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Rif1 regulates the replication timing domains on the human genome

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 07 February 2012

We have now heard back from the three expert that had agreed to review your study on human Rif1 and replication timing. As you will see from their reports copied below, all appreciate the novelty and potential importance of these findings. However, they also indicate that several of the main conclusions remain insufficiently supported by the current set of data, and also ask for a somewhat deeper investigation into some aspects of Rif1 function.

Given the overall interest in the topic, I would like to give you an opportunity to address these concerns in the form of a revised version of the manuscript. Nevertheless, I should make it clear that substantial further efforts may be required in order for this revision to be successful. In particular, it will be important to better define the link between Rif1 and licensing (see Ref 1 point 3, Ref 2 point 1), and the effect of Rif1 depletion on replication origins (see Ref 2 point 5, Ref 3 point 1). All three referees further request more precise definition of the BrdU foci pattern variation during S-phase, and better description of the DNA fiber experiments. Referee 3 (point 2) also asks for determination of genomic Rif1 binding sites in order to better understand its molecular function; while such analyses may not be absolutely essential within the scope of the current manuscript, it is clear that any such data you could contribute to address this aspect would greatly increase the impact of your present findings.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Therefore, please do not hesitate to get back to me should you have any question regarding the referee requests and/or the revision requirements.

We generally allow three months as standard revision time. As a matter of policy, competing

manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript reports a series of interesting and potentially important observations suggesting that altered levels of Rif1, an ortholog of the yeast telomere binding protein, affect the spatial distribution of replication foci and alters replication timing concomitant with affecting the global organization of chromatin loops.

These studies suggest that when Rif1 is depleted, the spatial distribution of replication foci in mid-S-phase resembles that of replication foci typically observed in early S-phase. Direct evidence for changes in replication timing is shown for one region of chromosome 5. For that chromosome, Rif1 depletion was shown to change the temporal order of replication, delaying replication of some regions and advances replication of another region. Unlike in yeast, the studies reported in the current submission do not seem to indicate that the changes in replication timing are restricted to or more prominent in subtelomeric regions. This is consistent with evidence suggesting that the human Rif1 does not bind to telomeres.

The observations reported in the current submission are novel and represent an important contribution to the literature. Some minor concerns about the data are listed below. One critical issue is that the abstract and some parts of the paper as currently written might be interpreted to suggest that the studies reported in the paper have directly tested the involvement on Rif1 in establishment of replication licensing during the timing decision point. Although the conclusion that Rif1 is involved in replication licensing is plausible, there are alternative explanations and the paper (especially the abstract) should be rewritten to clarify that licensing and the TDP were not directly tested.

Critique and suggestions

1/The evidence for altered replication timing is solid and well controlled and is consistent with a change in chromatin loop size. As briefly discussed in the current submission, replication timing correlates with chromatin accessibility on a global scale. Hence, it is possible that the changes in replication timing reflect altered chromatin condensation. Does the pattern of DNA sequences that exhibit DNAse sensitivity on the region tested on chromosome 5 reflect the changes in replication timing?

2/ The halo experiments are interesting and suggestive of a global change in chromatin structure. However, wouldn't one expect a more prominent change in replication timing than that observed on

chromosome 5, in which most replication domains seem similar in Rif depleted cells and unaltered cells? This question should be discussed.

3/ As mentioned above, the abstract should report the actual observations described in the paper. As currently written, the abstract suggests that Rif1 affects the licensing process at the timing decision point although no direct examination of the TDP was reported in the paper. This statement might be misleading and should be corrected.

4/ Figure S1 demonstrates a notable increase in DNA synthesis accompanying changes in replication timing. Is this observation reproducible and can the authors suggest an underlying mechanism?

5/ Related to the above, it is important to evaluate how transfection might affect the response of cells to thymidine-block associated synchronization. This issue might affect the conclusion regarding the involvement of cell cycle checkpoints in the response to chromatin decondensation and should be discussed.

6/ Figure 1D: Rif1 depletion seems to decrease overall MCM4 levels while increasing phosphorylation of MCM4. Is this reproducible?

7/ The image shown to illustrate Mid-late-S patterns of BrdU foci in Figure 2 does not seem to represent typical mid-S patterns. Is this a consistent observation in the cells used for the study, or do the authors typically observe a distribution similar to the pattern shown in Figure 4E?

Minor suggestions

1/ The abstract should mention the currently known function of Rif1 (at least, mention that it is a telomere associated protein) for the benefit of a wide audience.

2/ The legend to Figure 2: line 8 - do the authors mean (B) instead of (C)?

Referee #2 (Remarks to the Author):

Factors that determine the replication program of eukaryotic genomes are largely elusive. In the article in press accompanying this submission, Hayano et al show that in S. pombe loss of the rif1+ gene restores the growth of a null mutant of the Cdc7 kinase involved in activation of prereplication complexes (preRCs) during S phase and that the Rif1 protein binds chromatin close to some replication origins and affects the firing time of several (though not all) early and late origins both negatively and positively.
In this submission, Yamazaki et al explore a potential role for the human Rif 1 protein in regulating replication timing in the human genome. The authors report that siRNA depletion of Rif1 in HeLa cells (i) does not affect S phase progression but somewhat increases BrdU incorporation early in S phase; (ii) increases the phosphorylation level of MCM proteins in a Cdc7-dependent manner and the subsequent chromatin binding of Cdc45 and PCNA specifically during early S phase; (iii) leads to the disappearance of the mid-S phase pattern of BrdU and PCNA foci with extended persistence of the early-S pattern and unchanged advent of the late-S phase pattern; (iv) affects the replication timing profile of a 42 Mb region of human chromosome V in an ATR-independent manner, with regions 2-4 Mb in length replicating either earlier or later than normal; (v) increases the size of DNA loops measured by the nuclear halo assay and inter-origin distances measured on spread DNA fibers. They further report that Rif1 (vi) is present throughout interphase in insoluble, DNaseI-resistant nuclear structures, to which a large fraction of Lamin B1 also binds; (vii) dissociates from chromosomes in mitotic prophase to rebind in late telophase; (viii) colocalizes with foci of EdU incorporation during mid- but not early- or late-S phase.
Overall, the experimental data shown in the paper are of good quality and do suggest a role for Rif1 in regulating the structure and replication time of specific chromosomal domains in human cells, which is an important and novel finding. However, several points remain insufficiently investigated and/or need clarification.

1. The authors report as an unpublished observation that Cdc7 kinase activity is unaffected when Rif1 is depleted. This suggests that the increased phosphorylation of MCM proteins observed in

early S phase when Rif1 is depleted results from increased accessibility of preRCs to Cdc7 kinase, a potentially important insight on the mechanism by which Rif1 may regulate replication time. I think this claim should be substantiated by the presentation of appropriate data.

2. Hayano et al have shown that in S. pombe growth of hsk1-null cells can be rescued by insertion mutagenesis of the rif1 gene. Since the authors can simultaneously deplete Cdc7 and Rif1 have they observed a similar rescue of S phase progression in human cells ?

3. I am not convinced that the replication timing profiles of the 42 Mb region of chromosome V in control and Rif1-depleted cells clearly support the interpretation proposed by the authors, that the observed changes are as expected if mid-S phase domains become early and replicate over a somewhat broadened time window. First, both early-to-late and late-to-early changes are observed (Figure 3B and S4), as reported in S. pombe. Second, there is no quantitation of replication time dispersion and therefore no support for homogeneization by abolition of distinct timing patterns. First, it is crucial to show the sorting gates chosen to isolate early and late-S phase cells as well as a post-FACS control showing the actual DNA content distribution of the two selected cell populations. Second, the authors should show a histogram of replication timing ratios in control and Rif1-depleted cells as well as a histogram of replication timing changes by 100 kb windows for the entire 42 Mb region as well as for sorted early-, mid- and late-replicating windows. If the authors' interpretation is correct, (i) the Rif1 histogram of replication timing ratios should be shifted toward high early-to-late ratios with respect to the control, (ii) the mid-S windows should show much larger changes than the early- or late-S windows, and (iii) these changes should be predominantly toward earlier replication.

4. Figure 4E clearly shows that Rif1 localizes at the nuclear and nucleolar periphery at the time these regions replicate (in mid-S phase), but whether Rif1 is similarly located at other times in S phase is not so obvious from the only two early and late-S phase nuclei shown. Could it be that the location of Rif1 is actually more dynamic that the authors suggest, and that it coincides with mid-S phase foci only when these sequences replicate ?

5. The most striking effect of Rif1 depletion shown by DNA fibre-FISH (Figure S8), aside from the mentioned (but rather modest) increase in interorigin distances, is an almost 2-fold increase in replication fork speed. Obviously, this observation is quite important to correctly interpret the role of Rif1 in the replication timing program, but this is not even mentioned nor commented in the main text. Does this increase in fork speed occur throughout the genome or only at specific DNA regions, and if so are these the same regions that show replication timing changes ? Since this increase is not fully compensated by a similar increase in interorigin distances, why does not this accelerate S phase progression ? Couldn't this explain at least some of the late-to-early shifts in replication time ?

6. Measurement of interorigin distances on spread DNA fibres can be strongly biased by finite fibre size and varying fibre stretching. It is important to show whether fiber size distributions and fiber extensions measured by FISH with the human BAC clone are comparable in the control and the Rif1 depleted cells.

Referee #3 (Remarks to the Author):

Yamazaki et al. report on the role of Rif1 in the regulation of the replication programme in human cells. This work is closely related to recent publications showing that the absence of the Rif1 orthologue in Saccharomyces cerevisiae and in Schizosaccharomyces pombe modifies the timing and efficiency of replication origins and the genomic replication pattern. The results in this manuscript support the notion that Rif1 is also required for the same function in human cells, although the level of resolution is significantly lower than in the studies on yeasts.
In my opinion, these results are potentially interesting but I find them somewhat preliminary at this stage.

Specific comments:

1. One important point not addressed is whether the specification, activity or timing of relication origins is modified in the absence of Rif1. The increase in the phosphorylation of MCM2 and

MCM4 and the enhanced binding of Cdc45 and PCNA to chromatin in the early S phase caused by depletion of Rif1 (Figure 1) and the small difference in thymidine incorporation at the beginning of the S phase (Suppl. Figure 1) are not sufficient to support the title of the first section of the Results "Depletion of human Rif1 protein leads to increased initiation events at the early S phase".

2. Given the tight association between Rif1 and insoluble, DNAse I-resistant nuclear structures, it would be important to determine where Rif1 binds along the genome (by ChIP/chip, for example) to address what is the link between the changes in the replication profile (Figure 3), the sites of Rif1 binding and the sites of binding of other proteins involved in the organization of chromatin loops. This would complement the results based on the nuclear halo assay (Figure 5), which is suggestive but of low resolution.

3. I am surprised that the DNA fiber analysis of inter-origin distances and fork rates is only briefly commented (page 9) and shown in Suppl. Figure 8. This is an important assay and the results, along with some picture of the original fiber hybridizations, should be integrated and discussed in the main text in the context of the pattern of replication foci and the length of the S phase in the absence of Rif1.

4. Figure 2A should also include the BrdU and PCNA focus patterns in Rif1-depleted cells for direct comparison with the control nuclei. The authors do not explain why the pattern of BrdU foci is divided into four periods across the 8 hours of the S phase while the pattern of PCNA foci is split into 3 periods across only 6 hours.
Figure 2B shows that a very large fraction of Rif1-depleted cells maintain the early BrdU focus pattern during the entire S phase, indicative of a continuous low rate of DNA synthesis. How can this pattern be reconciled with the fact that the length of the S phase is the same as in the control cells, where the intensity of BrdU foci during early-mid and mid-late S phase indicates a more active BrdU incorporation?

5. Perhaps the previous point could be clarified by determining the rate of 3H-thymidine incorporation per unit of time at specific times during the S phase in cells with and without Rif1.

6. Are the replication profiles in Figure 3B the average of the two biological duplicates? Although different cell types could have different replication profiles, it would be interesting (although not essential) to hybridize the same microarray in Figure 3A with early- and late-replicated DNA from the NHDF dermal fibroblast cell line tested in Figure 1D in order to monitor the effect of Rif1 in an independent system that is probably closer to normal human cells than HeLa cells.

1st Revision - authors' response 04 May 2012

Title: **Rif1 regulates the replication timing domains on the human genome**
Authors: Satoshi Yamazaki, Ai Ishii, Yutaka Kanoh, Masako Oda, Yasumasa Nishito and Hisao Masai

Thank you very much for your letter of February 7 on our above manuscript. We have carefully studied the comments from you and the referees and conducted a series of experiments to address the issues raised by them. On the basis of the new results, we have revised the manuscript, as explained in detail below.

The revised manuscript contains six figures and fourteen supplementary figures. Among them, Figure 2, Supplementary Figure S1, S4, S5, S6, S8, S11, S13 and S14 contain new data.

In the following, our responses are in green. In the revised manuscript, the modified or added texts are shown in red.

Editor comments.
In particular, it will be important to better define the link between Rif1 and licensing (see Ref 1 point 3, Ref 2 point 1), and the effect of Rif1 depletion on replication origins (see Ref 2 point 5, Ref 3 point 1). All three referees further request more precise definition of the BrdU foci pattern variation during S-phase, and better description of the DNA fiber experiments. Referee 3 (point 2) also asks for determination of genomic Rif1 binding sites in order to better understand its molecular function; while such analyses may not be absolutely essential within the scope of the current manuscript, it is clear that any such data you could contribute to address this aspect would greatly increase the impact of your present findings

In response to your comments above—
1 We have presented new data showing that licensing (chromatin loading of Mcm) is not affected by depletion of Rif1.
2 We have examined replication from the selected replication origins and showed that mid~lateS origins are activated early in Rif1-depleted cells.
3 We have analyzed the replication timing pattern in a single cell level by sequential labeling, and presented clearer evidence of selected loss of mid-S replication foci pattern in Rif1-deleted cells.
4 We have conducted more detailed analyses of DNA fiber experiments and presented the data supporting our original observations.

Referee #1 (Remarks to the Author):

Although the conclusion that Rif1 is involved in replication licensing is plausible, there are alternative explanations and the paper (especially the abstract) should be rewritten to clarify that licensing and the TDP were not directly tested.

I believe that the referee meant that"Rif1 is involved in establishment of replication timing" when he/she said "Rif1 is involved in replication licensing", since we did not believe that licensing is affected by Rif1.

Nevertheless, we have examined the licensing in Rif1-depleted cells by two different methods, staining of Mcm in the detergent-prewashed cells and biochemical fractionation. In both methods, the chromatin binding of Mcm was not affected by Rif1 depletion, indicating that licensing is not affected by Rif1 (new Figure S11).
We did mean to speculate that TDP is regulated by Rif1. However, the referee is correct in that we did not measure TDP in the original assays. Therefore, we modified some of our statement. (We removed "at the time of TDP" from the abstract.)

Critique and suggestions

it is possible that the changes in replication timing reflect altered chromatin condensation. Does the pattern of DNA sequences that exhibit DNAse sensitivity on the region tested on chromosome 5 reflect the changes in replication timing?

This is an interesting possibility that will need to be examined, but will require rather extensive analyses and we decided to save it as our future experiments.

2/ The halo experiments are interesting and suggestive of a global change in chromatin structure. However, wouldn't one expect a more prominent change in replication timing than that observed on chromosome 5, in which most replication domains seem similar in Rif depleted cells and unaltered cells? This question should be discussed.

The change we observe on the chromosome 5 segment is indeed quite large. The replication timing pattern in a given cell line is very reproducible, and shows almost identical pattern in each experiment. Indeed, we have examined the effect of another chromatin binding factor on the replication timing in the same cell line on the same segment. The pattern in the absence and presence of this factor was almost identical. During the differentiation of ES cells, about 20% of the genome exhibits the change of replication timing domain, which has been regarded as quite significant. Thus, we believe that the changes we observe in Rif1-depleted cells are significant. We speculate that other factors are also likely to be involved in regulation of the timing domains, and as we show here, the late replication domains are not affected by Rif1 depletion. Therefore, we still see the domains that are not affected. We have some more discussion on this issue in the Discussion (page 11, middle)

the abstract suggests that Rif1 affects the licensing process at the timing decision point although no direct examination of the TDP was reported in the paper. This statement might be misleading and should be corrected.

We did not mean to state that Rif1 affects the licensing process. We would like to apologize if our description somehow caused misunderstanding. Indeed, we meant the opposite. Therefore, we examined the licensing (chromatin binding of Mcm) with two methods as stated above (staining of Mcm in the detergent-prewashed cells and biochemical fractionation). The results from both methods (Fig. S11) indicate that licensing is not affected by Rif1 depletion, while it is lost by Cdt1 depletion. We speculate that Rif1 may be involved in TDP but have not directly tested it in the licensing assays, as this referee pointed out. We therefore corrected our statement so that it would not mislead the readers. (We removed "at the time of TDP" from the abstract.) (see page 10, bottom ~ page 11, top)

4/ Figure S1 demonstrates a notable increase in DNA synthesis accompanying changes in replication timing. Is this observation reproducible and can the authors suggest an underlying mechanism?

We have conducted this experiment at least three times and the results were reproducible. We have repeated this experiment again and have presented new data (Figure S1), which convincingly show that DNA synthesis is indeed stimulated at 1 hr after release from double thymidine block. (see page 7, middle)

it is important to evaluate how transfection might affect the response of cells to thymidine-block associated synchronization. This issue might affect the conclusion regarding the involvement of cell cycle checkpoints in the response to chromatin decondensation and should be discussed.

We have checked whether Rif1 siRNA affect the HU-induced checkpoint responses, and showed that Chk1 activation is not affected by Rif1 depletion (Fig. S6). We also showed in the same figure that double-thymidine block release does not activate checkpoint either in the presence or absence of Rif1. Thus, we can conclude that checkpoint pathway is not involved in Rif1-mediated regulation of replication timing. (see page 8, top)

6/ Figure 1D: Rif1 depletion seems to decrease overall MCM4 levels while increasing phosphorylation of MCM4. Is this reproducible?

Hyperphosphorylation of Mcm4 causes retardation of the mobility of Mcm4 on SDS-PAGE, resulting in smeared bands migrating above the non-phosphorylated form. This generally decreases apparent intensity of the bands. This hyperphosphorylation of Mcms in Rif1-depleted cells is very reproducible (shown also in Fig. S2 and S3).

7/ The image shown to illustrate Mid-late-S patterns of BrdU foci in Figure 2 does not seem to represent typical mid-S patterns. Is this a consistent observation in

the cells used for the study, or do the authors typically observe a distribution similar to the pattern shown in Figure 4E?

This is due to the cell-types used in the study. Generally, H1299 exhibit clearer and more typical mid-S foci patterns compared to HeLa cells which are used for siRNA. We have redone the experiments in Figure 2 and present entirely new data, which show better mid-S patterns.

Minor suggestions

1/ The abstract should mention the currently known function of Rif1 (at least, mention that it is a telomere associated protein) for the benefit of a wide audience.
We modified the abstract in accordance with this comments.

2/ The legend to Figure 2: line 8 - do the authors mean (B) instead of (C)?
We would like to thank for the correction. We have revised the Figure 2 and removed the data for PCNA staining.

Referee #2 (Remarks to the Author):

1. The authors report as an unpublished observation that Cdc7 kinase activity is unaffected when Rif1 is depleted. This suggests that the increased phosphorylation of MCM proteins observed in early S phase when Rif1 is depleted results from increased accessibility of preRCs to Cdc7 kinase, a potentially important insight on the mechanism by which Rif1 may regulate replication time. I think this claim should be substantiated by the presentation of appropriate data.

We have shown in Figure S4 the data of the Cdc7 kinase assays in the untreated and Rif1-depleted cells. The data show that Cdc7 kinase activity is unaffected by depletion of Rif1. (see page 7, bottom)

2. Hayano et al have shown that in S. pombe growth of hsk1-null cells can be rescued by insertion mutagenesis of the rif1 gene. Since the authors can simultaneously deplete Cdc7 and Rif1 have they observed a similar rescue of S phase progression in human cells ?

It is certainly of interest whether Cdc7 deficiency can be rescued by loss of Cdc7. This experiment needs to be conducted by using genetically modified mutant mice/ cells and will be a major undertake, and we still do not have an answer for it.

3. I am not convinced that the replication timing profiles of the 42 Mb region of chromosome V in control and Rif1-depleted cells clearly support the interpretation proposed by the authors, that the observed changes are as expected if mid-S phase domains become early and replicate over a somewhat

broadened time window. First, both early-to-late and late-to-early changes are observed (Figure 3B and S4), as reported in S. pombe. Second, there is no quantitation of replication time dispersion and therefore no support for homogeneization by abolition of distinct timing patterns. First, it is crucial to show the sorting gates chosen to isolate early and late-S phase cells as well as a post-FACS control showing the actual DNA content distribution of the two selected cell populations.

We have shown the sorting gates used for the sorting and the results of the reanalyses of DNA content of the sorted cells in new Figure S13. The results indicate very clear separation of early- and late-S fractions.

Second, the authors should show a histogram of replication timing ratios in control and Rif1-depleted cells as well as a histogram of replication timing changes by 100 kb windows for the entire 42 Mb region as well as for sorted early-, mid- and late-replicating windows. If the authors' interpretation is correct, (i) the Rif1 histogram of replication timing ratios should be shifted toward high early-to-late ratios with respect to the control, (ii) the mid-S windows should show much larger changes than the early- or late-S windows, and (iii) these changes should be predominantly toward earlier replication

We have presented the early/late ratio of replication timing as a histogram on the entire 42 Mb in control and Rif1-depleted cells (Supplementary Figure S8A, upper). The results show that two early and late peaks in the control cells are lost and converged to one peak in the center. On the basis of these data, we realized that our previous statement was misleading. Our results clearly indicate that "mid-S replication foci pattern" is lost and this represents the disintegration of mid-S replication timing domains. However, that does not mean that replication during mid-S is reduced and that during early-S phase is increased. Indeed, the results show the opposite; the replication timing is shifted toward center, which is mid-S, with general decrease of replication in early and late S phase. This is due to loss of timing regulation (at least no mid-S domains) in Rif1-depleted cells, which will lead to more random timing centered to the mid-S. It should be noted this replication in the absence of Rif1 adopts early-S phase foci pattern throughout early to mid S phase. These are stated in the Result section (see page 9, middle and page 13, middle).

4. Figure 4E clearly shows that Rif1 localizes at the nuclear and nucleolar periphery at the time these regions replicate (in mid-S phase), but whether Rif1 is similarly located at other times in S phase is not so obvious from the only two early and late-S phase nuclei shown. Could it be that the location of Rif1 is actually more dynamic that the authors suggest, and that it coincides with mid-S phase foci only when these sequences replicate ?

This is an important issue regarding how Rif1 may regulates mid-S replication domains. We observe that Rif1 binds to nuclear insoluble structures at late M/early G1 and stays

bound during most of the interphase. At least during early and mid-S phase, Rif1 binds predominantly to nuclear and nucleolar periphery, reminiscent of mid-S replication pattern. It is rather difficult to determine precisely whether the location of Rif binding changes at late S phase even if we use high-resolution micrography. We plan to analyze this by using fluorescent protein-tagged Rif1, which turns out to be extremely difficult to manipulate at this moment due to its extremely large size (7.4kb cDNA).

5. The most striking effect of Rif1 depletion shown by DNA fibre-FISH (Figure S8), aside from the mentioned (but rather modest) increase in interorigin distances, is an almost 2-fold increase in replication fork speed. Obviously, this observation is quite important to correctly interpret the role of Rif1 in the replication timing program, but this is not even mentioned nor commented in the main text. Does this increase in fork speed occur throughout the genome or only at specific DNA regions, and if so are these the same regions that show replication timing changes ? Since this increase is not fully compensated by a similar increase in interorigin distances, why does not this accelerate S phase progression ? Couldn't this explain at least some of the late-to-early shifts in replication time ?

In order to answer this comment, we sorted the cells into early, mid and late S phase population and measured the fork rate and inter-origin distances (Supplementary Figure S13). Average fork rate and inter-origin distances increased in both early and mid S phases, but were unaltered in the late S phase. Although we were not able to determine whether the increase occurs throughout the genome or at specific regions, the results indicate that the replication fork speed increases in Rif1-depleted cells. This may in turn suppress some of the origins by passive replication and lead to increase of inter-origin distances. (see page 12, top) New data indicate that increase of fork speed appears to be compensated by increased inter-origin distances. Initial stimulation of initiation events observed in Rif1-depleted cells occur only at the early S phase, and does not contribute to the overall S phase progression. (see page 14, middle)

6. Measurement of interorigin distances on spread DNA fibres can be strongly biased by finite fibre size and varying fibre stretching. It is important to show whether fiber size distributions and fiber extensions measured by FISH with the human BAC clone are comparable in the control and the Rif1 depleted cells.

We are aware of this, and showed the actual data on the relation of fiber size and fiber extensions in Supplementary Figure S13D. The data show that correlation is almost identical between non-treated and Rif1-depleted cells.

Referee #3 (Remarks to the Author):

Specific comments:

1. One important point not addressed is whether the specification, activity or timing of relication origins is modified in the absence of Rif1. The increase in the phosphorylation of MCM2 and MCM4 and the enhanced binding of Cdc45 and PCNA to chromatin in the early S phase caused by depletion of Rif1 (Figure 1) and the small difference in thymidine incorporation at the beginning of the S phase (Suppl. Figure 1) are not sufficient to support the title of the first section of the Results "Depletion of human Rif1 protein leads to increased initiation events at the early S phase".

We have examined the replication of known replication origins in Rif1-depleted cells (Figure S5). Among the origins investigated, the replication timing of mid-late S origins changed to early. This indicates at least some origins are activated early in Rif1-depleted cells, consistent with this heading. However, to be more accurate we have changed the subheading "Depletion of human Rif1 protein leads to increased Cdc7-dependent phosphorylation and slight increase of DNA synthesis at the early S phase". (see page 7, bottom ~ page 8, top)

2. Given the tight association between Rif1 and insoluble, DNAse I-resistant nuclear structures, it would be important to determine where Rif1 binds along the genome (by ChIP/chip, for example) to address what is the link between the changes in the replication profile (Figure 3), the sites of Rif1 binding and the sites of binding of other proteins involved in the organization of chromatin loops. This would complement the results based on the nuclear halo assay (Figure 5), which is suggestive but of low resolution.

We agree completely with this reviewer that the determination of the genome-wide Rif1 binding sites and comparison with those of other related proteins will provide important information as to how Rif1 may regulate the replication timing domains. We are certainly planning these experiments, but it would not be possible to complete these experiments within the given revision timelimit, and we would like to respectfully suggest that this will be left for our future endeavor.

3. I am surprised that the DNA fiber analysis of inter-origin distances and fork rates is only briefly commented (page 9) and shown in Suppl. Figure 8. This is an important assay and the results, along with some picture of the original fiber hybridizations, should be integrated and discussed in the main text in the context of the pattern of replication foci and the length of the S phase in the absence of Rif1.

As described in the response to referee 2, comment 5, we show raw data for DNA fiber and new DNA fiber data for sorted S phase cells, and we now show this data as Supplementary Figure S13.

4. Figure 2A should also include the BrdU and PCNA focus patterns in Rif1-depleted cells for direct comparison with the control nuclei. The authors do not explain why the pattern of BrdU foci is divided into four periods across the 8

hours of the S phase while the pattern of PCNA foci is split into 3 periods across only 6 hours.

We have conducted additional experiments using EDU/Biotin labeling, and gave clear evidence that the early replication patterns persist in Rif1-depleted cells (new Figure 2). Since this data is quite strong, we have removed the PCNA data.

Figure 2B shows that a very large fraction of Rif1-depleted cells maintain the early BrdU focus pattern during the entire S phase, indicative of a continuous low rate of DNA synthesis. How can this pattern be reconciled with the fact that the length of the S phase is the same as in the control cells, where the intensity of BrdU foci during early-mid and mid-late S phase indicates a more active BrdU incorporation?

We did not state or imply that rate of DNA synthesis is low in Rif1-depleted cells due to loss of mid-S replication foci pattern. Rif1-depleted cells adopts early-S phase foci pattern, but the level of DNA synthesis is not necessarily low compared to that at the mid-S foci pattern (please see new Figure 2). The DNA fiber analyses show that fork rate is increased while inter-origin distances increases from early through mid-S phase. Although the initiation appears to be stimulated at early S phase, due to availability of mid-S replication domains for activation of pre-RC, it is only temporal, presumably because the availability of Cdc7-ASK is limited. Thus, replication proceeds in "early-S foci pattern" but initiation takes place all through the early-to-mid S phase, with tendency that more origins are fired toward the mid-S phase. As the new DNA fiber data indicate, faster fork rate appears to be compensated by the lower numbers for fired origins (Supplementary Figure S13), and thus overall S phase length may not change significantly. (see page 14, middle)

5. Perhaps the previous point could be clarified by determining the rate of 3H-thymidine incorporation per unit of time at specific times during the S phase in cells with and without Rif1.
The rate of [^{3}H]-thymidine incorporation is largely the same between untreated and Rif-depleted cells, except that slight increase is reproducibly observed at early S phase in Rif1-depleted cells (Fig. S1D). (see page 7, middle)

6. Are the replication profiles in Figure 3B the average of the two biological duplicates?
Yes, they are. The average of the two biological duplicates is shown.

Although different cell types could have different replication profiles, it would be interesting (although not essential) to hybridize the same microarray in Figure 3A with early- and late-replicated DNA from the NHDF dermal fibroblast cell line tested in Figure 1D in order to monitor the effect of Rif1 in an independent system that is probably closer to normal human cells than HeLa cells.

We agree with this comment and we do have preliminary Q-PCR data that the replication timing is altered in NHDF by Rif1 depletion. We are planning to determine the replication timing profiles in NHDF as well as in other cell lines. However, we did not have enough time to conduct all the necessary experiments before the due date and we would like to save this for our future experiments.

The raw and processed microarray data are now being deposited to GEO Datasets to obtain the accession numbers.

We hope that these explanation clarifies the changes made in the revised manuscript. We hope that the referees and you would agree that the new data and revision have improved our manuscript and that it is now acceptable for publication in EMBO Journal.

2nd Editorial Decision 01 June 2012

Thank you for submitting your revised manuscript for our consideration. Please excuse the slight delay in its re-revaluation, but all three original referees have now had a chance to look at it again (see comments below), and I am pleased to inform you that they all consider the manuscript significantly improved and thus in principle now suitable for publication in The EMBO Journal. Before acceptance, there are however a few remaining specific points, mostly regarding aspects of presentation and writing, which I would like to ask you to address in a final round of minor revision. When resubmitting the final version of the manuscript, please also take care of the following editorial points:
- please incorporate the GEO accession number that you have obtained in the meantime into the manuscript text
- please add a brief 'Author Contribution' statement, after the Acknowledgement section and before the Conflict of Interest statement
- please combine all supplementary information (figures, legends, etc) into one single supplementary PDF
- Supplementary Figure S3 seems to contain a blank division line in the MCM2 panel - I suspect this could be due to the contrast/brightness settings of the digital image, but please look into this and clarify. Also please make sure to re-check all other blot figure panels and, where necessary, re-adjust contrast/brightness settings in order to avoid obscuring the background signals. E.g. in the Cyclin B1 panel of Fig S3, where the background appears to be lost due to too much brightness/contrast.

Once we will have received your final version including the appropriate changes, we should hopefully be able to swiftly proceed with acceptance and production of the paper!

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors have addressed the previous review's concerns and I have no further major comments. Below are a few minor suggestions:

Abstract: the sentence "Overall replication timing is shifted toward mid-S in both directions" seems contradictory to the previous sentence, which specifies the disappearance of the mid-S pattern. This is clarified in the text, but it is confusing in the abstract as a stand-alone document. It would be good to clarify that the mid-S-phase pattern refers to a spatial pattern achieved by fluorescent imaging whereas the shift in replication timing refers to biochemical measurements of replication timing. One way to do this is to explicitly state that "whole genome analyses of replication timing show the replication of sequences that normally replicate early is delayed, whereas the replication time for sequences that normally replicate late is advanced".

Figure 2F could not be evaluated because the legend suggests it should show blue and red bars and I only see grey in the downloaded file. At least one of the referees should see a revised version.

Typo: What is the square in the halo formula (page16, last line)? Should it be a Greek letter?

Pages 5-6, "loss of rif+ " should it be "loss of rif"?

Referee #2 (Remarks to the Author):

This revised version has been significantly improved. However I still have a few minor concerns.

1. I do not fully agree with the statement that Rif1 depletion does not affect the chromatin loading of MCM proteins. What the authors show is that the total amount of MCM proteins loaded onto chromatin as seen by Western blotting is unchanged upon Rif1 depletion (FigS11). However, one current model for replication timing regulation is that different amounts of MCM proteins are loaded at early vs. late replication origins. It could be that Rif1 depletion abolishes this differential loading without affecting the total amount of loaded MCMs. I understand that specifically testing this hypothesis would be beyond the scope of this study, but I feel it should be discussed.

2. I do not agree that the data in Fig S5 show that med-late S origins are activated earlier in Rif1 depleted cells. The data do suggest that these origins are replicated earlier but they do not discriminate between passive replication and active initiation of these origins.

3. In response to my point 1, it seems to me that the new data in FigS4 show that Cdc7 activity is slightly decreased upon depletion of Rif1, rather than unchanged as suggested by the authors. Nevertheless, these data do support the authors'interpretation that increased phosphorylation of MCM proteins observed upon depletion of Rif1 cannot be explained by an increase in Cdc7 activity so I am satisfied with their response.

4. I am satisfied with the authors' responses to my other points and to the other referees' comments.

Referee #3 (Remarks to the Author):

Yamazaki et al. have addressed most of my comments in the revised version of their manuscript.

As regards Point 1, the new data (new Figure S5) showing that the firing of mid-late replication origins is advanced in Rif1-depleted cells, lend strong support to a role of Rif1 in the regulation of the temporal programme of replication. Also, results from of BrdU and thymidine incorporation (new Figure S1) support that the small increase in DNA synthesis in early S phase is consistently detectable in triplicate experiments. These experiments also address Point 5 of my comments.

As regards Point 3 (also raised by Referee#2), new data and a far more detailed description of the DNA fiber analyses (in early, mid and late S-phase) than in the previous version is provided in the text and in the new Figure S13.

As regards Point 4, diagrammes in the new Figure 2 show clearly the differences in replication foci between control and Rif1-depleted cells and the text addresses the apparent inconsistency I raised in my comment. However, the quality of the images of nucle° in panels A, B, D and E are poor, have a small size and they will hardly be visible in the final reduced Figure. Perhaps, panels C and D could be shown as a Supplementary Figure to make more room for larger and better quality images of nuclei foci.

Points 2 and 6 would certainly contribute to this story but are not essential at this stage for the main point of the manuscript. In particular, I think it is important to determine where Rif1 binds in the genome as a first step to disect its mechanism of action. However, I agree that this would represent a significant amount of new work to add to that already incorporated in the revised version in response to the Reviewers. These points perhaps could be addressed in future work.

Altogether, I believe that the revised version of the manuscript is stronger than the original submission and represents a significant contribution to the field of DNA replication.

2nd Revision - authors' response 05 June 2012

The EMBO Journal

Manuscript EMBOJ-2012-80717R
Title: Rif1 regulates the replication timing domains on the human genome
Authors: Satoshi Yamazaki, Ai Ishii, Yutaka Kanoh, Masako Oda, Yasumasa Nishito and Hisao Masai

Thank you very much for your mail of June 1, 2012 regarding our above manuscript. We are very pleased to learn that the manuscript can be acceptable after minor revision.

Below our responses are in blue.

In response to your request
- please incorporate the GEO accession number that you have obtained in the meantime into the manuscript text
We have added the GEO accession number on page 16.
- please add a brief 'Author Contribution' statement, after the Acknowledgement section and before the Conflict of Interest statement
We have added the statement for author contribution at the suggested location.
- please combine all supplementary information (figures, legends, etc) into one single supplementary PDF
We have combined all the supplementary information into one single supplementary PDF.
- Supplementary Figure S3 seems to contain a blank division line in the MCM2 panel - I suspect this could be due to the contrast/brightness settings of the digital image, but please look into this and clarify.
Also please make sure to re-check all other blot figure panels and, where necessary, re-adjust contrast/brightness settings in order to avoid obscuring the background signals. E.g. in the Cyclin B1 panel of Fig S3, where the background appears to be lost due to too much brightness/contrast.
We would like to thank the reviewer for pointing out this, which escaped our attention. We have not done any “cut and paste” but somehow this strange line was introduced when the original X-ray film was scanned. We are presenting the newly scanned data (MCM2S53 blot of Fig. S3). For cyclinB1, we are presenting the original data without any digital enhancement.
Additional changes are as follows. 1) Fig. S2; cleaner data for A, new scan for SMCM2 blot in B (there was a line again!) and longer exposure data for MCM4(S6T7) blot in B. 2) correction of errors in Rif1 blot of Figure 1G.

The followings are point-by-point responses to the referees' comments.

Referee #1 (Remarks to the Author):

The authors have addressed the previous review's concerns and I have no further major comments. Below are a few minor suggestions:

Abstract: the sentence "Overall replication timing is shifted toward mid-S in both directions" seems contradictory to the previous sentence, which specifies the disappearance of the mid-S pattern. This is clarified in the text, but it is confusing in the abstract as a stand-alone document. It would be good to clarify that the mid-S-phase pattern refers to a spatial pattern achieved by fluorescent imaging whereas the shift in replication timing refers to biochemical measurements of replication timing. One way to do this is to explicitly state that "whole genome analyses of replication timing show the replication of sequences that normally replicate early is delayed, whereas the replication time for sequences that normally replicate late is advanced".

We totally agree with the above comment by this reviewer that our statement could be confusing for the readers who only read the abstract, and would like to thank him/her for the proposal for how it could be stated. We have used this statement in the revised abstract.

Figure 2F could not be evaluated because the legend suggests it should show blue and red bars and I only see grey in the downloaded file. At least one of the referees should see a revised version.

We apologize for the inconvenience. It shows in blue and red in the Figure 2F in the PDF we had sent. There might have been some error during the data transfer or conversion. We double checked the file, and hopefully it could be confirmed.

Typo: What is the square in the halo formula (page16, last line)? Should it be a Greek letter?

This was supposed to be “The halo radius (R) =√(A/π) - √(B/π); A, total area of nucleus; B, central area.” Again this may not have been properly represented when the word file was converted to pdf.

Pages 5-6, "loss of rif+ " should it be "loss of rif"?

Yes, it should be loss of rif1. This was corrected.

Referee #2 (Remarks to the Author):

This revised version has been significantly improved. However I still have a few minor concerns.

1. I do not fully agree with the statement that Rif1 depletion does not affect the chromatin loading of MCM proteins. What the authors show is that the total amount of MCM proteins loaded onto chromatin as seen by Western blotting is unchanged upon Rif1 depletion (FigS11). However, one current model for replication timing regulation is that different amounts of MCM proteins are loaded at early vs. late

replication origins. It could be that Rif1 depletion abolishes this differential loading without affecting the total amount of loaded MCMs. I understand that specifically testing this hypothesis would be beyond the scope of this study, but I feel it should be discussed.

This argument pointed out by this reviewer is well-taken. The timing of MCM loading has been reported to affect the replication timing in fission yeast. Thus, we do need to measure the timing of MCM loading in control and Rif1-depleted cells to formally conclude that the Rif1 effect on replication timing is not through pre-RC assembly. We discussed this in Discussion as below (page 13 top)

"We show that Rif1 is not required for the pre-RC formation (Supplementary Figure S12) and think it is unlikely that Rif1 regulates replication timing through regulation of timing of pre-RC assembly (Wu and Nurse, 2009). However, the current result does not completely rule out this possibility."

2. I do not agree that the data in Fig S5 show that med-late S origins are activated earlier in Rif1 depleted cells. The data do suggest that these origins are replicated earlier but they do not discriminate between passive replication and active initiation of these origins.

The reviewer is correct in that the method in Figure S5 does not precisely measure the firing and we cannot distinguish the firing of the origin and passive replication, although it does show that the regions containing these origins are replicated earlier in Rif1-depleted cells. We therefore were careful about our statement and did not state that our data indicate the late origins are fired earlier in Rif1-depleted cells (page 6-7).

"The measurement of DNA synthesis at known replication origins in early or late S phase indicated that DNA replication at mid/late origins is enhanced in early S phase, showing that the sequences normally replicated late is replicated early in Rif1-depleted cells (Supplementary Figure S5)."

3. In response to my point 1, it seems to me that the new data in FigS4 show that Cdc7 activity is slightly decreased upon depletion of Rif1, rather than unchanged as suggested by the authors. Nevertheless, these data do support the authors'interpretation that increased phosphorylation of MCM proteins observed upon depletion of Rif1 cannot be explained by an increase in Cdc7 activity so I am satisfied with their response.

We stated in the text that activity may be slightly reduced in Rif1-depleted cells, as below. (page 6 lower)

"This is not due to the enhanced Cdc7 kinase activity in Rif1-depleted cells since we measured the Cdc7 kinase activity in control and Rif1-depleted HeLa cells, and showed that it is not affected or slightly reduced in Rif1-depleted cells (Supplementary Figure S4)."

4. I am satisfied with the authors' responses to my other points and to the other referees' comments.

Thank you very much for the positive comment.

Referee #3 (Remarks to the Author):

Yamazaki et al. have addressed most of my comments in the revised version of their manuscript.

As regards Point 1, the new data (new Figure S5) showing that the firing of mid-late replication origins is advanced in Rif1-depleted cells, lend strong support to a role of Rif1 in the regulation of the temporal programme of replication. Also, results from of BrdU and thymidine incorporation (new Figure S1) support that the small increase in DNA synthesis in early S phase is consistently detectable in triplicate experiments. These experiments also address Point 5 of my comments.

As regards Point 3 (also raised by Referee#2), new data and a far more detailed description of the DNA fiber analyses (in early, mid and late S-phase) than in the previous version is provided in the text and in the new Figure S13.

As regards Point 4, diagrammes in the new Figure 2 show clearly the differences in replication foci between control and Rif1-depleted cells and the text addresses the apparent inconsistency I raised in my comment. However, the quality of the images of nucle¡ in panels A, B, D and E are poor, have a small size and they will hardly be visible in the final reduced Figure. Perhaps, panels C and D could be shown as a Supplementary Figure to make more room for larger and better quality images of nuclei foci.
In accordance with the reviewer's suggestion, we have moved Figure 2C and D to supplementary Figure S7.

Points 2 and 6 would certainly contribute to this story but are not essential at this stage for the main point of the manuscript. In particular, I think it is important to determine where Rif1 binds in the genome as a first step to disect its mechanism of action. However, I agree that this would represent a significant amount of new work to add to that already incorporated in the revised version in response to the Reviewers. These points perhaps could be addressed in future work.
We thank the reviewer for kind understanding.

Altogether, I believe that the revised version of the manuscript is stronger than the original submission and represents a significant contribution to the field of DNA replication.

I hope that these changes adequately answer the reviewers' comments and that the revised manuscript is now acceptable for publication in EMBO Journal.

Acceptance letter 13 June 2012

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal

