THE EMBO JOURNAL

Manuscript EMBO-2009-72672

The NoRC complex mediates heterochromatin formation and stability of silent rRNA genes and centromeric repeats

Claudio Guetg, Philipp Lienemann, Valentina Sirri, Martin Fussenegger, Danièle Hernandez-Verdun, Michael Hottiger and Raffaella Santoro

Corresponding author: Raffaella Santoro, University of Zurich

Review timeline:

Submission date:	24 September 2009
1st Editorial Decision:	29 October 2009
1st Revision received:	08 January 2010
2nd Editorial Decision:	22 January 2010
2nd Revision received:	25 January 2010
Accepted:	26 January 2010

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 29 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I apologise that it has taken a little longer than usual to have your manuscript reviewed but it has now been evaluated by three referees and I enclose their reports below. As you will see from their comments the referees provide mixed recommendations regarding publication in the EMBO J. Referee#1 is clearly the more critical of the referees, but referee #2 and #3 express significant interest in the study. Nevertheless, all the referees raise a number of concerns especially surrounding the data in figure 2 and 3 that needs to be significantly strengthened to make the manuscript suitable for publication in the EMBO Journal. Should you be able to address these issues, we would be wiling to consider a revised manuscript.

I should remind you that it is EMBO Journal 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. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In mammalian cells a proportion of rDNA repeats, typically around 50%, are transcriptionally silent and packaged as heterochromatin. Work over the last 10 years has established that nucleolar remodeling complex, NoRC, comprising TIP5 and SNF2h, is recruited via association with TTF1 to rDNA promoters whereupon it induces formation of heterochromatin. How heterochormatic marks, including CgG methylation, spread across the rDNA repeat has not yet been determined. In this manuscript the consequences of shRNA knockdown of the NoRC component, TIP5, are examined. In agreement with previous work it is demonstrated that knock down of TIP5 results in derepression of rDNA repeats. In addition it is claimed here that establishment of heterochromatin at "centric and pericentric repeats" is TIP5 dependent. Moreover, it is argued that TIP5 is essential for maintenance of stability of silent rDNA repeats as well as that of major and minor satellites. The final claim is that deregulation of rDNA silencing contributes to cellular transformation. These are wide ranging and bold claims. Unfortunately these claims are not adequately supported by the data presented.
It is stated in the introduction that rRNA genes (NORs) are located on the long arms of chromosomes 12, 15 and 19. Laboratory strains of mouse and their derived cell lines contain more NORs than this. The authors should see Kurihara et al 1994, Mammalian Genome 5, 225.
The authors cite Akhmanova et al 2000 as evidence of an intricate relationship between perinucleolar heterochromatin and silent rDNA. The Akhmanova et al paper analyses a very particular cell type, neurons, where it has been observed that even non rDNA bearing chromosomes associate with nucleoli (Manuelidid and Borden 1988, Chromosoma 96, 397). This work should not be used to infer a general truism.
In reference to figure 1A the statement is made that CpG levels are reduced over the entire repeat. In fact only four sites were looked at.
Figure 1C is confusing. The figure legend suggests that individual cells were stained with DAPI, anit-Fib and anti-UBF, but left and right panels are clearly different cells.
Figure 1C. A commonly used method of analyzing the positioning of centromeres is to use ant-centromere antigens. This would have been useful.
Figure 1D. The quantitative description of CC and FC is not connected in anyway to the data in Fig 1D. What is interpreted as CC in this figure should be indicated.
Fig 2. The only data linking alterations in the status of heterochromatin on major and minor satellite sequences with loss of TIP5 is that presented in part C. It is claimed that TIP5 can be chipped onto satellite repeats. This seems to be an over interpretation of the data.
Fig3. The data in support of the claim that the number of rDNA repeats change as TIP5 levels are reduced is not sufficient. In yeast it is standard to use pulse field gel electrophoresis to examine changes in the size of the yeast rDNA array. Such a technique should be applied here. PCR is not sufficient, especially since timing of replication and cell cycle profiles are altered in TIP5 knock down.
Fig3C. I am surprised that there is no reference to the work of Hung Tseng who identified mouse rDNA variants.
Fig 3 E and F. Once rDNA repeats become derepressed presumably they can be transcriptionally activated. Consequently, how is it envisaged that previously silent repeats are targeted for rearrangement?
Insufficient data is presented to support the conclusion that loss of TIP5 induces cellular transformation. Do the authors believe that TIP5 is a tumor suppressor? This is the implication of their suggestion.
In the discussion it is stated that rDNA silencing in yeast prevents pol II transcription of a reporter gene integrated in the rDNA array. The fact is that Nomura and colleagues have demonstrated that Sir2 is an activator of rDNA transcription and that pol I and II transcription are incompatible (see Cioci et al 2003, Cell 12, 135).

Referee #2 (Remarks to the Author):

Using small RNA, genetics, qtPCR and cytological based approaches, the authors have effectively

demonstrated that TIP5 is important in regulating the epigenetic status of H4 and H3 at the rDNA loci, and that its depletion affects replication timing, genomic instability and cellular morphology. This is an exciting finding as previous studies have not successfully established a clear link between heterochromatic silent rDNA and cellular function. Here, the authors provide compelling evidence that these silent loci may play a role simply due to their close spatial proximity to pericentric heterochromatin and their ability to influence its morphology. The authors' data reveals that TIP5 establishes repressive histone marks at both major and minor satellite sequences, important for stabilizing silent rRNA genes, and TIP5 depletion affected both cellular morphology, upregulated rDNA transcription and loss of copies of all three types of DNA that may result in the transformation of these cells. Overall, this is a nice manuscript with some very novel observations. However, a number of clarifications and some additional data should be included to make this manuscripts findings more robust.

Major concerns
Figure 1 effectively confirms previous results (Santoro et al, 2002; Li et al, 2005) that TIP5 binds to and induces de novo rDNA methylation, but using shRNA-directed depletion of TIP5 revealed decreased percentage in CpG methylation in both shRNA-TIP5-1 and shRNA-TIP5-2 cell lines. It is also clear there is diminished number of nucleoli in the mutant cell lines. One concern is that the control shRNA clearly has an effect- about 65% of DNA methylation is lost in the control shRNA, and the rDNA expression has clearly increased. Either this is an inappropriate control, or there are pleiotropic effects that the authors have not accounted for. Please explain.
Also, the EM Figure is weak and should be removed. N=1 is quite insufficient to draw the conclusions the authors mention in text about repositioning of FC/CC boundaries. Furthermore, FC and CC are not labeled therefore it is unclear what the authors are referring to. Minor concern: Figure 1C should include a size bar and total number of cells scored must be included.
Figure 2 shows the interesting histone modifications changes that result from the TIP5 depleted cell lines compared to the control. However, the figure has a number of problems. First, b/input seems to me an inappropriate comparison- we are looking at 200 copies of rDNA locus over input, thus the composite changes reflect all loci. In order to know the per gene change, the figure should be modified to include accounting for copy number. In Figure 2D, authors show TIP5 depletion affects replication timing of rDNA, major & minor satellite DNA using semi-quantitative PCR. This is potentially a very interesting finding. However, again copy number must be accounted for, and relative intensity of the shRNA samples is similar to that of control. This is unintuitive, as if there was quantitative shift in replication timing on would expect a huge increase in amount of product obtained at 3-4hrs. Please explain. Authors explain in methods that PCR was normalized to amounts of B-lactamase using qRT-PCR. I'm confused as to why semi-quant PCR was utilized for this assay instead of using qRT-PCR (as has been used for the rest of the ms) for the whole assay instead of just to normalize for B-lactamase? Or, if B-lactamase is to be used for semi-quantitative PCR, I suggest that Figure 2D include a row where B-lactamase PCR amplified to show normalization. Minor concern: I would also suggest reconfiguring this data in a graphical format of increase vs cell cycle.

Figure 3- This is a nice set of experiments. The authors demonstrate quite convincingly that there are copy number changes in the shRNA and miRNA of TIP5 specifically targeting the rDNA and minor satellite. However, I think the interpretation is weak- the significant loss of minor satellite should be expected to have enormous consequences for centromeric function, since centromeric H3 assembles specifically on minor satellite in mouse. A simple CENPA-staining (or CREST) would yield deep insight into potential reduction of centromere staining, which could explain the transformation phenotype seen in the next figure. At the very least, the discussion should be a little bit bolder in addressing this possibility.
Figure 4: Authors make a bold leap here, and attempt to connect the TIP5 transformed phenotype to that of a phenotype from oncoprotein-like Ras (previously reported in Tognon et al, 1998). They also stated that depletion of TIP5 and impaired rDNA silencing can strengthen the intimate link between rDNA transcription and cancer presumably by both the loss of rDNA copies, increase in rDNA transcription. However, I am not thoroughly convinced of the connection, especially given the much larger loss of minor satellite DNA that most likely has a stronger effect on cellular transformation than just increased rDNA transcription. This should be discussed to make a better case for the authors' model.

Referee #3 (Remarks to the Author):

This manuscript describes the phenotypic effects of NoRC, a nucleolar chromatin remodelling complex, in terms of rRNA expression and heterochromatin formation. It presents the exciting and important observations that TIP5, a unique subunit of NoRC, may influence genomic stability, cell proliferation and cell transformation. If proven, these will be important discoveries of wide general interest. However, the manuscript requires improvement before it is acceptable for publication.

The abstract claims to have demonstrated a role of "rDNA silencing in controlling cell proliferation". This is not justified. The authors have demonstrated that TIP5 can influence rDNA silencing and can also influence cell proliferation. They have not established that the proliferative effect is caused by the effect on rDNA. There is a correlation, but it remains possible that TIP5 affects proliferation through some alternative target besides rDNA.

It is reported that shRNA-TIP5 causes a decrease in the number of nucleoli and an increase in their size. Figure 1C is not enough to convince one of this. Some quantitation is required, based on a significant number of cells.

In Figure 1B, there seems to be stronger staining in the nucleoplasm, as well as the nucleolus, for the shRNA-TIP5 cells. That is not expected if TIP5 only regulates rRNA gene expression.

Figure 1D should be annotated to make explicit the features that are being described.

Figure 2C is crucial, but is not convincing. The signal for the Satellite sequences is only marginally above background. That being the case, it is absolutely not sufficient to have only done this experiment twice. Indeed, error bars should not be used after a single repetition. This experiment needs to be repeated a sufficient number of times to allow assessment of statistical significance.

On the basis of Fig 2C, the authors claim that their "results indicated that TIP5 interacts with a minor fraction of centric-pericentric repeats". A weak interaction with a large fraction is just as compatible with their data as a strong interaction with a small fraction, assuming that it is statistically significant at all.

A negative control antibody should be included in these ChIP experiments, such as non-immune or preimmune serum, and signal should be normalised against this. Perhaps the satellites will give a higher background signal with any antibody? Because of their more condensed state, maybe they fragment less readily and immunoprecipitate more strongly in a non-specific manner, whatever antibody used. These possibilities need to be tested and excluded, if the authors wish to convince their readers of significant and specific binding by TIP5.

Figure 2D is unconvincing because the satellite panels have much weaker signals for shRNA-TIP5 than for shRNA-control. Are the cycle numbers/exposure times the same? For each DNA type, all 16 samples should be run on the same gel and presented in a single continuous panel, rather than two panels of eight.

I found Figures 3E and 3F rather confusing. It would be more appropriate to refer to methylated and unmethylated genes in Fig 3E, rather than silent and active, since methylation is what is being measured, if I understood correctly.

Please explain why expression of the A variant increases when TIP5 is depleted (Fig 3F) - I thought these were the active genes, which would imply that they are not bound/repressed by TIP5.

In Figure 3F, shRNA-TIP5-1 has a bigger effect on A expression than shRNA-TIP5-2. This could be explained if shRNA-TIP5-1 gives more efficient depletion. However, for T expression shRNA-TIP5-2 is more effective than shRNA-TIP5-1. The differences seem significant, judging by the error bars. Indeed, the differences here are much greater than many of the effects highlighted by the authors elsewhere in the paper.

Figure 4 is exciting. The authors should gate their FACS analyses and give the % cells in G0/G1, S and G2/M. Means and standard deviations are required. It is not enough simply to state that there are

"higher numbers of cells in S phase". In fact, the changes to the G0/G1 and G2/M populations appear more dramatic than S phase changes.

Error bars are required for the proliferation curves with shRNA cells.

The images in Figure 4C are not clear enough. They might be improved by being in colour, to show methylene blue staining.

The quality of English needs to be improved. It is not up to the necessary standard for publication.

1st Revision - authors' response 08 January 2010

Referee #1 (Remarks to the Author):

In mammalian cells a proportion of rDNA repeats, typically around 50%, are transcriptionally silent and packaged as heterochromatin. Work over the last 10 years has established that nucleolar remodeling complex, NoRC, comprising TIP5 and SNF2h, is recruited via association with TTF1 to rDNA promoters whereupon it induces formation of heterochromatin. How heterochormatic marks, including CgG methylation, spread across the rDNA repeat has not yet been determined. In this manuscript the consequences of shRNA knockdown of the NoRC component, TIP5, are examined. In agreement with previous work it is demonstrated that knock down of TIP5 results in derepression of rDNA repeats. In addition it is claimed here that establishment of heterochromatin at "centric and pericentric repeats" is TIP5 dependent. Moreover, it is argued that TIP5 is essential for maintenance of stability of silent rDNA repeats as well as that of major and minor satellites. The final claim is that deregulation of rDNA silencing contributes to cellular transformation. These are wide ranging and bold claims. Unfortunately these claims are not adequately supported by the data presented.

It is stated in the introduction that rRNA genes (NORs) are located on the long arms of chromosomes 12, 15 and 19. Laboratory strains of mouse and their derived cell lines contain more NORs than this. The authors should see Kurihara et al 1994, Mammalian Genome 5, 225.
Response: According to Kurihara et al 1994, we corrected the number of mouse chromosome containing NORs (pag. 2).

The authors cite Akhmanova et al 2000 as evidence of an intricate relationship between perinucleolar heterochromatin and silent rDNA. The Akhmanova et al paper analyses a very particular cell type, neurons, where it has been observed that even non rDNA bearing chromosomes associate with nucleoli (Manuelidid and Borden 1988, Chromosoma 96, 397). This work should not be used to infer a general truism.
Response: The work of Akhmanova was cited because this is the only study addressing the cellular localization of silent CpG methylated rDNA repeats. The finding of this study, i.e. the localization of methylated rDNA copies close to the centromere, is an important result for our work. In the revised version, we introduced in the text that this study was performed in neuronal cells. The localization of non rDNA bearing chromosomes associated with nucleoli has been already mentioned and discussed in the Introduction by reporting the results of the group of Carmo-Fonseca (Carvalho et al, 2001 and reference herein) pag. 2-3. The well known localization of centromeres near to nucleoli were cited by referring to Carvalho et al, 2001 and references herein, Haaf and Schmid, 1991; Pluta et al. 1995; Elsevier and Ruddle, 1975; Henderson et al, 1974).

In reference to figure 1A the statement is made that CpG levels are reduced over the entire repeat. In fact only four sites were looked at.
Response: The length of the rDNA repeat is about 40 Kb (about 13Kb representing the coding region). We analyzed the CpG methylation content of the intergenic (at -2080), the promoter (at -143), and the two coding rDNA regions (+672 and +8278/+8397, respectively). rDNA CpG methylation measurements from previous works (from our

group and from others) were generally limited to the rDNA promoter region. To our point of view, the analysis of four distinct rDNA regions can fully represent the CpG methylation state of the rDNA repeat.

Figure 1C is confusing. The figure legend suggests that individual cells were stained with DAPI, anit-Fib and anti-UBF, but left and right panels are clearly different cells.
Response: Clearly, these are different cells stained either with anti-Fibrillarin or with anti-UBF. We changed Fig. 1C legend accordingly

Figure 1C. A commonly used method of analyzing the positioning of centromeres is to use anti-centromere antigens. This would have been useful.
Response: As requested by the reviewer, we introduced a Supplementary Figure (Fig. S2) showing the cellular localization of the centromeric protein CENP-A and UBF in NIH3T3 cells. The data support the well documented localization of centromers at the nuclear periphery or around the nucleolus (Haaf and Schmid, 1991; Pluta et al. 1995).

Figure 1D. The quantitative description of CC and FC is not connected in anyway to the data in Fig 1D. What is interpreted as CC in this figure should be indicated.
Response: As requested by the reviewer, a detailed description was added to pages 5-6 and in the corresponding Figure legend.

Fig 2. The only data linking alterations in the status of heterochromatin on major and minor satellite sequences with loss of TIP5 is that presented in part C. It is claimed that TIP5 can be chipped onto satellite repeats. This seems to be an over interpretation of the data.
Response: According to the comments of the other reviewers, we repeated the ChIP experiments two more times, including the results obtained with chromatin incubated with the pre-immunoserum. Again, although the association of TIP5 (b/i) with major and minor satellite repeats is not very efficient, it remains always higher if compared to a preimmunoserum control and to three control sequences we used (Mariner, Charlie and globin). This result suggests that either the association is limited to a minor fraction of satellite repeats or that the interaction is weak and/or transient. To further analyze the relationship between TIP5 and centromere, we included a new experiment, which shows that TIP5 associates with the core kinetochore CENP-A protein (Fig. 2E), offering an explanation of how TIP5 can contact centric repeats. These data (ChIP and IP) are described on page 7.

Fig3. The data in support of the claim that the number of rDNA repeats change as TIP5 levels are reduced is not sufficient. In yeast it is standard to use pulse field gel electrophoresis to examine changes in the size of the yeast rDNA array. Such a technique should be applied here. PCR is not sufficient, especially since timing of replication and cell cycle profiles are altered in TIP5 knock down.
Response: Unfortunately higher eukaryotes cannot be always analyzed as yeast.
In yeast, the rDNA repeats (150 copies of the 9.1-kb rDNA unit) are present uniquely on chromosome XII. The size of chromosome XII is 2.5 Mb. Thus, significant changes in the number of repeats are generally detectable by analyzing the size of chromosome XII by use of pulse-field gel electrophoresis. In mouse, chromosomes bearing rDNA repeats have the following size: chr. 12: 121 Mb; chr.15: 103 Mb; chr.16: 98 Mb; chr.18: 91 Mb; chr.19: 61 Mb. Clearly, the size of these chromosomes is prohibitive to perform the requested analysis. To our experience, qPCR measurements are very precise and, considering that the levels of the repeats are normalized to globin gene amounts, alterations of replication timing and cell cycle cannot affect the measurements. Moreover, this assay was performed on stable shRNA-TIP5 cells and after 10 days TIP5 depletion (miRNA-TIP5) giving similar results. The same was true for the analysis of rDNA variants (Fig. 4).

Fig3C. I am surprised that there is no reference to the work of Hung Tseng who identified mouse rDNA variants.
Response: Polymorphisms in the mouse and human rDNA family have been revealed since more than 20 years (Arnheim, N. & Southern, E. M. (1977) Cell 11, 363-370;

Kominami et al., (1981) Nucleic Acids Res. 9, 3219-3233; and many others). We introduced these references at page 9.

Fig 3 E and F. Once rDNA repeats become derepressed presumably they can be transcriptionally activated. Consequently, how is it envisaged that previously silent repeats are targeted for rearrangement?
Response: The results presented in Figure 4 indicate that upregulation of rDNA transcription in TIP5 depleted cells does not depend on the de-repression of silent genes. We discussed this point at page 10 (Results) and at page 13 (Discussion).
Re-arrangement of rDNA repeats in the absence of silencing factors was previously observed in S.cerevisiae and Drosophila. As discussed on page 8, in Saccharomices cerevisiae, the stability of the rDNA repeats requires a Sir2-containing chromatin silencing complex (Straight et al, 1999). Similarly, Drosophila Su(var)3-9 and RNAi mutants caused increases in the amount of extrachromosomal circular rDNA, a typical result of rDNA recombination events (Peng and Karpen, 2007).
The data provided in Fig. 3A and 3B demonstrate that in the absence of TIP5 a large portion of silent genes are targeted for rearrangement, most likely due to loss of its heterochromatic structure. To our knowledge, this is the first time that shows that rearrangement of rDNA repeats in the absence of silencing factors (TIP5) occurs in mammalian cells.

Insufficient data is presented to support the conclusion that loss of TIP5 induces cellular transformation. Do the authors believe that TIP5 is a tumor suppressor? This is the implication of their suggestion.
Response: Tumor suppressor function is generally tested by cell foci formation and contact inhibition assays. According to this, the data of Figure 5 clearly indicated that cells lacking TIP5 proliferate aberrantly. However, although the data suggest that TIP5 can be a tumor suppressor gene, we believe that further investigations are required to dissect the role of TIP5 in the transformation process.

In the discussion it is stated that rDNA silencing in yeast prevents pol II transcription of a reporter gene integrated in the rDNA array. The fact is that Nomura and colleagues have demonstrated that Sir2 is an activator of rDNA transcription and that pol I and II transcription are incompatible (see Cioci et al 2003, Cell 12, 135).
Response: We deleted this sentence. Omission of this sentence does not affect the rest of the discussion, being the theme of this study the rRNA genes and not the Pol II reporter genes integrated within the rDNA locus. We would, however, like to point to the fact that Cioci et al. did not demonstrate that Sir2 is an activator but that silencing acts on reporter genes present in rDNA repeats that contain rRNA genes actively transcribed by Pol I.

Referee #2 (Remarks to the Author):

Using small RNA, genetics, qtPCR and cytological based approaches, the authors have effectively demonstrated that TIP5 is important in regulating the epigenetic status of H4 and H3 at the rDNA loci, and that its depletion affects replication timing, genomic instability and cellular morphology. This is an exciting finding as previous studies have not successfully established a clear link between heterochromatic silent rDNA and cellular function. Here, the authors provide compelling evidence that these silent loci may play a role simply due to their close spatial proximity to pericentric heterochromatin and their ability to influence its morphology. The authors' data reveals that TIP5 establishes repressive histone marks at both major and minor satellite sequences, important for stabilizing silent rRNA genes, and TIP5 depletion affected both cellular morphology, upregulated rDNA transcription and loss of copies of all three types of DNA that may result in the transformation of these cells. Overall, this is a nice manuscript with some very novel observations. However, a number of clarifications and some additional data should be included to make this manuscripts findings more robust.

Reference 2 Major concerns
Figure 1 effectively confirms previous results (Santoro et al, 2002; Li et al, 2005) that TIP5 binds to and induces de novo rDNA methylation, but using shRNA-directed

depletion of TIP5 revealed decreased percentage in CpG methylation in both shRNATIP5-1 and shRNA-TIP5-2 cell lines. It is also clear there is diminished number of nucleoli in the mutant cell lines. One concern is that the control shRNA clearly has an effect- about 65% of DNA methylation is lost in the control shRNA, and the rDNA expression has clearly increased. Either this is an inappropriate control, or there are pleiotropic effects that the authors have not accounted for. Please explain.
Response: Previous data showed that in NIH3T3 cell about 40-50% of the rDNA promoter region is CpG methylated. Similar values were measured at the rDNA promoter region in shRNA control cells (Fig. 1A). We clarified this point on page 4.

Also, the EM Figure is weak and should be removed. N=1 is quite insufficient to draw the conclusions the authors mention in text about repositioning of FC/CC boundaries. Furthermore, FC and CC are not labeled therefore it is unclear what the authors are referring to.
Response: The reviewer is right, this figure was not properly described. A detailed description (Fig 2A), including the number of analyzed cells, was added on pages 5-6 and in the corresponding figure legend.

Figure 2 shows the interesting histone modifications changes that result from the TIP5 depleted cell lines compared to the control. However, the figure has a number of problems. First, b/input seems to me an inappropriate comparison- we are looking at 200 copies of rDNA locus over input, thus the composite changes reflect all loci. In order to know the per gene change, the figure should be modified to include accounting for copy number.
Response: We agree with the reviewer that it would be important to show ChIP data accounting for copy number. However, the exact number of rRNA genes is not known (probably about 200 copies but the number of rDNA copies in NIH3T3 cells is not known). The same is true for the number of major and minor satellite repeats. Moreover, rDNA ChIP experiments of previous works were always determined using bound/input values. We would like to keep this format to allow direct comparisons with past and future works. Moreover, we think that normalizing the bound values to the input is more precise, taking into account of variations of the amounts of chromatin used for the immunoprecipitations.

In Figure 2D, authors show TIP5 depletion affects replication timing of rDNA, major & minor satellite DNA using semi-quantitative PCR. This is potentially a very interesting finding. However, again copy number must be accounted for, and relative intensity of the shRNA samples is similar to that of control. This is unintuitive, as if there was quantitative shift in replication timing on would expect a huge increase in amount of product obtained at 3-4hrs. Please explain. Authors explain in methods that PCR was normalized to amounts of B-lactamase using qRT-PCR. I'm confused as to why semiquant PCR was utilized for this assay instead of using qRT-PCR (as has been used for the rest of the ms) for the whole assay instead of just to normalize for B-lactamase? Or, if Blactamase is to be used for semi-quantitative PCR, I suggest that Figure 2D include a row where B-lactamase PCR amplified to show normalization. Minor concern: I would also suggest reconfiguring this data in a graphical format of increase vs cell cycle.
Response: We measured the BrdU immunoprecipitated DNA from two independent experiments by qPCR. The data are now showed in Figure 3C and discussed on pages 8-9. With this reconfiguration of the data it becomes clear that the majority of late replicating genes did not shift to early S phase but they are rather lost, a result that is consistent with data shown in Figure 3A,B (loss of silent rDNA, major and minor satellite repeats).

Figure 3- This is a nice set of experiments. The authors demonstrate quite convincingly that there are copy number changes in the shRNA and miRNA of TIP5 specifically targeting the rDNA and minor satellite. However, I think the interpretation is weak- the significant loss of minor satellite should be expected to have enormous consequences for centromeric function, since centromeric H3 assembles specifically on minor satellite in mouse. A simple CENP-A-staining (or CREST) would yield deep insight into potential reduction of centromere staining, which could explain the transformation phenotype seen

in the next figure. At the very least, the discussion should be a little bit bolder in addressing this possibility.
Response: As requested, we performed this experiment. shRNA-control and TIP5 cells were immunostained with anti-CENP-A (FIG. 1D). However, a change in the intensity for centromere staining was difficult to assess because of the different cellular distribution of CENP-A foci in shTIP5 cells. In cells during interphase, the CENP-A foci distribution is very similar to that one determined after DAPI staining: the number diminished, increased in their size and were preferentially localized in the inner part of the cells. Such a clustering indicates structural changes at the centric repeats. If this clustering is determined by the reduction of copy number or by change of chromatin structure will be aim of future work. Importantly, the levels of CENP-A expression between shRNA-control and TIP5 cells were unchanged, as determined by western blot (data not shown).

Figure 4: Authors make a bold leap here, and attempt to connect the TIP5 transformed phenotype to that of a phenotype from oncoprotein-like Ras (previously reported in Tognon et al, 1998). They also stated that depletion of TIP5 and impaired rDNA silencing can strengthen the intimate link between rDNA transcription and cancer presumably by both the loss of rDNA copies, increase in rDNA transcription. However, I am not thoroughly convinced of the connection, especially given the much larger loss of minor satellite DNA that most likely has a stronger effect on cellular transformation than just increased rDNA transcription. This should be discussed to make a better case for the authors' model.
Response: We discussed this important point on pages 12-13 accordingly.

Referee #3 (Remarks to the Author):

This manuscript describes the phenotypic effects of NoRC, a nucleolar chromatin remodelling complex, in terms of rRNA expression and heterochromatin formation. It presents the exciting and important observations that TIP5, a unique subunit of NoRC, may influence genomic stability, cell proliferation and cell transformation. If proven, these will be important discoveries of wide general interest. However, the manuscript requires improvement before it is acceptable for publication.

The abstract claims to have demonstrated a role of "rDNA silencing in controlling cell proliferation". This is not justified. The authors have demonstrated that TIP5 can influence rDNA silencing and can also influence cell proliferation. They have not established that the proliferative effect is caused by the effect on rDNA. There is a correlation, but it remains possible that TIP5 affects proliferation through some alternative target besides rDNA.
Response: We agree with the comment and deleted this sentence from the abstract.

It is reported that shRNA-TIP5 causes a decrease in the number of nucleoli and an increase in their size. Figure 1C is not enough to convince one of this. Some quantitation is required, based on a significant number of cells.
Response: We added data concerning the number of nucleoli and the nucleolus surface per cell (page 5). This analysis was performed on 100 shRNA-control and shRNA-TIP5 cells.

In Figure 1B, there seems to be stronger staining in the nucleoplasm, as well as the nucleolus, for the shRNA-TIP5 cells. That is not expected if TIP5 only regulates rRNA gene expression.
Response: As described (Leung A et al. 2004 JCB, 166 :787-800), by performing a short pulse-chase incorporation of BrUTP we can first observe the BrUTP-labeled rRNA within its site of synthesis. At longer times after removal of BrUTP, the labeled rRNA begins to leave the nucleolus and appears in the nucleoplasm and the cytoplasm. After a 30 minutes chase (as in Fig. 1B), detection of BrUTP-labeled rRNA in the nucleoplasm is expected, especially in shRNA-TIP5 cells in which the level of rRNA synthesis is higher with respect to control cells. However, we cannot completely exclude a possible role of TIP5 in nuclear gene expression.

Figure 1D should be annotated to make explicit the features that are being described.
Response: As suggested by the reviewer a detailed description was added on pages 5-6 and in the corresponding Figure legend.

Figure 2C is crucial, but is not convincing. The signal for the Satellite sequences is only marginally above background. That being the case, it is absolutely not sufficient to have only done this experiment twice. Indeed, error bars should not be used after a single repetition. This experiment needs to be repeated a sufficient number of times to allow assessment of statistical significance.
On the basis of Fig 2C, the authors claim that their "results indicated that TIP5 interacts with a minor fraction of centric-pericentric repeats". A weak interaction with a large fraction is just as compatible with their data as a strong interaction with a small fraction, assuming that it is statistically significant at all.
Response: We repeat the TIP5 ChIP experiments 2 more times (in total now 4 independent experiments). The data were similar to the previous results. We agree with the reviewer that this experiment can be interpreted as either a weak interaction with a large fraction of repeats or a strong association with a small fraction of repeats. We added this conclusion on page 7 (Results) and further discussed on page 12 (Discussion). In addition, to asses the relationship between TIP5 and the centromere, we analyzed whether TIP5 and CENP-A can associate by co-immunoprecipitation analysis. As shown in Fig. 2E, TIP5 and CENP-A interact, offering an explanation of how TIP5 can be associated with centromeric repeats.

A negative control antibody should be included in these ChIP experiments, such as nonimmune or preimmune serum, and signal should be normalised against this. Perhaps the satellites will give a higher background signal with any antibody? Because of their more condensed state, maybe they fragment less readily and immunoprecipitate more strongly in a non-specific manner, whatever antibody used. These possibilities need to be tested and excluded, if the authors wish to convince their readers of significant and specific binding by TIP5.
Response: All our ChIP experiments included always a control sample incubated with pre-immunoserum. We changed Figure 2C including now this control and the values of the two additional performed experiments (in total now 4 experimets).

Figure 2D is unconvincing because the satellite panels have much weaker signals for shRNA-TIP5 than for shRNA-control. Are the cycle numbers/exposure times the same? For each DNA type, all 16 samples should be run on the same gel and presented in a single continuous panel, rather than two panels of eight.
Response: We replaced Figure 2D with a bar-diagram representing qPCR measurements from two independent experiments (Fig. 3A). The samples showed in the previous Fig. 2D were amplified with the same cycle numbers and run on the same gel. The quantitative measurements of this experiments reveals clear that the majority of late replicating genes did not shift to early S phase but they are rather lost, a result that is consistent with data shown in Figure 3A,B (loss of silent rDNA, major and minor satellite repeats).

I found Figures 3E and 3F rather confusing. It would be more appropriate to refer to methylated and unmethylated genes in Fig 3E, rather than silent and active, since methylation is what is being measured, if I understood correctly.
Response: We agree. We replaced the labelling of the figure with CpG methylated and unmethylated.

Please explain why expression of the A variant increases when TIP5 is depleted (Fig 3F) - I thought these were the active genes, which would imply that they are not bound/repressed by TIP5.
Response: The data described indicate that rDNA transcription is upregulated in TIP5 depleted cells (Figure 1B, Supplementary Figures S1C,D), although the number of unmethylated, active genes is the same as in control cells (Figure 4C). These results suggest that the enhancement of rDNA transcription in TIP5-depleted cells does not

depend on the number of active genes. To further investigate this point, we compared the levels of rRNA transcripts synthesized by each class of rDNA variants. As shown in Figure 4D and Supplementary Figure S4D, all the variants, including the rDNA-A genes whose copy number was not affected by depletion of TIP5, transcribed at higher levels. These results strengthen the view that rDNA transcription is preferentially modulated by altering the transcriptional activity of each gene and not by altering the number of genes. Moreover, the data imply that TIP5 and the levels of rDNA silencing influence and modulate the transcription rate of active rRNA genes. However, we cannot exclude the possibility that upregulation of rDNA transcription is a consequence of genome instability that caused the acquisition of aberrant mechanisms of rDNA transcriptional regulation. We discussed this point on page 10 (Results) and on page 13 (Discussion).

In Figure 3F, shRNA-TIP5-1 has a bigger effect on A expression than shRNA-TIP5-2. This could be explained if shRNA-TIP5-1 gives more efficient depletion. However, for T expression shRNA-TIP5-2 is more effective than shRNA-TIP5-1. The differences seem significant, judging by the error bars. Indeed, the differences here are much greater than many of the effects highlighted by the authors elsewhere in the paper.
Response: We repeated the experiment twice again (now total number of independent experiment: 4) and we re-calculated the averages and standard deviations. The differences in transcription levels of A and T genes in shRNA TIP5-1 and TIP5-2 cells are now less marked but still present. The observed differences can be due to internal differences between the two cell clones which however is not affecting the conclusion of this result, i.e. after depletion of TIP5, rDNA transcription levels are enhanced among all the variants, independently by the content of CpG methylated repeats.

Figure 4 is exciting. The authors should gate their FACS analyses and give the % cells in G0/G1, S and G2/M. Means and standard deviations are required. It is not enough simply to state that there are "higher numbers of cells in S phase". In fact, the changes to the G0/G1 and G2/M populations appear more dramatic than S phase changes.
Response: We quantified the FACS analysis (Fig. 5A). We also provided novel data showing higher levels of BrdU incorporation and Cyclin A levels in shRNA-TIP5 and shRNA-control cells (Supplementary Figure 5).

Error bars are required for the proliferation curves with shRNA cells.
Response: Error bars are so small, that they are hidden by the symbols. We added this information in Figure 5 legend.

The images in Figure 4C are not clear enough. They might be improved by being in colour, to show methylene blue staining.
Response: As requested, we changed this Figure (Fig. 5C) in colour.

2nd Editorial Decision 22 January 2010

Your revised manuscript has been reviewed by two of the original referees. Both referees remain positive regarding the study and would like some additional changes to be made prior to publication in the EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
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 initiative, 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 reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

Although the authors have attempted to address concerns, the main novelty in the paper- the connection of cellular transformation to TIP5 depletion remains somewhat tenuous. The obvious changes in centromere reorganization and satellites are more likely to be responsible for the phenotype. If the paper were re-configured to perhaps discuss the transformation claim as a side note, but not make it the main novelty focus, I think the ms would be stronger.
I also think showing the Western blot that CENPA levels are indeed unchanged in Tip5 depletion is important.
I remain puzzled by the argument that not controlling for copy number in evaluating shRNA experiments is acceptable- clearly copy number matters, because doesn't input reflect mostly single copy genes?
That said, the authors have addressed some of my previous concerns about the data in the EM Figure, and the manuscript does clearly show TIP5 has effects on heterochromatin.

Referee #3 (Remarks to the Author):

The revised version of the manuscript is greatly improved and has largely addressed the concerns that I raised previously. I have just a few suggestions with regard to the text.

Title: it is not clear what is meant by "...mediates heterochromatin...".

Abstract, final sentence. A role of "...rDNA silencing in protecting genome stability..." has not been "demonstrated", as is claimed. It is suggested by the demonstrated role of TIP5, but a causal link has not been established between rDNA silencing and genome stability; it remains possible that these are two separate functions of TIP5.

Page 10, paragraph 1. I don't think it is true to claim that in Figure 4C "...amounts of unmethylated rDNA-A, -T and total genes remain unchanged..." It would be fairer to say that these amounts remain relatively unchanged.

Figure 5A. The percentages given for G2/M cells seem much too low, whilst the S phase fraction is over-represented. I don't think the FACS has been gated properly. Furthermore, the percentage of G2/M cells is reported as decreasing in miRNA-TIP5 cells, in contrast to what is stated in the text and to the appearance of the FACS trace, which shows a very large increase in the G2/M peak. There is clearly an error here that needs to be corrected.

Page 11, final sentence of discussion. The authors claim that their "...results indicate that..." "...impairment of rDNA silencing can contribute to cellular transformation..." As discussed above, as well as in my first review, demonstrating a role for TIP5 does not indicate a role for rDNA silencing - it may suggest one, but it does not exclude that TIP5 has more than one unrelated function. Large numbers of proteins are known to have more than one function, so it is blinkered of the authors to ignore the possibility that this is also the case for TIP5.

2nd Revision - authors' response 25 January 2010

Referee #2 (Remarks to the Author):

Although the authors have attempted to address concerns, the main novelty in the paperthe connection of cellular transformation to TIP5 depletion remains somewhat tenuous.

The obvious changes in centromere reorganization and satellites are more likely to be responsible for the phenotype
Response: To put much more emphasis that the centromere reorganization and satellites are more likely to be responsible for the transformed phenotype, we performed the following changes:
Last sentence of the abstract: These findings demonstrate a role of TIP5 in protecting genome stability and suggest that it can play a role in the cellular transformation process".
Beginning of the second-last paragraph of the discussion (page 12) "Cells in the absence of TIP5 proliferated beyond confluence and displayed a transformed phenotype, a likely result of the genome instability that we detected in TIP5-depleted cells. Loss of genome stability is known to be a key molecular step in cancer formation,.....".
End of the of the second-last paragraph of the discussion (page 13): "However, we cannot exclude the possibility that upregulation of rDNA transcription is a consequence of genome instability that caused the acquisition of aberrant mechanisms of rDNA transcriptional regulation, thus representing an advantage for the elevated protein synthesis necessary for high proliferative rates.

If the paper were re-configured to perhaps discuss the transformation claim as a side note, but not make it the main novelty focus, I think the ms would be stronger.
Response: We think that we configured the whole paper to make a central pont that TIP5 mediates heterochromatin formation of centromers and silent rDNA repeats and that it is involved in genome stability (see the title, the abstract, 5 Figures out of 6). The results concerning the transformed phenotype of cells depleted of TIP5 are important and they represent the final result of this study, i.e. the necessity of TIP5 to protect genome stability otherwise cells can undergo transformation. While the main novelty of our results (TIP5-mediated heterochromatin formation of centromeric repeats and genome instability) represents the core of our discussion, the discussion of cellular transformation was limited only at the beginning and at the end of the second-last paragraph of the discussion.

I also think showing the Western blot that CENPA levels are indeed unchanged in Tip5 depletion is important.
Response: As already mentioned in the previous rebuttal letter, this experiment was already performed, but not included in the revised manuscript. We added now the western blot analysis showing similar levels of CENP-A in both shRNA-control and shRNA-TIP5 cells (page 5 and Supplementary Figure S2A).

I remain puzzled by the argument that not controlling for copy number in evaluating shRNA experiments is acceptable- clearly copy number matters, because doesn't input reflect mostly single copy genes?
Response: Bound/input value used in the ChIP experiment of Figure 2B takes into account the copy number. For example, let's take two cell lines, A with 200 genes and B with 100 genes. Amplification of the inputs (usually in our experiments 5 ng of chromatin) will give a value for A cells that is double of the value of the input of B cells. Thus, if in a ChIP experiment the bound value is 100 for both cells A and B, normalization with the respective inputs will result in a bound/input value for A cells that is double of the bound/input value of B cells.

As said in the first rebuttal letter, we would like to keep this format to allow direct comparisons with past and future works. Moreover, we think that normalizing the bound values to the input is more precise than to normalize against a yet undefined absolute

number of genes (the absolute number of rDNA and satellite repeats is not known), taking also into account the variations of the amounts of chromatin used for the immunoprecipitations.

That said, the authors have addressed some of my previous concerns about the data in the EM Figure, and the manuscript does clearly show TIP5 has effects on heterochromatin.

Referee #3 (Remarks to the Author):

The revised version of the manuscript is greatly improved and has largely addressed the concerns that I raised previously. I have just a few suggestions with regard to the text.

Title: it is not clear what is meant by "...mediates heterochromatin...".

Response: We changed the title: "The NoRC complex mediates heterochromatin formation and stability of silent rRNA genes and centromeric repeats".

Abstract, final sentence. A role of "...rDNA silencing in protecting genome stability..." has not been "demonstrated", as is claimed. It is suggested by the demonstrated role of TIP5, but a causal link has not been established between rDNA silencing and genome stability; it remains possible that these are two separate functions of TIP5.
Response: We agree with the comment and deleted this sentence from the abstract (page 2).

Page 10, paragraph 1. I don't think it is true to claim that in Figure 4C "...amounts of unmethylated rDNA-A, -T and total genes remain unchanged..." It would be fairer to say that these amounts remain relatively unchanged.
Response: We changed this sentence accordingly (page 10).

Figure 5A. The percentages given for G2/M cells seem much too low, whilst the S phase fraction is over-represented. I don't think the FACS has been gated properly. Furthermore, the percentage of G2/M cells is reported as decreasing in miRNA-TIP5 cells, in contrast to what is stated in the text and to the appearance of the FACS trace, which shows a very large increase in the G2/M peak. There is clearly an error here that needs to be corrected.
Response: Clearly, there was a mistake. Cells were now properly gated and correct values were included in Figure 5. This correction does not affect the results shown in Figure 5A,B.

Page 11, final sentence of discussion. The authors claim that their "...results indicate that..." "...impairment of rDNA silencing can contribute to cellular transformation..." As discussed above, as well as in my first review, demonstrating a role for TIP5 does not indicate a role for rDNA silencing - it may suggest one, but it does not exclude that TIP5 has more than one unrelated function. Large numbers of proteins are known to have more than one function, so it is blinkered of the authors to ignore the possibility that this is also the case for TIP5.
Response: We agree with the comment and deleted this sentence (final sentence of Results, page 10).

