EMBO reports

Manuscript EMBOR-2011-34942

# Nucleolar protein Spindlin1 recognizes H3K4 methylation and stimulates the expression of rRNA genes

Weixiang Wang, Zhi Chen, Zhuo Mao, Huihui Zhang, Xiaojun Ding, She Chen, Xiaodong Zhang, Ruiming Xu and Bing Zhu

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**Review timeline:**

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| Submission date: | 30 March 2011 |
| Editorial Decision: | 15 April 2011 |
| Revision received: | 02 August 2011 |
| Editorial Decision: | 17 August 2011 |
| Revision received: | 24 August 2011 |
| Editorial Decision: | 24 August 2011 |
| Accepted: | 24 August 2011 |

## 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 15 April 2011

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, while the referees acknowledge that the findings are potentially interesting and novel, they also point out that the current experimental evidence is insufficient to support the conclusion that Spindlin1 facilitates rRNA gene transcription.

To strengthen this conclusion, referee 3 points out that colocalization of Spindlin1 with UBF or RNA polymerase I (for example by co-ChIP) needs to be demonstrated and referee 2 remarks that quantitative (real-time) PCR and proper statistics are required to demonstrate that the minor effects on rRNA expression are significant. Referee 1 adds that Spindlin1 and H3K4me3 levels should be determined at active ribosomal genes and that effects of tetracycline on rRNA expression need to be excluded. Both referees 1 and 2 further indicate that the hypothesis that Spindlin1 localization to rDNA depends on H3K4me3 (versus dsDNA or RNA) could be significantly strengthened by the use of a Spindlin1 mutant that does not bind H3K4me3. All referees also pinpoint a number of missing controls and statistic evaluations that would need to be included. However, nuclear run-on experiments and insight into how Spindlin1 distinguishes between H3K4me3 on ribosomal versus other genes would not be required for publication of the study in EMBO reports.

Given that the current data do not sufficiently support the main conclusion of the manuscript it is

 

clear that, as it stands, 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 reviewers 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 look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

RERE REE REPORTS:

Referee #1:

In the manuscript "Nucleolar protein Spindlin-1 recognizes H3K4me3 and facilitates rRNA gene transcription" Wang and colleagues identify a new facet of the meiotic spindle-associated protein Spindlin-1 in mouse embryonic fibroblast. The authors report that Spindlin-1 recognizes H3K4me3 and that Spindlin-1 facilitates the transcription of rRNA genes. These findings are definitely of interest, the data shown are generally convincing and the experiments have been well performed. However, the Spindlin family members are highly homologous and the authors need to include a number of additional controls to convincingly demonstrate that the effects they observe are mediated by Spindlin-1 and not another family member(s) prior to publication.

Specific comments:
Figure 1: siRNA against Spindlin-1 should be used to show specificity of the antibodies used. In addition, the specificity of the siRNAs must be tested by determining whether they affect the levels of other Spindlin family members (this point is also relevant to Figure 4c).

Figure 3: Other loci (i.e. non-rDNA regions) should be included in the ChIP analyses. Regions known to be lacking H3K4me3 should be used as controls. Moreover, to further assess the specificity of the Spinlin-1 antibody, ChIP experiment (using an anti-GFP antibody) should be performed with cells transfected with the GFP-Spindlin-1 construct (as shown in Figure 1). Ideally, ChIP experiments with HeLa cells depleted for Spindlin-1 should also be included.

Figure 4: The data presented in Figure 4 could be the result of a general effect of tetracycline on the cells. At the very least therefore, the authors need to show how tetracycline affects rRNA levels in control stable cells (e.g. stable cells containing just the empty vector or a control Flag-construct). Furthermore, the experiments in this figure should really be coupled to ChIP experiments so that the level of both Spindlin-1 and H3K4me3 can be assessed at the ribosomal genes upon induction.

General comment:
This work would have significantly benefited from the inclusion of a Spindlin-1 mutant that no longer binds to H3K4me3.

As another general remark, the authors might like to consider attempting to purify potential Spindlin-1 associated proteins at a more physiologically relevant salt concentration. Their attempt (documented in Figure S2) was performed at very high salt levels which would be destabilizing to most complexes.

Referee #2:

 

In this manuscript, the authors demonstrate that Spindlin1 co-localizes with the nucleolar marker B23 in MEFs. Additionally, they show evidence that Spindlin1 pulls down with H3Kc4me3-containing mono-nucleosomes and has affinity for H3K4me3-containing peptides, but not peptides with H3K9me3 or H3K27me3. They ChIP Spindlin1 and H3K4me3 to a human rDNA locus in Hela cells. Finally, they show that tet-inducible overexpression of Spindlin1 results in elevated pre-rRNA expression, while Spindlin1 knockdown has a negative effect on pre-rRNA expression. While there are some novel findings presented in this work, the manuscript would benefit greatly by addressing the following issues before it is suitable for publication:

1) Fig 2A is un-interpretable without showing nucleosome input controls. It is recommended to probe for the biotin tag with a streptavidin-conjugated antibody. Additionally, it would be helpful to add some data confirming the proper assembly of mononucleosomes and validation of the MLA reactions.

2) In Fig 2A, Spindlin1 also binds H3Kc4me2, and this observation is glossed over in the text. It is therefore important to do ITC analysis on an H3K4me2-containing peptide to determine the extent of this interaction.

3) Of note, binding of Spindlin1 to H3K4me3-modified nucleosomes was previously reported (Bartke T et al 2010, Cell). The authors do make reference to this finding in the text, but incorrectly state the substrate for this reaction as "nucleosomes containing H3Kc4me3."

4) In Fig 2B, the recorded Kd of 6 ...M is inconsistent with the Kd reported in the text (both the results section and the figure legend). Additionally, no error or N value is reported with the Kd measurement, and it is unclear how many times this experiment was repeated.

5) It is unclear how the ChIP data in Fig 3 was normalized or how the error bars were derived. This experiment is highly corollary and it is not convincing that localization of Spindlin1 to this rDNA loci is dependent on binding to H3K4me3. Moreover, a recent study (Zhao Q et al 2007, JBC) uses gel-shift assays to demonstrate Spindlin1 binding directly to dsDNA in vitro. This point of contention needs to be addressed. One way to address whether the binding of Spindlin1 is dependent on recognition of H3K4me3 would be to generate a Spindlin1 mutant insensitive to K4me3 and test this in ChIP assays. Candidate residues for mutagenesis could be derived from the crystal structure and potentially by sequence comparison to other tudor-domains known to bind histones. If Spindlin1 is a K4me3 reader, it should have a conserved aromatic cage that coordinates the methyl-lysine.

6) The statement in the text stating "the pattern of binding at these loci was similar to the profile of H3K4me3" is misleading. Additionally, the H3K4me3 antibody used in this experiment is not defined beyond the company in which it was purchased (Millipore). It is therefore unclear whether this antibody has specificity for H3K4me3, or might also recognize lower K4 methylation states.

7) In Fig 4, it is unclear how many times this experiment was repeated and what the error bars represent. Since the changes in rRNA expression are minimal, statistics should be applied to this data. Additionally, more quantitative measurements might be attained by real-time PCR.

8) The text describing an attempt to identify binding partners of Spindlin1 is not appropriate for a discussion section. This is an incomplete piece of data that does not add to the current story. The discussion section would greatly benefit from a thoughtful commentary on the implications of the findings presented in the paper.

Referee #3:

In recent years a wide variety of chromatin/histone binding proteins have been implicated in the regulation of ribosomal gene transcription. In this report Spindlin 1 is implicated. Spindlin 1 initially described as being associated with the meiotic spindle has been more recently described as a nuclear protein with oncogenic potential. An indication that it may bind chromatin came from structural work that identified tandem tudor domains. Tudor domains are known methylated histone binding proteins. Possibly of relevance here, RNA binding activity has also been attributed to these domains.

 

The initial experiments in this report indicate endogenous human Spindlin 1 is a nucleolar protein. This data is backed up my nucleolar localisation of GFP tagged Spindlin 1. Cause for concern is that localisation may be due to RNA binding rather than chromatin binding. The punctate patterning within nucleoli is suggestive of localisation the FC/DFC regions of nucleoli, the loacation of active rDNA repeats. A demonstration of precise colocalisation of Spindlin 1 with UBF or RNA polymerase I within nucleoli would further strengthen the claim of chromatin association.
The demonstration that Spindlin 1 can bind to H3K4me3 in the context of nucleosomes is convincing but raises the issue specificity. How does Spindlin 1 distinguish H3K4me3 on ribosomal genes versus the rest of the genome? No mechanism is proposed.
The Chip data presented in Figure 3 is of low quality and is lacking controls such as a positive control for binding to rDNA (UBF or RNA polymerase I) a negative control for rDNA, and a negative control for Spindlin 1 (other repeated elements in the genome).
Also problematical is the data presented in Figure 4. The RT PCR assay is a measure of steady state RNA levels NOT transcription. The location of the probe is of particular concern. A modest inhibition of pre-rRNA processing could easily lead to an increase in the signal and be misinterpreted as an upregulation of transcription. Other assays such as nuclear run-on or in vitro transcription with chromatinised templates would be required to confirm a role for Spindlin 1 in ribosomal gene transcription.
Finally, as Spindlin 1 is oncogenic the possibity exists that any affects on ribosomal gene transcription are indirect.
In conclusion, this work convincingly demonstrates that Spindlin 1 can bind to H3K4me3, and provides evidence tha it may be a nucleolar protein. However the main claim that Spindlin 1 'faciltates rRNA gene transcription' is not adequately supported by the data.

1st Revision - authors' response 02 August 2011

Point-by-point response to reviewers' comments

Referee #1:

In the manuscript "Nucleolar protein Spindlin-1 recognizes H3K4me3 and facilitates rRNA gene transcription" Wang and colleagues identify a new facet of the meiotic spindle-associated protein Spindlin-1 in mouse embryonic fibroblast. The authors report that Spindlin-1 recognizes H3K4me3 and that Spindlin-1 facilitates the transcription of rRNA genes. These findings are definitely of interest, the data shown are generally convincing and the experiments have been well performed. However, the Spindlin family members are highly homologous and the authors need to include a number of additional controls to convincingly demonstrate that the effects they observe are mediated by Spindlin-1 and not another family member(s) prior to publication.

Specific comments:
Figure 1: siRNA against Spindlin-1 should be used to show specificity of the antibodies used. In addition, the specificity of the siRNAs must be tested by determining whether they affect the levels of other Spindlin family members
The specificity issue for Figure 1A has been controlled by Figure 1B, where we used GFP-tagged Spindlin-1, which displayed similar nucleolar localization. Spindlin family has four proteins, 1, 2A, 2B and 3. Spindlin2A and 2B differ for only two nucleotides within the coding region (The alignment is shown in new Fig. S5). Nevertheless, siRNA specific for Spindlin1 can be designed. In fact, siRNA 1 and 3 used in this study can only target Spindlin1 according to their sequence. Indeed, siRNA3 efficiently knocked down Spindlin1, but not Spindlin2A/2B (Fig. S4B). This experiment also controls the specificity of the antibodies against Spindlin1, because although Spindlin2A/2B levels were unchanged (Fig. S4B), Spindlin1 protein level is significantly knocked down (Fig. 5C).

Figure 3: Other loci (i.e. non-rDNA regions) should be included in the ChIP analyses. Regions known to be lacking H3K4me3 should be used as controls. Moreover, to further assess the specificity of the Spinlin-1 antibody, ChIP experiment (using an anti-GFP antibody) should be performed with cells transfected with the GFP-Spindlin-1 construct (as shown in Figure 1). Ideally,

 

ChIP experiments with HeLa cells depleted for Spindlin-1 should also be included.
We have included two other regions that are inactive (PRM3, specifically expressed during spermiogenesis and MyoD, specifically expressed during myogenesis) and low at H3K4me3 as controls into Figure 3. In addition, ChIP experiments were also performed with Flag antibodies in the stable cells (Fig. 4C and Fig. S4A).

Figure 4: The data presented in Figure 4 could be the result of a general effect of tetracycline on the cells. At the very least therefore, the authors need to show how tetracycline affects rRNA levels in control stable cells (e.g. stable cells containing just the empty vector or a control Flag-construct). Furthermore, the experiments in this figure should really be coupled to ChIP experiments so that the level of both Spindlin-1 and H3K4me3 can be assessed at the ribosomal genes upon induction.
A mock stable cell line transfected with the empty vector and two stable cell lines expressing Spindlin1 mutants have been included as controls and the ChIP experiments were also performed. (Fig. 5B, Fig. 4C and Fig. S4A)

General comment:
This work would have significantly benefited from the inclusion of a Spindlin-1 mutant that no longer binds to H3K4me3.
Spindlin1 mutants with impaired H3K4me3 binding activity have been identified and the related data are included in the new Figure 2 and Figure 5.

As another general remark, the authors might like to consider attempting to purify potential Spindlin-1 associated proteins at a more physiologically relevant salt concentration. Their attempt (documented in Figure S2) was performed at very high salt levels which would be destabilizing to most complexes.
0.5 M KCl we used can disrupt weak transient interactions between proteins, but not the stable protein complexes. We also attempted to perform the purification with reduced stringency, which led to severely elevated background contaminations.

Referee #2:

In this manuscript, the authors demonstrate that Spindlin1 co-localizes with the nucleolar marker B23 in MEFs. Additionally, they show evidence that Spindlin1 pulls down with H3Kc4me3-containing mono-nucleosomes and has affinity for H3K4me3-containing peptides, but not peptides with H3K9me3 or H3K27me3. They ChIP Spindlin1 and H3K4me3 to a human rDNA locus in Hela cells. Finally, they show that tet-inducible overexpression of Spindlin1 results in elevated pre-rRNA expression, while Spindlin1 knockdown has a negative effect on pre-rRNA expression. While there are some novel findings presented in this work, the manuscript would benefit greatly by addressing the following issues before it is suitable for publication:

1) Fig 2A is un-interpretable without showing nucleosome input controls. It is recommended to probe for the biotin tag with a streptavidin-conjugated antibody.
(We replaced the gels shown in the Fig. 2A with their original full gels, which included the histones that migrated at the low molecular weight range to show that equal amounts of nucleosomal histones were bound to the beads. These data are included as the new Fig. 2A and Fig. S3A)
Additionally, it would be helpful to add some data confirming the proper assembly of mononucleosomes and validation of the MLA reactions.
(Agarose gel showing the shift of mononucleosomes is included in the new Fig. S2A; validation of the MLA reactions were shown in the new Fig. S2B by probing products with antibodies specific to the distinct methylation states)

2) In Fig 2A, Spindlin1 also binds H3Kc4me2, and this observation is glossed over in the text. It is therefore important to do ITC analysis on an H3K4me2-containing peptide to determine the extent of this interaction.
The ITC results of H3K4me2, H3K4me1 and H3K4me0 peptides are included in Fig. 2B.

3) Of note, binding of Spindlin1 to H3K4me3-modified nucleosomes was previously reported (Bartke T et al 2010, Cell). The authors do make reference to this finding in the text, but incorrectly state the substrate for this reaction as "nucleosomes containing H3Kc4me3."

 

We thank the reviewer's careful reading and the typo has been corrected.

4) In Fig 2B, the recorded Kd of 6 ...M is inconsistent with the Kd reported in the text (both the results section and the figure legend). Additionally, no error or N value is reported with the Kd measurement, and it is unclear how many times this experiment was repeated.
We apologize for the mistake, the Kd written in the text was incorrect and we have corrected the mistakes, the errors have been included in all figures and they were derived from three independent repeats. These information has been added into the Figure legends.

5) It is unclear how the ChIP data in Fig 3 was normalized or how the error bars were derived.
(The previous Fig. 3 has been replaced by new Fig. 3 because we added additional controls as reviewers requested. The ChIP data in new Fig. 3 are not normalized, which is a direct reflection of the percentage of input as we labeled on the figure. We have added a sentence into the Figure legend of Figure 2B, where we state that "Errors in these experiments and all subsequent experiments with error bars represent the standard deviation derived from three independent experiment repeats")
This experiment is highly corollary and it is not convincing that localization of Spindlin1 to this rDNA loci is dependent on binding to H3K4me3. Moreover, a recent study (Zhao Q et al 2007, JBC) uses gel-shift assays to demonstrate Spindlin1 binding directly to dsDNA in vitro. This point of contention needs to be addressed. One way to address whether the binding of Spindlin1 is dependent on recognition of H3K4me3 would be to generate a Spindlin1 mutant insensitive to K4me3 and test this in ChIP assays. Candidate residues for mutagenesis could be derived from the crystal structure and potentially by sequence comparison to other tudor-domains known to bind histones. If Spindlin1 is a K4me3 reader, it should have a conserved aromatic cage that coordinates the methyl-lysine. Spindlin1 mutants with impaired H3K4me3 binding activity have been identified and the related data are included in the Fig. 2, Fig. 5 and Fig. S4A. The rDNA localization of these mutants were slight reduced but not abolished (Fig. S4A). We explained in the text as such "First of all, the binding affinity between effector proteins and histone modifications are typically at the μM range, which suggests that the role of such interactions is to stabilize the complex, rather than to serve as the initial recruitment. In addition, the experiments shown in supplementary Fig. S4A were performed in the presence of endogenous Spindlin1, it is unclear whether Spindlin1 can form multimeric complex and such interaction might help maintaining the partial chromatin localization of the Flag-Spinlin1 mutants." Finally, we performed co-staining and sequential-ChIP to show Spindlin1 is localized to active rDNA repeats (Fig. 4)

6) The statement in the text stating "the pattern of binding at these loci was similar to the profile of H3K4me3" is misleading.
(We have deleted this sentence and performed co-staining and sequential-ChIP to show that Splindlin1 is localized to active rDNA repeats in Fig. 4)
Additionally, the H3K4me3 antibody used in this experiment is not defined beyond the company in which it was purchased (Millipore). It is therefore unclear whether this antibody has specificity for H3K4me3, or might also recognize lower K4 methylation states.
(The catalogue number of this antibody has been included into the methods section and we have shown the specificity of this antibody in Fig. S2B)

7) In Fig 4, it is unclear how many times this experiment was repeated and what the error bars represent. Since the changes in rRNA expression are minimal, statistics should be applied to this data. Additionally, more quantitative measurements might be attained by real-time PCR.
We have added a sentence into the Figure legend of Figure 2B, where we state that "Errors in these experiments and all subsequent experiments with error bars represent the standard deviation derived from three independent experiment repeats". Statistics have been applied to all rRNA expression measurements in Fig. 5.

8) The text describing an attempt to identify binding partners of Spindlin1 is not appropriate for a discussion section. This is an incomplete piece of data that does not add to the current story. The discussion section would greatly benefit from a thoughtful commentary on the implications of the findings presented in the paper.
According to the instruction of the editor in the decision letter, we have combined the Discussion part and Results part as "Results and Discussion". We kept the Spindlin1 purification as part of the results. Protein interactions can generally be classified as stable complex interaction and weak, transient interaction. Our attempt is an indication that Spindlin1 does not form a stable protein

 

complex with other proteins, which we think is valuable information for scientists who are interested in that direction.

Referee #3:

In recent years a wide variety of chromatin/histone binding proteins have been implicated in the regulation of ribosomal gene transcription. In this report Spindlin 1 is implicated. Spindlin 1 initially described as being associated with the meiotic spindle has been more recently described as a nuclear protein with oncogenic potential. An indication that it may bind chromatin came from structural work that identified tandem tudor domains. Tudor domains are known methylated histone binding proteins. Possibly of relevance here, RNA binding activity has also been attributed to these domains. The initial experiments in this report indicate endogenous human Spindlin 1 is a nucleolar protein. This data is backed up my nucleolar localisation of GFP tagged Spindlin 1. Cause for concern is that localisation may be due to RNA binding rather than chromatin binding. The punctate patterning within nucleoli is suggestive of localisation the FC/DFC regions of nucleoli, the loacation of active rDNA repeats. A demonstration of precise colocalisation of Spindlin 1 with UBF or RNA polymerase I within nucleoli would further strengthen the claim of chromatin association.
(Co-staining experiments were performed and Spindlin1 largely co-localize with Pol I subunit RPA194, Fig. 4A. In addition, we performed sequential-ChIP to show that Spindlin1 occupies active rDNA repeats.)
The demonstration that Spindlin 1 can bind to H3K4me3 in the context of nucleosomes is convincing but raises the issue specificity. How does Spindlin 1 distinguish H3K4me3 on ribosomal genes versus the rest of the genome? No mechanism is proposed.
(We think the specific nucleolus localization of Spindlin1 itself can ensure some part of the specificity. We did not perform additional experiments during revision in this direction, because we agree with the editor that this part is beyond the scope of the current manuscript. But we are quite interested in understanding more in this direction in our future work.)

The Chip data presented in Figure 3 is of low quality and is lacking controls such as a positive control for binding to rDNA (UBF or RNA polymerase I) a negative control for rDNA, and a negative control for Spindlin 1 (other repeated elements in the genome).
(Fig. 3 has been re-performed with all the above controls included.)

Also problematical is the data presented in Figure 4. The RT PCR assay is a measure of steady state RNA levels NOT transcription. The location of the probe is of particular concern. A modest inhibition of pre-rRNA processing could easily lead to an increase in the signal and be misinterpreted as an upregulation of transcription. Other assays such as nuclear run-on or in vitro transcription with chromatinised templates would be required to confirm a role for Spindlin 1 in ribosomal gene transcription.
(We did not perform the run-on experiments, because the editor specifically pointed out that it is not required in her decision letter)

Finally, as Spindlin 1 is oncogenic the possibity exists that any affects on ribosomal gene transcription are indirect.
We have added experiments showing that Spindlin1 point mutations lacking H3K4 methylation binding activity failed to stimulate the expression of pre-rRNA (Fig. 5B).

In conclusion, this work convincingly demonstrates that Spindlin 1 can bind to H3K4me3, and provides evidence tha it may be a nucleolar protein. However the main claim that Spindlin 1 'faciltates rRNA gene transcription' is not adequately supported by the data.

2nd Editorial Decision 17 August 2011

Thank you again for the submission of your manuscript to EMBO. We have now received the full set of reports from the referees. As you can see below, both reviewers are quite positive but some

 

concerns need to be addressed prior to acceptance.

While referee #1 is very positive towards you manuscript, referee #2 still has two concerns that need you attention. First, s/he considers that the Kd presented might be inaccurate and N values should be provided in order to properly assess the experimental information. Second, although we believe that further experiments in this direction are outside the scope of the manuscript, it should be further discussed that mechanisms other than binding to H3K4me most likely partially account for Spindlin1 residency at rDNA loci. In this direction, work by Zhao et al. (2007, J Biol. Chem.) demonstrating binding of Spindlin1 to DNA should be acknowledged and discussed.

Browsing through the manuscript myself, I have noticed that statistical analysis is not properly described. It must be described either in the Materials and Methods section for all the figures, or in the legend of every figure to which they apply. Please, state the method used to calculate statistical significance as well.

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

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

The revised manuscript is much improved. The authors have adequately addressed all of my concerns.

Referee #2:

Revised Manuscript Review: Wang W et al. "Nucleolar protein Spindlin1 recognizes H3K4me3 and facilitates rRNA gene transcription"

1) Figure 2 is significantly improved with the addition of proper controls as well as the ITC data for the aromatic cage mutants. The data strongly supports that Spindlin1 binds K4 methylation. However, the authors did not provide N values as requested. It is clear from the ITC traces that their N values are quite low (~0.6). It is assumed that the interaction they are observing has a 1:1 molar ratio and a 1-site binding model was used to fit the data. Therefore, their apparent Kd measurements are inaccurately calculated.

2) Figure 3 is significantly improved and Figure 4 is a good addition to support residence of Spindlin1 at this rDNA locus

3) I still have concerns regarding Figure 5. Multiple reviewers have pointed out that RT-PCR is not a quantitative measurement, and this point was not addressed. This is a problem, as the data for the cage mutants in Figure 5B suggesting diminished gene expression is very important to this story. Moreover, the ChIP data for Spindlin1 cage mutants in Supplemental FigS4A shows that residence of Spindlin1 at this locus is independent of binding to H3K4 methylation. Some hand-waving was done in the text to explain this discrepancy, but the way this story is packaged suggests that the stimulation of rRNA gene expression by Spindlin1 is dependent on the association with H3K4-methylated chromatin. Based on the mutant data, this is not the complete picture, and exploring other possible mechanisms for Spindlin1 association at this locus (i.e., DNA or RNA binding) may provide more clarity to this otherwise interesting study.

 

2nd Revision - authors' response 24 August 2011

Response to editor and reviewers' comments

Editor's comments

While referee #1 is very positive towards you manuscript, referee #2 still has two concerns that need you attention. First, s/he considers that the Kd presented might be inaccurate and N values should be provided in order to properly assess the experimental information. Second, although we believe that further experiments in this direction are outside the scope of the manuscript, it should be further discussed that mechanisms other than binding to H3K4me most likely partially account for Spindlin1 residency at rDNA loci. In this direction, work by Zhao et al. (2007, J Biol. Chem.) demonstrating binding of Spindlin1 to DNA should be acknowledged and discussed.

Browsing through the manuscript myself, I have noticed that statistical analysis is not properly described. It must be described either in the Materials and Methods section for all the figures, or in the legend of every figure to which they apply. Please, state the method used to calculate statistical significance as well.
In "material and methods", we have added a sentence in the section of ChIP and RT-PCR: "ChIP-PCR and RT-PCR experiments were performed in triplicates to calculate the standard deviation. T test was performed for all the RT-PCR experiments measuring the levels of pre-rRNA to calculate corresponding P values." We also described them in the figure legends of every relevant figures.

Referee #1:

The revised manuscript is much improved. The authors have adequately addressed all of my concerns.

Referee #2:

Revised Manuscript Review: Wang W et al. "Nucleolar protein Spindlin1 recognizes H3K4me3 and facilitates rRNA gene transcription"

1) Figure 2 is significantly improved with the addition of proper controls as well as the ITC data for the aromatic cage mutants. The data strongly supports that Spindlin1 binds K4 methylation. However, the authors did not provide N values as requested. It is clear from the ITC traces that their N values are quite low (~0.6). It is assumed that the interaction they are observing has a 1:1 molar ratio and a 1-site binding model was used to fit the data. Therefore, their apparent Kd measurements are inaccurately calculated.

The N values of our previous ITC results were around 0.6 as the reviewer noticed. For 1-site binding, the ideal N values should be between 0.8-1.2. Therefore, our previous Kd measurements were suboptimal as the reviewer criticized. We have re-performed the ITC experiments and obtained better N values and recalculated the Kd values. Indeed, the new ITC data reflects better the fact that Spindlin1 preferentially binds H3K4me3 than H3K4me2, in consistent with our pull-down results. We thank the reviewer's critical reading, which helps our manuscript in reporting the Kd values more accurately.

2) Figure 3 is significantly improved and Figure 4 is a good addition to support residence of Spindlin1 at this rDNA locus

3) I still have concerns regarding Figure 5. Multiple reviewers have pointed out that RT-PCR is not a quantitative measurement, and this point was not addressed. This is a problem, as the data for the cage mutants in Figure 5B suggesting diminished gene expression is very important to this story. Moreover, the ChIP data for Spindlin1 cage mutants in Supplemental FigS4A shows that residence of Spindlin1 at this locus is independent of binding to H3K4 methylation. Some hand-waving was done in the text to explain this discrepancy, but the way this story is packaged suggests that the stimulation of rRNA gene expression by Spindlin1 is dependent on the association with H3K4-methylated chromatin. Based on the mutant data, this is not the complete picture, and exploring

 

other possible mechanisms for Spindlin1 association at this locus (i.e., DNA or RNA binding) may provide more clarity to this otherwise interesting study. According to editors' suggestion, we have added a sentence after the mutant ChIP data: "In addition, other properties of Spindlin1, such as its interaction with DNA (Zhao *et al*, 2007), it's interaction with the endogenous Spindlin1 due to its dimeric structure (Zhao *et al*, 2007) and other potential protein partners may also contribute to the partial chromatin localization of these mutants."

3rd Editorial Decision 24 August 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

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

Yours sincerely,

Editor
EMBO Reports

 