High-resolution Analysis of DNA Synthesis Start Sites and Nucleosome Architecture at Efficient Mammalian Replication Origins

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Editor: Hartmut Vodermaier

1st Editorial Decision 05 April 2013

Thank you for submitting your manuscript on chromatin architecture at mammalian replication origins for consideration by The EMBO Journal. We have now received comments from three expert referees, which you will find copied below. I am afraid that these reports do not offer sufficient support for publication of this study in The EMBO Journal. As you will see, while all referees acknowledge the importance as well as the technical quality of this work, only one of them is currently supportive in principle. Referees 1 and 2, on the other hand, remain unconvinced that the presented data from a limited number of origins allow to draw sufficiently definitive novel insights or to strongly support the key conclusions of the manuscript. Major issues in this regard are the moderate and variable effects observed, as well as the lack of deeper functional analyses using nucleosome manipulations at initiation sites. Furthermore, the absence of complementary more genome-wide analyses, and comparison to available genome-wide ORC/nucleosome mapping studies, is of concern to the critical referees. In our view, these well-taken major concerns preclude publication of the study in The EMBO Journal, at least in the present form.

I would however not exclude the possibility of considering a new version of this work, should you be able to corroborate the key conclusions through future efforts guided by the comments of the referees. This would however require major extension to either add supporting functional analyses or complementary data from genome-wide data and from comparison with available nucleosome position maps and ORC binding sites (such as in Dellino et al 2013) to also arrive at a better distinction between ORC binding and initiation sites. Since this will obviously require substantial further efforts of uncertain outcome, I hope you understand that we would have to consider such an extended study as a new submission rather than a revision, and that we are at the present stage not
able to make strong commitments on whether or not the study would be sent back to our referees. Furthermore, it is only fair to say that the novelty of the findings at the time of resubmission would have to be taken into account.

In any case, thank you for having had the opportunity to consider this work for our journal. I am sorry we cannot be more positive at the present stage, but in the meantime hope that you will find our referees' detailed comments and suggestions helpful.

REFEREE REPORTS

Referee #1

Lombrana et al,

DNA replication initiates at discrete sites in genome termed origins of DNA replication. A major question in the field is the identification of cis- and trans-acting factors that function in specifying origin usage. Recent reports in S. cerevisiae, Drosophila and Chinese hamster ovary cells have found that the origin recognition complex (ORC) localizes to nucleosome free regions of the genome. Here the authors use a single locus nucleosome scanning assay (NuSA) to map the chromatin architecture of a handful of replication origins. The authors also examine the abundance of the short nascent strand DNA replication intermediates by NuSA and find that the nascent DNAs often but not always peak at nucleosome occupied sequences. I found the presentation of the data exquisite, but yet I am not quite convinced... Mnase digestion of chromatin and mapping of nucleosomes is tricky - confounded by dynamic and transient nucleosome occupancy, large TF complexes which may mimic nucleosome occupancy, and finally the strong sequence bias of MNase (MNase in effect does not cut at G or C's which can be problematic at CpG islands...). The authors highlight many of these problems and perform multiple digests at different concentrations of MNase and also perform ChIP against histone H3. The results presented are subtle at many of the loci and it is unclear that a few peaks of SNS intermediates at a handful of origins is significant. Perhaps this further emphasizes the diversity of mammalian origin initiation or perhaps it is noise? While I appreciate the effort involved in the NuSA assay, in this case, a genome wide approach analyzing 1000s to 10,000s of origins would be be more powerful and convincing. Even a few genome-wide plots in aggregate building on the author's prior SNS mapping and the recently published nucleosome mapping for mouse ES cells (Teif VB et al., 2012) would go far in supporting the claims of the authors that replication intermediates initiate from regions of high nucleosome occupancy.

Referee #2

DNA Synthesis Start Sites Occur at Positions of High Nucleosome Occupancy at Efficient Mammalian Replication Origins

by Rodrigo Lombrana et al.

In this manuscript, the authors investigated the relationship between nucleosome occupancy and replication initiation. On the basis of analyses of selected mouse replication origins, they concluded that the DNA replication start sites (as determined by short nascent strands (SNS) hybridization assays) map at the positions of high nucleosome occupancy.

Although the authors have generally conducted experiments with high precision and the data presented are reliable, I have some reservation with the conclusions with the data presented.

Although DNA replication initiation sites are mapped by analyses of short nascent DNA, some of the peaks do not seem to be reliable. For examples, the SNS peak of Hs6st2 maps at the +1 nucleosome, the summit is a single peak with weak intensity. The SNS peaks map at the unstable nucleosomes at the Vps45 and Hau7 oris, which may suggest that initiation sites may prefer the unstable nucleosome region at these origins. (see also my comment on Figure 3 below)

Thus, it could be rather premature to conclude that initiation sites generally map at the positions of high nucleosome occupancy, unless statistical analyses of genome-wide data are conducted.
Alternatively, the authors should show that manipulation of nucleosome positions changes the initiation sites. Since the authors have a Knock-in system for analyses of the lamin B2 origin, effects of the nucleosome manipulation on initiation sites could be examined.

Other comments:

All the analyses are conducted with asynchronous cultures. Nucleosome positioning or its stability may change during cell cycle (even during S phase) which may be linked to the difference of firing timing. It would be interesting to examine the correlation between nucleosome positioning and initiation sites, at early-firing origins and late-firing origins. The genome-wide replication timing data are available in mouse cells. Thus, cell cycle effects should be examined at least for some origins.

Table 1
The values for the transplanted human lamin B2 ori should also be shown for comparison.

Figure 3
The data on Pias3 and Slc7a14 rather indicate that initiation sites are located at the junctions of two nucleosomes. (In both cases, there are gaps of SNS enrichment data at potentially peak positions). For the Syde1 origin, the SNS peaks are unclear and it is hard to conclude on the relationship.

Typo: page 4, line 9
DNA synthesis start sites --> DNA synthesis start sites

Green and red histograms in Figure 4B are extremely hard to see for color-blind readers. A different color combination should be used.

Referee #3

Chromatin organization at metazoan replication origins remains poorly understood. In this manuscript, the position of nucleosomes was analyzed in mouse cells and the results reveal flexibility higher than in S. cerevisiae origins. This finding is well in agreement with the flexibility of origin positioning previously reported in mouse cells.

The authors mapped known replication origins using a nice high-resolution method (PCR scanning of the regions of interest). They also used ChIP assay with anti-H3 antibodies to complement and confirm their results.

The micrococcal nuclease assay was technically well performed, with either native or cross-linked chromatin. The correspondence with replication initiation sites was determined by mapping nascent strands, using three different samples of increasing length. This part of the work is also of high technical quality.

The authors convincingly show a correlation between a preferential nucleosome positioning at initiation sites and strong origins of DNA replication. They also report a lower nucleosome positioning when there is more variability in the initiation sites. The correlation between nucleosome positioning and initiation sites was further confirmed by the exploration of nucleosome position changes in regions that are differentially expressed in two different cell types. Finally the analysis was repeated in the known lamin B origin as well as in a transgene carrying origin sites. This study was also technically sound.

Overall, this is a solid piece of work that deserves publication. I have a few comments that might improve the manuscript.

- In Figure 2A, the amount of used MNase should be indicated in the figure for more clarity. Whether chromatin was cross-linked or not should also be indicated in the figure.
- In the Discussion, the authors comment on a recent work claiming 250 000 to 300 000 origins in the human genome (Besnard et al, 2012). I think this part should be deleted as most of the origins claimed in this paper will not be validated by a correct bioinformatic analysis. In this paper single isolated positive points were also scored as origins, but they probably represent background noise.
- I also would be happy if the authors could comment on their precise analysis of nascent strands at lamin B origins. It looks like the origin site is much larger than previously described. Is that because the analysis was at higher resolution in this paper, or should we envisage that the lamin B origin does not differ so much from other replication origins?
Thank you for your letter and the comments of the reviewers. We do agree with some of their criticisms and have initiated a series of experiments directed to solve the concerns they raised. This includes bioinformatics analysis of available genome-wide data of ORI peaks and nucleosomes in mouse ES cells and ORI peaks/ORC sites/stable and unstable nucleosomes in HeLa cells. We are also performing additional high-resolution analysis of nucleosome positioning and initiation sites at specific origins to provide further evidence, on one hand, of ORC mapping at sites normally occupied by unstable nucleosome particles and, on the other hand, of changes in the initiation sites when nucleosome positioning changed. We are getting encouraging results in both type of approaches and we hope these new data will substantially improve the current manuscript. Although this extended study will require a great deal of effort and major rewriting, we’ll try to get the new version ready within three months. If we manage in doing so, I hope you will reconsider a revision of our work and send it back to the referees.

Thank you very much in advance for giving us the opportunity to further support our findings. We believe they are timely, novel and relevant for the field.

1st Revision - authors' response 15 July 2013

Following our earlier communication, I’m enclosing the revised version of the manuscript EMBOJ-2013-84923 entitled “High-resolution analysis of DNA synthesis start sites and nucleosome architecture at efficient mammalian replication origins” authored by Rodrigo Lombraña, Ricardo Almeida, Isabel Revuelta, Sofia Madeira, Gonzalo Herranz, Néstor Saiz, Ugo Bastolla and María Gómez. We would like to thank you and the Reviewers for the exhaustive and constructive review and for the encouraging and positive opinion of our work.

Reviewer 3 unambiguously supports publication. In their introductory comments, Reviewers 1 and 2 also acknowledged the importance of our work and its technical quality but both show some concern about the generalization of our conclusions from a limited number of origins. We agree with those views and, in fact, we explicitly discussed that in the Discussion of the previous version. We have now addressed those main concerns by adding complementary analysis from available genome-wide data and novel analysis on ORC distribution related to histone variants and initiation sites. The text has been extensively rewritten to incorporate all the suggestions of the three Reviewers to stress or clarify specific points. They are detailed in our point-by-point comments on separate pages. In addition, we have carried out the analysis suggested by both Reviewers 1 and 2 on available genome-wide data of nascent strands abundance and nucleosome density to confirm and generalize our results on few individual replication origins (Figures 1 and 7). As you suggested in your letter, we have analysed the genomic distribution of ORC1 relative to replication initiation sites and found that they occupy adjacent but distinct positions within the origin region: ORC binds to the classically referred nucleosome-free region of active promoters, exactly at the position marked by the labile H3.3/H2A.Z-nucleosome containing particles, while the initiation sites occur at flanking sites bound by positioned nucleosomes (Figure 7). As required by Reviewer 2, we performed additional detailed analysis of another origin region that is differentially expressed in two cell types and confirm that variations in nucleosome positioning are reflected by variations in the replication profiles (Figure 4). Finally, we also investigated nucleosome dynamics upon origin activation at the laminB2 origin and found that, indeed, the labile nucleosome particle that coincides with the ORC binding site and the replication initiation site is rapidly removed before DNA synthesis starts (Figure 6).

The new results reinforce our previous conclusion that replication initiates at sites of high-nucleosome occupancy at efficient mammalian origins. We also provide novel evidence that ORC can occupy DNA sites that are transiently available shedding light into the mystery of origin specification in mammalian cells and adding interesting insights on the nucleosome architecture and
organization of these regulatory elements. Therefore, we have changed the title of our manuscript to reflect those findings.

Given that the conclusions of our work touch upon replication, transcription, nucleosome configuration and genome organization, we believe they will be of interest to a wide spectrum of scientists in these fields. We thank you and the Reviewers again for your advice and we hope you will find the revised manuscript suitable for publication.

Comments for Referee #1

- "The results presented are subtle at many of the loci and it is unclear that a few peaks of SNS intermediates at a handful of origins is significant. A genome wide approach analyzing 1000s to 10,000s of origins would be more powerful and convincing. Even a few genome-wide plots in aggregate building on the author’s prior SNS mapping and the recently published nucleosome mapping for mouse ES cells (Teif VB et al., 2012) would go far in supporting the claims of the authors that replication intermediates initiate from regions of high nucleosome occupancy”.

Following the reviewer advice we have carried out additional analysis on available genome-wide data on SNS abundance and nucleosome density both for mouse ES cells and human HeLa cells (new Figures 1 and 7). In both cases we found that the replication initiation sites at efficient CGI-ORIs peak at positions preferentially occupied by nucleosomes, confirming and extending our conclusions from detailed analysis at individual ORIs. For both groups of data we choose to analyse CGI promoter-ORIs for the following reasons:

1. Available genome-wide maps of ORIs are partial on mouse ES cells, comprising 60.4 Mbp from mouse chromosome 11 (Cayrou et al., 2011) or 10.1 Mbp from chromosomes 3 and X (Sequeira-Mendes et al., 2009). There is a complete genomic dataset derived from HeLa cells (Bensard et al., 2012), but the accuracy of the bioinformatics analysis used in that report is under question (see comment of Reviewer 3), and the overlap between those ORIs and the genome-wide map of ORC1 binding sites is quite low (Dellino et al., 2013). However, in all those studies, as well as on an earlier report focused on the ENCODE regions in HeLa cells (Cadoret et al., 2008), CGI-ORIs were found to be the most efficiently activated and more conserved between cells types. So, by analysing CGI-ORIs we make sure to select bona-fide efficient ORIs. The ORI regions whose composite patterns of SNS abundance and nucleosomal structure were analysed were: n=323 and n=36 for the Cayrou et al and Sequeira-Mendes et al data sets, respectively, and n=1,004 for the HeLa data set from Besnard et al. Numbers are indicated in the corresponding figure legends.

2. CGI-promoters have a characteristic nucleosomal array consisting in a NDR immediately upstream of the TSS flanked by positioned nucleosomes (Valouev et al., 2011; Fenouil et al., 2012; Teif et al., 2012). This allowed us to check the possible relationship between NDRs and ORIs, as reported for S. cerevisiae or Drosophila cells (Eaton et al., 2010; MacAlpine et al., 2010), as well as between positioned nucleosomes and initiation sites. In contrast, outside of promoter regions nucleosomes are loosely-positioned, meaning that a particular position that is occupied by a nucleosome is occupied in some genomes of the population but unoccupied in others, what would obscure any conclusion from the data.

The new results reinforce our previous conclusion and provide novel evidence that ORC can occupy DNA sites that are transiently available, shedding light into the mystery of origin specification in mammalian cells, as well as providing new insights into the architecture of efficient replication origins. Both the title and the discussion have been changed from the original version to reflect these findings.

Comments for Referee #2

- “It could be rather premature to conclude that initiation sites generally map at the positions of high nucleosome occupancy, unless statistical analyses of genome-wide data are conducted”.

As detailed in the answer to Reviewer 1, in the new version of the manuscript we have included genome-wide analysis of SNS abundance and nucleosome density both for mouse ES cells and human HeLa cells (Figures 1 and 7). In both cases we found that the replication initiation sites at
efficient CGI-ORIs peak at positions occupied by nucleosomes, confirming and extending our conclusions from detailed analysis at individual ORIs.

- “Alternatively, the authors should show that manipulation of nucleosome positions changes the initiation sites. Since the authors have a knock-in system for analysis of the LaminB2 origin, effects on of the nucleosome manipulation on initiation sites could be examined”.

Manipulating the nucleosome positions at the LaminB2 knock-in is a good suggestion but it would constitute a different long-term project. Instead, in this new version of our work we provide additional high-resolution analysis of another promoter-ORI region (Vps45) that is differentially expressed in mES cells and 3T3 fibroblasts. The new data are included in Figure 4 and show, similarly to the results found at Haus7 promoter-ORI region, that changes in nucleosome positioning are mirrored by changes at replication initiation sites.

- “Other comments:

All the analyses are conducted with asynchronous cultures. Nucleosome positioning or its stability may change during cell cycle (even during S phase) which may be linked to the difference of firing timing. It would be interesting to examine the correlation between nucleosome positioning and initiation sites, at early-firing origins and late-firing origins.”

We agree with the Reviewer comment and to address his/her point we have analysed nucleosome dynamics at the early-firing LaminB2 origin in synchronised HeLa cells upon origin activation (new Figure 6). By using very mild MNase digestions, we were able to detect an increased DNA accessibility at the region bound by ORC and the -2 nucleosome in early-S cells relative to G1/S-blocked cells, suggesting that nucleosomes located at initiation sites are specifically remodelled before DNA synthesis starts.

- “Table 1
The values for the transplanted human lamin B2 ori should also be shown for comparison”.

Knock-in LaminB2 origin efficiency values have been included in the new version (Table I).

- “Figure 3
The data on Pias3 and Slc7a14 rather indicate that initiation sites are located at the junctions of two nucleosomes. (In both cases, there are gaps of SNS enrichment data at potentially peak positions). For the Syde1 origin, the SNS peaks are unclear and it is hard to conclude on the relationship.”

The non-promoter origins located within the body of the genes Pias3, Slc7a14 and Syde1 are packed by loosely positioned nucleosomes (full MNase analysis is shown in Fig S4). This is interpreted as that every nucleosome on the region occupies a slightly different position in every cell (Sekinger et al., 2005). This makes, as the reviewer points out, very difficult to establish a relationship with the initiation sites at non-promoter origins. For the same reason, we have only analysed CGI promoters in the genome-wide comparisons of nucleosome density and initiation sites or ORC binding sites (see comment to Reviewer 1). In the new version of the manuscript we have moved the old Figure 3 to the supplementary information (new Figure S7) and added a sentence clarifying this point (page 12). Altogether, the new version of our work focus on highly efficient CGI-origins and the conclusions reached on origin architecture and initiation sites refer to this group of early firing-highly efficient origins. The Discussion has been completely re-written to stress these points and the title has been changed accordingly.

- “Typo: page 4, line 9
DNA synthesis star sites --> DNA synthesis start sites”

The typo has been corrected.

- “Green and red histograms in Figure 4B are extremely hard to see for color-blind readers. A different color combination should be used”.

Following the reviewer advice, we have changed the green and red histograms in Figure 4 for dark-
blue and light-blue colours. Same colour coding has been used in the new Figure 6 to align the nucleosome positioning of G1/S-blocked cells and early-S cells.

Comments for Referee #3

- In Figure 2A, the amount of used MNase should be indicated in the figure for more clarity. Whether chromatin was cross-linked or not should also be indicated in the figure.

The amount of MNase and chromatin preparation conditions used is now indicated in all main figures.

- In the Discussion, the authors comment on a recent work claiming 250 000 to 300 000 origins in the human genome (Besnard et al, 2012). I think this part should be deleted as most of the origins claimed in this paper will not be validated by a correct bioinformatic analysis. In this paper single isolated positive points were also scored as origins, but they probably represent background noise.

We have removed this paragraph, as suggested.

- I also would be happy if the authors could comment on their precise analysis of nascent strands at lamin B origins. It looks like the origin site is much larger than previously described. Is that because the analysis was at higher resolution in this paper, or should we envisage that the lamin B origin does not differ so much from other replication origins?

We have added a sentence on this regard on page 16 of the new version of the manuscript. The initiation site originally described by Abdurashidova et al., 2000 is the most efficient within the 826 bp region analysed, both in 293T cells (Figure 5) and HeLa (not shown). But we could reproducibly detect another two initiation sites at the location of the positioned nucleosomes in both cell types. Whether this is due to the fact that the whole region is imbibed within a CpG island, or this could be the case for most origins, we cannot tell from our data.

2nd Editorial Decision 05 August 2013

Thank you for submitting the new version of your manuscript on nucleosome architecture at mammalian replication start sites for our consideration. It has now been assessed once more by the three original referees, and I am pleased to inform you that they all consider the manuscript substantially improved and the major issues satisfactorily clarified. We should therefore be able to accept paper for publication, once the following minor/editorial points have been incorporated into the manuscript:

- Referees 1 and 2 both ask for some minor text/presentation changes and clarifications; please modify the manuscript accordingly.

- Please add a brief 'Author Contribution' description to the manuscript text, next to the acknowledgement section.

- Please combine all supplementary information (text and figures) into one single PDF of sufficiently high quality and sufficiently small size to facilitate download by our readers - keeping in mind that this part of the manuscript will not be type-set or copy-edited.

- Please complete and sign the necessary license forms (see information below) and upload them together with the re-revised final manuscript files, in order to expedite the production process.

I am therefore returning the manuscript to you for an ultimate round of minor revision, hoping you will be able to return the final version to us as early as possible.

REFEREE REPORTS
Referee #1

I'm pleased the authors examined their data in the context of available genome-wide chromatin sets. However, I was quite disappointed that the authors treated the enrichment of ORC at sites of dynamic chromatin (marked by enrichment of H3.3 and depletion of bulk nucleosomes) as a novel observation. This finding was first reported several years ago in the fruit fly -- Deal et al., Science 2010; MacAlpine et al., Genome Research 2010, and modENCODE et al., Science 2010. While the author's rediscovery of the link between ORC and H3.3 is not novel, I still think this is an important finding that emphasizes the similarity between the fly and mammalian systems and likely represents a conserved property of metazoan origins. The authors just need to cite the appropriate papers.

Referee #2

The authors made a good effort to answer the comments by the reviewers and I acknowledge that the revised manuscript is significantly improved.

The authors have changed the title to "High-resolution Analysis of DNA Synthesis Start Sites and Nucleosome Architecture at Efficient Mammalian Replication Origins" to reflect better the data presented. However, the abstract has not been modified in accordance with other changes. It states "Strikingly, despite this diversity initiation sites always occur at positions of high nucleosome occupancy." However, the authors focused on CGI-early origins and have not done genome-wide analyses on all the origins. This should be stated in the Abstract.

Figure 1 The red line representing nucleosome should be explained in more detail. The higher this value is, the more the nucleosome occupancy is. Is this correct? If that is the case, in A, the correlation between SNS peaks and high nucleosome occupancy does not look like very striking. Please clarify this.

Figure 6 The results of NuSA analyses of synchronized cells are nice, showing the changes of nucleosomal structures at the ORI after initiation.

Figure 7 It is clear that SNS and ORC peaks are distinct, and (A) shows that ORC peaks map at the labile histone segments. Are there nucleosome data as in Figure 1 that the authors can overlay in (B)?

Referee #3

In this revised version of this manuscript, the authors have greatly improved their data, which were already of high quality in the original manuscript. They answered all of my remarks, and also nicely answered the remarks of the other referees. In particular, they have performed an additional analysis on the available genome-wide data that confirms their main conclusion. They also performed an analysis of the nucleosome positioning in synchronized cells that nicely explains their dynamics. Overall this is manuscript clearly written, with solid data that clearly deserve to be published in this field.

2nd Revision - authors’ response 07 August 2013

Referee #1

I'm pleased the authors examined their data in the context of available genome-wide chromatin sets. However, I was quite disappointed that the authors treated the enrichment of ORC at sites of dynamic chromatin (marked by enrichment of H3.3 and depletion of bulk nucleosomes) as a novel observation. This finding was first reported several years ago in the fruit fly -- Deal et al., Science 2010; MacAlpine et al., Genome Research 2010, and modENCODE et al., Science 2010. While the author's rediscovery of the link between ORC and H3.3 is not novel, I still think this is an important finding that emphasizes the similarity between the fly and mammalian systems and likely represents a conserved property of metazoan origins. The authors just need to cite the appropriate papers.

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We agree with the comment of the reviewer that ORC enrichment at sites of dynamic chromatin was previously reported in Drosophila cells and indeed we did acknowledge these findings in the revised version of our manuscript (work from the MacAlpine lab was cited in the discussion, page 19 and from the Henikoff lab on page 20). Following the reviewer advice, however, we have further emphasised those original findings in the discussion of the final version of our manuscript and state that this similarity between the fly and mammalian systems might represent a conserved property of metazoan origins (page 19). The novelty of our observations resides indeed in the finding that ORC binding sites and initiation sites occupy adjacent but distinct locations within the origin and that both are linked to different features of the origin’s nucleosome architecture.

Referee #2

The authors made a good effort to answer the comments by the reviewers and I acknowledge that the revised manuscript is significantly improved. The authors have changed the title to "High-resolution Analysis of DNA Synthesis Start Sites and Nucleosome Architecture at Efficient Mammalian Replication Origins" to reflect better the data presented. However, the abstract has not been modified in accordance with other changes. It states “Strikingly, despite this diversity initiation sites always occur at positions of high nucleosome occupancy.” However, the authors focused on CGI-early origins and have not done genome-wide analyses on all the origins. This should be stated in the Abstract.

The sentence in the abstract has been changed by : ”Strikingly, initiation sites at efficient CpG island-associated origins always occur at positions of high nucleosome occupancy” to reflect the fact that, as mentioned by the referee, genome-wide analysis were only performed for CpG island-origins.

Figure 1 The red line representing nucleosome should be explained in more detail. The higher this value is, the more the nucleosome occupancy is. Is this correct? If that is the case, in A, the correlation between SNS peaks and high nucleosome occupancy does not look like very striking. Please clarify this.

Mammalian CGI-promoter regions are depleted from bulk nucleosomes relative to the genome average, as previously reported and reflected in the figure. However, positioned nucleosomes flanking the 200 bp NDR located just upstream of the TSS are detected at positions of approximately -400, +150, +300, and +450 (Barski et al, Cell 2007; Schones et al, Cell 2008; Jin et al., Nat Genet 2009). Our analysis on the genome-wide distribution of replication initiation sites and nucleosome positioning showed that the SNS peaks do not coincide with the NDR. Rather, the SNS profile is maximum at positions that are occupied by nucleosomes, although the sizes of the SNS used in those genome-wide analysis do not have enough resolution to relate the initiation sites to individual nucleosomes. The interpretation of the nucleosome profile (red lines in Figure 1) has been clarified in the figure legend.

Figure 6 The results of NuSA analyses of synchronized cells are nice, showing the changes of nucleosomal structures at the ORI after initiation.

We are glad that the reviewer found these results interesting.

Figure 7 It is clear that SNS and ORC peaks are distinct, and (A) shows that ORC peaks map at the labile histone segments. Are there nucleosome data as in Figure 1 that the authors can overlay in (B)?

To our knowledge there are not published reports on nucleosome positioning on HeLa cells. We agree with the referee that overlaying the SNS peaks and nucleosome density profiles would have been informative. However, we think that the fact that the biphasic replication profile of Hela cells peaked at both sides of the TSS, similar to the ES cells SNS profile shown in Figure 1, that coincide with regions that are normally occupied by nucleosomes, supports our claim that at efficient origins replication initiates from regions of high nucleosome occupancy.