important, particularly in the absence of an apparent phenotype and a clear understanding of the physiological relevance. Is it possible that Smc brings together the two dif sites on a chromosome dimer (by extrusion of an entire chromosome copy?) to promote chromosome dimer resolution?

The authors mention several times an apparently wrong suggestion put forward in previous work (lack of ScpB in archaeal

Smc complexes). While clarifying potentially misleading literature is important, the dominant appearance in the introduction and the first paragraph of the discussion somewhat distracts from the more important findings of this manuscript and generates a bit of a negative connotation. The authors may consider reorganizing the manuscript accordingly.

Fig 1C: Can the authors perform single-cell PCR to distinguish between the two models presented in this figure.

Since dif1 is not functional in T. acidophilum, it could be denoted in brackets in figure 3C and 3E.

Reviewer #2

(Remarks to the Author)
The article entitled 'Chromosomal domain formation by archeal SMC, a roadblock protein, and DNA structure' by Yamaura, Takemata and coll describes for the first time the interplay between the Smc-ScpAB complex and TrmBL2 and its role in genome architecture. This study reveals that in T. kodakarensis, Smc-ScpAB by interacting with TrmBL2 near the dif site generates a boundary. The authors propose that boundary formation is due to SMC complex stalling, and as a consequence, the chromosome of T. kodakarensis is organized into TAD-like structures. The study also correlates Smc-ScpAB stalling with AT rich DNA sequences and propose that this is linked to a specific DNA topology. Overall, the article is clearly written, findings are solid and highly interesting and represent a significant advance for the scientific community. Below a few questions and suggestions to improve the clarity of the article.

1. The MS clearly shows that both Smc-ScpAB and TrmBL2 are needed to promote boundary formation near dif site. The authors claim that these factors, together with a specific DNA sequence and topology are involved in TAD like formation. However, TAD-like structures are not highly visible on their contact matrices. In some matrices, it seems that the color scale used is too stringent and long-range contacts are not clearly observed. Could this be why TAD-like structures are not evident in contact maps? Could the authors use a tool to identify these domains in WT and mutant strains? The presence of a boundary does not necessarily mean that a domain is formed (see Bignaud A et al, 2024).
2. Since high-resolution contact matrices were obtained, do the authors observe whether a specific folding pattern is associated with boundaries, similar to what was recently shown in bacteria? (Bignaud A et al, 2024)
3. In Figure S4B, the WT contact matrix seems to show sliding loops at boundary 1, that seems to disappear in the smc mutant. Do the authors also observe this at boundary 2 ? Are these sliding loops also lost in the other mutants?
4. Although it is clear that long-range contacts seem to be increased in Figure 2, I am not able to see the increase at 200 kb. Could the authors find a way to quantitative determine this increase in long range contacts?
5. Figure 7E is highly complex, and the real added value of this panel (mainly for rows 5 to 17) is hard to get in the way this table is presented in the text (data presented on lines 307-336). Could the authors improve the clarity of the section? Are these correlations essential to reach the conclusions?

Minor points:
1. Through all the text, citations are always after a '.', they should be before.
2. Could the authors replace Line 55: "…. and they instead use the loop extrusion activity to resolve replicated DNA molecules for chromosome…"
By "…and they instead use presumably the loop extrusion activity to resolve replicated DNA molecules for chromosome…."
3. Line 54: "Smc-ScpAB and MukBEF," should also mention MksBEF
4. Line 124 & 125: "The insulation of local contacts was much weaker at other loci, suggesting that boundaries 1 and 2 are the major boundaries on the T. kodakarensis genome'
Considering the polyploidy of this model, it would be safer to state "…boundaries 1 and 2 are the most frequent boundaries on the T. kodakarensis genome'
5. Line 214: deletion mutants of trmBL2 and TK0795 using KU21
For consistence, it would be better to write mcrB instead of TK0795
6. Line 234: '… TrmBL2, in which two dimers of TrmBL2 interact with the DNA in a symmetric manner…'
Could the authors expand a bit more on why this do not fits well? Maybe a structure in supplementary data would be helpful.
7. Line 255: 'The two values showed a good correlation on average'
Could the authors give the value of this correlation?
8. Line 265: Authors might refer to Figure 6F?
9. Considering the importance of the massage, maybe Figure S6 should be on main text while Figure 4 on Supplementary.

Reviewer #3

(Remarks to the Author)
In this paper, the structure of chromosomes in the cells of one species of Archaea, Thermococcus kodakaraensis, was studied using the Hi-C method. Although Archaea are prokaryotes, they retain characteristics more closely related to eukaryotes than bacteria, making them extremely useful model organisms for elucidating the basic mechanisms of life. From this perspective, a small number of studies have been conducted on Archaea to understand the universal basic structure of eukaryotic chromosome architecture. The SMC complex is a fundamental building factor of chromosome structure and is common from prokaryotes to eukaryotes. However, it has been previously noted that they have different roles in chromosome structure in prokaryotes and eukaryotes. The SMC complex is conserved in Archaea, and the Hi-C method has been used to study chromosome structure. It was found that in the Archaea Halobacterium volcanii, chromosome domain boundaries are created by the SMC complex. Since the loop extrusion model with SMC complexes is currently being

discussed as a hypothesis to explain the formation of chromosome domain boundaries, it is thought that if the SMC complexes in Archaea do indeed create chromosome domain boundaries, then the loop extrusion model can be applied to the SMC complexes in Archaea. However, Halobacterium volcanii lacks one of the components of the SMC complex, which differs from the SMC complex common to prokaryotes and eukaryotes, making it impossible to necessarily incorporate the loop extrusion model as is.

To overcome this, the authors used T. kodakaraensis, which has an SMC complex consisting of components common to prokaryotes and eukaryotes, and succeeded in showing the boundaries of chromosomal domains by the SMC complex. The authors also found a DNA-binding protein that is essential for the boundary of the chromosomal domain and reported new findings, including that the binding region is an AT-rich sequence, among others. Specifically, the boundary was formed at the dif-adjacent region. A pull-down experiment uncovered that TrmBL2, a nucleoid-associated protein, accumulated on the dif site. ChIP-seq analysis again showed that the TrmBL2 protein was enriched on the dif-adjacent boundary region. ChIP-seq data also provide evidence of the co-localization of TrmBL2 and Smc protein. A slight gap between the peaks of TrmBL2 and Smc at the boundary 1 region led the authors to consider the mechanism forming the boundary: loop-extruding Smc protein stalls at the TrmBL2 accumulated zone. This mechanism is proposed as analogous to the TAD formation in eukaryotic chromosomes, which is supported by the SMC protein stalling at the CTCF site. The AT-rich sequences, especially the A-tracts, are responsible for the TrmBL2-mediated Smc stalling. The stiffness caused by A-tracts prohibits the progression of the loop extrusion, resulting in stalling. A gene disruption strain of the SMC complex factor was created, and the Hi-C method showed changes in chromosome structure in the mutant, providing sufficient experimental support.

In summary, a novel boundary formation mechanism in prokaryotes is proposed by multidimensional analyses. The loop extrusion model is still under discussion, as is whether the molecular movements actually occur in the cell. However, in the results section, it is not necessary to proceed with the results in line with the loop extrusion model. It is more valuable for the research results to be more objective in describing them. It should be added, however, that this is not a question of discussing the loop extrusion model in the Discussion.

It is also strange that the possible conformations of the AT sequence are considered and discussed, but the conformational changes due to DNA melting are not investigated for the AT sequence as the Tm value. The structural changes due to DNA melting should be eliminated after the analysis is examined.

Although the Δsmc, ΔscpA, ΔscpB, Δsmc ΔscpA, and Δsmc ΔscpB mutants showed changes in chromosome organization, the cell growth was almost as good as the wild-type cell. The presence of multiple chromosomes in T. kodakaraensis, which is a major difference from E. coli and Bacillus subtilis, is important in elucidating the biological significance of the multiple chromosomes and their relationship to chromosome structure. The relationship between the multiple chromosomes and chromosome structure is important in elucidating the biological significance of the Archaea SMC complex and its chromosome structure.

In addition, growth is typically observed using optical density (OD), but the actual rate of cell division cannot be observed with OD. Note that it merely reflects an increase in cell volume. Therefore, it is better to show observations of the morphology of the cells themselves during proliferation. Furthermore, if T. kodakaraensis proliferates logarithmically, Figure 3A should be presented as a single logarithmic graph.

Other comments
In the case of eukaryotic SMC proteins, it is generally thought that they function as dimers of dimers. To my knowledge, each SMC protein stalls at individual CTCF sites, forming a TAD structure. Does the Smc-ScpAB complex in T. kodakaraensis also form dimers of dimers? If so, when one of the two Smc-ScpAB complexes stalls at the TrmBL2 region, how does the other behave?

In Figure S2, the authors refer to all 5 mutants, including Δsmc, ΔscpA, ΔscpB, Δsmc ΔscpA, and Δsmc ΔscpB, as causing equivalent effects. Although I largely agree with this opinion, there seem to be slight differences in the Hi-C maps. Only when the Δsmc mutation is present can blue lines be seen at the position near 0.8 Mb. Is there a possibility that these differences reflect an ScpAB-independent function of the Smc core protein?

In Figure S5B, an increment of diagonal interactions around the 0.5-2 Mb region is seen in the ΔtrmBL2 mutant. Does this correspond to the juxtaposition of chromosomes observed in Bacillus subtilis Smc-ScpAB?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have performed additional experiments and made changes to manuscript text and figures in response to the comments raised by the reviewers. Altogether, the manuscript has been significantly improved. In particular, the experiment using the ectopically inserted boundary is a great addition. It clearly shows that the corresponding sequence is not only necessary but also sufficient for boundary formation. The authors also added paragraphs discussing possibly biological functions of the boundary, which I find helpful and stimulating.

This is a great paper; it is ready for publication in my opinion.
The authors may want to consider including the wild-type maps in fig 5 for direct comparison with the three mutant scenarios.

Reviewer #2

(Remarks to the Author)
The revised version of the article ‘Chromosomal domain formation by archeal SMC, a roadblock protein, and DNA structure’ by Yamaura, Takemata and coll addresses all the points raised by this reviewer. Furthermore, the new experiments, where the dif surrounding region was inserted into an ectopic location, have provided valuable insights, further strengthening the article. Overall, the article is solid and I do not have additional comments. I congratulate the authors on the effort they have put into this work.

Reviewer #3

(Remarks to the Author)
The authors have responded in good faith to our earlier comments and have revised the manuscript and figures accordingly. These are sufficient to satisfy us.

Reviewer #1 (Remarks to the Author):

The manuscript by Yamaura, Takemata et al. investigates the role of Smc and nucleoid-associated proteins in 3D chromosome architecture in archaea. The authors find Smc-dependent long-range DNA contacts throughout the T. kodakarensis chromosome, except across two boundary sites where such contacts are reduced. The major boundary is located at the dif site, with similar observations noted in other archaea. However, the boundary is largely unaffected by the removal of dif or XerA in T. kodakarensis. Instead, a NAP (TrmBL2) is shown to be crucial for Smc accumulation at the boundary site and for boundary formation. In addition to TrmBL2, DNA sequence features appear to contribute to boundary formation, likely due to altering DNA mechanics. Altogether, the work suggests that TrmBL2 bound to rigid/stretched DNA acts as a roadblock for Smc loop extrusion.

The study goes into impressive depth in its examination of T. kodakarensis chromosome organization (using various experimental approaches and simulation) and extrapolates some of the findings through additional experiments conducted in other archaea. The findings are striking and supported by convincing data. A minor drawback is the absence of an obvious phenotype and the lack of clarity on the physiological relevance of roadblocking/domain formation. Regardless, this is a strong study that appears essentially ready for publication. The following comments may be taken into consideration to further improve the manuscript:

Comments:

Is the dif-surrounding sequence sufficient for Smc roadblocking? Can the authors insert a second copy of this region at an ectopic location to test for roadblocking and to provide further support for the suggestion of bidirectional DNA motoring by Smc-ScpAB (by Hi-C)?

In response to this suggestion, we examined the sufficiency, and additionally the necessity, of the *dif*-surrounding intergenic sequence for boundary formation. We deleted the endogenous *dif*-containing intergenic region and/or inserted the sequence (but without *dif*) into an ectopic location. 3C-seq analysis using the constructed strains demonstrated that the *dif*-surrounding sequence is necessary and sufficient to form a boundary structure (please see lines 217-233 and Fig. 5 in the revised manuscript).

From these experiments, we have also obtained additional evidence for bidirectional DNA motoring by Smc-ScpAB. Since the insert was introduced outside the inverted segment, we were able to observe in detail how the ectopic boundary sequence formed long-range interactions that were not contaminated by the inversion described in Fig. 1. First, the ectopic boundary formed a stripe-type contact pattern as typically observed at strong cohesin-arresting sites in eukaryotes (Vian et al., *Cell*

2018). Second, the ectopic boundary engaged with boundary 2 to form a DNA loop. Although *T. kodakarensis* Smc-ScpAB needs to diffuse from the *dif*-associated boundary if the complex shapes the boundary as a unidirectional loop extruder, the observed stripe/loop formation suggests that *T. kodakarensis* Smc-ScpAB is rather stably retained at the boundary (please see lines 397-399 in the Discussion). We therefore believe that our additional experiments have provided further support for the bidirectional DNA motoring mechanism.

The main figures are highly accessible, but critical information might be lost to some readers by zooming in on selected genomic regions. Showing whole-genome Chip-seq and Hi-C profiles for selected experiments in some of the main figures might be useful. For example, the differential contact maps for smc, scpA… mutants to give the reader an indication of the overall impact of Smc activity.

We have moved most whole-genome ChIP-seq and 3C-seq profiles from Supplementary Figures to Main Figures. Please see Figs. 2a, 2b, 4a, 4b, 6d, 6e, 7a, and 7b in the revised manuscript.

In the manuscript, the authors prominently contrast the activity of Smc in domain formation and in sister chromosome resolution, while the two activities/functions may actually be identical. In T. kodakarensis, a chromosome domain is indeed visible in Hi-C maps, but the function of Smc might still be limited to supporting chromosome segregation. The appearance of a domain boundary might be an indirect consequence of removing Smc from the chromosome dimer resolution sites (to support chromosome segregation). With the currently available information, this option cannot be disregarded.

In response to this comment, we have suggested a speculative model where the archaeal *dif* boundary may promote chromosome segregation by serving as a decatenation hub that resolves DNA catenanes brought by loop-extruding Smc (lines 435-449 in the revised mauscript). This model is inspired by a recent study from Olivier Espéli's group (Colin et al., *NAR* 2022), where they suggest that *E. coli* MukBEF localizes DNA catenanes to the MukBEF-free *dif* zone possessing a dedicated decatenation system. We also note that bacterial Smc-ScpAB is implicated in minimizing sister DNA interconnections (Gruber et al., *Curr. Biol.* 2014). It is tempting to speculate that all of the three major SMC complexes in prokaryotes (archaeal Smc-ScpAB, bacterial Smc-ScpAB, and MukBEF) promote resolution and segregation of sister chromosomes despite their different roles in overall chromosome folding.

The fact that Smc is excluded from chr dimer resolution sites appears strikingly conserved in diverse prokaryotes. This is not well represented in the manuscript. The potentially related instance of MukBEF and MatP/matS appears entirely omitted from the manuscript. Additionally, suggestions/speculation on the putative function of Smc exclusion from these sites should be added. This is important,

particularly in the absence of an apparent phenotype and a clear understanding of the physiological relevance. Is it possible that Smc brings together the two dif sites on a chromosome dimer (by extrusion of an entire chromosome copy?) to promote chromosome dimer resolution?

We thank the reviewer for pointing out the missing but important information. According to this comment, we have described the common feature of the SMC flux in diverse prokaryotes—disfavoring SMC traversal across *dif*. Inspired by the reviewer's suggestion, we have also included a speculative model where two *dif* copies on a chromosome dimer are brought into proximity by loop extrusion due to the presence of the *dif*-associated boundary. Please see lines 414-420 and 428-434 in the revised manuscript for more detail.

The authors mention several times an apparently wrong suggestion put forward in previous work (lack of ScpB in archaeal Smc complexes). While clarifying potentially misleading literature is important, the dominant appearance in the introduction and the first paragraph of the discussion somewhat distracts from the more important findings of this manuscript and generates a bit of a negative connotation. The authors may consider reorganizing the manuscript accordingly.

We thank the reviewer for pointing out the unnecessary emphasis on the previous work (Jeon et al., *IUCrJ* 2020), whose suggestion of an archaeal binary Smc complex seems contradictory with our data at the moment. To focus on the more important findings as the reviewer suggested, we have revised the corresponding paragraphs in the Introduction and Discussion (lines 61-67 and 382-387, respectively).

Fig 1C: Can the authors perform single-cell PCR to distinguish between the two models presented in this figure.

We have been unsuccessful in amplifying the potential inversion breakpoints even in bulk PCR, possibly for the following reasons. First, since the breakpoints are located in highly homologous sequences of ~9 kb, a long DNA segment needs to be amplified to distinguish chromosome copies with and without the inversion. Second, PCR primers designed for the detection of either chromosome type also inevitably anneal to the other in a tandem orientation with perfect match. This annealing will strongly interfere with the intended PCR reaction. We are afraid that single-cell PCR detection of the inversion will be even harder and not feasible at the moment.

Since dif1 is not functional in T. acidophilum, it could be denoted in brackets in figure 3C and 3E.

According to the suggestion, *dif1* has been denoted in brackets in Figs. 3c-e in the revised manuscript.

Reviewer #2 (Remarks to the Author):

The article entitled 'Chromosomal domain formation by archeal SMC, a roadblock protein, and DNA structure' by Yamaura, Takemata and coll describes for the first time the interplay between the Smc-ScpAB complex and TrmBL2 and its role in genome architecture. This study reveals that in T. kodakarensis, Smc-ScpAB by interacting with TrmBL2 near the dif site generates a boundary. The authors propose that boundary formation is due to SMC complex stalling, and as a consequence, the chromosome of T. kodakarensis is organized into TAD-like structures. The study also correlates Smc-ScpAB stalling with AT rich DNA sequences and propose that this is linked to a specific DNA topology. Overall, the article is clearly written, findings are solid and highly interesting and represent a significant advance for the scientific community.
Below a few questions and suggestions to improve the clarity of the article.

1. The MS clearly shows that both Smc-ScpAB and TrmBL2 are needed to promote boundary formation near dif site. The authors claim that these factors, together with a specific DNA sequence and topology are involved in TAD like formation. However, TAD-like structures are not highly visible on their contact matrices. In some matrices, it seems that the color scale used is too stringent and long-range contacts are not clearly observed. Could this be why TAD-like structures are not evident in contact maps? Could the authors use a tool to identify these domains in WT and mutant strains? The presence of a boundary does not necessarily mean that a domain is formed (see Bignaud A et al, 2024).

A high-resolution Hi-C study done by Bignaud and colleagues showed that highly transcribed genes in *E. coli* form transcription-induced domains (TIDs), which are characterized by strong self-interactions separating the domains from neighboring loci. This TID insulation could explain the apparent formation of domain boundaries at highly transcribed genes at lower resolutions (Lioy et al., *Cell* 2018). However, as discussed below in our response to comment #2 from the same reviewer, our data suggest that boundaries 1 and 2 are different structural entities from TIDs. We therefore believe that boundaries 1 and 2 can be seen as canonical domain boundaries rather than TIDs.

To verify the presence of boundaries 1 and 2 using a bioinformatics tool as the reviewer suggested, we used the boundary-calling tool HiCDB (Chen et al., *NAR* 2018). This tool called both boundaries 1 and 2 from the parental strain (KU216) dataset, while it called neither of the two boundaries for Δ*smc* and only boundary 2 for Δ*trmBL2*. The results of the boundary calling has been included in the revised manuscript as Supplementary Table S1.

In summary, multiple lines of evidence support that boundaries 1 and 2 exist and are formed by SMC-dependent mechanisms.

2. Since high-resolution contact matrices were obtained, do the authors observe whether a specific folding pattern is associated with boundaries, similar to what was recently shown in bacteria? (Bignaud A et al, 2024)

The reviewer raises the possibility that boundaries 1 and 2 could correspond to TIDs recently described by Bignaud et al. TIDs are highly transcribed and display strong and dense short-range contact signals (called "bundled signals" in the authors). In the revised manuscript, we have shown that boundaries 1 and 2 are both transcriptionally inactive (Supplementary Fig. 2 in the revised manuscript). The two boundaries also do not display a bundle-like enrichment of local contacts (Supplementary Fig. 2 in the revised manuscript). Boundary 2 seems to be adjacent to a domain-like structure of ~5 kb, but this locus is not enriched for short-range interactions as remarkably as TIDs. Note that the contact maps shown in Supplementary Fig. 2 use a different color scale and a different color coding to focus on potential small differences in short-range interactions, which have made less visible the contact insulation across the boundaries. From these results, we conclude that boundaries 1 and 2 are different structural entities from TIDs.

3. In Figure S4B, the WT contact matrix seems to show sliding loops at boundary 1, that seems to disappear in the smc mutant. Do the authors also observe this at boundary 2 ? Are these sliding loops also lost in the other mutants?

Figure S4B in the first version of the manuscript (corresponding to Supplementary Fig. 5b in the revised manuscript) was generated from the published Hi-C data of *Haloferax volcanii* (Cockram et al., *Mol. Cell* 2021). The boundary shown in the figure is therefore not boundary 1.

4. Although it is clear that long-range contacts seem to be increased in Figure 2, I am not able to see the increase at 200 kb. Could the authors find a way to quantitative determine this increase in long range contacts?

To address this issue, we quantified the mean contact frequency for each genomic distance and plotted the log2 ratio of the mean values between the parental strain KU216 and derivative mutants (Supplementary Fig. 3c). The plots demonstrated that loss of either subunit of Smc-ScpAB led to a general increase in short-range interactions up to ~200 kb (more precisely ~150 kb). The description of the data is in lines 154-155 in the revised manuscript.

5. Figure 7E is highly complex, and the real added value of this panel (mainly for rows 5 to 17) is hard to get in the way this table is presented in the text (data presented on lines 307-336). Could the authors improve the clarity of the section? Are these correlations essential to reach the conclusions?

We thank the reviewer for pointing out the confusion that the figure caused. To improve the clarity, we have decided to only focus on the physical properties of DNA that are tightly related to A-tracts: persistence lengths and the width and electrostatic potential of minor groove. This change does not affect the conclusion of the paper. Please see lines 344-363 and Fig. 8e in the revised manuscript for more detail.

Minor points:
1. Through all the text, citations are always after a '.', they should be before.

We thank the reviewer for pointing out the misformatting. They have been corrected in the revised manuscript.

2. Could the authors replace Line 55: "…. and they instead use the loop extrusion activity to resolve replicated DNA molecules for chromosome…"
By "…and they instead use presumably the loop extrusion activity to resolve replicated DNA molecules for chromosome…."

The sentence has been modified as the reviewer suggested (line 56 in the revised manuscript).

3. Line 54: "Smc-ScpAB and MukBEF," should also mention MksBEF

We believe that, whereas the loop extrusion activities of bacterial Smc-ScpAB and MukBEF has been well documented (although still presumptive as the reviewer pointed out in the prior comment), that of MksBEF has been far less characterized. To maintain the stance that the loop extrusion model is still hypothetical in the Introduction (according to the suggestion by reviewer #3), and to avoid overgeneralization of the model from this standpoint, we would like to focus on Smc-ScpAB and MukBEF in this sentence.

4. Line 124 & 125: "The insulation of local contacts was much weaker at other loci, suggesting that boundaries 1 and 2 are the major boundaries on the T. kodakarensis genome'
Considering the polyploidy of this model, it would be safer to state "…boundaries 1 and 2 are the most frequent boundaries on the T. kodakarensis genome'

We believe that this comment refers to the potential structural heterogeneity between chromosome copies within the cell. It should also be noted that similar heterogeneity could exist even between chromosome copies in different cells, regardless of whether they are polyploid or not. To bring attention to these issues, we have added the sentence "However, given the ensemble nature of the 3C-seq contact matrix, it remains to be determined how frequently these boundaries are formed and

how stably they are maintained across individual chromosome copies and within individual cells." in lines 131-133 in the revised manuscript.

5. Line 214: deletion mutants of trmBL2 and TK0795 using KU21
For consistence, it would be better to write mcrB instead of TK0795

We apologize for the confusing use of the gene ID. We are afraid to treat TK0795 as *bona fide* McrB, because the molecular function of the TK0795 protein has never been characterized. In addition, TK0795 shares only weak homology with McrB (Fukui et al., *Genome Res.* 2005), and the AF2 database suggests that the TK0795 protein possesses a large N-terminal domain whose structure is quite dissimilar to those of characterized McrB homologs. In the revised manuscript, we have emphasized the possibility that TK0795 is not a functional homolog of McrB (lines 247-249).

6. Line 234: '… TrmBL2, in which two dimers of TrmBL2 interact with the DNA in a symmetric manner…'
Could the authors expand a bit more on why this do not fits well? Maybe a structure in supplementary data would be helpful.

We thank the reviewer for pointing out the vague description. A previous study showed that TrmBL2 binds DNA as a dimer-of-dimer whose overall structure is symmetric. This symmetry does not fit well with the assumed role of TrmBL2 as an asymmetric barrier for Smc-ScpAB. To clarify this point, we have revised the sentence as "However, this scenario does not fit well with the crystal structure of DNA-bound *Pyrococcus furiosus* TrmBL2, in which TrmBL2 forms a dimer-of-dimer whose overall structure is symmetric." The sentence is in lines 274-276 in the revised manuscript.

7. Line 255: 'The two values showed a good correlation on average'
Could the authors give the value of this correlation?

We have added the correlation coefficient in the main text (lines 297-298) and corresponding figure panel (Fig. 7j) in the revised manuscript.

8. Line 265: Authors might refer to Figure 6F?

By referring to the AT content profile in Figure 7A in the first version of the manuscript (now Fig. 8a in the revised manuscript), the corresponding sentence points to the high AT content of the intergenic region encompassing boundary 1. We have not discussed here the AT richness of the DNA motif found at TrmBL2/Smc-binding site as can be seen in Figure 6F in the first version of the manuscript (now Fig. 7h in the revised manuscript).

9. Considering the importance of the massage, maybe Figure S6 should be on main text while Figure 4 on Supplementary.

Figures S6A and B in the first version of the manuscript has been moved to Figs. 7a and b in the revised manuscript. Since Figure S6C in the first version describes essentially the same thing as Fig. 7c in the revised manuscript, we would like to keep it in Supplementary Fig.. We would also like to leave the 3C-seq data of Δ*dif* and Δ*xerA* (Fig. 4 in both the first and revised versions) in the main text because, although the data are essentially negative, clarification of this point is important given the concurrence of *dif* and boundaries in many archaea that is described in Fig. 3 in the revised manuscript.

Reviewer #3 (Remarks to the Author):

In this paper, the structure of chromosomes in the cells of one species of Archaea, Thermococcus kodakaraensis, was studied using the Hi-C method. Although Archaea are prokaryotes, they retain characteristics more closely related to eukaryotes than bacteria, making them extremely useful model organisms for elucidating the basic mechanisms of life. From this perspective, a small number of studies have been conducted on Archaea to understand the universal basic structure of eukaryotic chromosome architecture. The SMC complex is a fundamental building factor of chromosome structure and is common from prokaryotes to eukaryotes. However, it has been previously noted that they have different roles in chromosome structure in prokaryotes and eukaryotes. The SMC complex is conserved in Archaea, and the Hi-C method has been used to study chromosome structure. It was found that in the Archaea Halobacterium volcanii, chromosome domain boundaries are created by the SMC complex. Since the loop extrusion model with SMC complexes is currently being discussed as a hypothesis to explain the formation of chromosome domain boundaries, it is thought that if the SMC complexes in Archaea do indeed create chromosome domain boundaries, then the loop extrusion model can be applied to the SMC complexes in Archaea. However, Halobacterium volcanii lacks one of the components of the SMC complex, which differs from the SMC complex common to prokaryotes and eukaryotes, making it impossible to necessarily incorporate the loop extrusion model as is.

To overcome this, the authors used T. kodakaraensis, which has an SMC complex consisting of components common to prokaryotes and eukaryotes, and succeeded in showing the boundaries of chromosomal domains by the SMC complex. The authors also found a DNA-binding protein that is essential for the boundary of the chromosomal domain and reported new findings, including that the binding region is an AT-rich sequence, among others. Specifically, the boundary was formed at the dif-adjacent region. A pull-down experiment uncovered that TrmBL2, a nucleoid-associated protein, accumulated on the dif site. ChIP-seq analysis again showed that the TrmBL2 protein was enriched on the dif-adjacent boundary region. ChIP-seq data also provide evidence of the co-localization of TrmBL2

and Smc protein. A slight gap between the peaks of TrmBL2 and Smc at the boundary 1 region led the authors to consider the mechanism forming the boundary: loop-extruding Smc protein stalls at the TrmBL2 accumulated zone. This mechanism is proposed as analogous to the TAD formation in eukaryotic chromosomes, which is supported by the SMC protein stalling at the CTCF site. The AT-rich sequences, especially the A-tracts, are responsible for the TrmBL2-mediated Smc stalling. The stiffness caused by A-tracts prohibits the progression of the loop extrusion, resulting in stalling. A gene disruption strain of the SMC complex factor was created, and the Hi-C method showed changes in chromosome structure in the mutant, providing sufficient experimental support.

In summary, a novel boundary formation mechanism in prokaryotes is proposed by multidimensional analyses. The loop extrusion model is still under discussion, as is whether the molecular movements actually occur in the cell. However, in the results section, it is not necessary to proceed with the results in line with the loop extrusion model. It is more valuable for the research results to be more objective in describing them. It should be added, however, that this is not a question of discussing the loop extrusion model in the Discussion.

According to the reviewer's suggestion, we have made some revisions to emphasize that the loop extrusion model is currently prevalent but still hypothetical. We have also revised the result section to adopt a more descriptive tone, not presuming the loop extrusion model as a basic fact. For example, the word "stalling" has been rephrased as "positioning" to describe the TrmBL2-dependent enrichment of Smc at TrmBL2-binding sites, because "stalling" somewhat gives an impression of Smc as a loop extruder.

It is also strange that the possible conformations of the AT sequence are considered and discussed, but the conformational changes due to DNA melting are not investigated for the AT sequence as the Tm value. The structural changes due to DNA melting should be eliminated after the analysis is examined.

According to the comment, we estimated the T_m value of each TrmBL2/Smc-binding site of 200 bp using the nearest-neighbor method. The obtained T_m values and the Smc positioning efficiencies showed a Spearman correlation coefficient of –0.63 (Supplementary Fig. 8e in the revised manuscript). However, T_m and AT content showed an even stronger negative correlation with a Spearman correlation coefficient of –0.99, making it difficult to infer which of the two factors is more directly related to Smc positioning (lines 325-333 in the revised manuscript). Although the results do not completely rule out a potential role of ssDNA in Smc-ScpAB localization, we prefer the idea that other structural features of DNA play a more major role as discussed in the first version of the manuscript.

Although the Δsmc, ΔscpA, ΔscpB, Δsmc ΔscpA, and Δsmc ΔscpB mutants showed changes in

chromosome organization, the cell growth was almost as good as the wild-type cell. The presence of multiple chromosomes in T. kodakaraensis, which is a major difference from E. coli and Bacillus subtilis, is important in elucidating the biological significance of the multiple chromosomes and their relationship to chromosome structure. The relationship between the multiple chromosomes and chromosome structure is important in elucidating the biological significance of the Archaea SMC complex and its chromosome structure.

As the reviewer suggested, polyploidy may be key to understanding the physiological relevance of Smc-dependent chromosome organization in euryarchaea. Although future work is definitely needed to elucidate this conundrum, in the revised manuscript we have discussed a potential role of archaeal Smc-ScpAB in facilitating intra-molecular recombination for chromosome dimer resolution in the presence of multiple chromosome copies (lines 428-434).

In addition, growth is typically observed using optical density (OD), but the actual rate of cell division cannot be observed with OD. Note that it merely reflects an increase in cell volume. Therefore, it is better to show observations of the morphology of the cells themselves during proliferation. Furthermore, if T. kodakaraensis proliferates logarithmically, Figure 3A should be presented as a single logarithmic graph.

In response to the reviewer's comment, we compared the morphologies of unfixed cells for the parental strain (KU216) and derivative mutants (Δ*smc*, Δ*scpA*, and Δ*scpB*) under microscope (lines 170-174 and Supplementary Figs. 4c, d). All strains showed largely the same coccoid shape, although quantification revealed that the mutants had slightly larger cell areas. We additionally investigated the nucleoid distribution in the four strains by staining the DNA with Hoechst. Discernible differences, such as an increase in anucleate cells, were not observed in this experiment. These results corroborate that loss of Smc-ScpAB has only a minor impact on cell growth, at least in our experimental conditions.

We have also presented the growth curve data as single logarithmic graphs in the revised manuscript (Supplementary Figs. 4a and b in the revised manuscript). To comply with the editorial policy of Nature Portfolio, we have plotted individual data points instead of the mean values in the modified figure panel. Since plotting the data points from all strains in a single graph hiders their visibility, only the data points from the parental strain and one mutant are plotted per graph.

Other comments

In the case of eukaryotic SMC proteins, it is generally thought that they function as dimers of dimers. To my knowledge, each SMC protein stalls at individual CTCF sites, forming a TAD structure. Does the Smc-ScpAB complex in T. kodakaraensis also form dimers of dimers? If so, when one of the two Smc-

ScpAB complexes stalls at the TrmBL2 region, how does the other behave?

Currently, there is no experimental evidence that archaeal Smc-ScpAB forms a dimeric supercomplex. However, as mentioned in the first version of the manuscript, the study by Wang et al. suggested that bacterial Smc-ScpAB extrudes DNA loops as a dimeric supercomplexes (Wang et al., *Science* 2017). By performing time-course Hi-C experiments with an inducible Smc-ScpA loading system, they showed that Smc-ScpAB starts loop extrusion from a loading site while reeling in DNA segments on the left and right sides at the same constant rate of ~50 kb/min. Furthermore, extrusion of the DNA strand on one side can proceed at the same rate even if extrusion of the DNA strand on the other side is slowed down by a highly transcribed gene. From these data, Wang et al. proposed that two Smc-ScpAB rings form a handcuff-like structure in which each ring encircles the same DNA duplex and reels in the DNA independently but in the same direction. We surmise that *T. kodakarensis* Smc-ScpAB forms a similar dimer and, when one complex in the dimer is stalled by TrmBL2, the other will continue translocation as proposed for bacterial Smc-ScpAB. This idea is consistent with the observation of the stripe-type contact pattern extending from boundaries 1 and 2 (please see Figs. 1d, 1e, and 5d in the revised manuscript for more detail).

In Figure S2, the authors refer to all 5 mutants, including Δsmc, ΔscpA, ΔscpB, Δsmc ΔscpA, and Δsmc ΔscpB, as causing equivalent effects. Although I largely agree with this opinion, there seem to be slight differences in the Hi-C maps. Only when the Δsmc mutation is present can blue lines be seen at the position near 0.8 Mb. Is there a possibility that these differences reflect an ScpAB-independent function of the Smc core protein?

We thank the reviewer for carefully looking at the data. We surmise that this signal pattern is an artifact of the *smc* deletion, because the blue lines cross at a bin (genomic coordinate: 890,000-895,000) most part of which overlaps with the *smc* gene (genomic coordinate: 891,438-895,007). Since the length of the *smc* gene is close to the bin size, the *smc* deletion caused a substantial decrease in the number of reads mapped to the bin. Bins with extremely low read coverage often generate artifact signals in Hi-C/3C-seq analysis. We have indicated the potential artifact by asterisks in Fig. 2b and Supplementary Fig. 3b in the revised manuscript. They have also been mentioned in the corresponding figure legends.

In Figure S5B, an increment of diagonal interactions around the 0.5-2 Mb region is seen in the ΔtrmBL2 mutant. Does this correspond to the juxtaposition of chromosomes observed in Bacillus subtilis Smc-ScpAB?

We thank the reviewer for raising the interesting possibility. The blurred diagonal signal that the reviewer pointed out seems to emanate from the 1-1.5 Mb region. The total amount of Smc in this

region is apparently not increased in the Δ*trmBL2* mutant (Fig. 7a in the revised manuscript), suggesting that this phenomenon is mechanistically different from the Smc-mediated juxtaposition of the two chromosome arms in bacteria. To keep our focus on the boundary formation, we have not discussed this point in the revised manuscript.

Reviewer #1 (Remarks to the Author):

The authors have performed additional experiments and made changes to manuscript text and figures in response to the comments raised by the reviewers. Altogether, the manuscript has been significantly improved. In particular, the experiment using the ectopically inserted boundary is a great addition. It clearly shows that the corresponding sequence is not only necessary but also sufficient for boundary formation. The authors also added paragraphs discussing possibly biological functions of the boundary, which I find helpful and stimulating.
This is a great paper; it is ready for publication in my opinion.
The authors may want to consider including the wild-type maps in fig 5 for direct comparison with the three mutant scenarios.

We are deeply honored to receive the reviewer's compliment. According to the suggestion, we have added contact maps from wild type (KU216) to Figs. 5a and b.

Reviewer #2 (Remarks to the Author):

The revised version of the article 'Chromosomal domain formation by archeal SMC, a roadblock protein, and DNA structure' by Yamaura, Takemata and coll addresses all the points raised by this reviewer. Furthermore, the new experiments, where the dif surrounding region was inserted into an ectopic location, have provided valuable insights, further strengthening the article. Overall, the article is solid and I do not have additional comments. I congratulate the authors on the effort they have put into this work.

It is a great honor to receive the thoughtful compliment from the reviewer.

Reviewer #3 (Remarks to the Author):

The authors have responded in good faith to our earlier comments and have revised the manuscript and figures accordingly. These are sufficient to satisfy us.

We are pleased and honored to hear that our revisions and responses have addressed the earlier comments to the reviewer's satisfaction.