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Mouse Rif1 is a key regulator of the replication-timing program in mammalian cells.

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1st Editorial Decision 17 February 2012

Thank you for submitting your manuscript on mouse Rif1 and replication timing for consideration by The EMBO Journal. It has now been evaluated by three referees, whose reports are copied below. While the all consider your findings as potentially important, interesting and timely, they at the same time find the study presently still preliminary, and raise a number of very substantive concerns that in our view preclude publication at the current stage. In particular, all referees remain unconvinced that the Rif1 deletion effects on BrdU incorporation in relation to S-phase length and cell cycle/S-phase stages have been conclusively established. Another major issue is the unclear relation of Rif1 deletion to replication origin licensing or activity as possible causes for replication timing alterations.

Since it is unclear whether these, and several other substantial concerns could be satisfactorily addressed during a regular revision period, I am afraid I am not in a position to predict the outcome of an eventual re-review by our referees, and therefore I cannot make any strong commitments regarding suitability of this work for our journal. In light of the potential importance of your results, I would nevertheless offer you the opportunity to try and address the referees' criticisms through a single round of major revision. It is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. Still, I have to make it clear that we will only be able to ultimately consider the study for publication if the most pertinent issues are addressed to the referees' satisfaction during this single major revision round - in

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light of the competitive situation you mention, I would therefore also understand if you were to instead seek rapid publication without major changes elsewhere. Should you on the other hand be confident you may be able to address the various conceptual, experimental and presentational problems summarized in the referees' comments, then I would encourage you to attempt these improvements and to submit a revised version for our further consideration.

Should you have any questions regarding this decision or specific revision requirement, please do not hesitate to contact me for further discussion!

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This paper reports that depletion of the Rif1 protein in mice alters replication timing, interferes with cell cycle progression and results in chromatin reorganization. The main findings include: /1/ Rif1, a protein formerly known to associate with telomeres and participate in checkpoint mediated arrest of cell cycle progression in response to DNA damage, localizes to chromocenters during mid-late S-phase just prior to but not during chromocenter replication. /2/Although Rif1 does not cololcalize to active replication foci, Rif1 deficiency results in a distinct replication pattern similar to that found in cells with advanced replication time of major satellite DNA, suggesting replication domain reorganization. /3/Changes in replication timing in Rif1 deficient cells are detected in microarray-based analyses. /4/Rif1 deficiency is accompanied by changes in S-phase progression, consistent with the activation of a checkpoint previously reported by the authors' lab. /5/ Rif1 deficiency results in changes in chromatin packaging patterns and increased nuclease accessibility of newly replicated DNA.

The majority of the data reported in the paper are of high quality and are potentially very significant. The combination of cellular localization analyses and replication timing studies provides strong support to the main conclusions that Rif1 plays a role in replication timing and chromatin organization. However, some of the experimental findings need to be clarified and discussed, and there are some concerns about the data that should be addressed as listed below. In addition, the narrative could better explain the background and rationale underlying the studies and care should be taken to avoid over-interpretation. The following points should be addressed.

1/ Abstract: The abstract should describe the major findings in the paper. As written, the abstract states that Rif1 affects replication timing and that the accompanying chromatin reorganization is evidence that chromatin organization is "sensitive to altered replication timing". As discussed briefly in the paper, the results actually do not strongly show causation. Rif1's dynamic localization during the cell cycle is suggestive, but it is possible that the primary effect of Rif1 deletion is a change in replication timing, chromatin reorganization, or both. The abstract should be rewritten to clarify this point.

2/ Introduction: For the benefit of non-specialist audiences, a brief description of Rif1 and a short summary of prior knowledge about its known possible role(s) should be included. Such an introduction should help clarify why the experiments reported in the paper were performed.

Results:

3/ The paper relies on cyclin expression ratios for quantification of the fraction of cells in S-phase. Do these measurements conform to the fraction of cells in S-phase as measured by DNA content?

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4/ The results (Figure 2A) report fewer cells incorporating BrdU. Since the fraction of S-phase cells seems constant, it is curious that the overall level of BrdU incorporation as shown in Figure S3 seems unchanged. FACS analyses also do not seem to indicate that many cells with S-phase DNA content do not incorporate BrdU. Is there an explanation for these apparent discrepancies?

5/Data addressing the above question might be available in the measurements used for Figure 4. It would be more straightforward to include a table summarizing the relative fraction of cells with DNA content corresponding to the various stages of the cell cycle in association with Figure 2.

6/ For the benefit of the non-specialist reader, the narrative in the Results section describing DNA combing should clarify how replication forks were labeled.

7/ Similarly, the significance of the halo data in Figure 1E should be discussed. (Can the conclusions be more specific than "various distribution patterns?)

Conclusions:

8/ Although the overall transcriptome profile seems to be unchanged in Rif1 depleted cells, it is still possible that the effects of Rif1 deletion are mediated by a change in the expression of a small set of regulatory genes caused by the global changes in chromatin accessibility. This possibility should be discussed.

9/ Figure 1C: Why does the size of Rif1 look different in soluble and non -soluble fractions? Does this reflect a known post-translational modification?

(10/ The flipped image in Figure 2 - corrected in the image received on February 14.)

11/ Figure S3B: The numbers seem to be 25% for Rif+/+ and 32% for RifF/F, not a two fold reduction as stated text (page 6, line9 &10 from the bottom).

General:

12/Although checkpoint activation in the absence of Rif1 was demonstrated previously, this information is essential for the interpretation of the current data. Hence, it might be good to include an experiment exhibiting checkpoint activation under the conditions used in the current paper with a better marker (perhaps phosphorylation of Chk1).

13/How long is "chronic" Rif1 deficiency? Do cells under "chronic" depletion of Rif1 exhibit distinct cell cycle distributions as compared to shorter depletion?

14/ Cells do not seem to arrest in S-phase, cell cycle progression just seems to be delayed. Is there any indication on how they escape the delay? This should be discussed even if no experiments are shown.

Minor

Page3, line 2, a citation is needed for "correlation of early replication and active gene expression".
Page 5, line 13, (fig. S2A)
Page 7, line 6, it would be good to include a description of the main pertinent discrepancy that might be potentially resolved in Xu's paper.

Referee #2 (Remarks to the Author):

In this work Cornacchia et al suggest that the mouse Rif1 protein has a general role in the temporal regulation of origin firing, with potential consequences on chromatin organization and cell cycle progression. Although some results are clearly consistent with this view, several other results are

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unclear or even contradictory and the mechanism by which Rif1 may regulate replication timing is left unclear. I think a significant amount of work is required to clarify several points and provide a consistent ensemble of results.

1. The authors generated a knock-in mouse with an N-terminal FLAG-H2 tag of the Rif 1 gene. This allele is functional since Rif1 FH/FH mice from a Rif1 FH/+ x Rif1 FH/+ cross are born in normal health and in mendelian proportions and the tagged and untagged protein in heterozygous embryonic fibroblasts display identical intranuclear localization. Detailed studies reveal association of most of the protein with the insoluble nuclear scaffold and a dynamic intranuclear staining pattern that changes during S phase. Rif1 never colocalizes with replication forks but instead appears to anticipate them at least at pericentric heterochromatin in mid-S phase. This section is clear and convincing and the dynamic behavior of Rif1 in S phase is interesting. However, the authors should explain how they relate the seven different S phase patterns to S-phase progression. Has this been reported before and if so in which article ? Is it based on use of synchronized cells or on use of two sequential labeling pulses ? Also, it would be very interesting if the authors could compare the intranuclear staining pattern of Rif1 and MCM proteins, which mark domains whose origins have not yet been activated.

2. Using a conditional allele to induce acute Rif1 depletion in embryonic fibroblasts, the authors found by FACS that the proportion of BrdU-incorporating cells is reduced 2-fold with respect to the undepleted control (Fig 2A & S3B) but they suggest that the number of S-phase cells is the same because the amounts of Cyclin A and E are unchanged in the asynchronously growing population (Fig 2B). They conclude that "cells that are in S phase are incorporating BrdU less efficiently". However, the amounts of Cyclin A and E are only very indirectly related to the proportion of S phase cells and this claim is not substantiated by the FACS profile (Figure S3B) which suggests that if S phase cells are indeed less abundant these cells are incorporating BrdU at approximately the same rate than in the control. Furthermore, Rif 1 depletion induces no change in interorigin distances (Fig S3C) and a very small (<10%) increase in fork speed (Fig 2C&D -by the way the summarized data in 2C seem superfluous since the full data are shown in 2D). Since the rate of BrdU incorporation must be proportional to fork density and fork speed, this is again consistent with an overall similar rate of BrdU incorporation in control and depleted cells once they enter S phase. Overall, this section of the manuscript (p.6-7) is confusing and unconvincing.
Furthermore, Rif-/- cells have normal foci of EdU incorporation except for a very low percentage of cells showing a superposition of S2 and S4 patterns (Fig 3A). Therefore dramatic and systematic effects of Rif1 depletion on S phase progression at the level of replication foci are not observed, contrary to what would be expected if the global rate of DNA synthesis was largely perturbed.

3. Using genome-wide profiling of replication timing, the authors show that replication timing is profoundly affected by Rif1 depletion, with segments 2-10 Mb in length switching to either earlier or later replication than in the control (Fig 3C). Although the few examples shown are convincing, I was disappointed by the lack of genome-wide analysis. What is the overall percentage of loci that show significant timing changes ? What is the proportion and magnitude of early-to-late and late-to-early changes in replication time ? Are the few segments shown extreme examples of the timing changes or are they representative of an average behavior ? The authors conclude this section by suggesting "a lowered efficiency of overall origin firing" but again this seems to contradict the DNA combing data. Finally, how do they reconcile the massive replication timing changes suggested by this approach with the nearly normal patterns of EdU foci they observe ?

4. Figure 4A suggests that Rif1-/- cells accumulate in early S. It is neither explained in the Figure legend nor in the Materials and Methods how this result was obtained. Based on Figure S4 I guess this was obtained by scoring the number of cells showing the different S1-S7 patterns of EdU foci. However, the authors do not explain how they know the temporal order of S1 to S7 patterns (see point 1). Second, I do not understand why they chose to classify cells in early-, mid- or late S phase in Figure 4A & B rather than in the seven subtypes shown on Figure S4A. Third, and most importantly, it is unclear whether the pattern of EdU foci observed in Rif1-depleted cells reflects S phase progression as in wild-type cells (see point 3, last sentence). For example, could it be that the S3 and S4 patterns aberrantly appear in early S-phase cells when Rif1 is acutely depleted ?

5. When Rif1 deletion is induced in G0 (serum-starved) cells and BrdU incorporation is monitored in cells that are refed with serum the percentage of BrdU incorporating cells measured by FACS is

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reduced by half with respect to control (Fig 5A). Again, the authors argue that entry into S phase is not delayed because the amounts of Cyclins A and D1 are comparable through the release time course (page 10, Fig 5B). Why do not they simply use the FACS profile to measure the proportion of G0/G1 and S phase cells at different time points? How can they at the same time show that twice less cells are incorporating BrdU and say that S-phase entry is not affected ? Do they mean that some cells are in S phase but are not replicating DNA ? Isn't this contradictory with the usual definition of S phase ?
Furthermore, the authors suggest that once cells have entered S phase they progress through S phase in an unperturbed manner as judged by the proportion of the different patterns of replication foci (Fig 5C). However, this is not a measure of the rate of DNA synthesis, and there is no evidence that the temporal progression through the seven substages is not perturbed (see point 4). Again, this question must be addressed using the FACS data or some other direct measure of the rate of DNA replication in individual cells. Because of these uncertainties, the conclusion that S-phase progression is differently affected by chronic or acute Rif1 deficiency does not seem warranted.

6. A very recent article by Hayano et al (Genes Dev 2012) has reported a role for Rif1 in regulating replication timing in S. pombe. This study should be quoted and discussed.

Referee #3 (Remarks to the Author):

In this manuscript Cornacchia et al. describe the involvement of Rif1 in the regulation of the replication programme in mouse cells. They use two types of mouse embryonic fibroblasts (MEFs) as a model in their study. They derive from transgenic mice, where the Rif1 protein is tagged with FLAG-HA2 and from a previously generated transgenic strain harboring a Rif1 conditional allele. These are excellent models to study the role of Rif1 in DNA replication in an animal system under conditions as close as possible to the physiological situation.

The authors describe the nuclear distribution of Rif1 relative to the sites of DNA replication through a series of very good immunofluorescence analyses. The link between Rif1 and the regulation of the replication timing derives from the analysis of the replication profile in cells with and without Rif1. The main conclusion of the work is that Rif1 is required for the correct timing of replication. A similar function of Rif1 has been recently reported in fission yeast (Hayano et al. 2012, Genes Dev. 26: 137) However, unlike the case of the yeast, this manuscript does not provide much insight into the mechanism of action of Rif1, its genomic localization, or the physical or functional link with DNA replication origins. For this reason, I find these results potentially interesting but preliminary at this stage.

Specific comments:

1. If the seven substages S1-S7 into which the S phase is divided have been established in synchronous cultures, it should be indicated which time points do they correspond to in the context of the total length of the S phase.

2. The deletion of Rif1 reduces the incorporation of BrdU by half (Fig 2A). However, all the other parameters that were measured, such as the amount of Cyclins E and A, the interorigin distance and the speed of fork progression, are comparable regardless of whether Rif1 is present or not. Is the total length of the S phase different in both cases? Do Rif1 minus cells complete S phase and proceed to the next cell cycle? How does the pattern of EdU incorporation along the S1-S7 stages of the S phase compare with that of control cells in Fig 1A?

3. The experiments in Fig 5 are closely related to those in Fig 2 since they both study the progression of the S phase in the absence of Rif1. I believe that it would be clearer to present the two sets of results together or one immediately after the other.
Because the level of Cdc45, MCM3 and Cdc7 does not change in the absence of Rif1 (Fig 2E) it is inferred that the number of active origins is also maintained. On page 8 it is speculated that origins could be less efficient in Rif1 deficient cells. If this were the case, and given that the speed of replication forks is maintained, the completion of S phase should take longer in Rif1-deficient cells. However, from Fig 5A this does not seem to be the case. In this particular experiment (page 10, top)

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it is not clear whether only half of the Rif1 -/- cells enter S phase and/or whether those that do incorporate BrdU at half of the rate of the control cells. The rate of progression through S phase and its length in Rif1 -/- and control cells should be described more clearly.

4. A major limitation of the work is that although the authors speculate on several occasions about the activity of replication origins, they never actually test the activity of any of them. This is a crucial point in this work and it should be addressed to clarify whether the alteration in the replication programme in the absence of Rif1 is mediated by differences in the efficiency or time of activation of the replication origins.

5. Along the same lines, it would be important to determine where Rif1 binds along the genome (by ChIP/chip, for example) to address what the link between the changes in the replication profile (Figure 3C) and the sites of Rif1 binding is. This experiment would also be useful to determine the link between these sites and the replication origins.

6. The results in Fig 3C are essential to this work since they reveal dramatic differences in the replication profile in control cells relative to Rif1 -/- cells. Despite its importance, the experiment is only loosely described in the text, figure legend and materials and methods. A proper description of the experiment and what is actually measuring should be provided for readers to fully understand the relevance of the experiment.
The observed differences in replication timing (both early to late and late to early) should be validated at some sites along the genomic regions in Fig 3C by quantitative PCR to confirm and determine more accurately the magnitude of the differences. Is the firing of replication origins (some of which should be tested, as indicated in point 4) in the regions shown in Fig 3C advanced or delayed in parallel with the differences in the replication profile between control and Rif1 -/- cells?

7. It is not clear in Fig 4E why comparable amounts of total DNA (as shown in Fig S4 D) give a stronger signal of BrdU incorporation in Rif1 -/- cells relative to control cells if according to Fig 2A they incorporate half as much. Also, the use of a satellite DNA probe for normalization assumes that the sensitivity of these repeats to micrococcal nuclease should be the same in control and Rif1 -/- cells, which could not necessarily be the case.

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Response to Reviewer #1

Firstly, we would like to thank the Referee for his/her support of our manuscript and conclusions. Secondly, we are appreciative of the constructive criticism that has helped us improve analysis of data sets, support our claims and improve the presentation of our manuscript. Please find below a detailed response to the specific comments.

1/ Abstract: The abstract should describe the major findings in the paper. As written, the abstract states that Rif1 affects replication timing and that the accompanying chromatin reorganization is evidence that chromatin organization is "sensitive to altered replication timing". As discussed briefly in the paper, the results actually do not strongly show causation. Rif1's dynamic localization during the cell cycle is suggestive, but it is possible that the primary effect of Rif1 deletion is a change in replication timing, chromatin reorganization, or both. The abstract should be rewritten to clarify this point.
The abstract has been modified to address this important point.

2/ Introduction: For the benefit of non-specialist audiences, a brief description of Rif1 and a short summary of prior knowledge about its known possible role(s) should be included. Such an introduction should help clarify why the experiments reported in the paper were performed.
As indicated in bold in the text in page 4 and 5, we have expanded the introduction including more background information about Rif1 and the rationale that led us to study its function during S-phase progression.

3/ The paper relies on cyclin expression ratios for quantification of the fraction of cells in S-phase. Do these measurements conform to the fraction of cells in S-phase as measured by DNA content?
No, they did not. Indeed the source of the misunderstanding was the discrepancy between the measurements derived from the BrdU vs. Cyclins measurements. While BrdU incorporation showed that there were less Rif1 null cells in S-phase, cyclin A levels suggested that the amount of cells in S was the same for Rif1 wild type and null cells. In order to resolve the issue, we have now analyzed the G1/S transition in a synchronous cell cycle experiment, to gain a clearer picture in a cleaner system. We have examined the levels of p21 as G1 marker (Sherr & Roberts, 1999; Vogelstein et al, 2000) and the levels of chromatin-bound acetylated-Lys 12 histone H4 as independent marker of S-phase, given that H4 diacetylated on K5 and K12 is the form of newly incorporated histones (Loyola et al, 2006; Sobel et al, 1995). We found that p21 is induced upon Rif1 deletion, while the null cells show less chromatin-bound acetylated-Lys 12 histone H4. Based on these data we can conclude that the cells that do not incorporate BrdU are blocked at the G1/S transition, prior entry into S-phase. Since high levels of p21 indicate that CDK activity is inhibited, cyclin A levels become uninformative and we have therefore decided to take the specific cyclin A Western blotting out of the manuscript.

We have included the new data in Fig. 8C and Fig. S5B and proceeded to a major reorganization of the text. We hope these experiments and amendment to the text address this concern.

4/ The results (Figure 2A) report fewer cells incorporating BrdU. Since the fraction of S-phase cells seems constant, it is curious that the overall level of BrdU incorporation as shown in Figure S3 seems unchanged. FACS analyses also do not seem to indicate that many cells with S-phase DNA content do not incorporate BrdU. Is there an explanation for these apparent discrepancies?

The discrepancy noted here was generated by the contradictory data on the amount of cell in S-phase as judged from cyclin A levels and from BrdU incorporation, as discussed in the response to comment #3 above. However, for the approximately 50% of cells that do start DNA replication, we could detect no difference in BrdU incorporation between wild type and Rif1 null. DNA replication proceeds regularly, as judged by several parameters such as overall origin firing frequency, fork speed and also levels of BrdU incorporation per cell. Rif1 deletion affects some event preceding DNA syntheis, but after (or independently from) the assembly of the pre-RC. To further strengthen this point, we have now included the analysis of the chromatin-bound pre-RC from the synchronous cell cycle experiment (Fig. 8D). Also, we have used as an independent read-out of initiation of DNA replication the incorporation of newly synthesized histones, namely, histone H4K12Ac (Fig. 8D) (as explained before).

5/Data addressing the above question might be available in the measurements used for Figure 4. It would be more straightforward to include a table summarizing the relative fraction of cells with DNA content corresponding to the various stages of the cell cycle in association with Figure 2.

This section of the paper has been extensively reworked, as described in the detail in the responses to point #2 and 3.

6/ For the benefit of the non-specialist reader, the narrative in the Results section describing DNA combing should clarify how replication forks were labeled.

This information has now been added to the text with the reference of the original work and it is highlighted in bold in page 9.

7/ Similarly, the significance of the halo data in Figure 1E should be discussed. (Can the conclusions be more specific than "various distribution patterns?).

While we are not sure at this stage what the different patterns correspond to, a plausible explanation is that they could be associated with different cell cycle stages. However, this is difficult to address since we need to find markers for each cell cycle stage that would withstand the halo-preparation protocol. So far we have tried with MCM3, but without success. We have included a sentence in the text in bold in page 12 to explain this.

8/ Although the overall transcriptome profile seems to be unchanged in Rif1 depleted

cells, it is still possible that the effects of Rif1 deletion are mediated by a change in the expression of a small set of regulatory genes caused by the global changes in chromatin accessibility. This possibility should be discussed.

We have discussed this possibility in the revised text, in bold in page 8. We have also added the analysis of replication timing in Rif1 null cells during the first S-phase after deletion (Fig. 7A and B). As indicated in the text, these new data support the central hypothesis that Rif1 plays a direct role in replication timing determination.

9/ Figure 1C: Why does the size of Rif1 look different in soluble and non -soluble fractions? Does this reflect a known post-translational modification?

The insoluble fraction is solubilized by resuspending the pellets in Urea 8M. The presence of Urea causes a shift on SDS-PAGE that we have observed for other proteins. For this reason we believe that the higher mobility reflects the solubilization method. That said we cannot formally exclude that insoluble Rif1 also harbors secondary modifications we are not aware of at this point. We have added a note to clarify this in the figure legend 9, in bold.

11/ Figure S3B: The numbers seem to be 25% for Rif+/+ and 32% for RifF/F, not a two-fold reduction as stated text (page 6, line9 &10 from the bottom).

We apologize for this mistake, the wrong numbers were erroneously included. However, in the new version we have eliminated the FACS plots, since they are not as informative as the direct comparison of the percentage of BrdU positive cells in $Rif1^{+/+}$ + Cre versus $Rif1^{F/F}$ + Cre (Fig. 8A). By adding the P values, we have also strengthened the analysis of the percentage of decrease of BrdU incorporation shown now in Fig. S5A.

12/Although checkpoint activation in the absence of Rif1 was demonstrated previously, this information is essential for the interpretation of the current data. Hence, it might be good to include an experiment exhibiting checkpoint activation under the conditions used in the current paper with a better marker (perhaps phosphorylation of Chk1).

In the first version of the manuscript we interpreted the accumulation of early S-phase EdU pattern in Rif1 null cells as the result of checkpoint activation, since caffeine was able to partially revert this phenotype. However, referee #2 has raised the question of whether the spatial distribution of replication foci in Rif1 null cells still reflects the same temporal progression as in wild type cells. This suggestion prompted us to re-examine what we had scored as the accumulation of Rif1 null cells in early S-phase. Since the early S-phase pattern is diffuse, it could easily be an aberrant mixture of patterns, difficult to recognize. In order to test this possibility, we analyzed progression through different S-phase substages on FACS plots, rather then by EdU staining. In this way we rely on DNA content, rather then spatial distribution of replication foci. This analysis demonstrated that in fact it is likely that among what we have identified as early S-phase pattern there are aberrant patterns (see Fig. 4A and B). In the light of these results we feel that the data from the caffeine experiments are not easily interpretable and do not add any information, and we have therefore removed them. Also, as discussed in the response to point #4, we have found that Rif1 deletion in pMEFs causes

p21 stabilization (Fig. 8C and Fig. S5B). We had previously shown (Buonomo et al., 2009) that in MEFs immortalized by Large T infection, (that have therefore lost the G1/S checkpoint) the DNA replication checkpoint is instead activated by Rif1 deletion. We discuss this in the Discussion section.

13/How long is "chronic" Rif1 deficiency? Do cells under "chronic" depletion of Rif1 exhibit distinct cell cycle distributions as compared to shorter depletion?
"Chronic Rif1 deficiency" is defined after 36 hours of infection with Cre/empty virus and 72 hours of selection. We have only compared one cell-cycle deletion with this type of chronic deletion. As now explained in the text, the effect on replication-timing deregulation, p21 accumulation and decrease of the percentage of BrdU positive cells does not change in the two situations (compare Fig. 3C,D and E with Fig. 7A and B; Fig. 8C with Fig. S5B and Fig. 8A with Fig. 8B).

14/ Cells do not seem to arrest in S-phase, cell cycle progression just seems to be delayed. Is there any indication on how they escape the delay? This should be discussed even if no experiments are shown.
As discussed in the text, the progressive increase of the magnitude of the effect of Rif1 deletion on replication timing and p21 accumulation that we have shown in this revised version of the manuscript, suggests that cells do escape the checkpoint. However at this stage we do not know how. Amendments to the text have been done to clarify this (in bold in page 11).

Response to Reviewer #2

We would like to thank the reviewer for the thoughtful comments on our manuscript. We have addressed these by performing additional experiments and by including clarifications that have been highlighted in the text of the revised manuscript. Please see below a reply to each of the comments.

1)…. However, the authors should explain how they relate the seven different S phase patterns to S-phase progression. Has this been reported before and if so in which article? Is it based on use of synchronized cells or on use of two sequential labeling pulses? Also, it would be very interesting if the authors could compare the intranuclear staining pattern of Rif1 and MCM proteins, which mark domains whose origins have not yet been activated.

We have now reported the reference where the temporal appearance of the spatial patterns has been established (Dimitrova and Berezney JCS 115, 2002). Dimitrova had identified 6 patterns in MEFs. We identified 7 because we have subdivided stage 6 in two sub-stages. Since this distinction is irrelevant for our analysis, for the sake of simplicity we have now adopted Dimitrova's classification. We have also included an additional figure where Rif1 distribution is compared to MCM3's (Fig. 1).

2. Using a conditional allele to induce acute Rif1 depletion in embryonic fibroblasts, the authors found by FACS that the proportion of BrdU-incorporating cells is reduced 2-fold with respect to the undepleted control (Fig 2A & S3B) but they suggest that the number of S-phase cells is the same because the amounts of Cyclin A and E are unchanged in the asynchronously growing population (Fig 2B). They conclude that "cells that are in S phase are incorporating BrdU less efficiently". However, the amounts of Cyclin A and E are only very indirectly related to the proportion of S phase cells and this claim is not substantiated by the FACS profile (Figure S3B) which suggests that if S phase cells are indeed less abundant these cells are incorporating BrdU at approximately the same rate than in the control. Furthermore, Rif1 depletion induces no change in interorigin distances (Fig S3C) and a very small (<10%) increase in fork speed (Fig 2C&D -by the way the summarized data in 2C seem superfluous since the full data are shown in 2D). Since the rate of BrdU incorporation must be proportional to fork density and fork speed, this is again consistent with an overall similar rate of BrdU incorporation in control and depleted cells once they enter S phase. Overall, this section of the manuscript (p.6-7) is confusing and unconvincing.

Reviewer #1 also raised this important issue. The source of the misunderstanding was the discrepancy between the conclusions derived from the BrdU vs. Cyclins measurements. While BrdU incorporation showed that there were fewer Rif1 null cells in S-phase, cyclin A levels were the same for Rif1 wild type and null cells. In order to resolve the issue, we have now analyzed the G1/S transition in a synchronous cell cycle experiment, to gain a clearer picture in a cleaner system. We now have examined also

the levels of p21 as G1 marker and the levels of chromatin-bound acetylated-Lys 12 histone H4 as independent marker of S-phase. We found that p21 is induced upon Rif1 deletion, while the null cells show less chromatin-bound acetylated-Lys 12 histone H4. We feel that these new data allow us to conclude that the cells that do not incorporate BrdU are blocked at the G1/S transition, prior entry into S-phase. Since high levels of p21 indicate that CDK activity is inhibited, cyclin A levels become uninformative and we have therefore decided to take the specific cyclin A Western blotting out of the manuscript. We have included the new data in Fig. 8C and Fig. S5B. As the referee correctly points out, in the cells that do enter S-phase, DNA replication proceeds regularly, as judged by several parameters such as overall origin firing frequency, fork speed and also BrdU levels of incorporation per cell.

Furthermore, Rif-/- cells have normal foci of EdU incorporation except for a very low percentage of cells showing a superposition of S2 and S4 patterns (Fig 3A). Therefore dramatic and systematic effects of Rif1 depletion on S phase progression at the level of replication foci are not observed, contrary to what would be expected if the global rate of DNA synthesis was largely perturbed.
As this referee pointed out more in detail in both points #3 (*…..Finally, how do they reconcile the massive replication timing changes suggested by this approach with the nearly normal patterns of EdU foci they observe ?*) and #4 (*….Third, and most importantly, it is unclear whether the pattern of EdU foci observed in Rif1-depleted cells reflects S phase progression as in wild-type cells (see point 3, last sentence). For example, could it be that the S3 and S4 patterns aberrantly appear in early S-phase cells when Rif1 is acutely depleted?*) there was an inconsistency between the proportions of the S2-S4 aberrant mixed patterns and the extent of the replication timing deregulation revealed by the genome-wide analysis. Our explanation for this discrepancy was that probably the S2-S4 was the easiest recognizable aberrant pattern, thanks to the very characteristic EdU signal at the replicating chromocenters. By no means did we intend to conclude that it was the only spatial readout of the replication timing deregulation. However, in response to the reviewer's suggestion in point #4, we have considered the possibility that the accumulation of Rif1 null cells displaying an early-like S-phase spatial pattern (Fig. 4A) could instead be the result of the loss of spatial localization of sites of DNA synthesis. The early S-phase pattern is indeed characterized by a diffuse interior localization and it could therefore be difficult to distinguish from an aberrant mixture of patterns. In order to verify this possibility, we analyzed progression through different S-phase substages by flow cytometry, rather then by EdU staining. In this way we rely on DNA content, rather then spatial distribution of replication foci. This analysis showed that in fact the percentage of Rif1 null cells in early, mid and late S –phase is the same as in the wild type. Therefore, it is likely that among what we had identified as early S-phase pattern there are aberrant patterns (Fig. 4A and B). These data resolve the discrepancy between the extent of the alteration of timing and spatial organization of DNA replication in Rif1 null cells. We thank the referee for this inspiring suggestion.

3. Using genome-wide profiling of replication timing, the authors show that replication

timing is profoundly affected by Rif1 depletion, with segments 2-10 Mb in length switching to either earlier or later replication than in the control (Fig 3C). Although the few examples shown are convincing, I was disappointed by the lack of genome-wide analysis. What is the overall percentage of loci that show significant timing changes? What is the proportion and magnitude of early-to-late and late-to-early changes in replication time? Are the few segments shown extreme examples of the timing changes or are they representative of an average behavior?

We apologize for those omissions. A thorough analysis of the replication timing is now included in the manuscript. As the reviewer requests, we now have provided a series of analyses in Figures 4 and 7.

The authors conclude this section by suggesting "a lowered efficiency of overall origin firing" but again this seems to contradict the DNA combing data.

In the new version of the manuscript we show that Rif1 deficiency leads to a defective G1/S transition. However, the cells that do enter S-phase proceed normally. The new data resolve the contradiction pointed here.

Finally, how do they reconcile the massive replication timing changes suggested by this approach with the nearly normal patterns of EdU foci they observe?

We are also surprised by this result, which implies that the timing program is more extensively disrupted than is the 3D spatial patterns of replication. However, we did not intend to conclude that the mixed S2-S4 was the only aberrant pattern. It is only the easiest to score. However, in the revised version of the manuscript we show that the 50% increase of early S-phase-like pattern is probably the result of difficulties encountered in scoring aberrant patterns (see the second part of the response to point #2).

4. Figure 4A suggests that Rif1-/- cells accumulate in early S. It is neither explained in the Figure legend nor in the Materials and Methods how this result was obtained. Based on Figure S4 I guess this was obtained by scoring the number of cells showing the different S1-S7 patterns of EdU foci.

This information has been added in bold in the figure legend.

However, the authors do not explain how they know the temporal order of S1 to S7 patterns (see point 1).

The temporal progression through the patterns has been published (see response to point #1). However, we appreciate the fact that in the Rif1 null cells the correspondence between spatial distribution and temporal sequence could be lost. This important point was discussed in more detail in response to point #2, second part.

Second, I do not understand why they chose to classify cells in early-, mid- or late S phase in Figure 4A & B rather than in the seven subtypes shown on Figure S4A.

This was just out of simplicity. As a consequence of the extensive reorganization of the manuscript in this section, we adopted Dimitrova's published classification throughout.

We have left the classification early, mid and late S-phase only in Fig. 4 to render comparable the data between panel A and B (EdU counts versus flow cytometry data).

Third, and most importantly, it is unclear whether the pattern of EdU foci observed in Rif1-depleted cells reflects S phase progression as in wild-type cells (see point 3, last sentence). For example, could it be that the S3 and S4 patterns aberrantly appear in early S-phase cells when Rif1 is acutely depleted?
See response to point #2, second part.

5. When Rif1 deletion is induced in G0 (serum-starved) cells and BrdU incorporation is monitored in cells that are refed with serum the percentage of BrdU incorporating cells measured by FACS is reduced by half with respect to control (Fig 5A). Again, the authors argue that entry into S phase is not delayed because the amounts of Cyclins A and D1 are comparable through the release time course (page 10, Fig 5B). Why do not they simply use the FACS profile to measure the proportion of G0/G1 and S phase cells at different time points?
We did do this. The quantification by propidium iodide content of G0/G1, S and G2 throughout the time course was in agreement with the measures by BrdU. However this did not resolve the discrepancy with the cyclin A content. However, as discussed in the response to point #2, we have now used p21 as G1 marker and chromatin-bound acetylated Lys 12 histone H4 as S-phase marker to resolve the issue and we conclude that the half of Rif1 null cells show a defective G1/S transition.
How can they at the same time show that twice less cells are incorporating BrdU and say that S-phase entry is not affected? Do they mean that some cells are in S phase but are not replicating DNA? Isn't this contradictory with the usual definition of S phase?
See response to point #2, first part
Furthermore, the authors suggest that once cells have entered S phase they progress through S phase in an unperturbed manner as judged by the proportion of the different patterns of replication foci (Fig 5C). However, this is not a measure of the rate of DNA synthesis, and there is no evidence that the temporal progression through the seven substages is not perturbed (see point 4). Again, this question must be addressed using the FACS data or some other direct measure of the rate of DNA replication in individual cells. Because of these uncertainties, the conclusion that S-phase progression is differently affected by chronic or acute Rif1 deficiency does not seem warranted.
This criticism was very useful, as discussed in response to point #2, second part. It led us to revaluate the meaning of the data showing the accumulation of Rif1 null S-phase cells in early S.

6. A very recent article by Hayano et al (Genes Dev 2012) has reported a role for Rif1 in regulating replication timing in S. pombe. This study should be quoted and discussed.
The paper was published just few days before submission. That is why it had not been included. The reference has now been included.

Response to Reviewer #3

We would like to thank the reviewer for the fair and careful revision of our manuscript. We have addressed her/his concerns in a point-by-point response below, highlighting the new data and the changes made to the text accordingly.

1. If the seven substages S1-S7 into which the S phase is divided have been established in synchronous cultures, it should be indicated which time points do they correspond to in the context of the total length of the S phase.
The substages were originally determined by pulsing synchronous cultures with BrdU. To render this clearer we have included now the original reference (Dimitrova and Berezney, 2002). In our case the culture was not synchronized. Dimitrova had identified 6 patterns in MEFs. We have identified 7 because we subdivided stage 6 in two sub-stages. Since this distinction is irrelevant for our analysis, for the sake of simplicity we have now adopted Dimitrova's classification.

2. The deletion of Rif1 reduces the incorporation of BrdU by half (Fig 2A). However, all the other parameters that were measured, such as the amount of Cyclins E and A, the interorigin distance and the speed of fork progression, are comparable regardless of whether Rif1 is present or not. Is the total length of the S phase different in both cases?
In the previous version of the manuscript, we had shown in Fig. 2A that in Rif1 null half of the cells in the population incorporated BrdU compared to the wild type. Fig. S3B showed that each single Rif1 null cell incorporated the same amount of BrdU as in wild type. Judging from the dynamics of BrdU incorporation increase/decrease in the synchronous cell cycle experiment, the length of S-phase seems to be the same between Rif1 wild type and null cells (Fig. 8B). In the revised version of the manuscript we present a more detailed analysis of the G1/S transition in Rif1 null cells. By p21 stabilization (G1 marker) and decrease of acetylated K12 histone H4 incorporation into the chromatin (S-phase marker) we conclude that half of Rif1 null cells show a defective G1/S transition. We do not know at this stage why or how half of the cells manage to enter S-phase and proceed normally with DNA replication.

Do Rif1 minus cells complete S phase and proceed to the next cell cycle?
As discussed in the text, the progressive increase in magnitude of the effect of Rif1 deletion on replication timing that we have shown in this revised version of the manuscript suggests that cells do escape the checkpoint. However at this stage we do not know how. Amendments to the text have been done to highlight this (in bold in page 11). In order to thoroughly answer this question we would need a cell-based assay, such as live cell imaging of conditional cells stably expressing a marker such as histone H2B-GFP. Unfortunately generating a stable line in primary cells in combination with the Cre-mediated protocol it is not possible under the current time constraints.

How does the pattern of EdU incorporation along the S1-S7 stages of the S phase compare with that of control cells in Fig 1A?

The data relative to the pattern of EdU incorporation in the Rif1$^{-/-}$ cells was shown in Figure 4 and S4 of the old version of the manuscript, where we showed that Rif1 deficiency causes the appearance of an aberrant mixed S2-S4 replication pattern. However, there was an inconsistency between the proportions of the S2-S4 aberrant mixed patterns and the extent of the replication timing deregulation revealed by the genome-wide analysis. Our explanation for this discrepancy was that probably the S2-S4 was the easiest recognizable aberrant pattern, thanks to the very characteristic EdU signal at the replicating chromocenters. By no means did we intend to conclude that it was the only spatial readout of the replication timing deregulation. However, in response to a suggestion of reviewer #2, we considered the possibility that the seeming accumulation of Rif1 null cells displaying an early-like S-phase spatial pattern (Fig. 4A) could instead be the result of the loss of spatial localization of sites of DNA synthesis. The early S-phase pattern is indeed characterized by a diffuse interior localization and it could therefore be difficult to distinguish from an aberrant mixture of patterns. In order to test this possibility, we analyzed progression through different S-phase substages by flow cytometry, rather then by EdU staining. In this way we rely on DNA content, rather then spatial distribution of replication foci. This analysis showed that in fact the percentage of Rif1 null cells in early, mid and late S –phase is the same as in the wild type. Therefore, it is likely that among what we had identified as early S-phase pattern there are aberrant patterns (Fig. 4A and B). These data resolve the discrepancy between the extent of the alteration of timing and spatial organization of DNA replication in Rif1 null cells.

3. The experiments in Fig 5 are closely related to those in Fig 2 since they both study the progression of the S phase in the absence of Rif1. I believe that it would be clearer to present the two sets of results together or one immediately after the other.

In the new version of the manuscript we have replaced most of the data relative to the cycling cultures with data generated by the synchronized cultures, reducing the redundancy.

Because the level of Cdc45, MCM3 and Cdc7 does not change in the absence of Rif1 (Fig 2E) it is inferred that the number of active origins is also maintained. On page 8 it is speculated that origins could be less efficient in Rif1 deficient cells. If this were the case, and given that the speed of replication forks is maintained, the completion of S phase should take longer in Rif1-deficient cells. However, from Fig 5A this does not seem to be the case. In this particular experiment (page 10, top) it is not clear whether only half of the Rif1 -/- cells enter S phase and/or whether those that do incorporate BrdU at half of the rate of the control cells. The rate of progression through S phase and its length in Rif1 -/- and control cells should be described more clearly.

We realize that this should be clarified and we have proceeded to a major reorganization. Also, the new data obtained and included in the manuscript have been essential in resolving the discrepancies. In the new version of the manuscript we show

in Fig. 7C that in synchronized cells (as well as in cycling-Fig. S5B) the defect caused by Rif1 deletion induces the accumulation of p21. This data suggest that in the Rif1 null cells a signal is generated in G1 that blocks about half of the cells at the G1/S transition, after the pre-replication complex has been assembled. However, half of the cells proceed into S and do so firing origins at a normal frequency and show no defect in fork progression or BrdU incorporation.

4. A major limitation of the work is that although the authors speculate on several occasions about the activity of replication origins, they never actually test the activity of any of them. This is a crucial point in this work and it should be addressed to clarify whether the alteration in the replication programme in the absence of Rif1 is mediated by differences in the efficiency or time of activation of the replication origins.
We would love to be able to do this experiment, but in pMEFs this is unfortunately technically undoable. Only lamin B and IgH replication origins have been mapped to a sufficient degree of resolution. Their analysis requires the use of 2D gels that only few laboratories can perform in mammalian cells. Furthermore the amount of cells required for such experiment and the synchronization procedure are not compatible with pMEF culture. Moreover, as the reviewer is probably aware, replication origins are poorly defined in mammalian cells and are very inefficient, with different cells in the population using different subsets of the same very large set of potential origins. We hope the reviewer understands that this would be an impractical line of investigation at this point and would probably be very difficult to interpret even if executed.

5. Along the same lines, it would be important to determine where Rif1 binds along the genome (by ChIP/chip, for example) to address what the link between the changes in the replication profile (Figure 3C) and the sites of Rif1 binding is. This experiment would also be useful to determine the link between these sites and the replication origins.
We agree that this is the next most important experiment to do and we are preparing to do this experiment, but it is a big endeavor and we hope that the reviewer will allow us to report these results in subsequent work.

6. The results in Fig 3C are essential to this work since they reveal dramatic differences in the replication profile in control cells relative to Rif1 -/- cells. Despite its importance, the experiment is only loosely described in the text, figure legend and materials and methods. A proper description of the experiment and what is actually measuring should be provided for readers to fully understand the relevance of the experiment.
The observed differences in replication timing (both early to late and late to early) should be validated at some sites along the genomic regions in Fig 3C by quantitative PCR to confirm and determine more accurately the magnitude of the differences. Is the firing of replication origins (some of which should be tested, as indicated in point 4) in the regions shown in Fig 3C advanced or delayed in parallel with the differences in the replication profile between control and Rif1 -/- cells?
Replication-timing analysis was done as described (Ryba, Nature Protocols), with minor modifications described in Dileep et. al. (2012), but we agree that a brief outline should

be provided and now have done so in the Methods section. The protocol as described involves a routine evaluation of 10 loci by PCR as a quality control prior to array hybridization, and this is also described in the Methods section. The response to the question on origins is explained in response to point 4.

7. It is not clear in Fig 4E why comparable amounts of total DNA (as shown in Fig S4 D) give a stronger signal of BrdU incorporation in Rif1 -/- cells relative to control cells if according to Fig 2A they incorporate half as much. Also, the use of a satellite DNA probe for normalization assumes that the sensitivity of these repeats to micrococcal nuclease should be the same in control and Rif1 -/- cells, which could not necessarily be the case.

In the previous version of the manuscript, Fig. 2A showed that half of the cells incorporated, not that each cell incorporated half the amount of BrdU. This was even better appreciated in Fig. S3B. What this figure was showing is that half of the cells in the population incorporated a normal amount of BrdU. Also, the gel in Fig. 4E (now Fig. 5B) has been loaded with the same amount of DNA for all the cell lines. A different accessibility of the major satellites would have resulted in a different intensity of the radioactive signal. However, since in principle we could not exclude the possibility presented by the reviewer, we had included also the Ethidium bromide staining of the same gel in Figure S4C, which is the standard loading control for this type of assay. However, we have now moved the ethidium bromide staining as loading control to Fig. 5B and the major satellites Southern blotting to Fig. S4C. We hope this clarifies the confusion.

Additional correspondence (editor) 02 July 2012

Thank you for submitting your revised manuscript for our consideration. All three of the original referees have now assessed it once more, and all acknowledge the major improvements in response to their initial comments. They retain a few specific issues and questions in need of clarification, as you will see from the comments copied below. I think that these comments can probably be answered without the need to conduct further experiments; however, in order to avoid unnecessary delays before final acceptance, it would be helpful to know how you would respond to each of the remaining points. Therefore, I would appreciate if you could send me a considered point-by-point response letter at your earliest convenience, detailing how the remaining issues could be answered and clarified. On the basis of this response, we could then decide on the essential modifications to be incorporated in the final re-revised version of the manuscript.

Yours sincerely,

Editor
The EMBO Journal

Referee #1

This resubmitted paper addressed most of the comments from the previous round of review. Importantly, new data and interpretations clarify some of the discrepancies in cell cycle analyses by the important observation that many, but not all, cells subject to Rif1 elimination in G1 phase do not start DNA replication. As stated in the earlier version for the cells that do enter S-phase, Rif1 depletion results in spatially and temporally disorganized replication with fragmentation of replication domains. Consistent with this, Rif1 exhibits a focal distribution in S-phase cells in a pattern that resembles replication foci, although these foci appear before DNA replication and do not co-localize with the replication machinery except perhaps in chromocenters in mid-early S. The observations in the current version suggest two roles of Rif1: permitting progression into S-phase and delineating replication domains, likely facilitating appropriate genome organization associated with DNA replication. The new additions significantly improve the paper and provide important information. Several issues remain, and many other issues require further investigation in later stages. Some of the issues are listed below.

1. The revised paper reports that Rif1 depletion leads to a failure to progress into S-phase in many cells. This observation is supported by cell cycle studies but direct measurements of the number of cells at various stages of the cell cycle are not reported. The observations will be clarified by inclusion of a table reporting the number of cells at each cell cycle stage and appropriate statistics (how many times was the experiment performed with an independent cell population, what was the experimental variation, etc.).
2. Figure 6 shows that elimination of Rif1 does not seem to have a major effect on replication fork rates, but although overall rates seem similar the reduction in the abundance of slower replication forks, noted by the authors, seems significant. A subtle change in replication fork progression might be sufficient to affect genomic stability and is consistent with the DNA breakage associated with Rif1 deficiency. Is this observation reproducible - how many times was the experiment performed with independently isolated fibers and do the authors consistently see a reduction in the number of slow forks? If the observation is consistent, the possibility of an additional role of Rif1 during fork progression should be mentioned and discussed.
3. It is interesting that p21 levels increase after elimination of Rif1. As stated,

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p21 plays multiple roles in regulating DNA replication and is not limited to CDK inhibition in G1. Are other CDK inhibitors similarly affected?

Referee #2

I am satisfied with the novel analyses reported in this revised version, which resolve several questions I raised for the first version, but I still have a few concerns.

1) Introduction, page 5, line 4, which study are the authors referring to when they discuss "the effects of Rif1 deletion on S-phase progression" ?

2) Results, page 6, line 11, and Fig1A, panel S3 Rif1/MCM. I do not see any co-localization of Rif 1 and MCM3, contrary to what is stated in the text, even after enlarging the figure on my screen. The authors should either provide more convincing data or provide a faithful description of the picture shown.

3) Figure 7B is unsatisfactory. First, the differences between the red (Rif1-/-) and black (Rif1(+/+) profile are not very large; second, the segment shown is too large so that individual replication domains are not easily seen; third, the grey line (WT MEFs) is barely visible. Why not show the same segments as in Fig 3, so that the reader can really compare the effects of chronic and acute Rif1 depletion ?

4) Can the authors provide an interpretation for their observation that the percentages of EtoL and LtoE switching regions is equal during the first S phase after Rif1 depletion and that a bias to EtoL transition progressively emerges during subsequent cell cycles (p.10, bottom) ?

5) Typos:
p.3, line 6, should "entity" read "extent" ?

6) Reference Ryba et al 2012 is incomplete

Referee #3

Cornacchia et al. have addressed most of my comments in the revised version of the manuscript, which incorporates a significant amount of new data and clarifies several points as suggested by the Reviewers.

I still think that measuring the activity of some replication origins (point 4) (a few more than the two cited by the authors could be used as a test) would be an important point to analyze. Also, the distribution of Rif1 along the chromosomes (point 5) would be important but I understand that the revised version incorporates new material in response to the points raised by the Reviewers and that there is a limit to the information that reasonably fits into a manuscript.

I only have three points that the authors or the editor could consider:

1. In my previous point 6, I suggested that some of the differences observed in the replication timing in Rif1-/- cells could be validated by quantitative PCR. The authors reply that the protocol they used (now described in Materials and Methods) includes the evaluation of 10 loci as a routine control on the quality of the nascent strands. By itself, this does not address my question because it is not indicated whether they have used any of these 10 loci to confirm the differences in the LtoE or EtoL domains in replication profile shown in Figure 3D-E.
If the authors or the editor do not consider this control necessary, perhaps it could be replaced by showing in a Supplementary Figure how the duplicate replication profiles of Rif1 +/+ and -/- cells differ between them as an indication of the

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consistency of the differences detected.

2. I still do not understand the differences in the patterns generated by micrococcal nuclease (Figure 5). The same amount of total DNA is loaded into each lane and all this DNA must be present in the mononucleosome to tetra- or pentanucleosome since no DNA of higher molecular weight is left in the gel. If only half of the Rif1 -/- cells (which means half of the DNA in the preparation) incorporates BrdU, how is it possible that more BrdU is detected in the nucleosome ladder of Rif1 -/- than in Rif1 +/+ cells?

3. The grey line in Figure 3D-E (WT MEFs) is barely visible as it is. It will be completely invisible in the reduced printed Figure. Instead of making it thicker (it would make the black and red profiles more difficult to read), it could be shown (if necessary) as an independent Supplementary Figure.

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Additional correspondence (author) 05 July 2012

Referee #1

1. The revised paper reports that Rif1 depletion leads to a failure to progress into S-phase in many cells. This observation is supported by cell cycle studies but direct measurements of the number of cells at various stages of the cell cycle are not reported. The observations will be clarified by inclusion of a table reporting the number of cells at each cell cycle stage and appropriate statistics (how many times was the experiment performed with an independent cell population, what was the experimental variation, etc.).

If I understand correctly the referee is requesting a table with the number of cells in G1, S and G2. For S phase we already included in Fig. 8A the bar plot of the percentages from 3 independent $Rif1^{F/F}$ and $Rif1^{+/+}$ cell lines (one experiment, triplicate-standard deviation are shown) and in Fig. S5A from 6 independent $Rif1^{F/F}$ and $Rif1^{+/+}$ cell lines (two experiments, each with a triplicate, standard deviation and P values are shown). In Fig. 8B we show the average of a triplicate for each line from a single experiment (standard deviation are shown). We repeated the experiment more than three times. Would she/he like a table including the quantification of G1 and G2 from the FACS? With primary cells the ratios are more informative then absolute numbers or even percentages, as they can vary notably among experiments depending on the reaction to thawing, and, especially, efficiency of retroviral infection and consequent confluency. However, in the context of a single experiment, where we always have 3 $Rif1^{F/F}$ and $Rif1^{+/+}$ cell lines, also absolute numbers or percentages can be compared. We can provide these data if you think they will add information.

2. Figure 6 shows that elimination of Rif1 does not seem to have a major effect on replication fork rates, but although overall rates seem similar the reduction in the abundance of slower replication forks, noted by the authors, seems significant. A subtle change in replication fork progression might be sufficient to affect genomic stability and is consistent with the DNA breakage associated with Rif1 deficiency. Is this observation reproducible - how many times was the experiment performed with independently isolated fibers and do the authors consistently see a reduction in the number of slow forks? If the observation is consistent, the possibility of an additional role of Rif1 during fork progression should be mentioned and discussed.

Two independent $Rif1^{-/-}$ and two independent $Rif1^{+/+}$ were used. Each cell line (4 total) was treated to generate fibers. From each cell lines, fibers were independently isolated by combing at least 3 times on different glass slides. The observation that the abundance of slower

forks is reduced in Rif1$^{-/-}$ cells is reproducible. However, the reason we have not discussed it more extensively is that we do not know if the effect on fork progression is directly caused by Rif1 deletion or if it is an indirect consequence of how the cell adapts, or caused by the altered chromatin status. If necessary, it can be mentioned in the text like this.

3. It is interesting that p21 levels increase after elimination of Rif1. As stated, p21 plays multiple roles in regulating DNA replication and is not limited to CDK inhibition in G1. Are other CDK inhibitors similarly affected?

We have tried p27 but the antibody did not work. I also think it is an interesting question but beyond the scope of this manuscript.

Referee #2

1) Introduction, page 5, line 4, which study are the authors referring to when they discuss "the effects of Rif1 deletion on S-phase progression" ?

Buonomo et al 2009

2) Results, page 6, line 11, and Fig1A, panel S3 Rif1/MCM. I do not see any co-localization of Rif 1 and MCM3, contrary to what is stated in the text, even after enlarging the figure on my screen. The authors should either provide more convincing data or provide a faithful description of the picture shown.

Maybe we need to increase the contrast of the red to obtain a better yellow. In our opinion a relevant degree of co-localization is clearly visible.

3) Figure 7B is unsatisfactory. First, the differences between the red (Rif1-/-) and black (Rif1(+/+) profile are not very large; second, the segment shown is too large so that individual replication domains are not easily seen; third, the grey line (WT MEFs) is barely visible. Why not show the same segments as in Fig 3, so that the reader can really compare the effects of chronic and acute Rif1 depletion ?

We can show the same segments shown in Fig. 3 also in Fig. 7B, using a smaller scale.

4) Can the authors provide an interpretation for their observation that the percentages of EtoL and LtoE switching regions is equal during the first S phase after Rif1 depletion and that a bias to EtoL transition progressively emerges during subsequent cell cycles (p.10, bottom)?

This is an interesting point to discuss, but that there are many interpretations. For the sake of clarity and to avoid over-interpretation I would prefer not to comment about it in the text.

Referee #3

1. In my previous point 6, I suggested that some of the differences observed in the replication timing in Rif1-/- cells could be validated by quantitative PCR. The authors reply that the protocol they used (now described in Materials and Methods) includes the evaluation of 10 loci as a routine control on the quality of the nascent strands. By itself, this does not address my question because it is not indicated whether they have used any of these 10 loci to confirm the differences in the LtoE or EtoL domains in replication profile shown in Figure 3D-E. If the authors or the editor do not consider this control necessary, perhaps it could be replaced by showing in a Supplementary Figure how the duplicate replication profiles of Rif1 +/+ and -/- cells differ between them as an indication of the consistency of the differences detected.

If you think this is necessary we can show the replicate separately in a supplementary figure.

2. I still do not understand the differences in the patterns generated by micrococcal nuclease (Figure 5). The same amount of total DNA is loaded into each lane and all this DNA must be present in the mononucleosome to tetra- or pentanucleosome since no DNA of higher molecular weight is left in the gel. If only half of the Rif1 -/- cells (which means half of the DNA in the preparation) incorporates BrdU, how is it possible that more BrdU is detected in the nucleosome ladder of Rif1 -/- than in Rif1 +/+ cells?

As it is stated in the text, in the conditions used for this experiment about 4% of the cells are in S-phase, for both $Rif1^{+/+}$ and $Rif1^{-/-}$. Since $Rif1^{+/+}$ grow better than the $Rif1^{-/-}$ cells, they are more confluent and this makes the S-phase index comparable for the two genotypes. As a consequence, the proportion of BrdU-labeled DNA over total DNA is more or less the same in the two samples. In all the other experiments presented in the paper we have plated different number of cells for the two genotypes, in order to achieve a comparable degree of confluency at the moment of harvesting. I would like to point out also that this is a limited digest, and not all the chromatin is digested by the MNase, as it is visible in some of the wells. If there is a difference in the accessibility of the micrococcale specifically to the newly replicated DNA, the BrdU-containing DNA will be digested more readily in the KO. Therefore for the same amount of total DNA loaded there will be more BrdU positive DNA digested in the KO.

3. The grey line in Figure 3D-E (WT MEFs) is barely visible as it is. It will be completely invisible in the reduced printed Figure. Instead of making it thicker (it would make the black and red profiles more difficult to read), it could be shown (if necessary) as an independent Supplementary Figure.

We can do this if you think it is better. Or we could just change the

color to render the WT MEFs more visible.

2nd Editorial Decision 06 July 2012

Thank you for your response letter. In light of the referee comments and your answers, I would like to invite you to prepare a final minor revision of your manuscript, with attention on the following points listed below. Once we will have received this final version, we should then be able to swiftly move ahead with acceptance and production of the paper.

For Referee 1:
- I agree that adding a brief supplementary table as you offered would be helpful
- please briefly mention your thoughts in the referee's second point in the text as proposed

For Referee 2:
- please introduce the modifications 1-3 as proposed in your response letter, including attempts to get a better yellow in Figure 1A (I do agree with you that overlap is in principle visible)

For Referee 3:
- please show the replicate in the supplement as proposed
- for point 2, the clarification you provide is sufficient
- point 3, yes please simply choose a more visible color for the wt MEFs as you suggest

Editorial points:
- author names on the title page (and author initials in the Author Contribution section) should be in the order 'first name - last name'
- please make sure to list all authors' contributions in the Author Contribution section (I notice some are currently not mentioned there)
- please remove all supplementary text/legends from the main manuscript file; all supplementary material (text/figures/tables) should be combined in one single PDF
- please adjust the bibliography format, journal abbreviations and author listings to EMBO Journal reference formatting styles as explained in our Guide to Authors

I am looking forward to receiving your final version as early as possible

Yours sincerely,

Editor
The EMBO Journal

2nd Revision - authors' response 10 July 2012

We have re-submitted the manuscript Cornacchia et al. EMBOJ-2012-80948R with your requested modifications.

Specifically:

In Fig. 1 we have enhanced slightly the red channel to make clearer the co-localization in the merge.

In Fig. 3 we have changed the color of the wild type MEFs lines to green, to make it more visible.

In Fig. 7 we are showing the same chromosomal regions as in Fig. 3, with the same scale.

We have added in Fig. S3 the replication timing plots relative to Fig. 3, showing separately the biological replicates.

In Fig. S5 we have added tables to summarize the percentages of cells in G1 and G2 in the asynchronous population whose S-phase is quantified in Fig. 8A. As extra, for completion sake, we also added the curves of changes in the G1 and G2 percentages in the synchronous population shown in Fig. 8B.

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We have corrected the Ryba et al. (2012) reference that was incomplete.

In page 14 we have also included a sentence to comment on the reduction of the slower replication forks in Rif1 null pMEFs.

We have corrected the order of the names-last names in the author list and completed the author contribution section.

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