nature portfolio

Peer Review File

RIF1 regulates early replication timing in murine B cells

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Regulation replication timing is a critical for appropriate cell division and preservation of genomic integrity. The mechanisms that coordination of early versus late origin firing remain poorly understood. In this manuscript, the authors investigate the function of RIF1 in regulation of replication timing in activate B cells. They find that RIF1 controls early replicating regions and loss of RIF1 disrupts timing of these early replication sites to a greater extent that late replicating regions. This is a new and unexpected function of RIF1, which has been previously demonstrated to suppress late origins of replication in heterochromatin. The manuscript is well-written with well-controlled and complementary experiments. The authors do a very nice job distilling complex experiments and results into understandable and interpretable findings for readers. The results are novel and establish new insights into replication control in B cells as well as provide foundation for cell type-specific mechanisms for replication timing control.

A few minor comments:

1. In Figures 1 through 5, the green (Rif1-/-) and blue (WT) colors are difficult to distinguish in some of the figures, particularly in the UCSC browser tracings. More distinct color options would make it easier for readers to interpret results.

2. Line 183 on page 7, the text should reference Fig. 2G (rather than Fig. 2F).

3. Line 299 on page 9, text should reference Fig. 4A (rather than Fig. 4B).

4. Line 242-246, text states "The results showed that in WT cells, class 1 ISs were the most active (Fig. 4C)…whereas class 5 ISs were least active….(Fig. 4C-D)." It's not evident that Fig. 4C provides results on which ISs are most versus least active. The graphic under Fig. 4C-D delineates activity of ISs in WT versus Rif1-/- but the data in Fig 4C shows density of ISs in each class, which is not indicative of activity. Can the authors clarify this in the text?

5. In Figures 5-7, MCM6 has the dominant role in regulation of replication timing compared to RIF1. Loss of MCM6 has more significant impact on replication timing in all the assays. Loss of RIF1 in the MCM6-deficient cells does lead to additional changes supporting that the two proteins are complementary and non-epistatic. However, it should be stated/presented in these result sections that MCM6 has the dominant phenotype. The authors do address this in the Discussion but this information should also be included in the presentation and interpretation of the data in the Results.

Reviewer #2 (Remarks to the Author):

This manuscript investigates the role of Rif1 in activated B cells. The results presented are very unexpected, and potentially present a previously unknown function of Rif1.

Rif1 has been known to suppress the firing of replication origins by binding to chromatin in the vicinity of late-firing origins or in the late replicating regions. In this manuscript, authors show that Rif1 binds to early replicating domains and promotes early replication. The data supporting this conclusion appears to be robust and convincing. The data analyses have been conducted in a professional manner.

Major comments:
1 Looking at the data in Figure 1B, it appears that loss of Rif1 induces both E to L and L to E

conversions, resulting in RT with less dynamic range (toward flat RT), especially in primary B cells. This is similar to what has been observed in human iPS cells.

Does Rif1 binds selectively to early replicating domains in this chromosome region (chr2: 57,676,011-121,972,628)? It is known, in some cancer cells, that Rif1 binds to selective segments of chromosomes and RT of not all the chromosomes are regulated by Rif1. Do all the early replicating domains respond to Rif1 depletion by causing later replication? Or is there any segmental selectivity in RT regulation by Rif1 in activated B cells?

I see the transition of the late replicating domains to earlier-replicating in Rif1 -/- cells (Figure 1D). How is the Rif1 binding profile in those segments, where early replication is not affected or where late replication is converted to early by loss of Rif1?

2 In fission yeast Rif1 mutant, E to L transmission is observed at a number of early replicating regions (Hayano et al. 2012; Genes and Dev), and it has been explained that rate-limiting replication factors are utilized at activated late origins, and cannot be used for early origins, thus reducing origin firing at some early firing origins. A possibility remains that, due to activation of late origins which utilizes initiation factors, early origins cannot be fully activated due to the potential low abundance of initiation factors in activated B cells. This possibility needs to be discussed.

3 Authors state as follows (line 206). "Peaks in both cell types were enriched in early-replicating regions (Fig. 3C-D). However, although RADs in MEFs were mostly late-replicating, as described, in B cells, they were largely early-replicating (Fig. 3C-D)."
It is not intuitively clear that RADs (RIF1-associated domains) are late replicating, while Rif1 binding peaks are in early-replicating regions in MEFs. How are the peaks are enriched in the early-replicating regions, but Rif1 associates mainly in the late replicating regions?
Some more explanation is needed here for lay people to this field to be able to understand the points.

4 I suggest authors conduct several experiments described below to improve the manuscript before it is further considered for publication in NC.

4-1 Mechanistic insight into how Rif1 may directly regulate early firing origins is lacking in this manuscript.
It has been well established that Rif1's ability to recruit PP1 plays a major role in its ability to suppress origin firing. Authors need to examine the PP1 biding mutant of Rif1 in B cells to determine whether PP1 recruitment is required for early origin promotion in B cells. Authors also need to examine the phosphorylation states of MCM4 and Treslin, which are known to be enhanced in Rif1-depleted cells.

4-2 Figure 3E
The data in MEF needs to be shown in comparison.
H3K9 methyl pattern also needs to be shown.
It has been reported that Rif1 binding sites and H3K9me3 do not overlap.

4-3 Authors propose that Rif1 loss leads to reduced licensing, causing less efficient early origin firing. This possibility is testable by Mcm ChIP-seq, along with the data showing the extent of licensing in Mcm6 knockdown cells. This will also test the authors hypothesis that licensing is lost specifically in highly transcribed regions in the absence of Rif1.

4-4 Figure 5
The extent of Mcm6 depletion in both Rif1+ and Rif1-/- cells need to be shown by western analyses.

Minor comments:

It would be helpful for readers if authors clearly state, in each figure, which cells were used for analyses, primary B cells or CH12 cells.

Line 181; Fig2.E-F should be Fig2.E-G, Line 183; Fig2.F should be
Fig2.F-G, Line 191; Fig2.E-F should be Fig2.E-G,

Figure 1B, 2C, 5D, 6D
sMcm6 -> shMcm6

Fig.5C (lower panel)
It appears that Rif1-/- advances RT of mid-origins, consistent with other cell types. Authors need to
discuss this.

Fig. 1G
Y axis needs to be explained in the legend.

Fig. 3E
The correlation between Pro-seq peaks and RIF1-ChiP-seq peaks appears not to be very strong.
What is the extent of overlap between Pro-seq and Rif1-ChIP peaks?

Reviewer #3 (Remarks to the Author):

This manuscript describes the effect of Rif1 on DNA replication in murine B lymphocytes, and reports
the surprising finding that Rif1 associates most strongly with early-replicating chromosome domains,
and that removal of Rif1 delays the replication of these normally early regions.
Numerous previous studies have shown that Rif1 generally specifies late replication in both mouse and
human cells, at least partly by directing Protein Phosphatase 1 to dephosphorylate the MCM replicative
helicase and possibly other targets. In this study, the authors used the Repli-seq method to examine
replication timing (RT) in Rif1-/- mouse CH12 cells and in mouse primary B cells. In contrast to
previous findings in other cell lines and to the established model of its action, they find that Rif1 is
important for specifying the replication time of the early replicating regions. In some other cell lines
Rif1 has been implicated by 3C analyses in specifying chromosome architecture, but in mouse B cells
any effect on genome architecture is minor. In a particularly interesting and strong experiment, in Fig.
3 the authors use Repli-seq and ChIP-seq to show that in B cells Rif1 is specifically bound to early
domains, in complete contrast to the situation in MEFs where Rif1 associates with late domains as
previously reported in the literature. This surprising result certainly gives an entirely new view of how
Rif1 may affect DNA replication.
However, the authors' proposal that Rif1 directly regulates early origins is weaker: SNS-seq is a
controversial method and even accepting the data shown in Fig. 4 at face value, only a few of the very
strongest origins are affected. Nor is any data presented indicating that Rif1 interacts specifically or
directly with origin sites. Still, the interpretation of this SNS-seq experiment does not affect the main
conclusions of the paper concerning Rif1 association domains and effects on timing. The authors go on
to show that MCM and Rif1 act additively in B cells to affect early RT and early origin activity, with
MCM having a noticeably stronger effect. Perhaps surprisingly, even depleting both MCM and Rif1 does
not affect chromatin organisation. Finally, the authors make the interesting observation that in Rif1-/-
or shMcm6 B cells the regions whose replication timing is most compromised are those that show the
highest levels of transcription. Based on this last finding the authors provide an interesting Discussion
suggesting reasons why Rif1 might have been re-deployed in B cells to have this special effect on
replication, and relate their findings to an earlier study where human Rif1 was shown to stabilise Orc1
on chromatin to promote origin licensing. Overall, given the interesting new findings the paper is a
good candidate for Nature Communications. The following points should be addressed:

Major Points

1. In places the textual explanations are insufficient and/or opaque for the non-expert. In several

places more detailed explanation should be provided explaining the aspects on which the reader should focus (e.g., for Fig. 1C, Fig. 2E and Fig. 3E).

2. The Title and Abstract should specify that the study examines mouse cells.

3. Although it's clear that the major effect of Rif1 KO is on early regions, there is also some advancement on the replication time of late regions, seen for example in Fig. 1B and 1D. This effect, consistent with previous studies of Rif1, is overly downplayed in the main text.

4. Saddle plots in Fig. 2F-G seem to over-represent/exaggerate the changes in chromatin contacts that are seen in Fig. 2E. Therefore, Fig. 2F-G could be omitted.

5. The co-localisation of Rif1 peak intensity with transcription, chromatin accessibility and the H3K27ac mark is mild/weak in each case in Fig. 3E. This should be mentioned in the text.

6. Fig. 4B uses SNS-seq to identify sites of initiation, which is controversial as the technique may assign some sites of re-priming as origins of replication. Given this issue, the mildness of the effect in Fig. 4B, and the fact that Rif1 does not bind origin sites directly but rather early domains, the authors should alter their abstract to remove the words 'regulates early origin firing', and moderate their claims that Rif1 directly controls origin initiation.

7. The meaning of the vertical 'lines' observed through the centre of the DHS-seq and H3K36me3 experimental datasets in Fig. 4B is unclear. Could the authors elaborate on the reason for these low signal zones?

8. It would be helpful to move Fig. S4G into Fig. 5 to show the density-contour plots of Rif1-/- and Rif1-/- shMcm6 cells together with their effects on RT and chromatin architecture (using Hi-C).

9. The scatter plot in Fig. 6A regarding Rif1-/- shMcm6 cells is not accompanied by the same heatmaps as in Fig. 4B for Rif1-/- cells. This data should be included in Fig. 6.

10. Could the authors test whether the positive effect of Rif1 on early origin firing is dependent on PP1? This would further strengthen their model as outlined in the discussion section.

Minor Points

1. Often 'shMcm6' labelling on figures is mislabelled as 'sMcm6'. (e.g., Fig. 1B and Fig. 2C).

2. An experimental condition label (i.e., shMcm6 vs. shLacZ) on top of each panel would make it clearer what each experiment is showing (e.g., Fig. 1C).

3. Fig. S1A and S1C and Fig. S3A-B show the reproducibility of each Repli-seq, SNS-seq or ChIP-seq experiment, but are not particularly informative for the general reader and could be less prominent.

4. The authors could associate an 'Early' or 'Late' label with the positive or negative values in each figure where RT is examined (e.g., Fig. 1B). At least for this Reviewer, the graphs could be read more intuitively with the x-axis reversed so 'Early' is on the left and 'Late' on the right.

5. The blue and red colours used in Fig. 1F and 1G are confusing as they don't mean the same thing (i.e., one denotes RT and the other denotes an experimental condition e.g., shMcm6).

6. In Fig. 2C, given the fairly minor changes in RT that occur in Rif1-/- conditions (i.e. Early regions somewhat delayed, but still Early) we wouldn't expect much of a change in PC1 values. So, the conclusion drawn is perhaps not as surprising as the authors suggest and is somewhat overstated.

7. The lane order between Fig. 3C and Fig. 3D is changed, which is confusing when trying to compare RT and Rif1 localisation in the primary B and MEF cell lines.

8. The inclusion of the word 'decreasing' in the y-axis label of Fig. 3E is confusing. The authors should omit this word but keep the grey bar indicating peak intensity.

9. A couple of figures are mislabelled in the main text (e.g., Fig. 2F on page 7 should read Fig. 2G). Fig. 4A is not referred to in the main text.

10. The data in Fig. 4B is presented with the 'strongest' origins at the bottom, which is confusing, please invert these pile-up plots.

11. The black colour used in Fig. 6D to denote overlaps should be changed to be more translucent as it masks the data underneath it.

12. Fig. 7A can be omitted as Fig. 7B makes the point in a clearer and more intuitive manner.

13. The sentence in lines 385-388 is confusing and should be rewritten to clarify (do the authors mean to say 'earlier' rather than 'later' in line 388?)

Response to reviewers

We would like to thank the reviewers for their time, appreciation of our work and constructive comments on our manuscript. We are happy that all reviewers appreciated our new findings on the role of RIF1 in early RT in B cells. We have incorporated many of the suggestions from the reviewers which have considerably strengthened the manuscript. Please find below our point-by-point response to all comments in blue text. All new text in the main manuscript is also in blue text.

Reviewer #1 (Remarks to the Author):

Regulation replication timing is a critical for appropriate cell division and preservation of genomic integrity. The mechanisms that coordination of early versus late origin firing remain poorly understood. In this manuscript, the authors investigate the function of RIF1 in regulation of replication timing in activate B cells. They find that RIF1 controls early replicating regions and loss of RIF1 disrupts timing of these early replication sites to a greater extent that late replicating regions. This is a new and unexpected function of RIF1, which has been previously demonstrated to suppress late origins of replication in heterochromatin. The manuscript is well-written with well-controlled and complementary experiments. The authors do a very nice job distilling complex experiments and results into understandable and interpretable findings for readers. The results are novel and establish new insights into replication control in B cells as well as provide foundation for cell type-specific mechanisms for replication timing control.

A few minor comments:

1. In Figures 1 through 5, the green (Rif1-/-) and blue (WT) colors are difficult to distinguish in some of the figures, particularly in the UCSC browser tracings. More distinct color options would make it easier for readers to interpret results.

We have darkened the Rif1 track (from lighter to darker green) to make them more discernible from the blue WT and shLacZ tracks.

2. Line 183 on page 7, the text should reference Fig. 2G (rather than Fig. 2F).

Thank you, we have done it.

3. Line 299 on page 9, text should reference Fig. 4A (rather than Fig. 4B)

Thank you, we have changed it.

4. Line 242-246, text states "The results showed that in WT cells, class 1 ISs were the most active (Fig. 4C)…whereas class 5 ISs were least active….(Fig. 4C-D)." It's not evident that Fig. 4C provides results on which ISs are most versus least active. The graphic under Fig. 4C-D delineates activity of ISs in WT versus Rif1-/- but the data in Fig 4C shows density of ISs in each class, which is not indicative of activity. Can the authors clarify this in the text?

We apologize for the lack of clarity. By "density", we meant SNS-seq-derived read density (RPM) of the ISs, not the density of ISs. We have changed the axis label to "ISs (RPM)". The graphic under the figure shows the five classes based on fold-change of IS RPMs. Hence, class 1 ISs were most active in WT cells whereas class 5 were the least active in WT cells. We have changed the X axis to "Fold-change in IS read density (log2 WT/Rif1-/-)". We hope this is clear. We have also made the changes in the figure legends.

52
53 5. In Figures 5-7, MCM6 has the dominant role in regulation of replication timing compared to RIF1.
54 Loss of MCM6 has more significant impact on replication timing in all the assays. Loss of RIF1 in the
55 MCM6-deficient cells does lead to additional changes supporting that the two proteins are
56 complementary and non-epistatic. However, it should be stated/presented in these result sections that
57 MCM6 has the dominant phenotype. The authors do address this in the Discussion but this
58 information should also be included in the presentation and interpretation of the data in the Results.
59
60   We have made this conclusion more explicit in the results section.
61
62 **Reviewer #2 (Remarks to the Author):**
63
64 This manuscript investigates the role of Rif1 in activated B cells. The results presented are very
65 unexpected, and potentially present a previously unknown function of Rif1.
66
67 Rif1 has been known to suppress the firing of replication origins by binding to chromatin in the vicinity
68 of late-firing origins or in the late replicating regions. In this manuscript, authors show that Rif1 binds
69 to early replicating domains and promotes early replication. The data supporting this conclusion
70 appears to be robust and convincing. The data analyses have been conducted in a professional
71 manner.
72
73 Major comments:
74 1 Looking at the data in Figure 1B, it appears that loss of Rif1 induces both E to L and L to E
75 conversions, resulting in RT with less dynamic range (toward flat RT), especially in primary B cells.
76 This is similar to what has been observed in human iPS cells.
77 Does Rif1 binds selectively to early replicating domains in this chromosome region (chr2: 57,676,011-
78 121,972,628)? It is known, in some cancer cells, that Rif1 binds to selective segments of
79 chromosomes and RT of not all the chromosomes are regulated by Rif1. Do all the early replicating
80 domains respond to Rif1 depletion by causing later replication? Or is there any segmental selectivity
81 in RT regulation by Rif1 in activated B cells? I see the transition of the late replicating domains to
82 earlier-replicating in Rif1 -/- cells (Figure 1D). How is the Rif1 binding profile in those segments, where
83 early replication is not affected or where late replication is converted to early by loss of Rif1?
84
85   In B cells, RIF1 is present on all chromosomes indicating that there is no differential binding
86   between chromosomes. Please see the new analysis showing this (Fig. S3B-C).
87
88   Based on the scatter plots in Fig 1C for CH12 cells, most of the early bins show delayed replication
89   (note the red stripe consisting of early-replicating bins in Fig. 1C). The results are much more
90   striking in primary B cells (scatter plot in Fig. 1E) where all early-replicating bins show lower RT
91   values in $Rif1^{-/-}$ cells, implying that all early-replicating bins show later replication in the absence of
92   RIF1. In other words, there are almost no bins where early replication is not affected. This is also
93   apparent in Fig. 1I.
94
95   For the Rif1-/- primary B cells, there is indeed E to L and L to E, but in the heterozygous condition,
96   the E to L is very dominant (see also Fig. 1D). We infer from this that the E to L changes are the
97   direct effect of decreased RIF1 (an interpretation supported by the RIF1 ChIP-seq results) and the
98   L to E may be due to the indirect effect of having fewer early-firing origins in the full knockout that
99   leads to advanced firing of normally late origins. Accordingly, the size of the E to L shift in $Rif1^{-/-}$ is
100   stronger than the L to E shift (Fig. 1D and 1G).
101
102   Regarding RIF1 binding in late regions, our HMM analysis showed that L bins advanced their RT
103   but did not acquire positive RT values, but some L-like bins gained positive RT values (Fig. 1E).

104   When we looked at RIF1 binding in these bins, only ~10% of RIF1 sites were found in L-like bins
105   and even fewer in L bins (Fig. 3D).

106

107   2 In fission yeast Rif1 mutant, E to L transmission is observed at a number of early replicating regions
108   (Hayano et al. 2012; Genes and Dev), and it has been explained that rate-limiting replication factors
109   are utilized at activated late origins, and cannot be used for early origins, thus reducing origin firing at
110   some early firing origins. A possibility remains that, due to activation of late origins which utilizes
111   initiation factors, early origins cannot be fully activated due to the potential low abundance of initiation
112   factors in activated B cells. This possibility needs to be discussed.

113

114   Thank you. This is an important point and we have mentioned it in the revised Discussion (page
115   15).

116

117   3 Authors state as follows (line 206). "Peaks in both cell types were enriched in early-replicating
118   regions (Fig. 3C-D). However, although RADs in MEFs were mostly late-replicating, as described, in B
119   cells, they were largely early-replicating (Fig. 3C-D)." It is not intuitively clear that RADs (RIF1-
120   associated domains) are late replicating, while Rif1 binding peaks are in early-replicating regions in
121   MEFs. How are the peaks are enriched in the early-replicating regions, but Rif1 associates mainly in
122   the late replicating regions?
123   Some more explanation is needed here for lay people to this field to be able to understand the points.

124

125   We apologize for this lack of clarity, and we have explained it in the revised version (page 8).
126   Peaks reflect relatively stronger sites of RIF1 occupancy whereas RADs are larger domains where
127   RIF1 is more broadly distributed or where RIF1 peaks are clustered. Both peaks and RADs
128   constitute bona fide regions of RIF1 binding and are not defined based on location or RT, but
129   strictly using different bioinformatics tools for significant enrichment of signal over background.
130   Thus, although RIF1 is detected mostly as peaks in B cells (16,043 peaks and 289 RADs), in
131   principle, peaks or RADs could be located anywhere in the genome.

132

133   4 I suggest authors conduct several experiments described below to improve the manuscript before it
134   is further considered for publication in NC.

135

136   4-1 Mechanistic insight into how Rif1 may directly regulate early firing origins is lacking in this
137   manuscript.
138   It has been well established that Rif1's ability to recruit PP1 plays a major role in its ability to suppress
139   origin firing. Authors need to examine the PP1 biding mutant of Rif1 in B cells to determine whether
140   PP1 recruitment is required for early origin promotion in B cells. Authors also need to examine the
141   phosphorylation states of MCM4 and Treslin, which are known to be enhanced in Rif1-depleted cells.

142

143   We fully appreciate that the mechanism of RIF1 function in B cell RT is not addressed in this
144   study. As we have now discussed more clearly in the paper, the RIF1-PP1-mediated
145   dephosphorylation of MCM4 found in other cells, which is an inhibitory function as it suppresses
146   origin firing, cannot explain the RIF1-dependent firing of some early origins in B cells, which is an
147   activating function. It is more likely that the role of RIF1 at early origins, if PP1-dependent at all, is
148   to facilitate the firing or origins, perhaps akin to the PP2A and PP4-mediated dephosphorylation of
149   replication firing factors reported recently (Jenkinson et al. Mol. Cell 2023). However, we are
150   unaware of any positive function of PP1 in origin firing, and therefore, we do not have any
151   candidate protein whose dephosphorylation status we could test in $Rif1^{-/-}$ B cells.

152

153   However, addressing the potential role of RIF1-PP1 interaction in the regulation of early origin
154   firing is technically extremely complex and, even if successful, would take over a year of work.
155   RIF1 function is dosage-dependent, and therefore we cannot simply transfect a plasmid encoding
156   for $Rif1$-DPP1 (a RIF mutant deficient in PP1 binding) to rescue the $Rif1^{-/-}$ B cells. In the paper

157 from our co-author, Sara Buonomo (Gnan et al. Nature Comm. 2021), the authors had to: (1)
158 construct ES cells that carried one HA-tagged knock-in allele (*Rif1*-FH) and one floxed conditional
159 *Rif1* allele, and then target a mini-gene in the *Rif1*-FH allele to generate the DPP1 allele.
160 Moreover, they showed that *Rif1* hemizygotes already harbored some of the knock-out
161 phenotypes. In summary, to build a system so complex when we do not have candidate substrates
162 of PP1 to examine seems to us to go beyond the scope of this work.

164 Regarding Mcm4 phosphorylation, as found by our co-author, Sara Buonomo, there are no reliable
165 antibodies to detect phospho-Mcm4 in mouse cells. Importantly, however, please note that based
166 on the explanation above, it is unclear how a suppressive mechanism like the dephosphorylation
167 of Mcm4, even if present, would help to explain the positive regulation of origin firing by RIF1 in B
168 cells. Thus, we propose that the mechanism by which RIF1 regulates early RT is unlikely to be
169 linked to Mcm4-dephosphorylation. However, as mentioned above, it could still work via PP1-
170 mediated dephosphorylation of other replication proteins. We have mentioned this in the revised
171 Discussion.

173 4-2 Figure 3E
174 The data in MEF needs to be shown in comparison.

176 We have shown this in the revised version (new Fig. 3F). Thank you for pointing it out.

178 H3K9 methyl pattern also needs to be shown. It has been reported that Rif1 binding sites and
179 H3K9me3 do not overlap.

181 We have done the comparison with H3K9me3 ChIP-seq as requested (Fig. 3E-F).

183 4-3 Authors propose that Rif1 loss leads to reduced licensing, causing less efficient early origin firing.
184 This possibility is testable by Mcm ChIP-seq, along with the data showing the extent of licensing in
185 Mcm6 knockdown cells. This will also test the authors hypothesis that licensing is lost specifically in
186 highly transcribed regions in the absence of Rif1.

188 This is a good suggestion, but unfortunately, it is technically unfeasible. We have tried multiple
189 times with several antibodies, but Mcm ChIP-seq has never worked for us. We are aware that this
190 is a problem in the field and that many labs, including our co-authors, have struggled with it. We
191 believe that the problem, in large part, may be due to the underlying biology rather than the
192 antibodies. The current understanding in the field is that MCMs are loaded evenly throughout the
193 genome and can subsequently slide along the DNA without ATP. In this case, different cells have
194 different locations of MCMs at any given point of time, which would average out the signal in
195 population-based analyses like ChIP-seq resulting in poor enrichments over background.

197 4-4 Figure 5
198 The extent of Mcm6 depletion in both Rif1+ and Rif1-/- cells need to be shown by western analyses.

200 Please note that this was already shown in Fig. S1B and Fig. S4A.

202 Minor comments:

204 It would be helpful for readers if authors clearly state, in each figure, which cells were used for
205 analyses, primary B cells or CH12 cells.

207 We have now stated this in the figure legends for all figures,
208

209　Line 181; Fig2.E-F should be Fig2.E-G, Line 183; Fig2.F should be Fig2.F-G, Line 191; Fig2.E-F
210　should be Fig2.E-G,

211

212　　Thank you for pointing it out, we have made the changes.

213

214　Figure 1B, 2C, 5D, 6D sMcm6 -> shMcm6

215

216　　Thank you for pointing it out, we have corrected it.

217

218　Fig.5C (lower panel)
219　It appears that Rif1-/- advances RT of mid-origins, consistent with other cell types. Authors need to
220　discuss this.

221

222　　In this figure, we are comparing Mcm6 depletion in WT cells (shMcm6) to Mcm6 depletion in $Rif1^{-/-}$
223　　cells ($Rif1^{-/-}$ shMcm6). Hence, this analysis cannot be used to infer effects of Rif1. Rather, the
224　　advance is most likely the result of the exacerbation of the RT phenotype in $Rif1^{-/-}$ shMcm6 cells
225　　compared to shMcm6 alone and is reflected in the sign flips in RT values seen in Fig 5D. Please
226　　note that in Fig 1C, however, where we compare control and $Rif1^{-/-}$ cells, there is no major
227　　advance of mid-origins.

228

229　Lower panel is Fig. 1G Y axis needs to be explained in the legend.

230

231　　We have done so, thank you.

232

233　Fig. 3E, The correlation between Pro-seq peaks and RIF1-ChiP-seq peaks appears not to be very
234　strong. What is the extent of overlap between Pro-seq and Rif1-ChIP peaks?

235

236　　To address this point, also raised by another reviewer, we have provided a more detailed analysis
237　　of the RIF1 peaks in Fig. 3E-F and Fig. S3E.

238

239

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

241

242　This manuscript describes the effect of Rif1 on DNA replication in murine B lymphocytes, and reports
243　the surprising finding that Rif1 associates most strongly with early-replicating chromosome domains,
244　and that removal of Rif1 delays the replication of these normally early regions.
245　Numerous previous studies have shown that Rif1 generally specifies late replication in both mouse
246　and human cells, at least partly by directing Protein Phosphatase 1 to dephosphorylate the MCM
247　replicative helicase and possibly other targets. In this study, the authors used the Repli-seq method to
248　examine replication timing (RT) in Rif1-/- mouse CH12 cells and in mouse primary B cells. In contrast
249　to previous findings in other cell lines and to the established model of its action, they find that Rif1 is
250　important for specifying the replication time of the early replicating regions. In some other cell lines
251　Rif1 has been implicated by 3C analyses in specifying chromosome architecture, but in mouse B cells
252　any effect on genome architecture is minor. In a particularly interesting and strong experiment, in Fig.
253　3 the authors use Repli-seq and ChIP-seq to show that in B cells Rif1 is specifically bound to early
254　domains, in complete contrast to the situation in MEFs where Rif1 associates with late domains as
255　previously reported in the literature. This surprising result certainly gives an entirely new view of how
256　Rif1 may affect DNA replication.

257

258　However, the authors' proposal that Rif1 directly regulates early origins is weaker: SNS-seq is a
259　controversial method and even accepting the data shown in Fig. 4 at face value, only a few of the very
260　strongest origins are affected. Nor is any data presented indicating that Rif1 interacts specifically or

261 directly with origin sites. Still, the interpretation of this SNS-seq experiment does not affect the main
262 conclusions of the paper concerning Rif1 association domains and effects on timing. The authors go
263 on to show that MCM and Rif1 act additively in B cells to affect early RT and early origin activity, with
264 MCM having a noticeably stronger effect. Perhaps surprisingly, even depleting both MCM and Rif1
265 does not affect chromatin organisation. Finally, the authors make the interesting observation that in
266 Rif1-/- or shMcm6 B cells the regions whose replication timing is most compromised are those that
267 show the highest levels of transcription. Based on this last finding the authors provide an interesting
268 Discussion suggesting reasons why Rif1 might have been re-deployed in B cells to have this special
269 effect on replication, and relate their findings to an earlier study where human Rif1 was shown to
270 stabilise Orc1 on chromatin to promote origin licensing. Overall, given the interesting new findings the
271 paper is a good candidate for Nature Communications. The following points should be addressed:

272

273   We agree that RIF1 has a very weak effect on regulating origin activity. We have modified the text,
274   abstract and discussion accordingly. We also mention in the revised Discussion that SNS-seq
275   does not detect all origins, but probably only the stronger ones, meaning that there remains the
276   possibility that RIF1 may have a broader role in origin activity.

277

278 Major Points

279

280 1. In places the textual explanations are insufficient and/or opaque for the non-expert. In several
281 places more detailed explanation should be provided explaining the aspects on which the reader
282 should focus (e.g., for Fig. 1C, Fig. 2E and Fig. 3E).

283

284   We apologize for this. We have explained these in more detail in the revised version (see blue text
285   in pages 5, 7, 8 and 9).

286

287 2. The Title and Abstract should specify that the study examines mouse cells.

288

289   We have made changes to the title and abstract to specify the use of mouse cells.

290

291 3. Although it's clear that the major effect of Rif1 KO is on early regions, there is also some
292 advancement on the replication time of late regions, seen for example in Fig. 1B and 1D. This effect,
293 consistent with previous studies of Rif1, is overly downplayed in the main text.

294

295   We have explained this better in the revised version (pages 5-6), but in essence, we think that this
296   is an indirect effect of the decreased efficiency of early origins in the KO cells.

297

298 4. Saddle plots in Fig. 2F-G seem to over-represent/exaggerate the changes in chromatin contacts
299 that are seen in Fig. 2E. Therefore, Fig. 2F-G could be omitted.

300

301   We agree with the reviewer but given that such plots are widely used by the genome architecture
302   community to characterize changes in compartmentalization, we anticipate that several readers
303   would expect to see this, and therefore, we have retained it.

304

305 5. The co-localisation of Rif1 peak intensity with transcription, chromatin accessibility and the
306 H3K27ac mark is mild/weak in each case in Fig. 3E. This should be mentioned in the text.

307

308   We have provided a more detailed analysis of the heatmap in Fig. 3E using k-means clustering,
309   which better addresses the relationship of RIF1 peaks with transcription and chromatin features.

310

311 6. Fig. 4B uses SNS-seq to identify sites of initiation, which is controversial as the technique may
312 assign some sites of re-priming as origins of replication. Given this issue, the mildness of the effect in

313 Fig. 4B, and the fact that Rif1 does not bind origin sites directly but rather early domains, the authors
314 should alter their abstract to remove the words 'regulates early origin firing', and moderate their claims
315 that Rif1 directly controls origin initiation.
316
317   We have amended the abstract as requested and we have made it explicit in the revised text that
318   RIF1 only regulates ~5% of ISs and hence that it is not a major regulator of origin activity. See also
319   see our response in lines 273-276 above.
320
321 7. The meaning of the vertical 'lines' observed through the centre of the DHS-seq and H3K36me3
322 experimental datasets in Fig. 4B is unclear. Could the authors elaborate on the reason for these low
323 signal zones?
324
325   These low-signal regions are most likely the nucleosome-free regions where the ISs are most
326   abundant. This agrees with previous reports showing that ISs occur in the space between two
327   nucleosomes, which is also where OCCM complexes would reside.
328
329 8. It would be helpful to move Fig. S4G into Fig. 5 to show the density-contour plots of Rif1-/- and
330 Rif1-/- shMcm6 cells together with their effects on RT and chromatin architecture (using Hi-C).
331
332   We have done as requested.
333
334 9. The scatter plot in Fig. 6A regarding Rif1-/- shMcm6 cells is not accompanied by the same
335 heatmaps as in Fig. 4B for Rif1-/- cells. This data should be included in Fig. 6.
336
337   We have provided the heatmap as requested (new Fig, 6B).
338
339 10. Could the authors test whether the positive effect of Rif1 on early origin firing is dependent on
340 PP1? This would further strengthen their model as outlined in the discussion section.
341
342   Please see our explanation for reviewer 2, who also raised this point. We explain why testing the
343   requirement of PP1 is technically very challenging and would take well over a year, if successful.
344   More importantly, even if we were to find a PP1-dependency, we do not know of any candidate
345   RIF1-PP1 target of dephosphorylation in B cells, which is essential to address mechanistic
346   questions.
347
348 Minor Points
349
350 1. Often 'shMcm6' labelling on figures is mislabelled as 'sMcm6'. (e.g., Fig. 1B and Fig. 2C).
351
352   Thank you for pointing out, we have corrected this.
353
354 2. An experimental condition label (i.e., shMcm6 vs. shLacZ) on top of each panel would make it
355 clearer what each experiment is showing (e.g., Fig. 1C).
356
357   Please note that in all figures, the axes labels explicitly state the experimental condition.
358
359 3. Fig. S1A and S1C and Fig. S3A-B show the reproducibility of each Repli-seq, SNS-seq or ChIP-
360 seq experiment, but are not particularly informative for the general reader and could be less
361 prominent.
362
363   We agree that they may not be informative to general readers, but we know that there are many
364   who will want to see such analyses to judge the reproducibility for themselves. Indeed, many

365   papers have such analyses. Hence, we have retained it in the supplemental section where its
366   presence is not disruptive to the flow.
367
368 4. The authors could associate an 'Early' or 'Late' label with the positive or negative values in each
369 figure where RT is examined (e.g., Fig. 1B). At least for this Reviewer, the graphs could be read more
370 intuitively with the x-axis reversed so 'Early' is on the left and 'Late' on the right.
371
372   We generated these plots following the convention in the field (for example, all papers by our co-
373   authors and leading RT experts, D. Gilbert and S. Buonomo, use this layout for showing RT).
374   Hence, we have chosen to stick to this style.
375
376 5. The blue and red colours used in Fig. 1F and 1G are confusing as they don't mean the same thing
377 (i.e., one denotes RT and the other denotes an experimental condition e.g., shMcm6).
378
379   Thank you for raising this. We have changed the colors in Fig. 1F and 1H.
380
381 6. In Fig. 2C, given the fairly minor changes in RT that occur in Rif1-/- conditions (i.e. Early regions
382 somewhat delayed, but still Early) we wouldn't expect much of a change in PC1 values. So, the
383 conclusion drawn is perhaps not as surprising as the authors suggest and is somewhat overstated.
384
385   RT and genome organization are uncoupled, as we and others have shown earlier. Also, RIF1 was
386   shown to have more significant effects on genome organization in other cells. Given this
387   background, it was not predictable that loss of RIF1 in B cells would have the minor effects we
388   saw. In other words, it was theoretically possible that RIF1 could have had a more major effect on
389   genome compartmentalization even if the RT effect was small.
390
391 7. The lane order between Fig. 3C and Fig. 3D is changed, which is confusing when trying to compare
392 RT and Rif1 localisation in the primary B and MEF cell lines.
393
394   We have changed the order in Fig. 3C.
395
396 8. The inclusion of the word 'decreasing' in the y-axis label of Fig. 3E is confusing. The authors should
397 omit this word but keep the grey bar indicating peak intensity.
398
399   We have changed this heatmap with a new analysis of the same data, as mentioned above.
400
401 9. A couple of figures are mislabelled in the main text (e.g., Fig. 2F on page 7 should read Fig. 2G).
402 Fig. 4A is not referred to in the main text.
403
404   We have corrected this, thank you for pointing it out.
405
406 10. The data in Fig. 4B is presented with the 'strongest' origins at the bottom, which is confusing,
407 please invert these pile-up plots.
408
409   We have flipped the heatmaps as requested.
410
411 11. The black colour used in Fig. 6D to denote overlaps should be changed to be more translucent as
412 it masks the data underneath it.
413
414   As the reviewer correctly points out, the black proportion of the figure represents the overlapping
415   part of the signal of both tracks whereas the remaining color part shows the excess of one or the

416 other analyzed condition. Therefore, there is no underlying masked data within the black color
417 section (it simply represents the shared amount of the compared tracks).

418

419 12. Fig. 7A can be omitted as Fig. 7B makes the point in a clearer and more intuitive manner.

420

421 We understand the point, but we feel that Fig. 7A is very important to show how RT of the bins
422 within a given class are affected and to what degree, because all bins are shown separately and
423 the color change reflects the changes across the bins. This is not possible in Fig. 7B where all bins
424 are clubbed together. We do agree with the reviewer that Fig. 7A comes across as the more
425 intuitive analysis, but we feel that both representations are necessary for a comprehensive
426 analysis.

427

428 13. The sentence in lines 385-388 is confusing and should be rewritten to clarify (do the authors mean
429 to say 'earlier' rather than 'later' in line 388?)

430

431 Yes, indeed, we meant to say "earlier". Thank you for noticing it.

432

433

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors address all of this reviewer's comments/suggestions. The revised manuscript is well written and the findings will be important contribution to the field.

Reviewer #2 (Remarks to the Author):

Authors responded to most of the comments, but have decided not to conduct the experiments that were requested.

4-1 Mechanistic insight into how Rif1 may directly regulate early firing origins is lacking in this manuscript.
The authors state the reason for not conducting the Rif1-PP1 mutant experiments. The major excuse was the time required for construction of the mutant cells. I undestand the effort and time required for generation of the mutants. I believe that the effort is worth it to understand the mechanism of Rif1 mediated regulation of early replication in B cells and that the effect of PP1 mutation on B cell RT regulation would provide crucial information. However, in this case, I acknowledge the authors' rebuttal and would consent with authors' decision.

4-1 Regarding Mcm phosphorylation.
There are ways to detect the phosphorylation mediated by Cdc7, which would correlate with PP1 associated with Rif1. The followings are literatures detecting the phosphorylation of Mcm4 and Mcm2.

PMID: 28560864. Figure 2 Mcm4 is shifted upward (slow migration) by Cdc7 mediated phosphorylation.
PMID: 28273463. Figures 2 and 3 Mcm4 is shifted upward by Cdc7-mediated phosphorylation.
PMID: 25412417. Figure 5 shows that Mcm2 is shifted downward (fast migration) by Cdc7-mediated phosphorylation.
PMID: 34020950. Figure 4 Phosphorylated MCM2 at Ser40 (pMCM2) is used as Cdc7 phosphorylation target.
Basically, same patterns are observed for mice MCM as well.

Authors need to examine this by looking at MCM2 or MCM4. They could detect the phosphorylation of TMBP as well. The shift can be enhanced by using phosgel, if necessary. However, the shift should be visible on a regular gel.

S40,41 antibody of MCM2, can also be used as phosphorylated Cdc7 marker (PMID: 34020950).
If there is no enhancement of the phosphorylation in Rif1 depleted B cells, Rif1 may not interact with PP1 in B cells, which should also be examined.

4-3
"This is a good suggestion, but unfortunately, it is technically unfeasible. We have tried multiple times with several antibodies, but Mcm ChIP-seq has never worked for us."
Rif1 was previously reported to be required for efficient origin licensing (PMID: 28077461). Since authors propose that Rif1 loss leads to reduced licensing, causing less efficient early origin firing, I feel that this need to be tested. If the ChIP seq is difficult with currently available antibodies, authors could examine licensing by conducting immunostaining of Mcm after prewash with detergent and examine the effect of Rif1 depletion in B cells.
In both experiments described above, authors need to compare the results with a cell line that is known to undergo late to early changes of replication timing upon loss of Rif1.

Reviewer #3 (Remarks to the Author):

The authors have responded to the points raised and certainly adjusted the text and conclusions to take account of the major criticism that the original manuscript text was misleading given the mildness of any direct effect on early origins.

The additional k-means analysis of clustered data in Figures 3E-F is helpful, but in the case of the MEF data shown it is rather confusing whether the RIF1 peaks shown are within/correspond to the (generally late-replicating) RADs present in MEFS, particularly in light of the sentence "In agreement with the fact with most 254 RIF1 peaks in MEFs are in 255 early-replicating regions (Fig. 3C-D)…". So does this plot show analysis specifically of peaks that are not associated with the major RIF1 binding zones in MEFS?

It was disappointing that they dismissed many of the other suggestions that were aimed at clarifying the manuscript to make it more easily understood for the non-expert.

Response to Reviewer Comments

We thank the reviewers for their time and feedback. We have performed two new experiments to address Mcm4 phosphorylation in Rif1-KO B cells (new Fig. S1D) and whether RIF1 has a role in origin licensing in B cells (new Fig. S4B-C). We believe these new results have strengthened the paper by providing additional insights into the role of RIF1 in the B cell RT program. Please find below our point-by-point response in blue text. All new text in the main manuscript is in red.

Reviewer #1 (Remarks to the Author):

The authors address all of this reviewer's comments/suggestions. The revised manuscript is well written and the findings will be important contribution to the field.

Thank you for appreciating our work.

Reviewer #2 (Remarks to the Author):

Authors responded to most of the comments, but have decided not to conduct the experiments that were requested.

4-1 Mechanistic insight into how Rif1 may directly regulate early firing origins is lacking in this manuscript.

The authors state the reason for not conducting the Rif1-PP1 mutant experiments. The major excuse was the time required for construction of the mutant cells. I understand the effort and time required for generation of the mutants. I believe that the effort is worth it to understand the mechanism of Rif1 mediated regulation of early replication in B cells and that the effect of PP1 mutation on B cell RT regulation would provide crucial information. However, in this case, I acknowledge the authors' rebuttal and would consent with authors' decision.

Thank you for understanding this.

4-1 Regarding Mcm phosphorylation.

There are ways to detect the phosphorylation mediated by Cdc7, which would correlate with PP1 associated with Rif1. The followings are literatures detecting the phosphorylation of Mcm4 and Mcm2.

PMID: 28560864. Figure 2 Mcm4 is shifted upward (slow migration) by Cdc7 mediated phosphorylation. (MCM10A cells)

PMID: 28273463. Figures 2 and 3 Mcm4 is shifted upward by Cdc7-mediated phosphorylation. (Xenopus and HeLa)

PMID: 25412417. Figure 5 shows that Mcm2 is shifted downward (fast migration) by Cdc7-mediated phosphorylation. (both human cancer lines).

PMID: 34020950. Figure 4 Phosphorylated MCM2 at Ser40 (pMCM2) is used as Cdc7 phosphorylation target. (looks like all cancer cell lines).

Basically, same patterns are observed for mice MCM as well.

Authors need to examine this by looking at MCM2 or MCM4. They could detect the phosphorylation of TMBP as well. The shift can be enhanced by using phosgel, if necessary. However, the shift should be visible on a regular gel.

S40,41 antibody of MCM2, can also be used as phosphorylated Cdc7 marker (PMID: 34020950).

42 If there is no enhancement of the phosphorylation in Rif1 depleted B cells, Rif1 may not interact with
43 PP1 in B cells, which should also be examined.

44 As requested, we have probed for MCM4 phosphorylation in the chromatin fraction as done in
45 previous studies, and we found that there is indeed a slower-migrating population in Rif1-KO cells
46 but not in WT (new Fig. S1D). Moreover, this shift is seen in WT cells when the PP1 inhibitor.
47 Tautomycin is added. Finally, the RIF-PP1 interaction has been recently reported in murine B cells
48 (PMID: 35416772). These observations suggest that the absence of the late replication phenotype
49 in B cells is not associated with deficient MCM4 dephosphorylation via RIF1-PP1. Thank you for
50 suggesting this experiment.

51 4-3

52 "This is a good suggestion, but unfortunately, it is technically unfeasible. We have tried multiple times
53 with several antibodies, but Mcm ChIP-seq has never worked for us."

54 Rif1 was previously reported to be required for efficient origin licensing (PMID: 28077461). Since
55 authors propose that Rif1 loss leads to reduced licensing, causing less efficient early origin firing, I
56 feel that this need to be tested. If the ChIP seq is difficult with currently available antibodies, authors
57 could examine licensing by conducting immunostaining of Mcm after prewash with detergent and
58 examine the effect of Rif1 depletion in B cells.

59 In both experiments described above, authors need to compare the results with a cell line that is
60 known to undergo late to early changes of replication timing upon loss of Rif1.

61 To address this question, we have performed ChIP-qPCR for MCM5 using an antibody which we
62 have previously shown to work for ChIP-qPCR (but not ChIP-seq) (PMID: 36108018). Since only
63 5% of ISs show >1.5-fold change in activity in Rif1-KO cells, we selected five of the most
64 downregulated ISs and compared them with four unchanged ISs. We found that the
65 downregulated ISs had decreased MCM5 occupancy but that the unchanged ISs did not show any
66 change in MCM5 association (new Fig. S4B-C). Therefore, although RIF1 does not regulate
67 replication origin activity at the vast majority of origins in B cells, a small subset of origins requires
68 RIF1 for optimal firing efficiency. Thank you for prompting this experiment. It is a useful addition to
69 the study.

70 In the experiment discussed in response to the previous comment (lines 44-50; new Fig. S1D), we
71 find that the bulk levels of chromatin-associated MCM4 are not changed in Rif1-KO B cells,
72 arguing against a global licensing defect. This result is consistent with the SNS-seq data where
73 only 5% of origins show >1.5-fold changes in activity. Please note that although the study
74 mentioned by the reviewer (PMID: 28077461) did implicate RIF1 in licensing in 293 cells, there are
75 other reports, in addition to our present one, that did not observe such a relationship. For example,
76 studies from our co-author have shown that MCM3 levels were not changed on chromatin by
77 western blot in Rif1-KO MEFs (PMID: 22850673) or by immunofluorescence in Rif1-KO ES cells
78 (PMID: 34006872). Studies in RIF1-depleted HeLa cells (PMID: 28273463 and 22850674) also did
79 not report changes in MCM or ORC in the chromatin fraction by western blot. Thus, the effect of
80 RIF1 on global licensing is not generalizable across different systems and may be cell type-
81 dependent.

82

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

84 The authors have responded to the points raised and certainly adjusted the text and conclusions to
85 take account of the major criticism that the original manuscript text was misleading given the mildness
86 of any direct effect on early origins.

87 The additional k-means analysis of clustered data in Figures 3E-F is helpful, but in the case of the
88 MEF data shown it is rather confusing whether the RIF1 peaks shown are within/correspond to the
89 (generally late-replicating) RADs present in MEFS, particularly in light of the sentence "In agreement
90 with the fact with most RIF1 peaks in MEFs are in early-replicating regions (Fig. 3C-D)…". So does

91   this plot show analysis specifically of peaks that are not associated with the major RIF1 binding zones
92   in MEFS?

93   It was disappointing that they dismissed many of the other suggestions that were aimed at clarifying
94   the manuscript to make it more easily understood for the non-expert.

95   We apologize for the confusion, and we have clarified this in the revised manuscript. For the
96   analysis of peaks in Fig. 3C-F, we only define peaks as those that fall outside of RADs. If a peak
97   was called inside a RAD, it is considered as part of that RAD. In other words, peaks and RADs are
98   distinct, non-overlapping regions in our analyses. We have added this explanation to the revised
99   manuscript. In MEFs, the peaks are mostly early-replicating and RADs are mostly late-replicating.
100   Importantly, both peaks and RADs are bona fide RIF1 binding regions, but their distribution in the
101   genome is different between MEFs and B cells.

102   Regarding other suggestions, we believed we had addressed all the points made by the reviewer.
103   For example, we had provided more details for Fig.1C, Fig. 2E and Fig. 3E, as explicitly mentioned
104   by the reviewer (please see major point 1 from the reviewer in the first review). We went back and
105   checked all our responses, but we did not find anything that was not responded to.

106   The reviewer had asked us to remove some analyses, such as saddle plots and correlation
107   matrices, but we had also explained why these analyses were retained, namely, because they are
108   widely used and would be expected by many readers. We hope the reviewer understands these
109   points.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

Authors responsded to my comments by conducting additional experiments.
In Figure S4, authors showed significant decrease in MCM5
enrichment at all the five downregulated ISs, but not at the unchanged ISs. However, this has not
been discussed much in terms of effect of Rif1 on early RT.

Although only ~5% of ISs showed >1.5-fold change in read density upon loss of RIF, does this result
suggest that Rif1 does affect licensing in activated B cells? And could that be a part of the reason for
shift to later replication of early RT in Rif1 KO cells? The culmitative effects of Rif1KO on licensing over
the entire genome, whilst the effect at each origin may be small, could affect early replication, since
reduced (or delayed) licensing in G1 phase could lead to late firing.

I wish authors conducted MCM5 ChIP-seq and examine the correlation between MCM5 binding and Rif1
binding (as well as RT), which would provide more information on potential effect of Rif1 on licensing
efficiency and its relation to RT change. (I understand from the letter that the current MCM5 antibody
does not work for ChIP seq.)

At least authors could discuss the above possibility in the text.

Figure 5D legend
A magnified view of the boxed region in C.
->
There is no box in Figure 5C.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

Authors responsded to my comments by conducting additional experiments. In Figure S4, authors showed significant decrease in MCM5 enrichment at all the five downregulated ISs, but not at the unchanged ISs. However, this has not been discussed much in terms of effect of Rif1 on early RT.

Although only ~5% of ISs showed >1.5-fold change in read density upon loss of RIF, does this result suggest that Rif1 does affect licensing in activated B cells? And could that be a part of the reason for shift to later replication of early RT in Rif1 KO cells? The culmitative effects of Rif1KO on licensing over the entire genome, whilst the effect at each origin may be small, could affect early replication, since reduced (or delayed) licensing in G1 phase could lead to late firing.

I wish authors conducted MCM5 ChIP-seq and examine the correlation between MCM5 binding and Rif1 binding (as well as RT), which would provide more information on potential effect of Rif1 on licensing efficiency and its relation to RT change. (I understand from the letter that the current MCM5 antibody does not work for ChIP seq.)

At least authors could discuss the above possibility in the text.

> Please note that in the Discussion, we had mentioned the possibility that RIF1 may play a broader role in early origin licensing given that SNS-seq may not identify weak or infrequently used origins. However, we based our main conclusion on the ISs that we can detect (Fig. 4a-b). Here, it is apparent that the vast majority of origins do not show major changes in activity. For this reason, we concluded that RIF1 is not a major contributor to origin licensing in B cells. We have now added an additional line in the Discussion acknowledging the possibility that if RIF1 were to regulate more early origins that those detected by SNS-seq, then the early replication phenotype could be, in part, the result of decreased RIF1-mediated early origin licensing. Thank you for suggesting this.

Figure 5D legend
A magnified view of the boxed region in C.
-> There is no box in Figure 5C.

> We have corrected this. Thank you for pointing it out.