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dNTP pools determine fork progression and origin usage under replication stress

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Transaction Report:

(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 17 June 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I am very sorry that it has taken us much longer than usual to have it evaluated - the main problem here was finding suited referees able to take on the review of two back-to-back submissions at this time. We have finally received all their reports, which I am herewith transmitting to you.

As you will see, the referees in general express interest in your study, but especially referees 2 and 3 feel that the work has to be both substantiated and somewhat extended to become a strong candidate for EMBOJ publication. With regard to the latter issue, referee 3 asks for further mechanistic understanding of the effects of altered dNTP pools; and referee 2 (key point 6) asks for experiments to generalize the concepts using other mutants that affect the damage checkpoint with directly affecting replication. Additional concerns are raised regarding presentation and interpretation of the data.

I would like to give you an opportunity to address these, as well as the other more specific points raised, in the form of a revised version of the manuscript. Given the somewhat restrained referee enthusiasm at the current stage, I should however make clear that eventual publication of the manuscript will depend not only on consolidation of the present experiments and their presentation but also on a significant extension in this case. Therefore, should you have any concerns regarding the experiments requested by the reviewers, I would encourage you to get back to me for further consultations.

We generally allow three months as standard revision time, and it is our policy that competing

manuscripts published during this period will have no negative impact on our final assessment of your revised 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.

Best wishes,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Jerome Poli and others from Philippe Pasero's group describes the important role that dNTP pools play in determining replication fork movement in the yeast S. cerevisiae and the role that the number of ongoing forks plays in controlling subsequent activation of later firing origins. Some of the ideas in the paper have been floating around for a number of years, but this work crystallizes these two connections and clarifies the role of replication and damage checkpoint proteins in the regulation of S phase. They show quite convincingly that the major role that Mec1 and Rad53 play in regulating S phase is actually through their role in regulating nucleotide precursors rather than any direct role in origin regulation. The quality of the data is excellent and the range of experiments is impressive. I believe that this manuscript will become known as the definitive study on "replication in the presence of HU" as they so clearly establish the mechanisms that underlie a variety of observations in the literature.

One major concern:

Since the first timed sample from S phase is at 30 minutes, it is not clear how the initial fork rate of ~4 kb/min over the first 8 minutes is determined. (pages 6, 8 and elsewhere) This point needs some clarification as to how both numbers were derived.

A few minor concerns (in the order they occur in the manuscript):

1. I'm not sure that "HU-resistant DNA synthesis" is the best term to describe the synthesis that takes place in the presence of HU. It implies that some gene product is resistant to the effects of HU and that does not seem to be the case. (Abstract, page 10, 16, and elsewhere.)

2. "Moreover, it has been reported that the DRC pathway acts to slow down the replication program so that initiation from all origins is delayed." (Bottom of page 4) Technically, not ALL of the origins are slowed down in their initiation as replication occurs pretty normally from the earliest set of origins.

3. "Automated process" (page 6) needs a reference to see Galaxy in the Methods and Materials.

4. There is a distinction between median replication time and the time of activation for an origin. To date, there are no good measurements for activation time. The replication timing data from Yabuki et al. (2002) refers to the median replication time, a composite of both passive replication and origin firing. (page 7 and many other places throughout the manuscript)

5. ". . . these cells were unable to switch to slow elongation after 60 minutes." It isn't clear from where the data for this statement come and it is unclear to which cells (wild type or mec1/rad53) the authors are referring.

6. Where do the estimates of "a 10-fold reduction of elongation rate and a 30-fold reduction of the initiation rate" come from? (page 7/8)

7. I believe that Feng et al. (2009) first showed that forks proceed further in the mec1 sml1 mutant than in the rad53 mutant. It would be good to cite their paper at the top of page 8. ("The fact that replication forks progress further in HU-treated mec1 mutants . . .")

8. ". . . sml1 cells progressed slightly faster through S phase" could suggest two different things. (page 9) Either cells take less time to traverse S phase, or they take the same length of time but begin (and finish) earlier in the mutant than in the wild type. From my study of the FACs profiles (Figure 4), it looks like the latter is correct.

9. Is replication stress synonymous with 200 mM HU? If so, then it should be stated in the opening sentence on page 10.

10. Do early forks move farther because they started earlier than late forks, or is there really evidence from the work that the early forks were moving faster, exhausting the dNTP pools, and therefore contributed significantly to the differences in fork progression, or is it the combination of both? (top of page 15)

11. "We also noticed interesting differences between forks emanating from early and late origins in rad53-11 cells . . ." Please refer to Figures 2D and Supplementary Figure 2 so the reader can find on what this conclusion is based.

12. Is there a missing word on the 6th line of page 16? ". . . also show increased dNTP and HU-resistant DNA synthesis."

13. Please include the chromosome number as part of the figure (1, 3, 6, supp 1, 5, 6, 7) when showing the array profiles. This omission is particularly confusing since the same chromosome isn't shown throughout the paper.

14. The models in Figure 2 and 7 indicate that dNTP pools in G1 are equivalent to those in S phase and drop over S phase to a basal level. However, in HU, the cells enter S at basal levels of dNTPs and drop further below basal level. Also in Figure 1A it isn't clear to what the labels "early" and "late" are referring.

15. Figure 4 and legend: (C) I can't see the Red Lines in the figure. Also there is a missing period from the end of this sentence that describes this red line in the figure legend. One additional point- the dATP pools seem to be exceptionally high (usually it is the dTTP pools that much higher than all the rest). The authors don't mention this finding and I am wondering to what they attribute this novel finding.

Referee #2 (Remarks to the Author):

In this manuscript Poli et al. have extensively analyzed DNA replication profiles of a variety of yeast mutants, combining single-molecule analysis (DNA combing) with genomic approaches. This set of experiments leads in my view to two main conclusions: 1) dNTP pools are tightly regulated and minimal variations (2-fold) in their levels are sufficient to induce detectable differences in replication fork progression and origin usage; 2) yeast mutants chronically prone to accumulate low levels of replication stress, show increased dNTP levels, associated with altered patterns (fork rate, HU-resistance) of DNA replication. While the first conclusion is well supported by the data, the second may need additional experimental work (see below), especially if the authors wish to propose it as a general, cancer-relevant mechanism (as they do in the current manuscript). If and once properly supported, these hypotheses are in principle sufficiently interesting and provocative to

merit publication in the EMBO Journal.

Nonetheless, several aspects of the manuscript are at the moment not satisfactory. Besides the points highlighted above, many of the additional claims - mostly related to the checkpoint mutants and HU-response - appear to me rather technical, debatable and/or over-interpreted, diluting what I would consider an otherwise important take-home-message (see above). Furthermore, the whole structure of the manuscript is rather illogical and contributes to the confusion.

Only once a revised version of this manuscript - extensively restructured and integrated with additional experimental work (see below) - will be available, it will be possible to firmly establish how relevant and well-supported are the main conclusions of this work, thus reaching a decision on the suitability for publication in the EMBO Journal.

Major criticisms:

1) The authors should restructure their manuscript, starting off with the sml1/GAL-RNR data, and the effect that even slight perturbations of nucleotide levels can have on replication rate (Figure 4A-C). While these aspects are novel, surprising and important, most of the data shown in Figures 1-3 simply represent the combination of these effects with the well-known defects associated with checkpoint mutants. In this respect, while I could imagine that this figure order reflects the "chronological" development of the story, showing the checkpoint data before the detailed analysis of sml1/GAL-RNR is illogical and confusing. In this respect, I would also anticipate some of the results in Figure 7 (in particular those referring to the temperature effects), in order to expand on this first general part and discuss different ways by which the experimental set-up can influence fork rate and origin firing, by directly or indirectly affecting nucleotide levels.

2) One simple prediction of the model proposed by the authors is that any increase in cellular dNTP levels should phenocopy "replication stress" mutants in terms of effects on DNA replication (faster forks with and without HU). It would thus be important to design experimental strategies to increase dNTP levels in the cells without interfering with DNA damage checkpoints or nucleotide biosynthesis, i.e. providing nucleosides to the culture media of suitable yeast strains, similarly to what recently performed in human cells (Bester et al., Cell 2011).

3) Differently from the accompanying manuscript (Davidson et al.) the authors here show that increased nucleotide levels can alter the replication pattern even in the absence of exogenous sources of replication stress (i.e. HU). Still, most of the experiments included in the manuscript make use of an RNR inhibitor (HU) to suggest that increased nucleotide levels could also assist replication in face of stress and one could formulate the trivial alternative hypothesis that DDR-mediated RNR up-regulation makes the inhibitor ineffective in the first place (by excessive substrate levels), leading as a secondary effect to the increased relative level of dNTPs and thus extended incorporation. If basal activation of the DNA damage response really leads to faster replication via RNR upregulation, this should be visible also in the presence of different agents interfering with fork progression, such as MMS or UV. There could be reasons why results may be different upon DNA damage and upon nucleotide depletions, but I consider essential to address this point experimentally and discuss similarities and discrepancies with HU, in order to formulate a general model (implied even in their title) on the role of nucleotide levels in replication stress.

4) With even a 2-fold increase in nucleotide levels resulting in marked differences in fork rate (Fig. 4A), it is very likely that fork rate is non-linearly influenced by nucleotide levels, but is "suddenly" affected above/below a certain threshold. In this respect, I find inappropriate and misleading to use the terms "switch" and "replication modes" (fast vs slow) throughout the manuscript, as they imply an active process which is not necessarily supported by any of their data. Forks are expected to be fast when dNTP levels are still above that threshold, while they are expected to suddenly and "passively" become slow once that threshold has been reached. Overall, I would be much more careful using those terms, as opposed to interpreting all their observations (both in wt and checkpoint defective cells) as a secondary consequence of reaching that threshold earlier/later.

5) As mentioned above, I consider most of their data in checkpoint mutants as rather predictable consequences of the known checkpoint defects, associated with the altered nucleotide levels described here. In other words, once the reader is acquainted with the effects of nucleotide levels in

wt cells, all data in Figure 1, 2 and 3 are highly expected considering the known defects in origin firing and fork stability in the checkpoint defective background, leading to premature nucleotide depletion. In my view, this part does not significantly contribute to our knowledge on the role and mechanisms of the replication checkpoint during DNA replication stress. I also find misleading to include standard checkpoint mutants as Group3 in their fork rate analysis in Figure 5A, as at this point of the manuscript it should be obvious that all those effects are simply explained by the sml1 mutation (and thus the increased nucleotide levels).

6) The authors interpret their data in Figure 5A to suggest that any mutations affecting the maintenance of genome integrity may result in increased dNTP levels and thus increased fork rate in HU. As checkpoint mutants are not particularly informative in this respect (see above), the authors should test their general model on a panel of different genetic backgrounds known to be associated with genotoxic stress and DDR response (most replication/recombination mutants are). In my view it would be particularly important to test their working hypothesis on different homologous recombination mutants, which are known to show basal activation of the DNA damage checkpoint, but do not directly affect the replication process. It would be highly relevant in the field to show a possible effect of these mutations in accelerating replication fork progression even in the absence of HU.

7) The authors are most probably aware of a recent publication implying reduced nucleotide levels in oncogenic stress during early tumorigenesis (Bester et al., Cell 2011). The hypothesis formulated in that work (oncogenic replication stress leads to genome instability via depletion of the nucleotide pool) is apparently at odd with the ideas suggested here (cronic replication stress leads to increased dNTP pools). It will be particularly important to consider this new work while interpreting results, especially while discussing possible implications for higher eukaryotes and tumorigenesis.

Minor points:

1) It would be important to perform experiments at lower HU doses, especially for their experiments in Figure 7A and B. One of the predictions of their model is that this should change the proportion of active origins, as well as fork distance, giving intermediate results between untreated controls and HU 200mM.

2) Figure S3 is just quickly mentioned in the text, but aims, technicalities and conclusions of this experiment are not clear in the main text, nor in the Supplemental Figure Legend.

3) While in page 11 (lane 11) the authors refer to reproducible activation of Rad53 in ctf18 cells, a few lanes further (page 12, lane1) they claim the exact opposite. This point should be clarified.

4) It would be helpful to complete the panel in Fig 2B by including in the analysis sml1 and rad53 sml1 strains. Based on their interpretations, both curves should be similar to mec1, but sml1 should keep incorporating further at later time points.

Referee #3 (Remarks to the Author):

Poli et al. EMBO J.

In this paper, the authors investigated the regulation of S phase program, which includes origin usage and fork elongation, under low dNTPs conditions. With the use of BrdU-IP-chip and DNA combing analyses, they found that DNA replication transited from a fast- (1 kb/min) to slow (0.1 kb/min) mode when almost half of origins were activated. This transition of DNA replication mode appeared to be dependent on the intracellular concentration of dNTPs because upregulation of RNR, a rate-limiting enzyme of dNTPs synthesis, delayed this transition with higher fork rate. In addition, some of mutants defective in checkpoints and maintenance of fork integrity showed HU-resistant DNA synthesis, at least in part due to increased dNTPs levels by DUN1-dependent Sml1 degradation. From these results, the authors proposed that dNTPs levels regulated S phase program in the presence of chronic replication stress.
Overall, in my opinion, although this manuscript does contain some interesting issues (eg. role of dNTPs levels in the regulation of fork rate), there are various concerns to be addressed before

considering its publication.

Major points
1. The authors did not provide any insight into the mechanisms how dNTPs levels regulated fork rate. Although one could speculate that DNA polymerase might be a direct target of this system, the authors should provide some experimental evidence. In case of Fig. 7, higher temperature could easily affect the activities of many other enzymes than DNA polymerase.
2. Fig. 2D and 2E appeared to be one of the most important results in this manuscript. However, interpretation of the results and the figure legends were unclear and hardly understood. For example, the authors mentioned that mec1 and rad53 mutants were unable to switch to slow elongation after 60 minutes---. However, replication forks are unstable in these mutants under low dNTPs condition as the authors indicated in Fig. 2B. Thus, it appears to be difficult to draw a clear conclusion in transition of replication mode in these checkpoint mutants.
3. Fig. 2E is intriguing, but more detailed kinetic analysis within the first 8 min are critically required for clear conclusions. The concentrations of dNTPs pools should be directly determined. In addition, the rate of fork elongation should also be examined by pulse chase experiments.
4. In Fig. 2E, the authors proposed that slow replication mode constantly continued up to 8 hours, but there appeared no clear experimental data being able to support this idea.
5. Prolonged pre-incubation of wild-type cells with HU during G1 synchronization by alpha-factor could reduce the level of dNTPs at the onset of DNA replication. In such case, is first mode of DNA replication (1 kb/min) no longer observed?
6. Fig. 4: fork elongation in sml1 mutant appeared 1.5-fold faster than that in wild-type (Fig. 4A), but total duration of replication completion in sml1 mutant was almost same as that in wild-type. Why?

1st Revision - authors' response 10 October 2011

Point-by-point response to Referees:

We are grateful to the three Referees for their constructive comments, which have allowed us to significantly improve our manuscript. Detailed answers are indicated below.

Referee #1 (Remarks to the Author):

The manuscript by Jerome Poli and others from Philippe Pasero's group describes the important role that dNTP pools play in determining replication fork movement in the yeast S. cerevisiae and the role that the number of ongoing forks plays in controlling subsequent activation of later firing origins. Some of the ideas in the paper have been floating around for a number of years, but this work crystallizes these two connections and clarifies the role of replication and damage checkpoint proteins in the regulation of S phase. They show quite convincingly that the major role that Mec1 and Rad53 play in regulating S phase is actually through their role in regulating nucleotide precursors rather than any direct role in origin regulation. The quality of the data is excellent and the range of experiments is impressive. I believe that this manuscript will become known as the definitive study on "replication in the presence of HU" as they so clearly establish the mechanisms that underlie a variety of observations in the literature.

One major concern:

Since the first timed sample from S phase is at 30 minutes, it is not clear how the initial fork rate of ~4 kb/min over the first 8 minutes is determined. (pages 6, 8 and elsewhere) This point needs some clarification as to how both numbers were derived.

We have now clarified this issue in the text. Mean replication times of origins show a biphasic distribution from 17 to 25 minutes during a normal S phase, with a transition between early and late origins occurring precisely at t=25 minutes (Yabuki et al, 2002). In the presence of HU, our data indicate that cells switch to slow-replication mode concomitantly with the repression of late origins (t=25 min). We therefore estimated that cells replicate for 25-17= 8 minutes before running out of dNTPs. Fork rate during this period was determined by subtracting the distance covered by forks progressing from origins activated at t=17 min (~6.5kb) to the distance covered by forks progressing from origins activated at t=25 min (~1.5 kb) and dividing this difference (5 kb) by 8 minutes (0.6 kb/min). We also confirmed this value by determining precisely the time of *ARS305* activation in our experimental conditions and by measuring the distance covered by replication origins 30 minutes after release from the G1 arrest (Figure 1E). We now propose an estimate of 0.6 to 1 kb/min that is derived from these two types of experiments.

A few minor concerns (in the order they occur in the manuscript):

1. I'm not sure that "HU-resistant DNA synthesis" is the best term to describe the synthesis that takes place in the presence of HU. It implies that some gene product is resistant to the effects of HU and that does not seem to be the case. (Abstract, page 10, 16, and elsewhere.)

HU-resistant DNA synthesis does not necessarily imply an active process, but to alleviate any ambiguity, we have replaced “HU-resistant DNA synthesis” by “enhanced DNA synthesis in the presence of HU”.

2. "Moreover, it has been reported that the DRC pathway acts to slow down the replication program so that initiation from all origins is delayed." (Bottom of page 4) Technically, not ALL of the origins are slowed down in their initiation as replication occurs pretty normally from the earliest set of origins.

We agree on that and we have modified the text by replacing “all origins” by “late origins”.

3. "Automated process" (page 6) needs a reference to see Galaxy in the Methods and Materials.

References to Galaxy have been added to the text.

4. There is a distinction between median replication time and the time of activation for an origin. To date, there are no good measurements for activation time. The replication timing data from Yabuki et al. (2002) refers to the median replication time, a composite of both passive replication and origin firing. (page 7 and many other places throughout the manuscript)

We agree on that. Mean replication times from Yabuki *et al.* are now properly defined in the text and figure legends.

5. ". . . these cells were unable to switch to slow elongation after 60 minutes." It isn't clear from where the data for this statement come and it is unclear to which cells (wild type or mec1/rad53) the authors are referring.

We agree that the sentence is unclear. We have removed this sentence and transferred the corresponding figure to the Supplementary material.

6. Where do the estimates of "a 10-fold reduction of elongation rate and a 30-fold reduction of the initiation rate" come from? (page 7/8)

Elongation rates are derived from BrdU-IP-chip experiments (Fig 1) and from single-molecule analyses (Fig S2). Both approaches indicate a fork rate of about 100 bp/min (v=d/t). The initiation rate in HU was determined by measuring the number of origins that fire during the BrdU-IP-chip time-course experiment shown in figure 1 (#oris at 120 min - #oris at 60 min / 60). These calculations are now included in the text.

7. I believe that Feng et al. (2009) first showed that forks proceed further in the mec1 sml1Δ; mutant than in the rad53 mutant. It would be good to cite their paper at the top of page 8. ("The fact that replication forks progress further in HU-treated mec1 mutants . . .")

The reference has been added when *mec1-1 sml1-1* cells phenotype is discussed.

8. ". . . sml1Δ; cells progressed slightly faster through S phase" could suggest two different things. (page 9) Either cells take less time to traverse S phase, or they take the same length of time but begin (and finish) earlier in the mutant than in the wild type. From my study of the FACs profiles (Figure 4), it looks like the latter is correct.

To distinguish between the two possibilities suggested by this Referee, we analyzed the budding index of both cultures. We found that wild-type and *sml1Δ* cells start to bud at the same time. The FACs profiles indicate that both strains have a similar DNA content at 20 min post-G1 release but then, *sml1Δ* cells increase more rapidly their DNA content. Altogether, this suggests that both strains start to replicate at the same time and that *sml1Δ* cells progress slightly faster through S phase. Since this is not a major issue, we have moved these data to Supplementary material.

9. Is replication stress synonymous with 200 mM HU? If so, then it should be stated in the opening sentence on page 10.

In this study, we have mainly used 200 mM HU to induce replication stress. Other experiments have also been performed with lower concentration of HU (50 and 100 mM, Fig 3A-B) or with DNA damaging agents such as MMS & 4-NQO (Fig 6). We have now indicated the exact experimental conditions used whenever replication stress is induced.

10. Do early forks move farther because they started earlier than late forks, or is there really evidence from the work that the early forks were moving faster, exhausting the dNTP pools, and therefore contributed significantly to the differences in fork progression, or is it the combination of both? (top of page 15)

After 60 min in HU, we observed a strong correlation between BrdU tracks length and mean replication time for each replication forks. At later time points (90 and 120 min), all forks progressed at the same rate, regardless of their mean replication time (Fig. 1C). We therefore proposed that all forks move at the same rate until dNTP exhaustion, but early forks move farther because they started earlier. Although we cannot formally exclude the possibility that early forks move faster than late forks, we favor the hypothesis that differences in BrdU tracks length reflect differences in initiation time.

11. "We also noticed interesting differences between forks emanating from early and late origins in rad53-11 cells . . ." Please refer to Figures 2D and Supplementary Figure 2 so the reader can find on what this conclusion is based.

We have moved these data to Supplemental material and modified the text accordingly.

12. Is there a missing word on the 6th line of page 16? ". . . also show increased dNTP and HU-resistant DNA synthesis."

We have corrected this mistake.

13. Please include the chromosome number as part of the figure (1, 3, 6, supp 1, 5, 6, 7) when showing the array profiles. This omission is particularly confusing since the same chromosome isn't shown throughout the paper.

The chromosome number is now indicated above each replication profile.

14. The models in Figure 2 and 7 indicate that dNTP pools in G1 are equivalent to those in S phase and drop over S phase to a basal level. However, in HU, the cells enter S at basal levels of dNTPs and drop further below basal level. Also in Figure 1A it isn't clear to what the labels "early" and "late" are referring.

We now indicate in the model that in untreated cells (-HU), dNTP levels increase at the G1-S transition, as reported by Koç *et al*., 2004. In contrast, dNTP pools do not increase in the presence of HU and cells enter S phase with G_1 levels. Figure 1 has been extensively reorganized and “early and late” labels do no longer appear.

15. Figure 4 and legend: (C) I can't see the Red Lines in the figure. Also there is a missing period from the end of this sentence that describes this red line in the figure legend. One additional point-the dATP pools seem to be exceptionally high (usually it is the dTTP pools that much higher than all the rest). The authors don't mention this finding and I am wondering to what they attribute this novel finding.

The FACs profiles have been moved to Supplementary Figures. As pointed out by this Referee, dTTP is usually ~two-fold higher than dATP and dCTP. However, we have repeatedly seen that dATP increases more than the other three dNTPs in the strains overexpressing Rnr1. Most likely, this is due to the fact that when all dNTPs increase to a certain level, dATP starts to bind to the allosteric activity site in RNR to switch it off. Because we heavily overexpress Rnr1, and assuming then that both the allosteric activity and allosteric specificity sites will be dATP-bound, this will lead to the increased of dATP concentration.

Referee #2 (Remarks to the Author):

In this manuscript Poli et al. have extensively analyzed DNA replication profiles of a variety of yeast mutants, combining single-molecule analysis (DNA combing) with genomic approaches. This set of experiments leads in my view to two main conclusions: 1) dNTP pools are tightly regulated and minimal variations (2-fold) in their levels are sufficient to induce detectable differences in replication fork progression and origin usage; 2) yeast mutants chronically prone to accumulate low levels of replication stress, show increased dNTP levels, associated with altered patterns (fork rate, HU-resistance) of DNA replication. While the first conclusion is well supported by the data, the second may need additional experimental work (see below), especially if the authors wish to propose it as a general, cancer-relevant mechanism (as they do in the current manuscript). If and once properly supported, these hypotheses are in principle sufficiently interesting and provocative to merit publication in the EMBO Journal.

Nonetheless, several aspects of the manuscript are at the moment not satisfactory. Besides the points highlighted above, many of the additional claims - mostly related to the checkpoint mutants and HU-response - appear to me rather technical, debatable and/or over-interpreted, diluting what I would consider an otherwise important take-home-message (see above). Furthermore, the whole structure of the manuscript is rather illogical and contributes to the confusion.

Only once a revised version of this manuscript - extensively restructured and integrated with additional experimental work (see below) - will be available, it will be possible to firmly establish how relevant and well-supported are the main conclusions of this work, thus reaching a decision on the suitability for publication in the EMBO Journal.

Major criticisms:

1) The authors should restructure their manuscript, starting off with the sml1/GAL-RNR data, and the effect that even slight perturbations of nucleotide levels can have on replication rate (Figure 4A-C). While these aspects are novel, surprising and important, most of the data shown in Figures 1-3 simply represent the combination of these effects with the well-known defects associated with checkpoint mutants. In this respect, while I could imagine that this figure order reflects the "chronological" development of the story, showing the checkpoint data before the detailed analysis of sml1/GAL-RNR is illogical and confusing. In this respect, I would also anticipate some of the results in Figure 7 (in particular those referring to the temperature effects), in order to expand on this first general part and discuss different ways by which the experimental set-up can influence fork rate and origin firing, by directly or indirectly affecting nucleotide levels.

We have followed the Referee's suggestion to substantially restructure the manuscript. We now begin with a figure showing how wild type cells replicate under low dNTP conditions. Most of the data relative to checkpoint have been moved to Supplementary material. Then, we present the data showing that subtle changes in nucleotide levels affect the pattern of DNA replication, both in the presence or in absence of HU (Figure 2 and 3). We follow up with data indicating that chromosomal instability mutants show increased dNTP pools and altered replication profiles (Figure 4 and 5). Finally, we show that elevated dNTP pools promote bypass of DNA lesions and resistance to replication stress. We agree that this organization is more logical and clarifies the message of the manuscript.

2) One simple prediction of the model proposed by the authors is that any increase in cellular dNTP levels should phenocopy "replication stress" mutants in terms of effects on DNA replication (faster forks with and without HU). It would thus be important to design experimental strategies to increase dNTP levels in the cells without interfering with DNA damage checkpoints or nucleotide biosynthesis, i.e. providing nucleosides to the culture media of suitable yeast strains, similarly to what recently performed in human cells (Bester et al., Cell 2011).

In principle, the simplest strategy to increase intracellular dNTP pools would be to add precursors in the medium, as suggested by this Referee. However, this is not applicable to yeasts as they lack kinases that phosphorylate nucleosides to nucleotides (our stains only express Herpes

simplex thymidine kinase to incorporate BrdU). We have therefore increased dNTP levels by either overexpressing RNR or deleting the RNR inhibitor Sml1. This allowed us to increase dNTP levels and to maintain balanced pools, which is crucial to avoid defects in checkpoint activation (Kumar et al., 2010).

3) Differently from the accompanying manuscript (Davidson et al.) the authors here show that increased nucleotide levels can alter the replication pattern even in the absence of exogenous sources of replication stress (i.e. HU). Still, most of the experiments included in the manuscript make use of an RNR inhibitor (HU) to suggest that increased nucleotide levels could also assist replication in face of stress and one could formulate the trivial alternative hypothesis that DDR-mediated RNR up-regulation makes the inhibitor ineffective in the first place (by excessive substrate levels), leading as a secondary effect to the increased relative level of dNTPs and thus extended incorporation. If basal activation of the DNA damage response really leads to faster replication via RNR upregulation, this should be visible also in the presence of different agents interfering with fork progression, such as MMS or UV. There could be reasons why results may be different upon DNA damage and upon nucleotide depletions, but I consider essential to address this point experimentally and discuss similarities and discrepancies with HU, in order to formulate a general model (implied even in their title) on the role of nucleotide levels in replication stress.

As requested by this Referee, we have extended our analysis to other DNA damaging agents such as MMS and the UV-mimetic 4-NQO (Fig. 6). We now show that increased dNTP levels promote fork progression in the presence of MMS and prevent the activation of the DNA replication checkpoint, both in the presence of MMS and 4-NQO. These data support the view that increased nucleotide levels help yeast cells tolerate replication stress.

4) With even a 2-fold increase in nucleotide levels resulting in marked differences in fork rate (Fig. 4A), it is very likely that fork rate is non-linearly influenced by nucleotide levels, but is "suddenly" affected above/below a certain threshold. In this respect, I find inappropriate and misleading to use the terms "switch" and "replication modes" (fast vs slow) throughout the manuscript, as they imply an active process which is not necessarily supported by any of their data. Forks are expected to be fast when dNTP levels are still above that threshold, while they are expected to suddenly and "passively" become slow once that threshold has been reached. Overall, I would be much more careful using those terms, as opposed to interpreting all their observations (both in wt and checkpoint defective cells) as a secondary consequence of reaching that threshold earlier/later.

We agree that the term “switch” is misleading as it infers the existence of an active process that is not demonstrated here. We are now using the term of “transition” between regular and slow replication and we make clear that this transition is essentially determined by dNTP levels and not by checkpoint-mediated mechanisms.

5) As mentioned above, I consider most of their data in checkpoint mutants as rather predictable consequences of the known checkpoint defects, associated with the altered nucleotide levels described here. In other words, once the reader is acquainted with the effects of nucleotide levels

in wt cells, all data in Figure 1, 2 and 3 are highly expected considering the known defects in origin firing and fork stability in the checkpoint defective background, leading to premature nucleotide depletion. In my view, this part does not significantly contribute to our knowledge on the role and mechanisms of the replication checkpoint during DNA replication stress. I also find misleading to include standard checkpoint mutants as Group3 in their fork rate analysis in Figure 5A, as at this point of the manuscript it should be obvious that all those effects are simply explained by the sml1 mutation (and thus the increased nucleotide levels).

We have now moved most of the data on checkpoint mutants to supplementary material and we make it clear from the beginning that the large BrdU tracks in *mec1* mutants are due to the *sml1* mutation. However, we decided to keep the checkpoint mutants in Figure 4 as some readers might be interested to see that differences in track length between *mec1* and *rad53* mutants are due to dNTP levels and not to Mec1-dependent and Rad53-independent mechanisms.

6) The authors interpret their data in Figure 5A to suggest that any mutations affecting the maintenance of genome integrity may result in increased dNTP levels and thus increased fork rate in HU. As checkpoint mutants are not particularly informative in this respect (see above), the authors should test their general model on a panel of different genetic backgrounds known to be associated with genotoxic stress and DDR response (most replication/recombination mutants are). In my view it would be particularly important to test their working hypothesis on different homologous recombination mutants, which are known to show basal activation of the DNA damage checkpoint, but do not directly affect the replication process. It would be highly relevant in the field to show a possible effect of these mutations in accelerating replication fork progression even in the absence of HU.

We have now included additional mutants showing spontaneous DNA damage and DDR activation, including *rad52Δ*, *sgs1Δ*, *asf1Δ*, *rrm3Δ* and *rtt101Δ* to our analysis of dNTP levels and replication profile in HU. All these mutants exhibit larger BrdU tracks and higher dNTP levels compared to wild-type cells in HU (Fig. 4). We also observed that several of these mutants (*asf1Δ*, *sgs1Δ*, *rtt101Δ*) have faster forks in the absence of HU (data not shown and Versini *et al.* 2003).

7) The authors are most probably aware of a recent publication implying reduced nucleotide levels in oncogenic stress during early tumorigenesis (Bester et al., Cell 2011). The hypothesis formulated in that work oncogenic replication stress leads to genome instability via depletion of the nucleotide pool) is apparently at odd with the ideas suggested here (chronic replication stress leads to increased dNTP pools). It will be particularly important to consider this new work while interpreting results, especially while discussing possible implications for higher eukaryotes and tumorigenesis.

We now discuss this article, together with another related work showing that imbalanced dNTP pools contribute to genomic instability in Bloom syndrome (Chabosseau et al., 2011). We also comment on differences between yeast and higher eukaryotes regarding the regulation of dNTP pools in response to DNA damage.

Minor points:

1) It would be important to perform experiments at lower HU doses, especially for their experiments in Figure 7A and B. One of the predictions of their model is that this should change the proportion of active origins, as well as fork distance, giving intermediate results between untreated controls and HU 200mM.

We have now included the results obtained in wild-type cells treated with 50 and 100 mM HU in Figure 3A-B. Results are coherent with our model, showing that intermediate amounts of HU between 0 and 200 mM lead to proportional effects on fork progression and origin usage. This further suggests that the transition between regular and slow replication occurs at the time of dNTP pools exhaustion.

2) Figure S3 is just quickly mentioned in the text, but aims, technicalities and conclusions of this experiment are not clear in the main text, nor in the Supplemental Figure Legend.

We have now included additional information to explain how the experiment was performed and how the results are interpreted. As requested by Referee 3, we also performed 2h BrdU pulses during a prolonged exposure to HU. This experiment revealed that forks move at a slow but constant rate throughout the HU treatment (Figure S2 C and D).

3) While in page 11 (lane 11) the authors refer to reproducible activation of Rad53 in ctf18 cells, a few lanes further (page 12, lane1) they claim the exact opposite. This point should be clarified.

We now explain that the Mrc1 branch of the DRC is defective in *ctf18Δ* cells, but these mutants are still able to activate Rad53 through the DDC pathway because they accumulate DNA damage at arrested forks (Crabbé et al., 2010).

4) It would be helpful to complete the panel in Fig 2B by including in the analysis sml1 and rad53 sml1 strains. Based on their interpretations, both curves should be similar to mec1, but sml1 should keep incorporating further at later time points.

We have been unable to perform these experiments due to time constraints.

Referee #3 (Remarks to the Author):

Poli et al. EMBO J.

In this paper, the authors investigated the regulation of S phase program, which includes origin usage and fork elongation, under low dNTPs conditions. With the use of BrdU-IP-chip and DNA combing analyses, they found that DNA replication transited from a fast- (1 kb/min) to slow (0.1 kb/min) mode when almost half of origins were activated. This transition of DNA replication mode appeared to be dependent on the intracellular concentration of dNTPs because upregulation of RNR, a rate-limiting enzyme of dNTPs synthesis, delayed this transition with higher fork rate. In addition, some of mutants defective in checkpoints and maintenance of fork integrity showed HU-resistant DNA synthesis, at least in part due to increased dNTPs levels by DUN1-dependent

Sml1 degradation. From these results, the authors proposed that dNTPs levels regulated S phase program in the presence of chronic replication stress.
Overall, in my opinion, although this manuscript does contain some interesting issues (eg. role of dNTPs levels in the regulation of fork rate), there are various concerns to be addressed before considering its publication.

Major points
1. The authors did not provide any insight into the mechanisms how dNTPs levels regulated fork rate. Although one could speculate that DNA polymerase might be a direct target of this system, the authors should provide some experimental evidence. In case of Fig. 7, higher temperature could easily affect the activities of many other enzymes than DNA polymerase.

Our study aims at understanding better how dNTP pools affect DNA replication kinetics, both in the presence or the absence of replication stress. We provide direct evidence that physiological dNTP pools are limiting for DNA synthesis and that increased nucleotide levels accelerate fork progression. The mechanisms by which increased temperature or dNTP pools promote fork progression are probably very complex and deciphering these mechanisms goes beyond the scope of our work.

2. Fig. 2D and 2E appeared to be one of the most important results in this manuscript. However, interpretation of the results and the figure legends were unclear and hardly understood. For example, the authors mentioned that mec1 and rad53 mutants were unable to switch to slow elongation after 60 minutes---. However, replication forks are unstable in these mutants under low dNTPs condition as the authors indicated in Fig. 2B. Thus, it appears to be difficult to draw a clear conclusion in transition of replication mode in these checkpoint mutants.

We have extensively restructured this figure, which is now Figure 1. Data supporting the model are now better explained in the main text. We agree that the sentence on *mec1* and *rad53* mutants was unclear, as also pointed out by Referee 1. We have moved this part of the figure to the Supplementary material and removed this comment from the main text. The point that we wanted to make is that checkpoint-deficient cells are unable to sustain replication after the transition to slow replication.

3. Fig. 2E is intriguing, but more detailed kinetic analysis within the first 8 min are critically required for clear conclusions. The concentrations of dNTPs pools should be directly determined. In addition, the rate of fork elongation should also be examined by pulse chase experiments.

We have extended the time-course analysis of BrdU track length to earlier time points (30, 45 and 60 minutes after release from G_1. We have also used a qPCR-based approach to determine precisely when the early origin ARS305 fires in our experimental conditions. From these experiments, we conclude that the transition from regular to slow replication occurs around 30 min after release from G1.

4. In Fig. 2E, the authors proposed that slow replication mode constantly continued up to 8 hours, but there appeared no clear experimental data being able to support this idea.

As requested by this Referee, we have performed 2h BrdU pulses during prolonged exposure to HU. This experiment allowed us to determine that forks are moving at a slow but constant rate throughout the HU treatment (Figure S1 and S2 C,D), at least until 50% of genome duplication (i.e t=4h). This experiment is consistent with the time course in HU shown in Supplementary Figure S2A-B, showing a mean fork speed of 100 bp/min. After 6 hours in HU, DNA fibers are more frequently broken, indicating that prolonged HU exposure induces DNA breaks.

5. Prolonged pre-incubation of wild-type cells with HU during G1 synchronization by alpha-factor could reduce the level of dNTPs at the onset of DNA replication. In such case, is first mode of DNA replication (1 kb/min) no longer observed?

Koç et al (2004) have reported that continuous labeling studies, in which ^{14}C-Uracil incorporation into DNA was monitored, indicated that HU inhibits DNA synthesis in yeast within 10 min of its administration. For this reason, in our experimental design, we decided to incubate cells 20 mins in HU prior G1 release to ensure that HU had adequate time to enter the cell and inactivate RNR.

6. Fig. 4: fork elongation in sml1 mutant appeared 1.5-fold faster than that in wild-type (Fig. 4A), but total duration of replication completion in sml1 mutant was almost same as that in wild-type. Why?

This issue has also been raised by Referee 1. According to FACS profiles, we believe that S-phase is slightly shorter in *sml1Δ* cells. Since this is not a major issue, we have moved these data to Supplementary material.

2nd Editorial Decision 10 November 2011

Thank you for submitting your revised manuscript, which has now been re-reviewed by the original referees. All of them consider the study significantly improved, and feel that the presented data would now in principle justify publication in The EMBO Journal. Nevertheless, both referees 1 and 2 still retain a number of issues with the interpretation, presentation and restructuring of the manuscript. I am therefore returning it to you once more for a final round of modification, with the kind request to carefully take the points of the referees into account and to edit and restructure the manuscript in order to clarify all remaining concerns.

As additional editorial issues:
- please remove supplementary figures legends from the main text, and combine them with all supplementary figures and tables in one single supplementary information PDF
- please make sure to include a valid GEO accession number at this stage
- finally, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to ask you if you would be willing to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission selecting "Figure Source Data" as object type, and would be published online with the article as a supplementary "Source Data" file.

Once carefully revised, please re-submit the final version as usual through our website. I am hoping that following adequate re-revision, we should then be able to proceed with eventual acceptance and publication of the study. Should you have any further questions in this regard, please do not hesitate to contact me.

Sincerely,
Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Poli et al. (dNTP pools determine fork progression and origin usage under replication stress) has undergone substantial revision in response to the three reviewer's comments. Because it has been radically restructured, some deficiencies that were not apparent to us in the first version are now more obvious. We are sorry that we missed them or failed to weight them as strongly as we now do. However, we are enthusiastic about the data that relate to fork movement and are hopeful that our criticisms will lead to a strengthened story, by removing those aspects that we feel are over-interpreted or weak.

One of disappointments in this version is the lack of attention to detail that suggests the authors didn't take our previous concerns seriously or were too quick to return the manuscript for re-review (because of the co-submission from the Brown lab, possibly?). In particular, we are disappointed that they said they responded to our concerns in the new manuscript, when in fact they had not. A second disappointment stems from a lack of consistent detail in the figures and methods and their references in the text. We will break down this review into two areas: 1) interpretation of data and 2) attention to details.

1) Interpretation of data:

A) A concern that we had with the first version was that there was no clear explanation for how they calculated the early fork rate of ~4 kb/minute when their earliest sampling time was 60 minutes. In the new version, they reduce the estimate to 1.5 kb/min, by including a 30 minute sample, but the calculations rely on imputation from data obtained from Yabuki et al. (2002) (in a still rather unclear way). In addition, the description still does not adequately describe how this value is determined. If the values can change by a factor of almost 3 by adding a single timed sample, it makes us wonder whether the difference between slow and fast forks might disappear altogether if earlier times were sampled. This calculation should be made transparent in the text, methods or supplement.

B) We raised the problem with their reference to origins as early and late based on the Yabuki data. Replication time is a composite of both activation time and passive replication due to variable origin firing efficiencies. The authors said that they agreed that there were no good measurements of origin firing time and said they changed it in their text (6th line from the bottom of page 6), yet in the very next sentence there is a statement that yeast origins "fire from 17 to 33 minutes during a normal S phase and show a biphasic distribution . . .". Again on the top of the next page is the phrase that "track lengths in HU correlate with time of origin activation". In the next few sentences are references to "forks activated at 17 minutes", "forks generated at 8 minutes" and "regardless of their initiation time". Despite the initial caution, it is clear that the authors want to equate replication time with firing time. And the readers will certainly take away from their discussion that early and late refer to initiation times. In fact, we believe their BU-data provide some potential places to clarify this issue, but they didn't choose to describe how their data could be used to support the ideas of origins with different activation times.

C) The authors show very nicely that the core of ARS305 replicates at 25 minutes in their HU experiment using qPCR that measures the ~100 bp centered on the ARS. They also need to measure the time of replication in -HU experiments using the same methods instead of relying on the data from Yabuki et el. who measured ARS305 replication at 19.7 minutes in the absence of HU. The authors compare these results and conclude that ARS305 replicates at the same time in HU as it does in the absence of HU. Do the authors consider a 5.3 minute difference to be meaningless? If so, it is surprising that they place a lot of weight on an 8 minute difference in replication time from the Yabuki et al. data when making other arguments. Alvino et al. (2007) showed a much later time of half maximal replication of ARS 305 in HU (~80 minutes or so can be inferred from their data obtained at 23 C). Are there difference in strain background or culture conditions that would lead to these differences? Or in the way that replication is measured-copy number vs. shift in density of a large restriction fragment from HH to HL? Is the temperature or type of media playing a role in dNTP pools that are responsible for these timing differences? We are disappointed that the authors completely fail to discuss their results in light of this previous work of Alvino et al., (2007) that does not show a "fast vs. slow" rate of replication for different origins. What do the authors think was missing from the Alvino et al. study?

2) Lack of attention to detail:

A) "HU-resistant DNA synthesis" was said to have been stricken from the manuscript, but there it is several times on pages 11 and 12. (Also see the concern about the continued use of origin firing time imputed from replication time reported by Yabuki et al. in point 1B above.)

B) The "automated process" to determine rates of initiation and elongation (page 6) is said to be described in the methods and materials. There is a brief mention of how peaks are called (to identify origins) and that peaks that did not grow in size over time were eliminated from the calculation of fork progression. There is not enough information is this brief section to allow the reader of the manuscript to re-derive their rates. (Why were forks that didn't appear to move eliminated from fork rate estimates? It seems like this is an important part of the data that should be included in the average rate.) There is also no mention of which of the OriDB origins were detected in their BU-ChIP experiments. Did they ignore BU signals that didn't correspond to OriDB sites? How did their BU-positive peaks correspond to those of Yabuki et al. (2002), Feng et al. (2006), and Alvino et al. (2007)? Are the authors making their own calls on what is an active origin or just using the list from the Yabuki paper? Many of the BU-ChIP experiments (Figures 4D, S4, S5, S8B) don't show the significance cut-off value, so the reader is left to wonder why some peaks are significant and others are not; and the negative log2 values are such a light gray that they all but disappear. Finally, the way in which the Yabuki data was used is entirely mysterious to us.

C) The dGTP pool measurements in Figures 4B and 5C differ by almost an order of magnitude for the same strains. This lack of reproducibility makes me wonder whether the other differences noted are at all meaningful.

D) There are several model drawings (1F and 3E) that are not well described in either the figure legends or in the text (in the original version and in this version). We can't interpret them without some dialogue. Also missing from this version are the supplemental whole genome BU-ChIP analyses that were included in the first version. It is important for the reader to be able to flip through these to come to the same conclusions as the authors. The small pieces of just two chromosomes shown in 1A and 5D are fine for the main text, but only if the rest of the genome is shown in a similar format in the supplementary information.

E) Figures: There are no references in the text to Supplemental Figures 1D, 2C and D (and no conclusions as to the possibility that forks are speeding up the longer cells are kept in HU). What are the R2 values for all of the origins considered as a single class (Supplemental figures 1D and 6D)? I can't see the red lines in Supplemental Figure 3A that show the difference between wt and sml1Del FACS profiles. The legend to Supplement Figure 4 says there are two smlDel strains but I see only one but two wild types strains at different temperatures. There are two mysterious ARSs in Supplemental Figure 5B labeled .61 and .62. These are not annotations that I am familiar with. In Supplementary Figure 6A there appears to be a significant peak for ARS 425 (based upon the significance cut-off) that is not called as an active origin. What is the explanation for their call? Where is the vertical red line indicating the position of the centromere in this figure? What is the serial dilution factor for the plating experiment in Figure 8C? (It probably doesn't matter, but the lack of detail, when it is so easily given, is annoying.)

Overall Recommendations:

We think the manuscript could tell a great story about fork progression and the role of dNTP pools, but that the conclusions on early/late origins overreach the data. We don't want to be presumptuous, but feel that the manuscript could be edited successfully by beginning on page 8 with "We monitored the effect of increased RNR activity on replication fork progression" (line 6); eliminate any reference to the origin initiation program and the relevant figures; and keep the figures that relate to fork progression: Figures1A and E (but delete the dotted line to 17 minutes on the X axis), Figure 2, Figure 3A and B (but get rid of the gray and blue bars designating early and late firing origins), and Figures 4, 5 and 6. Finally, add a model figure that includes some of the features of the models presented in the earlier figures and describe the model in the discussion.

Referee #2 (Remarks to the Author):

In this revised manuscript Poli et al. have adequately addressed the most relevant concerns of this reviewer, by extensive restructuring of figures and text in their Results section. Now the paper is in my view much more logical and easier to follow, and conveys more effectively the most important messages. Two rather important criticisms, that would require further work on text and figures, remain to be addressed prior to publication in EMBO Journal:

1) The Discussion session has not been sufficiently restructured according to the new format of the manuscript, keeping too much emphasis on correlative and rather technical points (differences between checkpoint mutations, etc.) and omitting to highlight the most relevant observations impacting the fields of DNA replication and genome stability in general (effects on fork progression of minor changes in dNTP levels; general effects on fork rate in all mutants with elevated endogenous DDR; effects on fork progression extendible to various conditions of replication stress). The authors would make their message much stronger discussing first these central points and getting to the checkpoint connection only briefly towards the end (most of these data have anyway been moved to the Supplementary Material).

2) While most experiments are performed in HU (a possible caveat, as discussed in my previous comments, point 3), the authors have now solid data to confirm these observations in response to different types of replication stress (MMS, 4-NQO). I am convinced that this important validation of

their system should be much anticipated, to avoid similar scepticism by the readers until the end of the Results section. I would thus strongly suggest that Fig. 6 should become Fig. 3, especially considering that the format of this figure is quite similar to the data presented in Fig. 2 (easy to establish an immediate connection in the text).

A few minor points should also be considered while finalizing the manuscript for publication:

1) Most data in Fig. S1 are not mentioned/described anywhere in the manuscript. There is also no mention of panels C and D of Fig. S2.

2) Page 10, the list of mutants analyzed for intra-S checkpoint includes a strain (mec1tel1sml1) that is no longer depicted in Figure 4A, where instead rad53sml1 is included (and not mentioned in the text)

3) The FACS profiles in Fig. S3 were probably meant to show some overlays of the wt profile within the sml1 panel, but those are not properly visible. This may require some graphical modifications.

Referee #3 (Remarks to the Author):

The authors addressed previous my concerns in the revised version. Especially, the revised Fig 1 is now much more convincing than the previous Fig. 2. I recommend its publication in EMBO J.

2nd Revision - authors' response 29 November 2011

Detailed response to Referees – Re-revision of ms #EMBOJ-2011-78050

Referee #1 (Remarks to the Author):

The manuscript by Poli et al. (dNTP pools determine fork progression and origin usage under replication stress) has undergone substantial revision in response to the three reviewer's comments. Because it has been radically restructured, some deficiencies that were not apparent to us in the first version are now more obvious. We are sorry that we missed them or failed to weight them as strongly as we now do. However, we are enthusiastic about the data that relate to fork movement and are hopeful that our criticisms will lead to a strengthened story, by removing those aspects that we feel are over-interpreted or weak.

We thank this Referee for his/hers general comments and further suggestions to improve our manuscript.

One of disappointments in this version is the lack of attention to detail that suggests the authors didn't take our previous concerns seriously or were too quick to return the manuscript for re-review (because of the co-submission from the Brown lab, possibly?). In particular, we are disappointed that they said they responded to our concerns in the new manuscript, when in fact they had not. A second disappointment stems from a lack of consistent detail in the figures and methods and their references in the text. We will break down this review into two areas: 1) interpretation of data and 2) attention to details.

We are sorry if this Referee felt that we didn't take his/hers comments seriously as this was not our intention. During this revision process, we have addressed over 30 specific issues raised by the three Referees and the manuscript has undergone substantial modifications. We apologize if some issues have not been properly addressed during this extensive revision process. We are thankful to Referee #1 for pointing out several points that still needed to be improved. Yet, we would like to mention that the only major issue raised by this Referee was based on an incorrect interpretation of our data (see below).

1) Interpretation of data:

A) A concern that we had with the first version was that there was no clear explanation for how they calculated the early fork rate of ~4 kb/minute when their earliest sampling time was 60 minutes. In the new version, they reduce the estimate to 1.5 kb/min, by including a 30 minute sample, but the calculations rely on imputation from data obtained from Yabuki et al. (2002) (in a still rather unclear way). In addition, the description still does not adequately describe how this value is determined. If the values can change by a factor of almost 3 by adding a single timed sample, it makes us wonder whether the difference between slow and fast forks might disappear altogether if earlier times were sampled. This calculation should be made transparent in the text, methods or supplement.

An early fork rate of 4 kb/min was never mentioned anywhere in the manuscript. In the first version, we indicated a fork rate of 1 kb/min (e.g. Fig. 2E in the first version). We also indicated at the bottom of page 6 that "Analysis of fork progression revealed that cells replicate ~4 kb per fork on average and then switch to a slow replication mode". We assume that this 4 kb value has been misinterpreted by this Referee as "4 kb/min". Along the same line, we never "reduced this estimate to 1.5 kb/min" as stated above by this Referee. Although we mentioned page 7 that "forks generated 8 minutes later covered less than 1.5 kb", the fork rate of 1.5 kb/min is never mentioned in the revised manuscript (same mistake). In conclusion, there is no significant value change between the two versions of the manuscript despite the addition of earlier time point. The calculations are made perfectly clear in the text, methods and supplement.

B) We raised the problem with their reference to origins as early and late based on the Yabuki data. Replication time is a composite of both activation time and passive replication due to variable origin firing efficiencies. The authors said that they agreed that there were no good measurements of origin firing time and said they changed it in their text (6th line from the bottom of page 6), yet in the very next sentence there is a statement that yeast origins "fire from 17 to 33 minutes during a normal S phase and show a biphasic distribution . . .". Again on the top of the next page is the phrase that "track lengths in HU correlate with time of origin activation". In the next few sentences are references to "forks activated at 17 minutes", "forks generated at 8 minutes" and "regardless of their initiation time". Despite the initial caution, it is clear that the authors want to equate replication time with firing time. And the readers will certainly take away from their discussion that early and late refer to initiation times. In fact, we believe their BU-data provide some potential places to clarify this issue, but they didn't choose to describe how their data could be used to support the ideas of origins with different activation times.

As requested by this Referee, we defined the "mean replication time" of origins in the revised version (page 6) that "as the time of 50% replication along the origin nucleotide sequence (Yabuki et al., 2002)". We agree with this Referee that this is distinct from an initiation time, but since initiation times for origins are not available, we used the "mean replication time" as the best-available approximation for initiation time. We have now made this statement even clearer in the main text and in the Materials and Methods section. Using our BrdU-IP data to clarify the difference between "replication time" with "firing time" is certainly an interesting suggestion, but this analysis goes beyond the scope of this study.

C) The authors show very nicely that the core of ARS305 replicates at 25 minutes in their HU experiment using qPCR that measures the ~100 bp centered on the ARS. They also need to measure the time of replication in -HU experiments using the same methods instead of relying on the data from Yabuki et el. who measured ARS305 replication at 19.7 minutes in the absence of HU. The authors compare these results and conclude that ARS305 replicates at the same time in HU as it does in the absence of HU. Do the authors consider a 5.3 minute difference to be meaningless? If so, it is surprising that they place a lot of weight on an 8 minute difference in replication time from the Yabuki et al. data when making other arguments.

Indeed, we consider that a 5.3 min difference between replication times measured in different laboratories with different techniques is meaningless. This is essentially due to the fact that the time of S-phase entry after release from an alpha-factor block depends on multiple parameters, including the quality and the concentration of alpha-factor used, the technique used to wash out or degrade alpha-factor, the growth medium or the cell concentration. In contrast, considering an 8 min difference within the same time course experiment makes perfect sense to us.

Alvino et al. (2007) showed a much later time of half maximal replication of ARS 305 in HU (~80 minutes or so can be inferred from their data obtained at 23˚C). Are there difference in strain background or culture conditions that would lead to these differences? Or in the way that replication is measured-copy number vs. shift in density of a large restriction fragment from HH to HL? Is the temperature or type of media playing a role in dNTP pools that are responsible for these timing differences? We are disappointed that the authors completely fail to discuss their results in light of this previous work of Alvino et al., (2007) that does not show a "fast vs. slow" rate of replication for different origins. What do the authors think was missing from the Alvino et al. study?

This study of Alvino and colleagues is a major contribution to the field and we repeatedly refer to it in the manuscript. Yet, we haven't discussed this issue in the previous version of the manuscript and we are grateful for this Referee for pointing this out. As suggested by Referee #1, we assume that the reason why the Alvino study did not detect this "fast vs. slow" rate of replication is due to the fact that the density shift assay used by these authors is not adapted to measure fork rates. This comment is now included in the discussion.

2) Lack of attention to detail:

A) "HU-resistant DNA synthesis" was said to have been stricken from the manuscript, but there it is several times on pages 11 and 12. (Also see the concern about the continued use of origin firing time imputed from replication time reported by Yabuki et al. in point 1B above.)

We apologize for these omissions. We have now replaced "HU-resistant DNA synthesis" by "Enhance DNA synthesis" in page 11 and 12.

B) The "automated process" to determine rates of initiation and elongation (page 6) is said to be described in the methods and materials. There is a brief mention of how peaks are called (to identify origins) and that peaks that did not grow in size over time were eliminated from the calculation of fork progression. There is not enough information is this brief section to allow the reader of the manuscript to re-derive their rates. (Why were forks that didn't appear to move eliminated from fork rate estimates? It seems like this is an important part of the data that should be included in the average rate.) There is also no mention of which of the OriDB origins were detected in their BU-ChIP experiments. Did they ignore BU signals that didn't correspond to OriDB sites? How did their BU-positive peaks correspond to those of Yabuki et al. (2002), Feng et al. (2006), and Alvino et al. (2007)? Are the authors making their own calls on what is an active origin or just using the list from the Yabuki paper? Many of the BU-ChIP experiments (Figures 4D, S4, S5, S8B) don't show the significance cut-off value, so the reader is left to wonder why some peaks are significant and others are not; and the negative log2 values are such a light gray that they all but disappear. Finally, the way in which the Yabuki data was used is entirely mysterious to us.

We found this particular comment unfair. As indicated in the manuscript, this assay is extensively described in a previous report from our lab (Crabbé et al., 2010). The exact number of "confirmed", "likely" and "dubious" OriDB origins in our BrdU-IP experiment is indicated in this article. We also explain why BrdU peaks that do not correspond to OriDB origins and that do not grow in size during time course experiments were not considered as potentially novel origins. These data are available as supplementary information in the Crabbé et al. paper and original BrdU-IP files can be downloaded on GEO. The way how cut-off values are defined for all samples is indicated in Materials and Methods. So is the method we used to automatically measured fork rates using P-values calculated with the TAS software (Affymetrix) to precisely define the edges of BrdU tracks. All the parameters used to calculate P-values are indicated in the Materials and Methods section and all the files will be made available on GEO after acceptance of the paper.

C) The dGTP pool measurements in Figures 4B and 5C differ by almost an order of magnitude for the same strains. This lack of reproducibility makes me wonder whether the other differences noted are at all meaningful.

There is indeed a mistake in figure 5C (now 6C). We used an average sum of the four dNTPs pools values instead of mean dGTP pools and the figure was mislabeled. New Figure 6C shows dGTP fold change in relative units in the different mutants. We apologize for this mistake and we thank the Referee for pointing this out.

D) There are several model drawings (1F and 3E) that are not well described in either the figure legends or in the text (in the original version and in this version). We can't interpret them without some dialogue.

These models are now more extensively described in figure legends.

Also missing from this version are the supplemental whole genome BU-ChIP analyses that were included in the first version. It is important for the reader to be able to flip through these to come to the same conclusions as the authors. The small pieces of just two chromosomes shown in 1A and 5D are fine for the main text, but only if the rest of the genome is shown in a similar format in the supplementary information.

We removed these data from the revised version as Referee #2 requested that we remove data on *rad53* and *mec1* mutants. We have now reintegrated whole-genome BrdU-IP maps in supplemental materials.

E) Figures: There are no references in the text to Supplemental Figures 1D, 2C and D (and no conclusions as to the possibility that forks are speeding up the longer cells are kept in HU).

These figures are now referred to in the text.

What are the R2 values for all of the origins considered as a single class (Supplemental figures 1D and 6D)?

The R2 values for all the origins considered as a single class are now indicated in Supplemental figures 1D and 6D.

I can't see the red lines in Supplemental Figure 3A that show the difference between wt and sml1Del FACS profiles.

The red line has now been made darker and is more visible.

The legend to Supplement Figure 4 says there are two smlDel strains but I see only one but two wild types strains at different temperatures.

This error has now been corrected.

There are two mysterious ARSs in Supplemental Figure 5B labeled .61 and .62. These are not annotations that I am familiar with.

There was a mistake in the labeling of this figure. These two BrdU peaks do not correspond to OriDB origins. As described in Materials and Methods, we have consistently labeled these BrdU peaks with the chromosome number and the position of the peak in kb (here 16-553 and 16-583). These peaks show marked differences between 25°C and 30°C samples, suggesting that they correspond to uncharacterized replication origins.

In Supplementary Figure 6A there appears to be a significant peak for ARS 425 (based upon the significance cut-off) that is not called as an active origin. What is the explanation for their call?

As indicated in Materials and Methods, BrdU peaks were scored as active origins when signal intensity was above a threshold arbitrarily determined as 50% of the signal range and when BrdU tracks were larger than 1 kb. Based on these parameters, ARS425 is considered as active at 90 and 120 minutes, but not at 60 minutes.

Where is the vertical red line indicating the position of the centromere in this figure? What is the serial dilution factor for the plating experiment in Figure 8C? (It probably doesn't matter, but the lack of detail, when it is so easily given, is annoying.)

These issues have been corrected in the text and figures.

Overall Recommendations:

We think the manuscript could tell a great story about fork progression and the role of dNTP pools, but that the conclusions on early/late origins overreach the data. We don't want to be presumptuous, but feel that the manuscript could be edited successfully by beginning on page 8 with "We monitored the effect of increased RNR activity on replication fork progression" (line 6); eliminate any reference to the origin initiation program and the relevant figures; and keep the figures that relate to fork progression: Figures1A and E (but delete the dotted line to 17 minutes on the X axis), Figure 2, Figure 3A and B (but get rid of the gray and blue bars designating early and late firing origins), and Figures 4, 5 and 6. Finally, add a model figure that includes some of the features of the models presented in the earlier figures and describe the model in the discussion.

We disagree with Referee #1 that our conclusions on early/late origins overreach the data. Our observation that different sets of origins fire in HU in different strains and/or growth conditions is a fact and not an over-interpretation of the data. This was the basis of a study that allowed us to identify new mediators of the DNA replication checkpoint (Crabbé et al., 2010). Here, the fact that the pattern of origin usage in HU is modulated by dNTP pools is an important observation that deserves to be presented in the manuscript.

Referee #2 (Remarks to the Author):

In this revised manuscript Poli et al. have adequately addressed the most relevant concerns of this reviewer, by extensive restructuring of figures and text in their Results section. Now the paper is in my view much more logical and easier to follow, and conveys more effectively the most important messages.

We thank this Referee for acknowledging our effort to extensively reorganize the manuscript according to his/her request.

Two rather important criticisms, that would require further work on text and figures, remain to be addressed prior to publication in EMBO Journal:

1) The Discussion session has not been sufficiently restructured according to the new format of the manuscript, keeping too much emphasis on correlative and rather technical points (differences between checkpoint mutations, etc.) and omitting to highlight the most relevant observations impacting the fields of DNA replication and genome stability in general (effects on fork progression of minor changes in dNTP levels; general effects on fork rate in all mutants with elevated endogenous DDR; effects on fork progression extendible to various conditions of replication stress). The authors would make their message much stronger discussing first these central points and getting to the checkpoint connection only briefly towards the end (most of these data have anyway been moved to the Supplementary Material).

We have now made further changes to the Discussion to follow this referee's advice.

2) While most experiments are performed in HU (a possible caveat, as discussed in my previous comments, point 3), the authors have now solid data to confirm these observations in response to different types of replication stress (MMS, 4-NQO). I am convinced that this important validation of their system should be much anticipated, to avoid similar scepticism by the readers until the end of the Results section. I would thus strongly

suggest that Fig. 6 should become Fig. 3, especially considering that the format of this figure is quite similar to the data presented in Fig. 2 (easy to establish an immediate connection in the text).

We thank this referee for this suggestion. We agree that moving the MMS/4-NQO experiments just after HU confirms that the mechanisms that we describe are not restricted to HU-induced replicative stress. Figures 3 and 6 have now been swapped in the new version of the manuscript.

A few minor points should also be considered while finalizing the manuscript for publication:

1) Most data in Fig. S1 are not mentioned/described anywhere in the manuscript. There is also no mention of panels C and D of Fig. S2.

We have now addressed this issue.

2) Page 10, the list of mutants analyzed for intra-S checkpoint includes a strain (mec1tel1sml1) that is no longer depicted in Figure 4A, where instead rad53sml1 is included (and not mentioned in the text)

We have also addressed this issue.

3) The FACS profiles in Fig. S3 were probably meant to show some overlays of the wt profile within the sml1 panel, but those are not properly visible. This may require some graphical modifications.

We have now used a darker red color to enhance the visibility of the overlay.

Referee #3 (Remarks to the Author):

The authors addressed previous my concerns in the revised version. Especially, the revised Fig 1 is now much more convincing than the previous Fig. 2. I recommend its publication in EMBO J.

We are grateful to this Referee for his/her support.