EMBO
reports

Manuscript EMBOR-2012-36891

The chromatin remodelling complex NoRC safeguards genome stability by heterochromatin formation at telomeres and centromeres

Anna Postepska-Igielska, Damir Krunic, Nina Schmitt, Karin M. Greulich-Bode, Petra Boukamp and Ingrid Grummt

Corresponding author: Ingrid Grummt, DKFZ

Review timeline:

Submission date:	26 November 2012
Editorial Decision:	19 December 2012
Revision received:	17 April 2013
Editorial Decision:	10 May 2013
Revision received:	17 May 2013
Accepted:	22 May 2013

Editor: Esther Schnapp

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 19 December 2012

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports on your study that is copied below.

As you will see, while the referees acknowledge that the findings are potentially interesting, they also point out that the data are not fully convincing due to the many rather minor effects of unclear significance, the several missing important controls, and the missing analysis of whether TRF2 and CENP-A are indeed required for the recruitment of TIP5 to telomeres and centromeres. It is also unclear why some results are in contrast to the previous study and why TIP5 only localizes to 30% of the telomeres. Referee 2 further requests that the data on telomeric and centromeric noncoding RNA expression in response to TIP5 knockdown and overexpression need to be shown.

Based on these comments, publication of the study in our journal cannot be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the

understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. I would like to stress that all the missing controls, missing statistical analyses and missing causality in TIP5 recruitment must be addressed experimentally, and the data on noncoding RNAs must be included. It would also make the study much stronger, if interactions between endogenous proteins (TIP5, TRF2, CENP-A) could be shown.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 figures plus 5 supplementary figures, which should directly relate to their corresponding main figure. Please also specify the number (n) of experiments and the error bars and statistical tests used to calculate p-values for all quantifications in the corresponding figure legends. This information is currently incomplete.

When submitting your revised manuscript, please include:

A Microsoft Word file of the manuscript text, editable high resolution TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referee comments.

We also recently decided to offer the authors the possibility to submit "source data" with their revised manuscript that will be published in a separate supplemental file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) of your key experiments together with the revised manuscript.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready.

REFEREE REPORTS:

Referee #1:

This paper addresses the potential role of the NoRC complex beyond its well established function in the formation of nucleolar heterochromatin and the silencing of rDNA. Overall the paper defines the various properties that distinguish cells in which TIP5 (an essential NoRC component) has been depleted by siRNA, from mock-depleted controls. Overall the findings allow the conclusion that TIP5, through its various protein-protein interactions, plays an important role in the formation and maintenance of centric and telomeric heterochromatin and, perhaps as a consequence, in genomic stability, including the behaviour of chromosomes through mitosis.
Many of the effects described are subtle. The growth effects (Fig.S1A) are very small (sigificant?)

and surely likely to be a consequence of the small increase in apoptosis (Fig.1A)? The slight prolongation of mitosis is also of doubtful significance (Fig.1B). ChIP assays showing TIP5 at centromeres are more convincing, though the authors must explain how chromatin immunoprecipitated specifically from the inactive X was assayed (Fig.S2). Perhaps the most convincing data comes from the reciprocal changes in H3K9me3 and H4K20me3 in cells in which TIP5 is knocked down or over-expressed, an observation consistent with the enzymes with which it is known to be associated (Fig.1F).
A modest increase in size of the mitotic spindle is seen in TIP5 depleted cells (Fig.2B). Why does this suggest a role for NoRC in "proper kinetochore structure" (Fig.2B, page 5).
The authors show several examples of association of TIP5 with various proteins by co-immunoprecipitation (eg. Figs.2A, 3E), and use this to speculate on how it might be targeted to certain regions or chromatin types. The impression is that TIP5 is a rather sticky protein and some more negative controls would make the positive findings more convincing.
Overall the accumulated evidence is consistent with a role for NoRC and TIP5 in maintenance of genome stability and heterochromatin formation beyond the nucleolus. A role in centric heterochromatin formation and possibly genomic stability has been described previously by Guetg et al (ref.17), but the present results extend these findings. Also, as the authors point out, previous results were obtained from a cell line in which TIP5 was stably depleted, raising the possibility that the line had adapted to the absence of TIP5, thereby disguising or modifying possible effects.
The greatest weakness of this paper is the lack of any mechanistic insight into how TIP5, and NoRC might exert their functions. There is some interesting speculation on the possible role of RNA, but no data.

Referee #2:

In eukaryotes, pericentromeres, centromeres and telomeres are described as constitutive heterochromatin, a condensed type of chromatin associated with repressing gene expression, and characterized by specific markers (post-translational modification of histones, DNA methylation, chromatin-binding proteins). Each of these domains are unique in their structure and function, and their integrity is essential for genome stability. In this manuscript, Postepska-Igielska et al. provide a description of the role of TIP-5 (TTF-I interacting protein 5), the large subunit of the Nucleolar Remodelling complex (NoRC), in the establishment and maintenance of the heterochromatic state at pericentromeres, centromeres, subtelomeres and telomeres. The data suggest that NoRC, which was previously known for its role in nucleolus integrity, is essential for structure and function of constitutive heterochromatin. The same authors have previously described a role for NoRC in centromeric chromatin structure (Grummt lab, EMBO, 2010), and this study is essentially an extension of the work into telomeres

Overall, this manuscript is nicely written, and the goal of this study is well described. Generally, the data are convincing and of interest to the chromatin field. However, there are several issues that must be addressed to highlight the novelty of the manuscript and effectively make the points stated by the authors, as listed below.

Major comments:
1. Some figures of the manuscript (parts of figures 1 and 2) are only partially new. After getting a feeling of déjà vu, this reviewer looked back in the literature- the results contained herein concerning pericentromeric and centromeric chromatins were published previously by the same lab in EMBO in 2010 (see Guetg et al Figures 1 and 2). Although these are previously published data (not the same figures, but same content), nowhere is it mentioned in the manuscript and the previous data was not cited clearly. Is it meant to be a control in this ms? If so, authors should move what was already published into supporting figures or simply cite their previous paper, and clearly state in the text if they confirmed Guetg's work (e.g. TIP-5 interaction with CENP proteins) or if they obtained a different result (e.g. cell cycle progression, TIP-5 accumulation at centromeres). In the second case, authors must discuss why their results are different. For example, authors may (i) comment the decrease of TIP5-CENPs containing spots (figure 1D) with the drop of CENP-A stained foci in number and the increase in size in mouse TIP-5 depleted cells (Guetg et al. 2010 - Figure 1D); (ii) or discuss the difference in cell cycle progression (Figure 1C) between their current model and their original model as published in Guetg et al 2010.

2. The section on telomeres and subtelomeres is indeed novel and interesting. Focusing the manuscript on these data should be considered to improve the novelty and interest, condensing the ms into a short report only on this subject.

To further buttress the role of TIP-5 at telomeres and subtelomeres, several experiments need to performed (i) Regarding the last sentence on page 5, the decreased level of acetylated histone H4 when TIP-5 is over-expressed, is expected to be seen on Figure 3. Authors may consider adding this panel. (ii) On Figure 3D, authors analyzed H3K9 acetylation and Suv4-20h2 association at subtelomeres only. They may consider studying also these two marks at telomeres when TIP-5 is knock-downed, and at telomeres and subtelomeres when TIP-5 is over-expressed. I would have liked to see a control with H3K4Me2 or some other euchromatic mark, to substantiate the claim that only heterochromatin is affected by TIP-5 over-expression or siRNA (iii) The figure S3 should perhaps be incorporated in the corpus of the manuscript. (iv) Data on non-coding RNA expression when TIP-5 is down-regulated or over-expressed (page 7) and TIP-5 - non-coding RNAs interaction have to be shown to emphasize the importance of this protein in heterochromatin structure. Authors may also discuss more on how TIP-5 could regulate TERRA RNAs expression (e.g. indirect effects resulting from the modification of heterochromatin structure).

4. A concern is some experiments are not well explained in the text (type of cells, methods used). The authors used three different cell lines (two mouse and one human)- often the same figure has different panels with different cell lines. The co-staining of TIP-5 with TRF2 (Figure 3A) and the M-FISH (Figure 4C) are not described in the text or not enough explained to give the opportunity to the reader to interpret the result.
Another example is the "data not shown" for the co-staining 53BP1 - TIP-5 (page 6), however this immuno-fluorescence experiment is presented on Figure 4A. In addition, why some proteins are studied is not well explained (e.g. RNA Polymerase I, 53BP1). Authors should provide some context- is it a control or is this factor involved in the analyzed pathway? Finally, the discussion of results is timid and diffuse. Consequently, focusing on telomeres and subtelomeres section may give the authors greater freedom in both the description and discussion of telomere data to enhance the manuscript and its interest.

5. Pericentromericic and centromeric heterochromatin are two distinct chromatin domains. Their structure and function are different. In mammals, pericentromeric heterochromatin is marked by an accumulation of H3K9me2/3, heterochromatin protein HP1, histone H3K9 methyl-transferase (SUV39H1 in human) (Probst & Almouzni 2008, Almouzni & Probst 2011). In contrast, centromeres exhibit post-transcriptional modification associated with euchromatin such H3K4me2, but lack H3K4me3 and H3 and H4 acetylation found in euchromatin (Sullivan & Karpen 2004). Additionally, Sullivan et al. showed alternative domains of the histone variant CENP-A and H3K4me2 at centromere (Sullivan & Karpen 2004). However, this organization is not perfectly conserved in eukaryotes. In mice, H3K9me3 is enriched at pericentromeric (major) satellite DNAs and to a lesser extend at centromeric (minor) satellite DNAs (Guenatri et al. 2004). Studies on rice centromeres also revealed the presence of H3K9me2 within these regions (Nagaki et al. 2004). These data suggest centromeric chromatin structure is dependent on the studied model. Because of the difference of centromere structure, authors may consider describing and discussing more precisely their results from mouse and human cells for centromeres vs. for pericentric heterochromatin.

6. The authors state that NoRC is recruited by CENP-A to centromeres and by telomere-binding proteins to telomeres. This claim is not justified: co-IPs included in the data simply demonstrate physical association, not causality. For epistasis mapping, the authors would need to knock down CENP-A or TRF and demonstrate that NoRC binding is consequently abrogated in the appopriate chromosomal domain. Unless they intend to show such data, the claim for recruitment must be rephrased as a hypothesis.

Minor comments:
1. There are some editing issues that authors may be fix (e.g. abbreviations, spelling mistake, consistency in nomenclature).

2. In the introduction (page 3), authors should consider clarifying the sentence starting with "Cells

© European Molecular Biology Organization

deficient". Do the authors suggest that CENP-A is a modification or affect other histone modifications? The link described in this sentence is intriguing because references 12 and 13 do not described CENP-A deficiency nor its potential effects on histone modifications.

3. On page 5, line 3, authors may clarify the first sentence starting with "Moreover". HP1 is associated with pericentromeric heterochromatin, but not with centromeres. Authors should explain how HP1 is involved in NoRC recruitment to centromeres.

4. On page 6, line 22, the sentence starting with "However" is confusing because it is not clear if the described process is the association of PML bodies with telomeres or PML bodies by themselves.

5. In figures, the signification of the symbol "***" is not explained.

6. It is unclear why for some Chromatin Immuno-Precipitation (ChIP) the data were normalized to input (e.g. figure 1E, figure 3C), and for others to relative occupancy (e.g. figure 1F, figure 3D). An explanation would be appreciated.

7. Several figures and figures legends (e.g. 2C-E, 3A, S1) are incomplete (e.g. scale bar, staining).

8. On Figure 1A, it is difficult to figure out what is the morphology of apoptotic HeLa/Kyoto cells. Authors may consider changing it for a higher resolution picture.

9. In figure 1E, authors showed a clear association of TIP-5 with centromeres and rDNA promoter, whereas in Guetg et al. (2010) TIP-5 binding to centromere was described as "much lower than that with rDNA sequences" (figure 2E). Can authors discuss this difference? Additionaly, the centromeric probe is not known and its specificity (particularly for human cells) is not show

10. In figure 3C, the standard error bar for H4K20me3 level at subtelomeres is large and the lower value is under the control value. Authors should consider highlighting this point in the text.

11. On figure legend 3D, Suv4-20h2 is described as "ectopic". Can authors explain why they used this adjective to describe this factor?

12. On figure 4C (the 2 pictures on the left), the legends regarding the red and green signals are missing- it's not all clear what we are looking at unless we go into the methods and materials. In addition, the M-FISH experiment is not explained.

13. In methods and supplementary methods, protocols of ChIP are different. Authors might wish to indicate when each one is used specifically.

Referee #3:

In this manuscript, Postepska-Igielska et al show that TIP5, the large component of NoRC, regulates centromere and telomere structures using a knockdown approach in human cells. The authors show that TIP5 is targeted to centromere by CENP-A and TIP5 deletion leads disorganized mitotic spindle and genome instability. In addition, the author suggested that TIP5 is localized to telomere through TRF2 interaction and the deletion of TIP5 is associated with the increase in telomere recombination. The authors propose that repressive chromatin (increased level of H3K9me3 and K4K20me3 at centromere and telomere) in the absence of TIP5 is the key process to regulate centromere and telomere structure. The biggest drawback to this study is the correlative nature of data presented and not enough mechanistic insights into NoRC function at heterochromatins.

1. In Fig1A, the % of apoptotic cell is rather small (4-8%) and this result is contradictory to previous report (ref 17) showing that deletion of NoRC promotes cellular proliferation. This discrepancy is not well addressed. Is this apoptosis p53-dependnent? Is there increased cellular proliferation in long term NoRC knockdown cultures? Could it be possible other subunits in NoRC or other chromatin remodeling complexes play a role substituting for TIP5 during long term culture?

2. In Fig3, to conclude that TRF2 is targeting TIP5 to telomere, (reduced) TIP5 localization at telomere when TRF2 is absence will be necessary.

3. It would be important to show immunostaining analyses for methylated histone to support the ChIP data on histone methylation status at telomere (Fig3C).

4. Who does TIP5 localize to only 30% of telomeres? Are these newly replicated telomeres? The TIP5-TRF2 IP experiments are poorly done. Where are the input controls? Do the two proteins interact in a domain specific manner? Is TIP5 localization to telomeres TRF2 dependent? Do TIP5 preferentially localize to functional or dysfunctional telomeres? In TIP5 depleted cells, if you remove TRF2, is telomere dysfunction enhanced?

5. The authors show increased T-SCEs in TIP5 depleted cells and conclude that telomere recombination is impacted. Is general DNA recombination affected in TIP5 depleted cells?

Minor point
6. Although TIP5 is the major component of NoRC, authors should use "TIP5" instead of NoRC to avoid misunderstanding.

1st Revision - authors' response 17 April 2013

The following is a point-by-point response to the referees' concerns (marked in blue):

Referee #1:

1. Many of the effects described are subtle. The growth effects (Fig.S1A) are very small (sigificant?) and surely likely to be a consequence of the small increase in apoptosis (Fig.1A)? The slight prolongation of mitosis is also of doubtful significance (Fig.1B).

The increase in apoptosis (3-5%) does not explain the observed growth retardation in TIP5-depleted cells (10-20% after 96h in three cell lines). The proliferation rate of TIP-deficient cells has been measured many times (10 times for U2OS, 6 times for NIH3T3 and 3 times for HeLa/Kyoto cells), reproducibly demonstrating growth retardation. We have referred to this in the legend to Fig. S1A.

- Moreover, we have repeated the time lapse experiment in HeLa/Kyoto cells two more times and have consistently observed prolonged mitosis in TIP5 depleted cells. These data are presented in Fig. 1B. The figure legend has been modified accordingly and includes information concerning statistical significance.

2. ChIP assays showing TIP5 at centromeres are more convincing, though the authors must explain how chromatin immunoprecipitated specifically from the inactive X was assayed (Fig.S2). Perhaps the most convincing data comes from the reciprocal changes in H3K9me3 and H4K20me3 in cells in which TIP5 is knocked down or over-expressed, an observation consistent with the enzymes with which it is known to be associated (Fig.1F).

- Obviously, we did not explain this ChIP assay well enough. The primers used to control ChIP specificity will of course amplify the respective region at the X chromosome, regardless of X chromosome activity. As the inactive X chromosome is enriched in heterochromatic histone modifications, we have used this region as a positive control. We have modified the text (page 4) and the respective figure legend and included the appropriate reference in the Supplements.

© European Molecular Biology Organization

3. A modest increase in size of the mitotic spindle is seen in TIP5 depleted cells (Fig.2B). Why does this suggest a role for NoRC in "proper kinetochore structure" (Fig.2B, page 5).

- TIP5-deficient cells exhibit clear aberrations in the mitotic spindle structure which very likely result from inappropriatly assembled kinetochores. Using the size of the spindle as an indicator of such aberrations, we found that the area occupied by a spindle increased on the average by 30% upon depletion of TIP5. This information is now included in the text (page 4). We also show more examples of mitotic cells in Fig. 2A (previous Fig. 2B) to demonstrate how metaphases of TIP5-deficient cells differ from wildtype cells, illustrating the requirement of TIP5 for "proper kinetochore structure".

4. The authors show several examples of association of TIP5 with various proteins by co-immunoprecipitation (eg. Figs.2A, 3E), and use this to speculate on how it might be targeted to certain regions or chromatin types. The impression is that TIP5 is a rather sticky protein and some more negative controls would make the positive findings more convincing.

- We understand the reviewer's concern about the potential stickiness of TIP5. However, having studied NoRC function for many years, we know that TIP5 selectively interacts with many - but not all - chromatin modifiers that establish heterochromatic features. We have added a sentence (and the corresponding references) referring to previous studies showing that TIP5 does NOT interact with HDAC4 nor with Mi-2 (page 5). In addition, a negative control was already included in the primary version, showing that TIP5 does not associate with the transcription factor TIF-IA (Fig. 3C).

5. The greatest weakness of this paper is the lack of any mechanistic insight into how TIP5, and NoRC might exert their functions. There is some interesting speculation on the possible role of RNA, but no data.

- We do not agree with this comment. Having shown that NoRC targets chromatin modifying enzymes to centromeres and telomeres, establishing heterochromatic features that are essential for genome stability is an important ‚mechanistic insight'. In the revised version, we provide data showing TIP-mediated recruitment of Suv4-20h2 and SIRT6 to telomeres and subtelomeres (replacing previously presented data about SIRT6-mediated deacetylation H3K9Ac). However, the reviewer's comment did prompt us to analyse the role of TERRA in NoRC function at telomeres. We have added new data (Fig. S5) showing that depletion of TIP5 leads to decreased levels of TERRA and satellite RNA. Moreover, we now show by RNA immunoprecipitation (RIP) experiments that TIP5 is associated with TERRA (Fig. 3G). These new data add important and novel mechanistic insight into how NoRC may exert its function at centromeres and telomeres.

Referee #2:

1. Some figures of the manuscript (parts of figures 1 and 2) are only partially new. After getting a feeling of déjà vu, this reviewer looked back in the literature- the results contained herein concerning pericentromeric and centromeric chromatins were published previously by the same lab in EMBO in 2010 (see Guetg et al Figures 1 and 2). Although these are previously published data (not the same figures, but same content), nowhere is it mentioned in the manuscript and the previous data was not cited clearly. Is it meant to be a control in this ms? If so, authors should move what was already published into supporting figures or simply cite their previous paper, and clearly state in the text if they confirmed Guetg's work (e.g. TIP-5 interaction with CENP proteins) or if they obtained a different result (e.g. cell cycle progression, TIP-5 accumulation at centromeres). In the second case, authors must discuss why their results are different. For example, **authors may (i) comment the decrease of TIP5-CENPs containing spots (figure 1D) with the drop of CENP-A stained foci in number and the increase in size in mouse TIP-5 depleted cells (Guetg et al. 2010 - Figure 1D);** (ii) or discuss the

difference in cell cycle progression (Figure 1C) between their current model and their original model as published in Guetg et al 2010.

- Although we appreciate the reviewer's concern, we have not merely repeated previous results, but rather got different results with a different approach. Guetg et al. used a NIH3T3 cell line in which TIP5 was stably knocked down. This cell line was established a few years ago by Raffaella Santoro, a former postdoc in my lab. After long-term cultivation, these cells show enhanced proliferation, loss of growth control, decreased number and increased size of CENP-A stained foci, indicating that enhanced growth might have been transformed. Considering the substantial conceptual difference between the Guetg et al. and our data, we decided to show the changes of histone modifications at centromeres both after overexpression and after knockdown of TIP5, even at the risk of a déjà vu feeling. If requested, we will transfer the overexpression data to the Supplement. However, as stated above, we would prefer to show the opposite effects in gain-of-function and loss-of-function experiments side-by-side.

- Regarding the interaction of TIP5 with CENP-A, we have moved the co-IP experiment (although more convincing than the one in the Guetg et al. paper) to the supplement (Fig. S3A). Our study definitely goes beyond the previous one in which an association of TIP5 with centromeres was not seen and the conclusion of TIP5/NoRC promoting pericentromeric heterochromatin formation was based only on the decreased number and increased size of CENP-A stained foci and histone ChIPs in TIP5-deficient cells. We have added a few sentences to the text referring to the different results.

2. The section on telomeres and subtelomeres is indeed novel and interesting. Focusing the manuscript on these data should be considered to improve the novelty and interest, condensing the ms into a short report only on this subject.

- We are pleased that the referee acknowledged the significance and novelty of NoRC-dependent heterochromatin formation at telomeres. However, we decided to keep the centromere data as it would be difficult to explain the abnormal mitotic phenotype and growth defects exclusively by aberrant telomeric chromatin organisation.

3. Regarding the last sentence on page 5, the decreased level of acetylated histone H4 when TIP-5 is over-expressed, is expected to be seen on Figure 3. Authors may consider adding this panel.

- New data showing changes in acetylation of histone H4 (H4Ac) in response to TIP5 overexpression and knockdown have been added to Fig. 3D.

(ii) On Figure 3D, authors analyzed H3K9 acetylation and Suv4-20h2 association at subtelomeres only. They may consider studying also these two marks at telomeres when TIP-5 is knock-downed, and at telomeres and subtelomeres when TIP-5 is over-expressed.

- As suggested, we now show changes in Suv4-20h2 and SIRT6 (which deacetylates H3K9Ac) at telomeres and subtelomeres both after overexpression and knockdown of TIP5 (in Fig. 3E).

I would have liked to see a control with H3K4Me2 or some other euchromatic mark, to substantiate the claim that only heterochromatin is affected by TIP-5 over-expression or siRNA

- ChIP data for H4Ac have been added to Fig. 3D, showing the expected inverse change of H4Ac compared to heterochromatic histone marks upon overexpression and depletion of TIP5, respectively.

(iii) The figure S3 should perhaps be incorporated in the corpus of the manuscript.

© European Molecular Biology Organization

- The experiments showing the interaction of TIP5 with different chromatin-associated proteins relate to TIP5 binding to proteins that function at telomeres, centromeres, or both. Therefore, these co-IPs would need to be shown and explained in separate figures. Considering the space restrictions, we have incorporated data showing interaction of TIP5 with Suv4-20h2 and SIRT6 in Fig 3C, but prefer to leave the rest of Fig. S3 in the Supplement.

Data on non-coding RNA expression when TIP-5 is down-regulated or over-expressed (page 7) and TIP-5 - non-coding RNAs interaction have to be shown to emphasize the importance of this protein in heterochromatin structure. Authors may also discuss more on how TIP-5 could regulate TERRA RNAs expression (e.g. indirect effects resulting from the modification of heterochromatin structure).

- In Fig. S5 we present data showing that downregulation of TIP5 leads to decreased levels of TERRA and major satellite RNA. We also demonstrate that TIP5 is associated with TERRA in vivo. These data are referred to on pages 5/6 and in the Discussion (page 7).

4. A concern is some experiments are not well explained in the text (type of cells, methods used). The authors used three different cell lines (two mouse and one human) often the same Fig. has different panels with different cell lines. The co-staining of TIP-5 with TRF2 (Fig. 3A) and the M-FISH (Fig. 4C) are not described in the text or not enough explained to give the opportunity to the reader to interpret the result

- Because of space limitations, we may not have adequately described some methodological details. We have used three different cell types to exclude cell type-specific results and to demonstrate changes in cell physiology by using life cell microscopy. The corrected figure legends indicate which cell type has been used.

- In Fig. 4C we describe a co-FISH experiment, commonly used to demonstrate homologous recombination between telomeres. M-FISH, on the other hand, is demonstrated in Supplemental Table 2. The legend to Fig. 4C and the description of the methods have been improved, the text has been modified to provide methodological details. We have also added a scheme to Fig. 4C explaining the Co-FISH experiment.

Another example is the "data not shown" for the co-staining 53BP1 - TIP-5 (page 6), however this immuno-fluorescence experiment is presented on Fig. 4A.

- Thank you for pointing this out. Indeed, we overlooked the "data not shown" and have corrected the text accordingly.

In addition, why some proteins are studied is not well explained (e.g. RNA polymerase I, 53BP1). Authors should provide some context - is it a control or is this factor involved in the analyzed pathway?

- Agreeably, the explanation for using p53BP1 and γH2AX staining was unsatisfactory. This has been corrected (page 6).

Finally, the discussion of results is timid and diffuse. Consequently, focusing on telomeres and subtelomeres section may give the authors greater freedom in both the description and discussion of telomere data to enhance the manuscript and its interest.

- Please see Point 2.

5. Pericentromericic and centromeric heterochromatin are two distinct chromatin domains. Their structure and function are different. In mammals, pericentromeric heterochromatin is marked by an accumulation of H3K9me2/3, heterochromatin protein HP1, histone H3K9 methyl-transferase (SUV39H1 in human) (Probst & Almouzni 2008, Almouzni & Probst 2011). In contrast, centromeres

© European Molecular Biology Organization

exhibit post-transcriptional modification associated with euchromatin such H3K4me2, but lack H3K4me3 and H3 and H4 acetylation found in euchromatin (Sullivan & Karpen 2004). Additionally, Sullivan et al. showed alternative domains of the histone variant CENP-A and H3K4me2 at centromere (Sullivan & Karpen 2004). However, this organization is not perfectly conserved in eukaryotes. In mice, H3K9me3 is enriched at pericentromeric (major) satellite DNAs and to a lesser extend at centromeric (minor) satellite DNAs (Guenatri et al. 2004). Studies on rice centromeres also revealed the presence of H3K9me2 within these regions (Nagaki et al. 2004). These data suggest centromeric chromatin structure is dependent on the studied model. Because of the difference of centromere structure, authors may consider describing and discussing more precisely their results from mouse and human cells for centromeres vs. for pericentric heterochromatin.

- We have described the distinct domains of centric and pericentric chromatin in the Introduction (page 3) and discuss our findings in the Results section (page 7). We regret that, in a few cases, we did not use the respective term correctly and have modified the text accordingly.

6. The authors state that NoRC is recruited by CENP-A to centromeres and by telomere-binding proteins to telomeres. This claim is not justified: co-IPs included in the data simply demonstrate physical association, not causality. For epistasis mapping, the authors would need to knock down CENP-A or TRF and demonstrate that NoRC binding is consequently abrogated in the appropriate chromosomal domain. Unless they intend to show such data, the claim for recruitment must be rephrased as a hypothesis.

- We agree with the referee that the interaction data are consistent with – but do not prove - that NoRC is recruited to centromeres by CENP-A and to telomeres by TRF2 and have modified the text accordingly. The suggested experiment, i.e., knocking down CENP-A and TRF2, is not feasible because depletion of TRF2 causes extensive telomere disruption, as evidenced by massive end-to-end fusions and loss of the G-strand overhang and TIFs (van Steensel et al. 1998; Celli and de Lange, 2005). Moreover, even after double knockdown TRF2 can be detected at telomeres in some cells. Therefore, ChIP or IF data of TIP5 after knockdown of TRF would be of little significance. However, our finding that TIP5 interacts with TRF2 and is associated with TERRA (new Fig. S5) suggests a protein- and RNA-based mechanism that guides NoRC to telomeres.

Minor comments:

1. There are some editing issues that authors may be fix (e.g. abbreviations, spelling mistake, consistency in nomenclature).

2. In the introduction (page 3), authors should consider clarifying the sentence starting with "Cells deficient". Do the authors suggest that CENP-A is a modification or affect other histone modifications? The link described in this sentence is intriguing because references 12 and 13 do not described CENP-A deficiency nor its potential effects on histone modifications.

- The paragraph has been rephrased and a new, more accurate reference has been added.

3. On page 5, line 3, authors may clarify the first sentence starting with "Moreover". HP1 is associated with pericentromeric heterochromatin, but not with centromeres. Authors should explain how HP1 is involved in NoRC recruitment to centromeres.

- On page 5, we have incorrectly used the term ‚centromere‘ rather than ‚pericentro-meric repeats‘. This has been corrected. As HP1α is also a component of telomeric heterochromatin, we have rephrased the text postulating that the observed interaction between TIP5 and HP1α may contribute to NoRC targeting to chromosome ends (page 5).

4. On page 6, line 22, the sentence starting with "However" is confusing because it is not clear if the described process is the association of PML bodies with telomeres or PML bodies by themselves.

- The sentence has been rephrased to avoid confusion.

5. In figures, the signification of the symbol "***" is not explained.

- The symbols „*“ or „***“ expressing the significance of data are explained in each figure legend.

6. It is unclear why for some Chromatin Immuno-Precipitation (ChIP) the data were normalized to input (e.g. figure 1E, figure 3C), and for others to relative occupancy (e.g. figure 1F, figure 3D). An explanation would be appreciated.

- All ChIPs have been normalized to input. The ‚relative occupancy‘ compares different conditions, e.g. control with TIP5-depleted cells. The ChIP data (normalized to input) are presented as the relative change under experimental conditions; binding in control cells was set to 1. This is described in the Methods section.

7. Several figures and figures legends (e.g. 2C-E, 3A, S1) are incomplete (e.g. scale bar, staining).

- We have corrected the respective figures and their legends.

8. On Figure 1A, it is difficult to figure out what is the morphology of apoptotic HeLa/Kyoto cells. Authors may consider changing it for a higher resolution picture.

- We have replaced the image of the apoptotic cell by another, better image. Furthermore, as the figures are generally very small, we have added a movie showing a cell undergoing apoptosis (please see Supplemental movie 1).

9. In figure 1E, authors showed a clear association of TIP-5 with centromeres and rDNA promoter, whereas in Guetg et al. (2010) TIP-5 binding to centromere was described as "much lower than that with rDNA sequences" (figure 2E). Can authors discuss this difference?

- Please see Point 1

Additionaly, the centromeric probe is not known and its specificity (particularly for human cells) is not shown.

- The probe has been published and is referred to in the Methods section.

10. In figure 3C, the standard error bar for H4K20me3 level at subtelomeres is large and the lower value is under the control value. Authors should consider highlighting this point in the text.

- To strengthen the significance of our data, we have monitored histone modifications at subtelomeres upon overexpression of TIP5 in two more experiments. These additional data are now included in Fig. 3D.

11. On figure legend 3D, Suv4-20h2 is described as "ectopic". Can authors explain why they used this adjective to describe this factor?

- To monitor the association of Suv4-20h2 with subtelomeres, a cell line was used that expresses ER-tagged Suv4-20h2 in response to tamoxifen treatment (see Supplemental Methods). The word ‚ectopic‘ is commonly used to illustrate that the ChIP has been performed with tagged rather than with endogenous Suv4-202h.

© European Molecular Biology Organization

12. On figure 4C (the 2 pictures on the left), the legends regarding the red and green signals are missing- it's not all clear what we are looking at unless we go into the Methods and Materials.

- We apolgize for this mistake. The information has now been added to the legend of Fig. 4C.

In addition, the M-FISH experiment is not explained.

- A more detailed explanation of the M-FISH experiment is provided in the legend to Supplementary Table 2.

In methods and supplementary methods, protocols of ChIP are different. Authors might wish to indicate when each one is used specifically.

- All ChIPs were performed using the protocol described in Supplementary Methods. The protocol in the Methods section applies to the co-IPs, not to alternative ChIPs. We regret this misunderstanding. ChIPs and co-IPs are now described side by side in the Supplementary Methods section.

Referee #3:

1. In Fig1A, the % of apoptotic cell is rather small (4-8%) and this result is contradictory to previous report (ref 17) showing that deletion of NoRC promotes cellular proliferation. This discrepancy is not well addressed. Is this apoptosis p53-dependent? Is there increased cellular proliferation in long term NoRC knockdown cultures? Could it be possible other subunits in NoRC or other chromatin remodeling complexes play a role substituting for TIP5 during long term culture?

- As pointed out in our response to Reviewer 1, Guetg et al. worked with a TIP5-deficient cell line that was established years ago. In contrast to the results obtained with this cell line, in our current study, we observe decreased proliferation, increased apoptosis and aberrant mitotic phenotypes. However, we cannot exclude that other chromatin remodeling complexes may substitute for TIP5 during long term culture. We have addressed this point in the text (page 7).

2. In Fig3, to conclude that TRF2 is targeting TIP5 to telomere, (reduced) TIP5 localization at telomere when TRF2 is absence will be necessary.

- Please see our response to Point 6 of referee 2

3. It would be important to show immunostaining analyses for methylated histone to support the ChIP data on histone methylation status at telomere (Fig3C).

- Immunofluorescence can reveal global changes of histone modifications. As we are dealing with relatively subtle changes at defined genomic loci, ChIP is the most sensitive and precise way to measure histone modifications at specific genes, and is therefore the method of choice to monitor chromatin changes at telomeres. Nevertheless, in response to the referee’s request, we tried to visualize H3K9me3 at telomeres by high-resolution microscopy after double-staining with anti-H3K9me3 antibodies and a telomere FISH probe. However, the results are not satisfactory because of high background staining of H3K9me3.

4. Who does TIP5 localize to only 30% of telomeres? Are these newly replicated telomeres? The TIP5-TRF2 IP experiments are poorly done. Where are the input controls? Has this been improved?

© European Molecular Biology Organization

- We have replaced the blot by a better one.

Do the two proteins interact in a domain specific manner? Is TIP5 localization to telomeres TRF2 dependent? Do TIP5 preferentially localize to functional or dysfunctional telomeres? In TIP5 depleted cells, if you remove TRF2, is telomere dysfunction enhanced?

- We found no telomeric aberrations (fusions, telomeric duplications, telomeric losses) +/- TIP5 or aggregation of gammaH2A.X and p53BP at telomeres (new Fig. 4A, S4B and C), indicating that NoRC is not linked to dysfunctional telomeres. We did not analyze which domain of TIP5 interacts with TRF2, nor did we analyze the effect of TRF2 knockdown in TIP5-deficient cells (see our response to referee 2, point 6), as we feel that this is beyond the scope of this study.

5. The authors show increased T-SCEs in TIP5 depleted cells and conclude that telomere recombination is impacted. Is general DNA recombination affected in TIP5 depleted cells?

- The increased association with PML bodies, correlating with a 2-fold increase in telomeric sister chromatid exchange, is a sign of extended recombination. In accord with NoRC safeguarding genome stability, we find a two to three-fold increase in chromosomal translocations as early as 32 h after TIP5 knockdown.

6. Although TIP5 is the major component of NoRC, authors should use "TIP5" instead of NoRC to avoid misunderstanding.

- Done

2nd Editorial Decision 10 May 2013

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the enclosed reports from the referees. As you will see, referee 3 still does not support publication of the study here. However, her/his concerns mainly regard the mechanism by which NorC regulates heterochromatin formation, and this information, beyond what you show already, is not required for publication by EMBO reports.

The other two referees are more positive. If their remaining concerns can be successfully addressed, especially concerning the statistical analyses, we can accept the manuscript for publication.

I need to tell you that it is EMBO reports policy that manuscripts must be accepted latest 6 months after a first decision was made. In your case this was the 19th of December, so your manuscript needs to be accepted latest in 5 weeks. It is therefore important that you submit the newly revised version as soon as possible.

I also noticed that the majority of the figure panels with statistical analyses lack information on n, the number of experiments the calculated averages are based on, and do not define the error bars. Please include the missing information for each figure panel, including the supplementary ones.

I look forward to seeing a new revised version of your manuscript as soon as possible.

© European Molecular Biology Organization

REFEREE REPORTS:

Referee #1:

Overall, the authors have addressed the issues raised in my original review and the new data on the involvement of TERRA RNA do indeed provide some additional mechanistic insights. However, I remain concerned by the authors' reluctance to use statistics to back up some of their contentions. I accept that the growth rate experiments have been repeated multiple times and that this is outlined in a Figure legend (Fig.S1A), but why can these multiple repeats not be converted into a statistical analysis confirming the small differences observed? Confirming this growth effect is particularly important in view of a previous report, highlighted by reviewer 3 (ref 17 in the original version), showing enhanced growth in NoRC deficient cells. Similarly, there is no statistical analysis to support the differences shown for the new TERRA data shown in Figure S5. Given that histograms and standard errors are shown a statistical comparison should be possible.
I these issues can be taken care of I think the paper represents an extensive and useful contribution to understanding the possible roles of chromatin remodelling and, heterochromatin in genome stabibility, a topic that is of general interest in the field.

Referee #2:

The new version of the manuscript EMBOR-2012-36891V is very compelling indeed. I appreciate that the authors went to considerable length to address all issues raised by the reviewers. Technically the ms is improved significantly, controls and missing experiments have been provided, and the quality of their figures are now consistent with publication in this journal. They did a good editing job by explaining abbreviations, using constant nomenclature, rephrasing confusing sentences. Finally, they clarified why they observed differences with their previous article (Guetg et al. 2010) and made a compelling argument as to why they wish to include the centromere data.
Their overall effort has led to a nicely written manuscript with persuasive evidences for the role of TIP5 in heterochromatin stability at telomeres and centromeres. Consequently, I strongly recommend rapid publication of this revised manuscript.

Three minor editing corrections may be considered:
(i) The figure S5 may be renamed S4 (and vice versa) to follow the order of their appearance in the text;
(ii) On page 7, references describing the interaction of TIP5 with TTF-I and SET-DB1 may be remind;
(iii) Several typos are still observed (e.g., page 5: H4 in place of K4, page 6: Q-FISH in place of qFISH, page 14: in figure legends 3A antibodies in place of antibody, page 15: scale in place of sale, in figure legend S3 , but not GFP-tagged TIF-IA in place of and GFP-TIF-IA).

Referee #3:

The manuscript by Postepska-Igielska et al has been improved technically following reviewers' suggestions. However the lack of mechanism still plagues this paper. For example, the authors describe increased TERRA transcripts by TIP5. What does this mean mechanistically? TERRA has been shown to participate with RPA to regulate POT1 access to telomeres. Does NoRC play a role in this? Along this line, what does Co-IP of TRF2 with TIP5 mean functionally? Which domain of TRF2 is required for this? The TRFH domain has been shown to recruit a large number of proteins to telomeres. Is NuRD one of them? Finally, from Fig 3A, it is difficult to say that TIP5 localizes to telomeres, since TIP5 generates such huge patches of staining that telomere signals may simply overlap them.

2nd Revision - authors' response 17 May 2013

I am happy to sent the new revised version of our manuscript *„The chromatin remodelling complex NoRC safeguards genome stability by heterochromatin formation at telomeres and centromeres“*. I believe we have addressed all the remaining concers of the referees and yourself, in particular:

- all information about statistical analysis (n, error bars, statistical test used) has been included, for both main figure legends and supplements, please find them listed in the table below;
- statistical analysis has been provided for the experiments in Fig S1A and Fig S4 (former Fig S5), in response to Referee#1 request;
- minor editing corrections, pointed out by Referee#2, were introduced.

I very much hope you will now find the manuscript suitable for publication in EMBO Reports.

Figure	**New information provided**
1A	Error bars description
1B	Number of experiments performed, number of cells analyzed
1D	Number of experiments performed, number of cells analyzed
1E	Number of experiments performed
1F	Number of experiments performed
2A	Number of experiments performed, number of cells analyzed
2B-D	Number of experiments performed, number of cells analyzed
3A	Number of experiments performed, number of cells analyzed
3B	Number of experiments performed
3E	Number of experiments performed
4A	Number of experiments performed, number of cells analyzed
4B	Number of experiments performed, number of cells analyzed
4C	Number of experiments performed, number of cells analyzed
S1B	Number of experiments performed
S2	Number of experiments performed, error bars description
S5A	Error bars description

3rd Editorial Decision 22 May 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a

© European Molecular Biology Organization

Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Finally, we provide a short summary of published papers on our website to emphasize the major findings in the paper and their implications/applications for the non-specialist reader. To help us prepare this short, non-specialist text, we would be grateful if you could provide a simple 1-2 sentence summary of your article in reply to this email.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

© European Molecular Biology Organization