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Fission yeast Cactin restricts telomere transcription and elongation by controlling Rap1 levels

Luca E. Lorenzi, Amadou Bah, Harry Wischnewski, Vadim Shchepachev, Charlotte Soneson, Marco Santagostino & Claus M. Azzalin

Corresponding author: Claus Azzalin, ETHZ

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Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below

As you will see from the three reports, all referees express interest in the findings reported in your manuscript; however, they also all point out the need for extensive revision and restructuring of the study before they can support publication in The EMBO Journal.

I understand that addressing the concerns raised will require an extensive effort on your side and I would understand it if you would rather wish to seek rapid publication in a less demanding venue. However, if you would be willing to undertake the effort to revise the manuscript along the lines laid out by the referees we would be happy to consider a revised version. In light of the need for extensive revision we can also offer to extend the deadline for the revised manuscript.

I would particularly ask you to focus your efforts on the following points:

-> Restructure the manuscript to present a role for Cay1 in telomere biology as a clear, coherent story; however, we would encourage you to keep the data on transposon activation in cay1 deletion strains in the manuscript.

-> Please provide clarification/further experimentation to address the RNA quantification issues raised by both refs #1 and #3

-> Please also expand on the mechanistic basis for Rap1 regulation by Cay1 (ref #2 and #3)

Given the referees' positive recommendations, I would thus like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS:

Referee #1:

Lorenzi et al
Fission yeast Cactin coordinates silencing of telomeres and retrotransposable elements

The aim of the study by Lorenzi et al. was to identify new regulators of telomere transcription in fission yeast. Through screening of the S. pombe gene deletion collection, the authors report on the identification of fission yeast Cactin (Cay1) as a new major regulator of telomere biology in S. pombe. Deletion of cay1+ results in telomere over-elongation associated with increased H3K9 acetylation, a phenotype reminiscent to rap1+ deletion. Accordingly, the authors show that the impact of cay1+ deletion on telomere length results from the degradation of Rap1 in cay1 cells. Although telomere defect phenotype of cay1 cells was complemented by Rap1 overexpression, not all growth defects were reverted, suggesting that Cay1 plays additional important roles in the cell. One possible important role of Cay1 outside telomeres may stem from its ability to silence retrotransposons as authors further identified Cay1 as a regulator of various fission yeast noncoding RNA species, including Tf2 retrotransposons.

A huge amount of experiments has been carried out in this study that, however, would require additional analysis/interpretation and data organization prior to publication. I also feel that the two parts of the story (Rap1 stability on one hand and retrotransposon silencing on the other hand) are difficult to put together in a same story without apparent link between them. By the end of the manuscript, we do not have any clear idea about the possible overlap of function of Cay1 in these two phenomena. Discussion does not help much and no model is proposed.
I would also suggest to present Cay1 as a new regulator of Rap1 abundance in S. pombe rather than as a regulator of telomere transcription. The role of Cay1 in retrotransposon silencing seems to be unrelated to that of telomere silencing.

Major concerns.

1) Authors used a double-stranded probe to detect both TERRA and ARIA transcripts. What is the rationale behind this? Why not looking at TERRA and ARIA independently? In support of this, Figure 7d and E9 suggest that TERRA and ARIA may, under some circumstances, display opposite regulation. Indeed, authors showed that, while global levels of combined TERRA and ARIA were down-regulated in cay1 cph2 cells compared to cay1Δ cells (northern blot-Fig 7d), TERRA levels (measured by qRT-PCR) were up-regulated (Fig E9).

2) How do authors explain the discrepancies between dot-blot data (Fig. 1a upper panel and Fig E1) and northern blot (Fig 1a lower panel)? For example, the very high levels of TERRA/ARIA detected by dot blot in poz1Δ cells do not match with northern blot data in the lower panel (same holds true for northern blot of Fig 3b). Authors even state in the text (page 9) that poz1+ deletion only mildly affects TERRA/ARIA levels while, based on the screen shown in Fig E1, poz1+ deletion had a drastic effect on TERRA/ARIA. This should be clarified.

3) In addition, how do authors explain the discrepancies between their new northern blots and the ones they published previously in NAR (Bah et al, 2012)? Indeed, in their previous study, authors reported TERRA or ARIA molecule length of about 5 kb in rap1Δ cells (Fig. 1 in NAR paper). They clearly stated in the NAR manuscript that the smear coming from telomeric RNAase-sensitive species was between 0.1 and 2 kb in WT cells but extended up to about 5 kb in rap1 Δ cells. This should be clarified. Is it possible that TERRA/ARIA molecules (possibly in duplexes) are processed in S. pombe cells? In this case, is there any difference in the experimental conditions that may explain the differences between the two studies? Because most experiments in this study rely on the use of telomeric probes, I think that this is a very important issue to address as we still don't know whether longer fission yeast telomeres result in an increased production of telomeric RNA repeats due to longer TERRA/ARIA molecules. Data are still controversial in human cells but, to my knowledge, this has not been addressed in fission yeast. One way to circumvent this issue and to monitor telomere transcription more accurately would be to rely on qRT-PCR (TERRA molecules only). This has been done in Fig 3c and revealed only mild increase in TERRA levels (about 1.5 fold) upon cay1+ deletion, thus suggesting that the huge increase in TERRA/ARIA levels observed by northern blot is mostly due to increased telomere length in these cells. I do agree however that, based on qRT-PCR data, there is an increased transcription of telomeres but all this should be properly discussed in the text. This would as well help the reader understanding the apparent discrepancies between Fig 3b (northern blot) and Fig 3c (qRT-PCR).

4) Regarding ARRET up-regulation in cay1Δ cells, data are not convincing as, while in Fig 1b or Fig 3b, up-regulation is barely detectable, a strong up-regulation is observed in Fig 5c (assuming that the first lane corresponds to WT cells and the last lane to cay1Δ cells). Did authors quantify ARRET levels in their various northern blot experiments? This should be done and appear in the manuscript.

5) Authors show that H3K9 acetylation marks are increased at cay1Δ subtelomeres to a similar extent than what is observed at rap1Δ subtelomeres (Fig 3d). To test whether subtelomeric chromatin alterations in cay1Δ cells result from Rap1 degradation, they should perform ChIP experiments in cay1Δ cells overexpressing Rap1(-YFP) as authors showed that, in these conditions, the residual Rap1-YFP levels were similar to Rap1 levels in WT cells. This experiment would tell us whether Rap1 loss is responsible for increased subtelomeric H3K9ac in cay1Δ cells. ChIP against H3K9me3 should also been performed in these conditions.

6) Fig 6d suggests that TF2 LTR up-regulation does not result from altered H3K9 marks as ChIP data reveal similar H3K9ac/H3 and H3K9me3/H3 ratio at LTR loci in WT and cay1Δ cells. Hence, I am not convinced that transcriptional activation of retrotransposons results from altered chromatin properties. Although I agree that rap1Δ cells do not show any up-regulation of Tf2 transcription in Fig 3b, it would be interesting to test the impact of Rap1(-YFP) overexpression on Tf2 transcription in cay1Δ cells to formally exclude the possibility that Rap1 degradation in cay1Δ cells is involved.

7) Although the two-hybrid screen with Cay1 is interesting, I do not feel that the actual data are convincing enough to be included in this study. The impact of cph2+ deletion is confusing as, as stated above, this results in i) down-regulation of TERRA/ARIA (northern blot of Fig 7d), ii) up-regulation of TERRA (qRT-PCR in Fig E9) and iii) no impact on subtelomeric H3K9ac levels (data not shown). What can we conclude from all this? What about the other interactants that were

identified by the 2-hybrid screen?

So, overall, I believe that there are interesting data that warrant publication but that the focus of the paper should be changed in order to put more emphasis on the important role of Cay1 as Rap1 regulator. If authors show that subtelomeric H3K9ac alterations are indeed due to Rap1 loss, then they can conclude that all telomeric defects in cay1 cells result from Rap1 degradation. The authors should as well discuss about the link between subtelomeric H3K9ac/H3K9me3 levels and telomere transcription in S. pombe in reference to previous observations regarding human TERRA regulation. This does not appear in the discussion part. Discussion should also be adapted to take into account comment #3 in "Major concerns" section.

Minor concerns

-Fig 1d: do not mention "tel ChIP" as, in fact, this is a subtelomeric ChIP. Confusions between telomeres and subtelomeres appear throughout the manuscript. This should be changed.

-Fig 3f: please explain the L, M, C and I probes. Not all readers are familiar with J. Cooper's nomenclature...

-Authors refer to Fig 3A-C before Fig 2 in the results section. This should be re-organized.

-Fig 5c: does the first lane correspond to WT cells and the last one to cay1Δ cells? This should be mentioned more clearly.

-How do human and fission yeast cactin proteins compare? How conserved are these proteins?

-Could authors speculate about the drastic telomere-independent cold sensitivity phenotype of cay1 cells?

-Deletion of either spbp8b7.08c+ or vip1+ has a drastic impact on telomere length (Fig. 1c). Have authors looked at Rap1 levels in these mutant cells? In fact, the screen that initially aimed at identifying new TERRA regulators turned out to be successful in identifying new fission yeast telomere length regulators..

Referee #2:

Lorenzi et al. described the role of fission yeast cactin, whose molecular function had not been understood well even in other species, in telomere homeostasis and retrotransposon repression. They isolated cay1 gene encoding fission yeast cactin as a factor modulating the levels of telomere transcripts in a genetic screening of deletion library. Deletion of cay1 resulted in not only accumulation of telomeric lncRNA but also lengthening of telomere, which caused chromosome abnormalities. In addition, deletion of cay1 caused derepresion of Tf2 retrotransposons. Further analysis revealed that the defects of telomere homeostasis were largely caused by destabilization of telomeric protein Rap1, while Tf2-deprepression was partly caused by de-regulation of Clr6 histone deacetylase complex, whose subunit Cph2 interact with Cay1. These findings revealed the new role of cactin family proteins and introduced a new telomere regulating protein.
The involvement of cactin in telomere homeostasis and retropson repression is new and interesting, but I feel the overall presentation is rather descriptive and several important issues that deepen the understanding of molecular mechanism of Cay1 function should be answered.

Major points

1. What is the mechanism for the destabilization of Rap1 in the cay1-deleted cells? This is the main cause of the telomeric defects. Authors speculated that Cay1 regulated modifications of Rap1 that might affect protein stability. More investigation in this line should be done. As authors indicated

that Rap1 was degraded via proteasome pathway and Cay1 interact with E4 ubiquitin ligase Ufd2, the role of Ufd2-Cay1 interaction should be examined.
2. What recruits Cay1 to telomere and Tf2 retrotransposons? Does Cay1 have intrinsic DNA binding activity? Since one of the main issues in this manuscript is crosstalk between telomere regulation and reterotransposon repression via Cay1 that localized both loci, the localization mechanism is important to understand molecular nature of Cay1.

3. Authors proposed the idea that Cay1 participates in clustering of telomere and/or Tf2 and the disruption of clustering caused defects observed in cay1-deleted cells. I feel this idea is relatively easily tested by fluorescent-microscopic observation.

4. Tf2 retroposons in fission yeast are shown to be repressed by HDACs including Clr6 that is recruited by CENP-B homologues that binds to the reterotransposons. The relationship between this repression system and Cay1-dependet system should be addressed.

Minor points
1. In page 7 and Figure E2C, they described Cay1-YFP accumulated in the nucleus and often formed a single dot. What does the dot represent? Telomeres are known to form clusters and are found as multiple nuclear dots, like Rap1 dots in Fig. 5G. In contrast Tf2 reteroposons are known to form cluster that are recognized as 1-3 dots that are distinct from telomere clusters. Since Cay1 seems to binds both telomere and Tf2 elements, it is curious to me that Cay1 forms single dot.

2. HDAC Clr6 forms complex with Cph2. However, the amounts of Clr6 at telomere and Tf2 did not change in cay1-deleted cells though the amounts of Cph2 were significantly reduced. In addition, the levels of H3K9ac at both loci were increased. How these phenomena are explained?

Referee #3:

The manuscript by Lorenzi and colleagues describes three sets of results:
1. A screen of the pombe deletion collection for factor that modulate the abundance of telomeric transcripts.
2. Characterization of how cay1, one of the genes identified above, affects telomere biology.
3. Description of other non-telomeric effects of deleting cay1 in fission yeast.

The work harbors a wealth of important and interesting information that opens up a whole new direction for the telomere field and will undoubtedly stimulate many follow-up studies. My main concern at this point is that the manuscript provides many insights, but falls short on a clear message. I would advise the authors to drill deeper on one of the above areas and omit other results to improve clarity and not to dilute the message. The comments below are intended to help the authors make their work more accessible, tie up loose ends and publish a manuscript that will be read widely, cited frequently and stimulate follow-up studies.

Although the authors include the results for the entire screen, the description is brief and incomplete. This screen is an important contribution to the field, which has been struggling to gain an understanding of the functions and implications of telomere transcription. The results in this section should be described in more detail. Why were 18s and U6 chosen as loading controls? How many deletions differentially affect these controls? Going from 31 to 8 up-TERRA strains, is this a result of 23 genes giving a false positive as deletions down-regulate 18s instead of increasing TERRA? What were the results for the down-TERRA genes in the secondary screen?
It is unclear how the quantification of the blots shown in Figure 1a was obtained. There appears to be little correlation between fold increase and the signals on the blot.

The characterization of the telomere phenotypes leaves several basic questions unanswered, and remains speculative on an underlying mechanism for cay1 action. The authors may already have the answers to many of the questions below as a by-product of the experiments described. Specifically, the manuscript would be strengthened considerably by a more detailed analysis of the effects of

cay1 deletion on all of the core telomere proteins. Including these and omitting the Tf2 data would make this a more rounded piece of work with a clear message as to how cay1 affects telomere biology.

Cay1-YFP forms a single dot in many cells. Does this colocalize with telomeres?

The authors state that Rap1 mRNA levels are not reduced in cay1 delta cells, but the figure shows highly variable increase in Rap1 mRNA levels. In light of concerns about bortezomib (see below) and the previously reported role of cactins in splicing, the mRNA analysis should be carried out in more depth. Would the RT-PCR primers amplify pre-mRNA as well as mature message?

How Bortezomib affects S. pombe physiology is to my knowledge poorly characterized. A role in inhibiting the proteasome is largely inferred from experiments involving 1 mM concentrations (Takeda et al. DOI: 10.1371/journal.pone.0022021; 10x of what the authors used). The drug clearly has pleiotropic effects in other organisms. Since the authors show that a significant portion of the cay1 delete phenotypes is mediated via Rap1 protein levels, how Cay1 affects Rap1 levels ought to be examined in more detail.

While a number of the results support that cay1 affects telomere biology via its effect on Rap1 protein levels, other observations suggest that cay1D effects are independent of Rap1: a cay1D taz1D mutant looses telomeres, a rap1D taz1D does not, hence telomere loss and end fusions must be mediated through something else than the reduced levels of Rap1. Similarly, a cay1D rap1D has the same telomere length phenotype as a cay1D, but shorter than a rap1D. Rap1 levels can therefore not be the primary determinant of telomere length in this experiment. This is at odds with the rescue observed in the Rap1 overexpression experiment, but such results must be interpreted with caution as promiscuous overexpression of Rap1 may simply bypass a regulatory process involving cay1. In addition, the rescue shown in Figure 5 is only partial. I would not be surprised if cay1 affects the levels of other telomeric proteins in addition to Rap1. The sole piece of data that argues against a role of cay1 in splicing is the global analysis of normalized intron and exon expression base don tiling arrays. Such global expression analysis may mask even dramatic effects that specifically occur on a subset of RNAs.
The two hybrid results should be discussed in more detail, but this could be part of another manuscript focused on the Tf2 results. A ranking can be established based on how many different inserts represent each of the 48 proteins. Interactions can be verified and quantified by cloning the candidate genes into the bait vector and examining the strength of interaction using galactosidase assay.

Minor comments:

page 8:
the result that cay1 taz1 deletion results in chromosome circularization may be a critical piece to understanding the mechanism by which cay1 affects telomere biology. The result should be included, not described as an unpublished observation.
second to last line: correct "chromosome ands. taz1"
page 9 and 10:
delete question mark after Rap1D.
page 11:
"Taz1-YFP also induced telomere repeat loss" is confusing in this context, using the term telomere shortening would help distinguish from telomere loss observed in the cay1D taz1D strain.
page 15:
correct: "molecular functions associated to a Cactin protein"

1st Revision - authors' response 23 September 2014

Point-by-point response to the Reviewers' comments

Referee #1:

Major concerns.

1) Authors used a double-stranded probe to detect both TERRA and ARIA transcripts. What is the rationale behind this? Why not looking at TERRA and ARIA independently? In support of this, Figure 7d and E9 suggest that TERRA and ARIA may, under some circumstances, display opposite regulation. Indeed, authors showed that, while global levels of combined TERRA and ARIA were down-regulated in cay1Δcph2Δ cells compared to cay1Δ cells (northern blot-Fig 7d), TERRA levels (measured by qRT-PCR) were up-regulated (Fig E9).

We used a double-stranded radioactive probe for screening for telomeric RNA regulators because of the high specific activity of the probe. We have prepared RNA in 96-well plates and the final amount of total RNA was therefore limited. As our and Julie Cooper's laboratories have previously shown (Bah et al. *Nucleic Acids Res.* 40: 2995-3005, 2012; Greenwood and Cooper *Nucleic Acids Res*. 40: 2956-2963, 2012), telomeric transcripts are barely detectable in wt cells and this, together with the limited amount of total RNA per well, impedes the use of strand-specific telomeric oligonucleotides as probes (C-rich to detect TERRA and G-rich to detect ARIA). We have better explained this rationale in the Results section. We now also show oligonucleotide hybridizations of northern blots of total RNA from wt, *cay1*D, *rap1*D and *taz1*D cells (Figure E6C). When we perform northern blots we can load much higher amounts of RNA than the one present in one single well of a 96-well plate. These experiments reveal that ARIA is the RNA species that primarily accounts for hybridization signals obtained with double stranded telomeric probes and we now clearly explain this in the text. To help following this argument, we have now indicated as 'ARIA' the signal stemming from hybridizations with double-stranded telomeric probes in Figures 4D, 7B, and E6D while we have used the nomenclature 'telomeric signal' in Figure 1A. As for the data relating to *cph2*D, they have now been removed (see below, Major concern 7).

2) How do authors explain the discrepancies between dot-blot data (Fig. 1a upper panel and Fig E1) and northern blot (Fig 1a lower panel)? For example, the very high levels of TERRA/ARIA detected by dot blot in poz1Δ cells do not match with northern blot data in the lower panel (same holds true for northern blot of Fig 3b). Authors even state in the text (page 9) that poz1+ deletion only mildly affects TERRA/ARIA levels while, based on the screen shown in Fig E1, poz1+ deletion had a drastic effect on TERRA/ARIA. This should be clarified.

The discrepancies between the results obtained in dot-blot and northern blot experiments possibly derive from different growth conditions. For dot-blot experiments all strains were collectively grown for the same amount of time in 96-well plates, thus not necessarily allowing the same number of generations or growth phase; on the contrary, for northern blots, strains were grown individually to exponential phase. Moreover, while in dot-blot experiments RNA is concentrated in one spot, in northern blot experiments RNA is electrophoresed prior to hybridization and TERRA/ARIA signals are therefore spread throughout the gel lanes. Finally, the differences between the levels of stabilization of telomeric transcripts in *poz1*D strains presented in Figure 1A and current Figure 4D might also stem from their different genetic backgrounds (see below, Major concern 3). Nevertheless, in both cases, transcripts comprising telomeric sequences are stabilized over wt, consistently indicating that Poz1 contribute to telomere silencing. These points are now better explained in the Results section.

3) In addition, how do authors explain the discrepancies between their new northern blots and the ones they published previously in NAR (Bah et al, 2012)? Indeed, in their previous study, authors reported TERRA or ARIA molecule length of about 5 kb in rap1Δ cells (Fig. 1 in NAR paper). They clearly stated in the NAR manuscript that the smear coming from telomeric RNAase-sensitive species was between 0.1 and 2 kb in WT cells but extended up to about 5 kb in rap1Δ cells. This should be clarified. Is it possible that TERRA/ARIA molecules (possibly in duplexes) are processed in S. pombe cells? In this case, is there any difference in the experimental conditions that may explain the differences between the two studies? Because most experiments in this study rely on the use of telomeric probes, I think that this is a very important issue to address as we still don't know whether longer fission yeast telomeres result in an increased production of telomeric RNA repeats due to longer TERRA/ARIA molecules. Data are still controversial in human cells but, to my knowledge, this has not been addressed in fission yeast. One way to circumvent this issue and to

monitor telomere transcription more accurately would be to rely on qRT-PCR (TERRA molecules only). This has been done in Fig 3c and revealed only mild increase in TERRA levels (about 1.5 fold) upon cay1+ deletion, thus suggesting that the huge increase in TERRA/ARIA levels observed by northern blot is mostly due to increased telomere length in these cells. I do agree however that, based on qRT-PCR data, there is an increased transcription of telomeres but all this should be properly discussed in the text. This would as well help the reader understanding the apparent discrepancies between Fig 3b (northern blot) and Fig 3c (qRT-PCR).

This is a very good point that we have not properly discussed in the original version of our manuscript. Indeed, whether TERRA ~~length is~~levels are influenced by telomere length is still controversial. Different laboratories, including ours, have proposed different hypothesis using different model organisms. Yet, our data using Cay1-YFP expression in *cay1*D cells suggest that long telomeres do not produce more telomeric RNA. Indeed, at early generations when telomeres are still very long, ARIA levels are brought back to wt levels. We explain this point in the Discussion and reference specific papers dealing with the relationship between telomere length and telomere transcription both in human and budding yeast cells. As for the differences in TERRA/ARIA hybridization pattern observed in this current manuscript and in our previous paper (Bah et al. *Nucleic Acids Res.* 40: 2995-3005, 2012), they mostly derive from different genetic backgrounds. Indeed, in fission yeast, expression of the telomeric transcriptome is partly affected by the strain genetic background, an issue that we have already faced in our previous paper. We have now explained this point in the introduction. Yet, although some differences might be generated according to the used yeast genetic background, we also think the trend is always maintained, thus the final message is not altered. The possibility that TERRA/ARIA molecules (possibly in duplexes) are processed in *S. pombe* cells is very intriguing and we have been looking into that by inactivating different RNA nucleases including Dicer. Nevertheless, until now, we have not found any evidence for TERRA/ARIA duplex processing. More experiments to clarify this point will be performed in the future.

4) Regarding ARRET up-regulation in cay1Δ cells, data are not convincing as, while in Fig 1b or Fig 3b, up-regulation is barely detectable, a strong up-regulation is observed in Fig 5c (assuming that the first lane corresponds to WT cells and the last lane to cay1Δ cells). Did authors quantify ARRET levels in their various northern blot experiments? This should be done and appear in the manuscript.

As for TERRA and ARIA, ARRET and aARRET levels are also dependent on the genetic background of the used strains. This might explain why *cay1*+ deletion impacts on subtelomeric RNA species differently. Yet, as for telomeric repeat-containing species the trend is always maintained as *cay1*D cells have increased levels of ARRET and aARRET than wt counterparts. We think that the most important experiments to define to what extent Cay1 suppresses ARRET are the ones utilizing our Cay1-YFP and Rap1-YFP complementation system (Figure 7B). ARRET quantifications are now shown in the figure and discussed in the text.

5) Authors show that H3K9 acetylation marks are increased at cay1Δ subtelomeres to a similar extent than what is observed at rap1Δ subtelomeres (Fig 3d). To test whether subtelomeric chromatin alterations in cay1Δ cells result from Rap1 degradation, they should perform ChIP experiments in cay1Δ cells overexpressing Rap1(-YFP) as authors showed that, in these conditions, the residual Rap1-YFP levels were similar to Rap1 levels in WT cells. This experiment would tell us whether Rap1 loss is responsible for increased subtelomeric H3K9ac in cay1Δ cells. ChIP against H3K9me3 should also been performed in these conditions.

We sincerely thank this Reviewer for this crucial suggestion. We have now performed anti H3K9ac ChIPs using chromatin from early generation *cay1*D strains expressing Cay1-YFP and Rap1-YFP. Both Cay1-YFP and Rap1-YFP readily restored normal subtelomeric H3K9ac levels in *cay1*D cells (Figure 7C), validating two crucial conclusions of our work: *i*) accumulation of subtelomeric H3K9ac in *cay1*D cells is a true outcome of *cay1*+ deletion; *ii*) accumulation of subtelomeric H3K9ac in *cay1*D cells is due to insufficient Rap1 protein levels. This second conclusion is in complete agreement with the lack of additivity between *rap1*+ and *cay1*+ deletions in stabilizing subtelomeric H3K9ac (Figure 4D). We have also performed anti H3K9me3 and anti total H3 ChIPs in the same strains. Interestingly, defects in subtelomeric H3K9me3 and total H3 density associated to *cay1*+ deletion were not resolved by Cay1-YFP nor by Rap1-YFP, at least at early generations

(Figure 7C). These data indicate that Cay1 and Rap1 do not directly restrict subtelomeric H3K9me3 and total H3 (as it is the case for H3K9ac) but these defects most probably derive from altered telomere length, as early generation cells still carry very long telomeres as compared to wt cells (Figure 7A). We have discussed these points in the Results and Discussion sections.

6) Fig 6d suggests that TF2 LTR up-regulation does not result from altered H3K9 marks as ChIP data reveal similar H3K9ac/H3 and H3K9me3/H3 ratio at LTR loci in WT and cay1Δ cells. Hence, I am not convinced that transcriptional activation of retrotransposons results from altered chromatin properties. Although I agree that rap1Δ cells do not show any up-regulation of Tf2 transcription in Fig 3b, it would be interesting to test the impact of Rap1(-YFP) overexpression on Tf2 transcription in cay1Δ cells to formally exclude the possibility that Rap1 degradation in cay1Δ cells is involved.

We completely agree with the interpretation of our ChIP results and we have now re-phrased the relative discussion in the text and do not suggest anymore that *Tf2* stabilization largely derive from altered chromatin composition. The suggested experiment is undoubtedly interesting and we have tried it. Yet, the experimental set-up that we are using poses a number of problems. First of all, Cay1-YFP over-expression in wt cells stabilizes *Tf2* transcripts. Similarly, although at much lower levels, also Rap1-YFP and Taz1-YFP over-expression in wt cells lead to stabilization of *Tf2s*. We believe that this effect is not direct, as we clearly show that *rap1+* and *taz1+* deletions do not alter *Tf2* transcript levels in cells (Figure 4D). We therefore prefer not to present these experiments, as their interpretation seems to be complicated and possibly misleading. On the other side, we now show that pre-mRNA splicing of *rap1+* and of other cellular transcripts is altered in *cay1*D cells (Figure 3B and Figure 6A; see response to Reviewer 3 below), linking Cay1 to pre-mRNA splicing pathways. Following this new line of evidence, we also show that unprocessed *Tf2* transcripts accumulate in the two independent pre-mRNA splicing mutants *prp1-1* and *mpn1*D (Figure 8). We thus propose that the defects in *Tf2* transcript accumulation and processing observed in *cay1*D cells derive from pre-mRNA splicing inefficiency. These new data are discussed in the Results and Discussion sections.

7) Although the two-hybrid screen with Cay1 is interesting, I do not feel that the actual data are convincing enough to be included in this study. The impact of cph2+ deletion is confusing as, as stated above, this results in i) down-regulation of TERRA/ARIA (northern blot of Fig 7d), ii) up-regulation of TERRA (qRT-PCR in Fig E9) and iii) no impact on subtelomeric H3K9ac levels (data not shown). What can we conclude from all this? What about the other interactants that were identified by the 2-hybrid screen?

We agree with this point and we have removed the 2-hybrid screening and Cph2/Clr6 data from the manuscript. We will use these data as a starting point for further experiments that will be presented in a new report.

So, overall, I believe that there are interesting data that warrant publication but that the focus of the paper should be changed in order to put more emphasis on the important role of Cay1 as Rap1 regulator. If authors show that subtelomeric H3K9ac alterations are indeed due to Rap1 loss, then they can conclude that all telomeric defects in cay1Δ cells result from Rap1 degradation. The authors should as well discuss about the link between subtelomeric H3K9ac/H3K9me3 levels and telomere transcription in S. pombe in reference to previous observations regarding human TERRA regulation. This does not appear in the discussion part. Discussion should also be adapted to take into account comment #3 in "Major concerns" section.

We thank this Reviewer for recognizing the general interest of our data and for his/her suggestions that have allowed us to restructure our manuscript into a much coherent story focused on Cay1-mediated regulation of Rap1 and telomere homeostasis. In the Discussion section, we have also elaborated more in depth on the connection between subtelomeric H3K9 modifications, TERRA transcription and telomere length regulation, an aspect that indeed was missing in our previous version. To clearly represent the novel focus of our manuscript, we have changed its title into '*Fission yeast Cactin restricts telomere transcription and elongation by promoting Rap1 pre-mRNA splicing and protein stabilization*'.

Minor concerns

-Fig 1d: do not mention "tel ChIP" as, in fact, this is a subtelomeric ChIP. Confusions between telomeres and subtelomeres appear throughout the manuscript. This should be changed.

We have changed 'telChIP' into 'subtel ChIP' in the different Figures showing ChIP results obtained using subtelomeric oligonucleotides for quantitative real time PCRs. We have also better clarified the differences between subtelomeric loci and telomeric loci throughout the text.

-Fig 3f: please explain the L, M, C and I probes. Not all readers are familiar with J. Cooper's nomenclature.

We have added a specific description of L, M, C and I probes in the Materials and Methods section describing PFGE procedures. Moreover, a sketch of the terminal *Not*I fragments detected by the different probes is presented in Figure E7A.

-Authors refer to Fig 3A-C before Fig 2 in the results section. This should be re-organized.

The order of the Figures is now different as compared to the previous version of the manuscript and this issue has been fixed.

-Fig 5c: does the first lane correspond to WT cells and the last one to cay1Δ cells? This should be mentioned more clearly.

We have fixed this issue in the current Figure 7, which corresponds to the original Figure 5.

-How do human and fission yeast cactin proteins compare? How conserved are these proteins?

We have added a brief description of Catin protein domains and conservation between Cay1 and human Cactin in the Discussion section.

-Could authors speculate about the drastic telomere-independent cold sensitivity phenotype of cay1Δ cells?

In the Discussion section, we now propose that aberrant pre-mRNA splicing or protein stability of factors other than Rap1 could explain the inability of Rap1-YFP to rescue the proliferation defects of cay1D mutants in the cold.

-Deletion of either spbp8b7.08c+ or vip1+ has a drastic impact on telomere length (Fig. 1c). Have authors looked at Rap1 levels in these mutant cells? In fact, the screen that initially aimed at identifying new TERRA regulators turned out to be successful in identifying new fission yeast telomere length regulators.

We have performed western blot analysis of protein extracts from *spbp8b7.08c*D and *vip1*D cells using antibodies against endogenous Rap1 and Taz1 proteins (Act1 was used as loading control). As shown in the referee figure below (removed from Peer Review Process File), while *spbp8b7.08c+* deletion does not affect the Rap1 and Taz1, *vip1+* deletion, similarly to *cay1+* deletion, strongly suppresses Rap1 total levels while leaving Taz1 unchanged. These data are very intriguing and even more exciting if one considers that the RNA binding protein Vip1 is very poorly characterized. Yet, at this stage, we feel that these data are too preliminary and would need further validation experiments before publishing. Hence, we would prefer not to include them in the final version of our manuscript. We will for sure follow up this observation in the future.

Referee #2:

Lorenzi et al. described the role of fission yeast cactin, whose molecular function had not been understood well even in other species, in telomere homeostasis and retrotransposon repression. They isolated cay1 gene encoding fission yeast cactin as a factor modulating the levels of telomere transcripts in a genetic screening of deletion library. Deletion of cay1 resulted in not only accumulation of telomeric lncRNA but also lengthening of telomere, which caused chromosome abnormalities. In addition, deletion of cay1 caused derepresion of Tf2 retrotransposons. Further analysis revealed that the defects of telomere homeostasis were largely caused by destabilization of telomeric protein Rap1, while Tf2-deprepression was partly caused by de-regulation of Clr6 histone deacetylase complex, whose subunit Cph2 interact with Cay1. These findings revealed the new role of cactin family proteins and introduced a new telomere regulating protein. The involvement of cactin in telomere homeostasis and retropson repression is new and interesting, but I feel the overall presentation is rather descriptive and several important issues that deepen the understanding of molecular mechanism of Cay1 function should be answered.

We thank this Reviewer for recognizing the general interest and novelty of our work. We are confident that the new version of our manuscript presents solid mechanisms explaining how Cay1 functions in assuring proper Rap1 cellular levels and therefore telomere maintenance.

Major points

1. What is the mechanism for the destabilization of Rap1 in the cay1-deleted cells? This is the main cause of the telomeric defects. Authors speculated that Cay1 regulated modifications of Rap1 that might affect protein stability. More investigation in this line should be done. As authors indicated that Rap1 was degraded via proteasome pathway and Cay1 interact with E4 ubiquitin ligase Ufd2, the role of Ufd2-Cay1 interaction should be examined.

We now present compelling evidence showing that *rap1+* pre-mRNA is inefficiently spliced in *cay1*D cells (Figure 3B and Figure 6A). This, together with published data reporting physical interactions between Cactins and spliceosomal proteins in different organisms, indicate a direct role for Cay1 in promoting efficient splicing of at least a set of pre-mRNAs. It remains that also Rap1 protein stability is diminished in *cay1*D cells. Thus, Cay1 assures proper Rap1 cellular levels through regulation of post-transcriptional and post-translational mechanisms. As for the connection between Cay1 and proteasome-mediated degradation, following the suggestions of Reviewers 1 and 3, we have removed data on the 2-hybrid screening. Therefore we are not showing nor discussing the Cay1 interaction with Ufd2 any longer. For this reason and for the concerns about Bortezomib raised by Reviewer 3 (see below), we have also removed the analysis of Rap1 protein levels in cells treated with Bortezomib, while we still show that Rap1-YFP half-life is diminished when *cay1+* is deleted. Results and Discussion have been changed accordingly.

2. What recruits Cay1 to telomere and Tf2 retrotransposons? Does Cay1 have intrinsic DNA binding activity? Since one of the main issues in this manuscript is crosstalk between telomere regulation and retrotransposon repression via Cay1 that localized both loci, the localization mechanism is important to understand molecular nature of Cay1.

Although this point is very interesting, we feel that it would take very long to establish *in vitro* systems to test whether Cay1 binds directly to nucleic acids. Setting up such protocols would somehow constitute a novel line of research going beyond the scope of our current story.

3. The authors proposed the idea that Cay1 participates in clustering of telomere and/or Tf2 and the disruption of clustering caused defects observed in cay1-deleted cells. I feel this idea is relatively easily tested by fluorescent-microscopic observation.

Indeed we have proposed this idea in the Discussion section of the original version of our manuscript. In the current version, the focus has clearly been shifted towards the molecular mechanisms through which Cay1 assures sufficient Rap1 levels in cells (pre-mRNA splicing and protein stabilization). We have therefore removed the reference to telomere and *Tf2* clustering from the Discussion as we feel that this point is not of primary importance. We will for sure follow this

line of research in the future.

4. Tf2 retrotransposons in fission yeast are shown to be repressed by HDACs including Clr6 that is recruited by CENP-B homologues that binds to the reterotransposons. The relationship between this repression system and Cay1-dependet system should be addressed.

As suggested by Reviewers 1 and 3 we have removed data on the 2-hybrid screening and Cph2/Clr6 interactions with Cay1. We will use these data as a starting point for new research that will be presented in a new report and that will for sure take into account the suggestion of this Reviewer.

Minor points

1. In page 7 and Figure E2C, they described Cay1-YFP accumulated in the nucleus and often formed a single dot. What does the dot represent? Telomeres are known to form clusters and are found as multiple nuclear dots, like Rap1 dots in Fig. 5G. In contrast Tf2 retrotransposons are known to form cluster that are recognized as 1-3 dots that are distinct from telomere clusters. Since Cay1 seems to binds both telomere and Tf2 elements, it is curious to me that Cay1 forms single dot.

Our ChIP experiments indicate that binding of Cay1 to telomeres and even more to Tf2s is not very strong. Thus it seems that Cay1 associates to chromatin at low levels or in a transient way, rendering unlikely that the single Cay1-YFP focus observed in our experiments could represent telomeres or *Tf2* loci. Indeed, we have now performed co-localization experiments between Cay1-YFP and Pot1-RFP and no co-localization between the two proteins has been detected (Figure 6C). GFP-tagged, *Arabidopsis thaliana* Cactin was found to accumulate within nuclear speckles containing known splicing factors such as SR45 and RSP31 (Baldwin et al. *FEBS Lett*. 587: 873-879, 2013). By analogy, we now propose in the Discussion section that the single focus formed by Cay1-YFP might represent a nuclear center containing spliceosomal factors.

2. HDAC Clr6 forms complex with Cph2. However, the amounts of Clr6 at telomere and Tf2 did not change in cay1-deleted cells though the amounts of Cph2 were significantly reduced. In addition, the levels of H3K9ac at both loci were increased. How these phenomena are explained?

As suggested by Reviewers 1 and 3 we have removed data on the 2-hybrid screening and Cph2/Clr6 interactions with Cay1. We will use these data as a starting point for new research that will be presented in a new report and that will for sure take into account the suggestion of this Reviewer.

Referee #3:

The manuscript by Lorenzi and colleagues describes three sets of results:

1. A screen of the pombe deletion collection for factor that modulate the abundance of telomeric transcripts. 2. Characterization of how cay1, one of the genes identified above, affects telomere biology. 3. Description of other non-telomeric effects of deleting cay1 in fission yeast.

The work harbors a wealth of important and interesting information that opens up a whole new direction for the telomere field and will undoubtedly stimulate many follow-up studies. My main concern at this point is that the manuscript provides many insights, but falls short on a clear message. I would advise the authors to drill deeper on one of the above areas and omit other results to improve clarity and not to dilute the message. The comments below are intended to help the authors make their work more accessible, tie up loose ends and publish a manuscript that will be read widely, cited frequently and stimulate follow-up studies.

We thank this Reviewer for recognizing the importance of our study and the impact that it will have on future research in the field. We admit that the original version of the manuscript was not

organized in a very coherent way and the main take-on message was diluted away by a number of results that could have been omitted. We have now substantially restructured our manuscript into a much coherent story clearly focusing on the fact that Cay1 stabilizes cellular Rap1, thereby assuring proper silencing of telomeric RNA and telomere length homeostasis. To clearly represent the novel focus of our manuscript, we have changed its title into '*Fission yeast Cactin restricts telomere transcription and elongation by promoting Rap1 pre-mRNA splicing and protein stabilization*'.

Although the authors include the results for the entire screen, the description is brief and incomplete. This screen is an important contribution to the field, which has been struggling to gain an understanding of the functions and implications of telomere transcription. The results in this section should be described in more detail. Why were 18s and U6 chosen as loading controls? How many deletions differentially affect these controls? Going from 31 to 8 up-TERRA strains, is this a result of 23 genes giving a false positive as deletions down-regulate 18s instead of increasing TERRA? What were the results for the down-TERRA genes in the secondary screen?

18S rRNA and U6 were chosen as loading controls as they are abundant in cells and therefore possibly less likely to be strongly affected by a large number of gene deletions. Moreover, using two alternative loading controls to normalize the same samples should limit the number of false positive. For this first story, we have arbitrarily chosen to perform our secondary screening only on strains where telomeric RNA was up-regulated as compared to wt cells and not to follow strains were telomeric RNA was down-regulated. Those strains are anyway presented in Table E2 and we hope that they will represent a starting point for future investigations performed by us and other laboratories. We have now expanded the description of the screening procedures in the Results section.

It is unclear how the quantification of the blots shown in Figure 1a was obtained. There appears to be little correlation between fold increase and the signals on the blot.

As explained for Reviewer 1's Major concern, the discrepancies between the results obtained in dot-blot and northern blot experiments possibly derive from different growth conditions. For dot-blot experiments, strains were grown collectively for the same amount of time in 96-well plates, thus not necessarily allowing the same number of generations or growth phase; on the contrary, for northern blots, strains were grown individually to exponential phase. Moreover, while in dot-blot experiments RNA is concentrated in one spot, in northern blot experiments RNA is electrophoresed prior to hybridization and TERRA/ARIA signals are therefore spread throughout the gel lanes. Nevertheless, both results indicate stabilization of telomeric transcripts in the identified strains. These points are now better explained in the Results section.

The characterization of the telomere phenotypes leaves several basic questions unanswered, and remains speculative on an underlying mechanism for cay1 action. The authors may already have the answers to many of the questions below as a by-product of the experiments described. Specifically, the manuscript would be strengthened considerably by a more detailed analysis of the effects of cay1 deletion on all of the core telomere proteins. Including these and omitting the Tf2 data would make this a more rounded piece of work with a clear message as to how cay1 affects telomere biology.

We have now better characterized the molecular mechanisms that lead to Rap1 reduced levels in *cay1*D cells (see below), and expanded our experimental analysis and discussion to other core telomeric factors. Data on *Tf2* transcript stabilization are still present in the manuscript, as they show that Cay1 is essential not only at telomeres but also at other genomic loci. Yet, the current version of our manuscript seems to us way more rounded than the original one and, thanks to the Reviewers' comments, conveying a much clearer message on how Cay1 is involved in telomere biology.

Cay1-YFP forms a single dot in many cells. Does this colocalize with telomeres?

We have now performed co-localization experiments using Cay1-YFP and Pot1-RFP, and no co-localization between the two proteins has been detected (Figure 6C). GFP-tagged, *Arabidopsis thaliana* Cactin was found to accumulate within nuclear speckles containing known splicing factors

such as SR45 and RSP31 (Baldwin et al. *FEBS Lett*. 587: 873-879, 2013). By analogy, we now propose in the Discussion section that the single focus formed by Cay1-YFP might represent a nuclear center containing spliceosomal factors.

The authors state that Rap1 mRNA levels are not reduced in cay1 delta cells, but the figure shows highly variable increase in Rap1 mRNA levels. In light of concerns about bortezomib (see below) and the previously reported role of cactins in splicing, the mRNA analysis should be carried out in more depth. Would the RT-PCR primers amplify pre-mRNA as well as mature message?

This is a very important point and we are extremely thankful to this Reviewer for bringing it up. We have now performed a PCR-based analysis of *rap1*+ transcripts and we show that indeed *rap1*+ pre-mRNA is inefficiently spliced in *cay1*D cells, largely explaining Rap1 protein insufficiency. These data are presented in Figure 3B and Figure 6A. Still, it remains that also Rap1 protein half-life is diminished in *cay1*D cells (Figure 3C). Thus, Cay1 assures proper Rap1 cellular levels by promoting pre-mRNA splicing and, apparently to a lower extent, protein stabilization. We have also analyzed pre-mRNA splicing of other transcripts (encoding both telomeric and non telomeric factors) and we now show that splicing of different substrates is executed with different efficiencies in *cay1*+-deleted strains (Figure 3B). As mentioned by this Reviewer, published reports based on interaction and co-localization studies have suggested that Cactins could be part of spliceosome machineries. Nevertheless, to the best of our knowledge, this is the first time that an actual defect in pre-mRNA splicing has been demonstrated in cells lacking Cactin (Cay1). Inspired by this new line of evidence we have also tested whether impairing with global pre-mRNA splicing affects *Tf2* transcript levels. We now show that unprocessed *Tf2* RNA accumulates in the two independent pre-mRNA splicing mutants *prp1-1* and *mpn1*D (Figure 8). We thus propose that *Tf2* transcript accumulation and processing defects observed in *cay1*D cells derive from pre-mRNA splicing inefficiency. We elaborate on all these new data in the Results and Discussion sections.

How Bortezomib affects S. pombe physiology is to my knowledge poorly characterized. A role in inhibiting the proteasome is largely inferred from experiments involving 1 mM concentrations (Takeda et al. DOI: 10.1371/journal.pone.0022021; 10x of what the authors used). The drug clearly has pleiotropic effects in other organisms. Since the authors show that a significant portion of the cay1 delete phenotypes is mediated via Rap1 protein levels, how Cay1 affects Rap1 levels ought to be examined in more detail.

We agree with this Reviewer that how Bortezomib affects proteasome-mediated degradation in cells has been poorly characterized. Before resorting to this specific drug, we have tried several different proteasome inhibitors, but in our hands Bortezomib is the only one that reproducibly stabilizes total ubiquitinated proteins. Taking into account this specific concern and the new data on *rap1*+ pre-mRNA splicing, we have decided to remove the Bortezomib experiments as we feel that this change does not affect the main take-on message of our story. We have adjusted Results and Discussion accordingly.

While a number of the results support that cay1 affects telomere biology via its effect on Rap1 protein levels, other observations suggest that cay1D effects are independent of Rap1: a cay1D taz1D mutant looses telomeres, a rap1D taz1D does not, hence telomere loss and end fusions must be mediated through something else than the reduced levels of Rap1. Similarly, a cay1D rap1D has the same telomere length phenotype as a cay1D, but shorter than a rap1D. Rap1 levels can therefore not be the primary determinant of telomere length in this experiment. This is at odds with the rescue observed in the Rap1 overexpression experiment, but such results must be interpreted with caution as promiscuous overexpression of Rap1 may simply bypass a regulatory process involving cay1. In addition, the rescue shown in Figure 5 is only partial. I would not be surprised if cay1 affects the levels of other telomeric proteins in addition to Rap1. The sole piece of data that argues against a role of cay1 in splicing is the global analysis of normalized intron and exon expression base don tiling arrays. Such global expression analysis may mask even dramatic effects that specifically occur on a subset of RNAs.

As above, we totally agree with this concern and we now show that Cay1 promotes splicing of *rap1*+ pre-mRNA as well as, to a lower extent, of other pre-mRNAs that encode telomeric factors. In particular, we show that *poz1*+ pre-mRNA is also spliced inefficiently in *cay1*D cells, although

the effect of *cay1*+ deletion is not as dramatic as for *rap1*+ pre-mRNA: approximately 50% of mature *poz1*+ mRNA is present in *cay1*D mutants (Figure 3B and 6A). Decreased levels of Poz1 protein in *cay1*D strains might explain why expressing Rap1-YFP is not sufficient to re-establish normal telomere length and to repress ARRET (Figure 7A and B). We now discuss these points in the Discussion section. Also, we now discuss the results obtained using our tiling array data in the context of the results obtained by RT-PCR and clearly state that Cay1 seems to affects pre-mRNA splicing of different transcripts to different extents, thus explaining why we could not detect any major alteration of global splicing efficiencies.

The two hybrid results should be discussed in more detail, but this could be part of another manuscript focused on the Tf2 results. A ranking can be established based on how many different inserts represent each of the 48 proteins. Interactions can be verified and quantified by cloning the candidate genes into the bait vector and examining the strength of interaction using galactosidase assay.

As suggested by Reviewers 1 and 3 we have removed data on the 2-hybrid screening and Cph2/Clr6 interactions with Cay1. We will use these data as a starting point for new research that will be presented in future reports.

Minor comments:

page 8:

the result that cay1 taz1 deletion results in chromosome circularization may be a critical piece to understanding the mechanism by which cay1 affects telomere biology. The result should be included, not described as an unpublished observation.

We have performed PFGE of late generation *cay1*D*taz1*D cells (referee figure removed from Peer Review Process File). The PFGE was performed using DNA from wt, circular *trt1*D (O*trt1*D, positive control for chromosome circularization), *cay1*D and double mutants deleted for *cay1*+ in combination with *rap1*+, *taz1*+ and *poz1*+. DNA was hybridized with a mix of C, M, L and I probes. It is clear that *cay1*D*rap1*D and *cay1*D*poz1*D chromosome ends are linear. On the contrary, the results obtained for *cay1*D*taz1*D cells are quite strange: while chromosome II has circularized (complete disappearance of fragments C and M, appearance of fragment C+M), chromosome I is still linear (presence of fragments L and I, absence of fragment L+I). This might indicate that circularization of chromosome II occurs faster than the one of chromosome I, yet we are not aware that this was previously reported. It is also possible that more complicated mechanisms, at play in *cay1*D*taz1*D cells, restrict the ability of chromosome I to circularize. We feel that these data, although intriguing, might be too preliminary and would complicate our story rather than helping to understand the biology of Cay1. We have therefore removed the mention to chromosome circularization in *cay1*D*taz1*D cells. Of course we would agree to include these data as supplementary information in case this Reviewer requested that.

second to last line: correct "chromosome ands. taz1"

We have corrected this mistake in the text.

page 9 and 10:

delete question mark after Rap1D.

We have corrected this mistake in the text.

page 11:

"Taz1-YFP also induced telomere repeat loss" is confusing in this context, using the term telomere shortening would help distinguish from telomere loss observed in the cay1D taz1D strain.

We have substituted ‘telomere repeat loss’ with ‘telomere shortening’ in the text.

page 15:

correct: "molecular functions associated to a Cactin protein"

We have corrected this in the text and now we write: ‘molecular functions associated to a member of the conserved family of Cactins’.

2nd Editorial Decision 14 October 2014

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by all three original referees (comments included below).

As you will see the referees all find that the revised manuscript has been considerably strengthened and that the removal of the Y2H data has made the story much clearer. However, while they are thus supportive of publication they do still raise a few minor points that should be discussed more extensively before the manuscript can be officially accepted. I would therefore ask you to comment on the role for Cay1 localization to subtelomeric regions (ref #1 and #2) as well as the possible nature of Rap1 regulation (ref #1) in a final revision of the manuscript text. A model figure as suggested by ref #1 would be helpful for the study but is not an absolute requirement from our side.

In addition, I would ask you to include a few minor editorial points:
-> Please provide data base accession number for the tiling array data.

-> Based on the comments from ref #3, I would suggest changing the title along the lines of 'Cactin restricts telomere transcription and elongation by controlling Rap1 levels'.

-> We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

-> As of Jan 1st 2014 every paper published in The EMBO Journal include a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis will include a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points.

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE REPORTS

Referee #1:

Lorenzi et al
Revised manuscript

The manuscript by Lorenzi et al has been substantially revised and additional experiments asked by reviewers were performed. Explanations have also been added in the text to help reader's understanding of the manuscript. Removal of 2-hybrid screen data is as well appreciated and reduces confusion.
In spite of this, I found the Results part still very difficult to follow and many questions arose during the reading that, hopefully, were partially answered by the -much improved- Discussion part. In particular, I was concerned by the discrepancy between ChIP data that show Cay1 protein binding to subtelomeres (Fig. 1D) while fluorescent microscopy data from Fig. 6C show that Cay1-YFP does not co-localize with telomeres (and authors state in the text that Cay1-YFP NEVER co-localized with Pot1-RFP). Adding a possible explanation for this discrepancy in the Results section would be useful to fully/peacefully appreciate the manuscript. My hypothesis while reading this new version of the Results was that Cay1 may rather impact on pre-mRNA splicing of an unknown protein that, itself, impacts on Rap1 stability, rather than being directly involved in Rap1 protein stability. I found this hypothesis to be indeed mentioned by the authors in the Discussion part. Based on this and on the new data authors got on pre-mRNA splicing, I would suggest to draw a model that would include possible scenarios related to Cay1 function in S. pombe. This model should be presented and explained in the Discussion part.

Minor concerns:

1) Figure 3B. RT-PCR experiments have been performed to show that cay1+ deletion impacts on Rap1 pre-mRNA splicing. Additional pre-mRNA splicing alterations are shown. It is unclear to me whether these RT-PCR were performed in a semi-quantitative manner? How many PCR cycles have been performed? If 40 cycles, then one cannot quantify band intensity on the gel and quantifications should be done by qRT-PCR instead. I do not feel however that quantification is required at this stage as data are already convincing in a qualitative way. Same comments hold true for Fig. 6A.
2) Figure 2D: change "tel cay1D" to "subtel cay1D" in the legend

Referee #2:

Revised manuscript by Lorenzi et al. has been restructured and includes new data that Cay1, a fission yeast homologue of cactin, contributes the maintenance of proper amount of Rap1 by regulating splicing and protein stabilization. This explains the telomere defects observed in cay1-deleted cells. In addition, accumulation of un-spliced form of Tf2 retrotransposon transcripts were observed in cay1-deleted cells, suggesting a role of Cay1 in Tf2 RNA splicing.

Major points
Comparing with the previous version, the revised manuscript well focused on the role of Cay1 in the Rap1 regulation, which explains the initial observation of telomere defects in cay1-deleted cells. I feel removal of 2-hybrid data and addition of new data related improved understandability of the manuscript. Indication of Cactin in splicing is very interesting and will contribute to understand the role of still-mysterious protein Cactin.

Minor points
1. The reason for choosing cay1 mutant among other mutants that compromise telomere transcriptome for further analysis was the localization of Cay1 at telomere. But this localization seems to be unnecessary for regulation of cellular level of Rap1. What is the role of telomere-binding Rap1? As Cay1 also localizes at Tf2, the same question about Tf2-assoicated Cay1 arises. These point should be discussed.

2. Authors suggest the single focus formed by Cay1-YFP might represent a nuclear center containing spliceosomal factors. This is a very important possibility. Indeed, some of splicing factor

such as Prp11 and Cwf16 formed a focus at SPB (Orfeome Localization Data in SPD/RIKEN). The comparison of the localization of Cay1 and these splicing factors may further strengthen the author's arguments.

Referee #3:

The manuscript has undergone major revisions in response to the editor's and reviewers' comments. These revisions have addressed my concerns and I now think that the work represents an important and well-presented contribution to the field. Although the mechanisms by which cay1 affects splicing and protein stability remain elusive, the results presented constitute a milestone and should be made available to the broader scientific community at this point. Undoubtedly the paper will stimulate future work in several laboratories.

The new title seems overly long and specific. It does not do justice to the observation that Rap1 is not the only factor affected by cay1 deletion and ignores the Tf2 results altogether. Following removal of the bortezomib data, the protein stability angle seem rather weakly supported by the nmt1-Rap1-YFP result and should perhaps not be part of the title.

Regarding the PFG, I agree with the authors that the data is intriguing but preliminary. Considering that the double mutants examined here are not the main focus of the manuscript I concur with the removal of the reference to unpublished data rather than presenting what would create a new "loose end" at this point. The most likely explanation for circular chrII and linear ChrI are differences in kinetics of circularization. While this could be addressed experimentally, it would further delay publication without a high chance of adding substantial new insights.

2nd Revision - authors' response 19 October 2014

Point-by-point response to the Editor's and Reviewers' comments

Editor:

In addition, I would ask you to include a few minor editorial points:

-> Please provide data base accession number for the tiling array data.

The tiling array data have been submitted to the GEO repository and have been assigned the database number GSE61792. This information is now included in the Materials and Methods section.

-> Based on the comments from ref #3, I would suggest changing the title along the lines of 'Cactin restricts telomere transcription and elongation by controlling Rap1 levels'.

We have now changed the title into *'Fission yeast Cactin restricts telomere transcription and elongation by controlling Rap1 levels*'.

-> We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

The majority of our electrophoretic blots (telomeres and TERRA/ARIA) are essentially representing the entire region of the membranes showing a signal. As I mentioned to you in our previous e-mail conversation, we have not included original source data and I hope that this is acceptable.

-> As of Jan 1st 2014 every paper published in The EMBO Journal include a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis will include a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points.

We have included 4 bullet points on the first page of the manuscript.

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

The image for the synopsis has been included.

Referee #1:

The manuscript by Lorenzi et al has been substantially revised and additional experiments asked by reviewers were performed. Explanations have also been added in the text to help reader's understanding of the manuscript. Removal of 2-hybrid screen data is as well appreciated and reduces confusion.

In spite of this, I found the Results part still very difficult to follow and many questions arose during the reading that, hopefully, were partially answered by the -much improved- Discussion part. In particular, I was concerned by the discrepancy between ChIP data that show Cay1 protein binding to subtelomeres (Fig. 1D) while fluorescent microscopy data from Fig. 6C show that Cay1-YFP does not co-localize with telomeres (and authors state in the text that Cay1-YFP NEVER co-localized with Pot1-RFP). Adding a possible explanation for this discrepancy in the Results section would be useful to fully/peacefully appreciate the manuscript. My hypothesis while reading this new version of the Results was that Cay1 may rather impact on pre-mRNA splicing of an unknown protein that, itself, impacts on Rap1 stability, rather than being directly involved in Rap1 protein stability. I found this hypothesis to be indeed mentioned by the authors in the Discussion part. Based on this and on the new data authors got on pre-mRNA splicing, I would suggest to draw a model that would include possible scenarios related to Cay1 function in S. pombe. This model should be presented and explained in the Discussion part.

In the Results section we have now added that the fact that Cay1-YFP never co-localizes with Pot1-RFP, together with our Cay1-myc ChIP results, indicates that Cay1 binds to telomeres at low levels or transiently thereby impeding cytological detection of the protein at telomeric loci.

As for the suggested model, we find it quite difficult at this point to draw a coherent and simplified model that would take into consideration all the aspects touched upon in our manuscript. We would therefore prefer not to include this model.

Minor concerns:

1) Figure 3B. RT-PCR experiments have been performed to show that cay1+ deletion impacts on Rap1 pre-mRNA splicing. Additional pre-mRNA splicing alterations are shown. It is unclear to me whether these RT-PCR were performed in a semi-quantitative manner? How many PCR cycles have been performed? If 40 cycles, then one cannot quantify band intensity on the gel and quantifications should be done by qRT-PCR instead. I do not feel however that quantification is required at this stage as data are already convincing in a qualitative way. Same comments hold true for Fig. 6A.

The PCR experiments have been performed in a semi-quantitative manner. Indeed, no more than 31

cycles have been used. The information is now included in the Materials and Methods section.

2) Figure 2D: change "tel cay1D" to "subtel cay1D" in the legend

We have changed the figure accordingly.

Referee #2:

Revised manuscript by Lorenzi et al. has been restructured and includes new data that Cay1, a fission yeast homologue of cactin, contributes to the maintenance of proper amount of Rap1 by regulating splicing and protein stabilization. This explains the telomere defects observed in cay1-deleted cells. In addition, accumulation of un-spliced form of Tf2 retrotransposon transcripts were observed in cay1-deleted cells, suggesting a role of Cay1 in Tf2 RNA splicing.

Major points

Comparing with the previous version, the revised manuscript well focused on the role of Cay1 in the Rap1 regulation, which explains the initial observation of telomere defects in cay1-deleted cells. I feel removal of 2-hybrid data and addition of new data related improved understandability of the manuscript. Indication of Cactin in splicing is very interesting and will contribute to understand the role of still-mysterious protein Cactin.

Minor points

1. The reason for choosing cay1 mutant among other mutants that compromise telomere transcriptome for further analysis was the localization of Cay1 at telomere. But this localization seems to be unnecessary for regulation of cellular level of Rap1. What is the role of telomere-binding Rap1? As Cay1 also localizes at Tf2, the same question about Tf2-assoicated Cay1 arises. These points should be discussed.

We have extended the already present discussion of this issue in the Discussion section of the manuscript. We now state that although transiently or at low levels, Cay1-Myc localizes to telomeres, suggesting that Cay1-mediated post-translational stabilization of Rap1 might occur in the context of telomeric chromatin. Moreover, Cay1 physical association with telomeres and *Tf2s* suggests that Cay1 might also regulate expression of these loci *in cis*, for example by participating in chromatin remodeling processes.

2. Authors suggest the single focus formed by Cay1-YFP might represent a nuclear center containing spliceosomal factors. This is a very important possibility. Indeed, some of splicing factor such as Prp11 and Cwf16 formed a focus at SPB (Orfeome Localization Data in SPD/RIKEN). The comparison of the localization of Cay1 and these splicing factors may further strengthen the author's arguments.

This suggestion is very interesting and we will for sure try to co-localize Cay1-YFP with the SPB or splicing factors in the future.

Referee #3:

The manuscript has undergone major revisions in response to the editor's and reviewers' comments. These revisions have addressed my concerns and I now think that the work represents an important and well-presented contribution to the field. Although the mechanisms by which cay1 affects splicing and protein stability remain elusive, the results presented constitute a milestone and should be made available to the broader scientific community at this point. Undoubtedly the paper will

stimulate future work in several laboratories.

The new title seems overly long and specific. It does not do justice to the observation that Rap1 is not the only factor affected by cay1 deletion and ignores the Tf2 results altogether. Following removal of the bortezomib data, the protein stability angle seem rather weakly supported by the nmt1-Rap1-YFP result and should perhaps not be part of the title.

We have now changed the title into *'Fission yeast Cactin restricts telomere transcription and elongation by controlling Rap1 levels'*.

Regarding the PFG, I agree with the authors that the data is intriguing but preliminary. Considering that the double mutants examined here are not the main focus of the manuscript I concur with the removal of the reference to unpublished data rather than presenting what would create a new "loose end" at this point. The most likely explanation for circular chrII and linear ChrI are differences in kinetics of circularization. While this could be addressed experimentally, it would further delay publication without a high chance of adding substantial new insights.

The PFGE data have not been included.