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# The chromatin remodelling factor ATRX suppresses R-loops in transcribed telomeric repeats

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First Editorial Decision from EMBO reports 21 July 2016

Thank you for the submission of your manuscript and proposed point-by-point response to EMBO reports and for your enquiry about the potential suitability of your study for our journal.

We certainly recognize that you will provide data on ATRX binding to and R-loop formation at endogenous telomeres, and that you will use a second shRNA to deplete ATRX as well as ATRX KO cells. However, your revised model that ATRX does not directly recognize R-loops but rather binds to G4s whose formation is potentiated by R-loops (as far as I understand your explanation), does not seem to be supported by additional experimental evidence. The data in your manuscript show a correlation between ATRX binding to DNA repeat sequences and transcription, and ATRX-induced inhibition of R-loops. It remains unclear though how ATRX exerts its effect.

If you could provide additional evidence for your model, I would certainly send your revised study back to the referees. Given the current point-by-point response I have to say that I am not sure that it will convince the referees. EMBO reports usually does not ask for extensive mechanistic insight but we also do not publish papers that report correlations and interesting observations (like ATRX inhibiting R-loops) without further insight. We would also require some data that indicate how ATRX acts. That said, I would discuss your revised manuscript with the referees to see what they think, but you would need to convince at least 2 of the 3 referees in order to proceed with the manuscript here.

 

Another option I can offer is a fresh round of peer-review of your revised manuscript, but my prediction is that the outcome will be very similar. I would finally like to add that 3 independent biological repeats are sufficient for statistical analyses for your ATRX ChIP experiments, and that such analyses should be performed. I hope that my response was helpful; please let me know if you have any questions.

1st Revision - authors' response 05 January 2017

Thank you for an opportunity to submit a revised version of our manuscript The chromatin remodelling factor ATRX suppresses R-loops in transcribed telomeric repeats for consideration by EMBO Reports. As you know, over the past few years work based on our findings and those of others have shown that ATRX acts as an important tumour suppressor in a substantial group of malignancies which rely on the so-called alternative pathway of telomere maintenance (ALT). This is therefore a major current topic of interest. The aim of the field is now to understand how ATRX is recruited to telomeres and what role it normally plays at these repeats. In the current work, we have made significant progress in understanding how ATRX is recruited both in vivo and in a well controlled experimental system. This has enabled us to propose a model of how ATRX might normally resolve G4 structures at telomeres, consistent with our previously reported observation that ATRX can recognise such secondary DNA structures (Law et al Cell 2010). Our new findings also suggest how an absence of ATRX may promote homologous recombination and the ALT pathway.

To provide some more detail, in the past it has not been clear how and under what circumstances ATRX binds to G-rich tandem repeats as this varies from one cell type to another. We now show that transcription is an important factor. We have previously shown that these targets can form G quadruplex (G4) structures in vitro and that ATRX can bind these. However G4 can only form if the DNA is single stranded and one way by which this can occur is via transcription. It has previously been shown by others that R-loops, RNA-DNA hybrids, can also arise at G-rich sequences when transcribed and these stabilise G4 structures when the non-template strand is G-rich to form G-loops. We show that R-loops form at the G-rich repeats in our study and although they form in either orientation of the repeat they are more stable when the G-rich strand is the non-template. Critically we show that ATRX only binds when the G-rich strand is non-template, the orientation which would allow G4 to form, consistent with a model by which ATRX binds G4 at these repeats. Manipulating R-loop levels through treatment with the topoisomerase 1 inhibitor, camptothecin, leads to changes in ATRX binding at ectopic and endogenous telomeric repeats consistent with the role of R-loops in promoting ATRX binding. We finally demonstrate using 3 independent shRNAs against ATRX that loss of ATRX results in an increase in R-loop formation. Furthermore, re-expression of ATRX in ATRX null cells (U-2 OS), which use the ALT pathway, leads to a decrease in R-loops, strongly suggesting a role for ATRX in resolving R-loops or suppressing their formation. For the first time, I think, this gives us an indication that ATRX has a role helping process DNA secondary structures.

The referees' comments for our previous EMBO submission were very insightful and helpful in indicating how the manuscript could be strengthened. We have now addressed their concerns by completing experiments to provide the additional supporting data they required. Attached is a point-by-point response indicating how we have addressed the referees' comments. We have refined our model for how ATRX interacts with tandem repeats including telomeres and in response to the referees' points have clarified some of the ideas and associated text. These changes have strengthened the manuscript substantially and I hope that you will consider this manuscript for publication in EMBO reports and indicate how we might make the revised submission.

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RESPONES TO REFEREES

**The chromatin remodelling factor ATRX suppresses R-loops in transcribed telomeric repeats**

Referee #1:

The mechanisms by which ATRX interacts with telomeres are not understood nor have its precise functions been defined. Strikingly, however, ATRX is generally lost in cancer cells that maintain their telomeres by the ALT-pathway, which involves homologous recombination. In the current

paper, the authors attempt to elucidate the interaction of ATRX with telomeric repeats and they suggest functions for ATRX in resolving R-loops.

The authors report ATRX ChIP-seq experiments, which suggest that ATRX binds largely to different genes in embryonic stem cells, fibroblasts and fetal liver. It is concluded from the data that ATRX is recruited more prominently to transcribed genes but the differences seem not very striking as the boxes in the Figures overlap to large extents (Figure 1).

A statistical test was applied and the p values show that the difference is significant. These preliminary observations prompted the detailed functional analysis, which supports our original hypothesis.

In order to study ATRX recruitment to telomeric repeats, TTAGGG-repeats are fused to an inducible strong promoter and inserted into the genome of 293T-Rex cells. Upon induction of transcription, ATRX is recruited to the trans-gene (Figure 2). Additional experiments indicate that the transcribed telomeric repeats accumulate R-loops (Figure 4) in consistency with previous reports on R-loops at telomeres. Transcription of the other telomeric strand (TTAGGG-template) did not induce recruitment of ATRX to the transgene. However, R-loops were still formed even with this template and only somewhat reduced (Figure EV4). Camptothecin treatment, which inhibits topoisomerase I increased R-loops at the TTAGGG-ectopic locus and at the same time increased ATRX recruitment. The authors take this as an argument that R-loops recruit ATRX but this correlation may be fortuitous. It is also reported that ATRX-KD leads to R-loop accumulation but the experiment is incomplete as only one shRNA was used (see specific comments). It is also not investigated if and how ATRX cooperates with RNaseH1 or other factors to remove R-loops. Overall, the concepts in the paper are not sufficiently developed and the conclusions are not convincingly supported by the data. The mechanisms of ATRX recruitment remain fuzzy and the roles of ATRX for R-loop resolution uncertain and uncharacterized.

These points are addressed below.

Specific critique points:

1. Inversion of the telomeric repeats leads at most to a 50% reduction in R-loops (Figure EV4) but ATRX binding was reduced much more strikingly to levels that were observed without transcription (Figure 4D). If R-loops were recruiting ATRX as proposed, it seems that ATRX binding to the TTAGGG-template construct should have been stronger with than without transcription in Figure 4D. Thus, it seems equally or more plausible that ATRX is recruited by other mechanisms. For example, the UUAGGG-containing RNA or TTAGGG-containing displaced telomeric DNA both of which could adopt G-quadruplex conformations might recruit ATRX. Indeed the authors previously published interactions of ATRX with DNA G-quadruplexes in vitro. If they now want to propose that ATRX binds R-loops this should be tested in vitro.

We agree with the reviewer that it is most likely that ATRX is binding G4 on the non-template strand consistent with our in vitro studies. The observation that ATRX is not recruited with the repeat in the reverse orientation strongly supports this since in this situation the non-template strand, being C-rich would no longer form G4. It is possible that ATRX binds G4 RNA but the presence of R-loops at the repeat sequences make this less likely as hybridization of the nascent RNA into an R-loop structure is presumably unfavourable for the formation of RNA secondary structures.
Our results which show a reduction rather than abolition of RNA-DNA hybrids detected by the antibody S9.6 at the inverted repeats compared to the original orientation are consistent with the observation in Arora R. et al. 2014 Nat Comms. RNA:DNA hybrids can form in the reverse orientation (TTAGGG on the template strand) but are not as stable as the original orientation with CCCTAA on the template strand (Ratmeyer L et al 1994 Biochem). We think it most likely that stable R-loops promote the recruitment of ATRX to G4 as these secondary structures are mutually reinforcing as described by Duquette ML et al 2004 Genes & Development. We have previously shown that ATRX binds G4 DNA *in vitro* but it has hitherto not been clear how the G4 DNA might be generated. To form G4, a G-rich DNA sequence has to be single-stranded. Here we show the importance of transcription in the recruitment of ATRX and this suggests that stable R-loops formed during transcription extend the life-time of the non-template single-stranded DNA which potentiates G4 formation (Duquette ML et al 2004). We have rewritten this part to make this model clearer.

 

In addition, the authors should test if under mutant situations that are known to promote R-loops (e.g. RNase H1 depletion, THO-depletion, mRNA cleavage and polyadenylation mutants, etc) ATRX becomes recruited to sites of R-loops under these circumstances. At this stage, it can only be firmly stated that recruitment of ATRX to TTAGGG-repeats in the transgene correlates with induction of transcription.

We have attempted numerous times to manipulate the levels of RNase H1 including siRNA, knockout and overexpression with no success in affecting R-loop levels. This is consistent with the difficulties described in the study by Arora R. et al 2014 Nat Comms in which the authors show that RNaseH1 depletion stabilised telomeric R-loops in U-2 OS (ALT cells) but not in HeLa cells (non-ALT cells). Furthermore, they showed that RNaseH1 depletion did not affect R-loop abundance at the actin locus in either U-2 OS or HeLa, suggesting that RNaseH1 controls hybrids only at specific loci. We therefore used an alternative approach involving the use of Camptothecin (CPT).
CPT perturbs R-loops to different levels at different loci (as shown before (Marinello et al 2016 PLoS ONE) as well as in our system), which gives us an opportunity to see if ATRX binding faithfully correlates with R-loop enrichment. We have added data looking at changes in R-loop abundance and ATRX binding at endogenous telomeres by slot blot in the CPT experiment. The results show a decrease in R-loops upon treatment with CPT, which correlated with a reduction in ATRX binding at endogenous telomeres thus reinforcing our observations.

2. In the ATRX-KD experiments, only one shRNA was used. Therefore, off-targets effects are not excluded. Consequently, the reported effect of ATRX-KD is anecdotal. At least two shRNAs must be tested and give the same phenotype. Alternatively, the phenotype must be rescued upon expression of shRNA-resistant ATRX from a cDNA.

We have tested another two shRNAs against ATRX (shATRX90 and shATRX91). The results show consistent increase in R-loops in ATRX depleted cells compared to the control, strengthening our initial observation. Furthermore, we have added new data using our inducible ATRX expression system in the U-2 OS 22/3 cell line that was previously published (Clynes D et 2015 Nat Comms) and the results show that re-introduction of ATRX reduced R-loop levels in these cells, strongly supporting our hypothesis that ATRX suppresses R-loop formation.

3. In Figure 6D-G, it is reported that ATRX depletion reduces expression of the GFP reporter. A second population of low GFP expressing cells appears. It remains unclear if there is an effect of the ATRX knock down on splicing, transcription or DNA repeat stability. The RNA transcripts have not been characterized. At this stage, this experiment does not provide mechanistic insights.

We agree that the reason for reduced GFP expression is not determined sufficiently to give mechanistic insights. Nevertheless, these data show that there is no *increase* in transcription when ATRX is depleted, which rules out the possibility that the increase in R-loops upon loss of ATRX is due to increased transcription. We have now moved this into the appendix.

4. On page 18, the authors state that nascent transcripts with high G content thread back and invade the open DNA duplex, giving rise to R-loops. This statement does not appear to explain how transcription of TTAGGG-templates should give rise to R-loops (reported in Figure EV4) as in this experiment, the nascent RNA contains no G's at all.

Please see our answer above to point 1.

Referee #2:

ATRX, which is mutated in a rare form of alpha thalassaemia associated with mental retardation, has emerged as a crucial and interesting chromatin-remodelling enzyme. In concert with the histone chaperone DAXX, it mediates the incorporation of the variant histone H3.3 at genomic sites with a tendency to form secondary structures, notably G quadruplexes. Current thinking suggests that this promotes the continued chromatinisation of these genomic regions, preventing secondary structure formation and consequent genetic and epigenetic instability. How ATRX is recruited to the sites at which it acts remains incompletely understood. Thus far, it is known to be recruited to heterochromatic regions through its ADD domain, which can 'read' H3K9me3 in the context of

 

unmodified H3K4 and it is able to bind G-rich, quadruplex-forming DNA directly.

This new manuscript reports data suggesting that the recruitment of ATRX is also promoted by the formation of co-transcriptional R-loops, RNA:DNA hybrids that form between the nascent mRNA and DNA template. The authors report that the recruitment of ATRX to telomeric repeats, and a previously characterised ATRX substrate - the pseudo-repeat GC-rich VNTR minisatellite - is dependent on transcription. To do this they use a split GFP reporter construct recombined into a defined locus. They show that this recruitment correlates with the induction of R-loops and that, in the absence of ATRX, R-loop formation increases. The authors propose that ATRX suppresses R-loop formation and thus reducing the formation of G quadruplex DNA leading to replication stalling and genetic instability.

This paper contains some very interesting and elegant experiments. However, I think there are a number of points that require further attention as I am not convinced that the relationship between potential G quadruplex formation and R-loop formation, and hence the role of ATRX in suppressing these structures, is sufficiently clear. The hypothesis that R-loops per se are deleterious and whether they are directly or indirectly responsible for ATRX recruitment could be more clearly addressed. Two aspects of the study, in particular, could be extended to address these concerns.

A key prediction of the proposed model is that reducing R-loops in vivo would reduce ATRX recruitment. The standard approach to this question is to overexpress RNAseHI. This experiment is reported not to have been possible for technical reasons. The alternative approach, to treat cells with camptothecin to inhibit TopI, provides two data points with either an increase or decrease in R-loops correlating with ATRX recruitment. However, this evidence is not as strong as removing R-loops themselves. The camptothecin approach might be more statistically persuasive at a genome wide level, allowing correlation of the change in DIP signal with ATRX binding across a much wide range of sites.

We have strengthened the data in our CPT experiment by looking at the effect of CPT on R-loop formation and ATRX recruitment at endogenous telomeric sequences using slot blot, this confirms that the levels of both are correlated. This is of particular relevance regarding the role of ATRX at telomeres and how it might suppress the alternative lengthening of telomere pathway. These data are now included in a revised manuscript.

It is unclear whether ATRX actually binds R-loops directly, or whether recruitment is still via G quadruplex binding, the DNA structures being promoted by R-loop formation. Have the authors examined whether artificial R-loops can shift ATRX, in a similar manner to G quadruplex-forming sequences, as they have previously reported (Law et al. Cell 2010)?

Please see the answer to this point above in comments to Ref 1, point 1.

Other points:

Why is there such a sharp cut off in the transcription-dependent recruitment of ATRX between 42 and 71 repeats?

It's interesting that there is a threshold for ATRX recruitment for the sizes of the telomeric repeats. We don't have any explanation at the moment. However, such a threshold effect has been reported for the phenotypes of expanded repeats diseases. See http://www.nature.com/nrg/journal/v6/n10/fig_tab/nrg1691_T2.html. It might be associated with the likelihood of G4 structures forming. Alternatively, it might possibly be related to whether there is sufficient room for a nucleosome to be inserted. This point is rather too speculative to be included in the discussion.

What is known about the reporter integration site in the cells, and in particular the surrounding chromatin environment?

According to the manufacturer (Invitrogen) of the host cell line, the integration site (FRT) is in an active chromatin region, based on the expression of the control gene, which is integrated in the same FRT site.

 

Some statistical tests on the ATRX ChIP experiments are needed. In many cases the trend looks significant (e.g. EV2) but the difference between individual repeat lengths may not be.

We have now applied statistical tests. The difference in ATRX binding at different repeat telomeric repeat lengths is shown to be significant (Fig 1). In Figure EV2 although there looks to be a trend in ATRX binding with increased repeat length, only the difference between the longest repeat and no-repeat is significant and this is now indicated

The ChIP for H3 modifications (Figure 3A) is somewhat problematic. H3K4me3 is usually fairly tightly restricted around the TSS, which is consistent for the induced state. However, H3K4me3 enrichment in the uninduced state at the D4 primer pair is similar to that at exon 1 in the induced state. It is not clear why this is the case.

We agree with the reviewer that the profile of H3K4me3 is somewhat unexpected, however, this is very reproducible and we are confident that this accurately reflects the distribution of H3K4me3 at this ectopic repeat. Nonetheless, this experiment makes it clear that K4me3 is not a recruiter of ATRX although it might modulate ATRX binding level at this ectopic locus. Since the binding pattern of ATRX is anti-correlated with the distribution of K4me3 across the repeat. Furthermore, it has been reported that K4me3 inhibits ATRX binding to histone H3 (Eustermann S. et al 2011).

It would also be expected that H3K36me3 would increase on transcription induction, but this is not seen. Is this problem related to the chromatin fragmentation size used?

No, the K4me3 ChIP and K36me3 ChIP were performed on the same sonicated chromatin sample. It may be that the pCMV TetO2 inducible promoter is sufficiently leaky in the basal state for H3K36me3 to be induced.

The experiments showing a population of GFP-low cells in cells depleted of ATRX with a repeat sequence is interesting. By analogy with the proposals of the Proudfoot group, does this population exhibit H3K9me3 and DNA methylation?

We are omitting these data as the mechanism by which ATRX depletion affects gene expression is not defined (See ref 1. Point 3)

The proposal (p19) that ATRX may bind upstream of R-loops and then translocate in a 5'-3' direction is intriguing. Is there any existing evidence that ATRX could do this?

Yes, ATRX has been shown to have a translocase activity, displacing the third strand of a triple helix (Xue and Gibbon et al. 2003 PNAS) but the direction of this activity has not been determined so this is raised as a discussion point.

Referee #3:

The study by Nguyen et al. reports on the interesting observation that ATRX chromatin remodeler may be specifically targeted to genomic loci that are actively transcribed and comprise G-rich repeats, like the ones found at telomeres. Authors propose that ATRX recruitment may depend on the formation of RNA-DNA hybrids (R-loops) that were previously reported to be abundant at telomeres (TERRA-DNA hybrids). Conclusions from the study are drawn from a combination of genome-wide analyses in mouse cells and studies of ectopic telomeric repeats inserted into an artificial inducible system in human 293T cells. Although the abstract mostly refers to ATRX loss in ALT cells and how this may impact on R-loop formation at telomeres of these cells, no attempt has been done to test this hypothesis that remains speculative. Overall, although data are interesting and promising, I find the study mostly descriptive and speculative, with no attempt to look at endogenous telomeres (qPCR against 16p actually reflects subtelomeres, not telomeres), neither in mouse, nor in human cells. I also feel that the authors did not sufficiently acknowledge previous relevant studies in the field and fail to discuss their data in view of the existing literature.

Major comments:

 

- Figure 2. The text mentions that several stable 293T clones with integrated G-rich constructs were obtained and were all shown to have a single integration site (Southern blots are provided as Suppl info). This, it is claimed, is important to reduce the possible bias coming from the chromatin environment properties of the integration sites. Good, but then, we have absolutely no idea about how many stable clones were actually analyzed. The figure legend states "ATRX ChIP analysis in stable clones", with no further indication. Was the analysis performed on a pool of stable clones? I think that it is necessary to show data separately for independent stable clones. And this, for all three constructs: no repeats, (TTAGGG)42 and (TTAGGG)71. Like this, we cannot be convinced that the number of repeats matters. It would be convincing if, based on the analysis of, at least 5 independent clones for each construct, differences were statistically significant. This should be done.

The analysis was done in separate independent stable clones, not a pool of clones. The analysis was done in 4 independent experiments of 3 independent clones for the construct of 71 units, 3 independent experiments of 2 independent clones of the construct of 42 units and 2 independent experiments of 2 independent clones for the construct of No repeat. We have clarified this in the revised manuscript.

It is also important to check transcription-dependent recruitment of ATRX at endogenous telomeres. One possible experiment would be to compare ATRX recruitment (and R-loop formation-see below) at telomeres of isogenic human cell lines displaying various transcriptional activity at telomeres (HCT116 and DNMT KO for instance, see Nergadze et al, RNA, 2009) using a dot-blot assay with telomeric probe to monitor endogenous TTAGGG repeats (and not the 16p subtelomeric PCR). This technique has been extensively used previously to analyze endogenous telomeres and should be used here too.

We thank the referee for this suggestion. We obtained the cell lines and tried to do the experiment. However, we discovered that ATRX protein level is dramatically reduced in the HCT116 DKO compared to the control. We also observed an increase in expression levels of other proteins such as alpha-tubulin and vinculin (that we used as loading control). We think that as DKO of DNMT1 and DNMT3b reduces global methylation this affects global gene expression, including ATRX. Therefore, since many factors apart from TERRA expression are perturbed in these cells this is, unfortunately, not an appropriate model to see study ATRX recruitment.

- Figure 3. Same comment as above: how many clones were analyzed?

For K9me3 ChIP, four independent experiments were done on 2 independent clones. For K4me3, K36me3 and H3 ChIPs, three independent experiments were done on 2 independent clones. We have clarified this in the revised manuscript.

- Figure 4. Panel B: I do not understand why qPCR data are not shown for U1, D3, 16p-tel. They should appear on the graph. Can authors comment on why there is no decrease in R-loops at rDNA locus upon RNAseH treatment? Cfr comment above, authors should compare R-loop formation at endogenous telomeres of isogenic cell lines with various levels of TERRA expression using the dot-blot assay.

U1 is only 100bp from U2 and D3 is only 150bp from D2 as shown on the scheme. Initial experiments involving ATRX ChIP included U1 and D3 but they were subsequently omitted as they showed similar levels to U2 and D2.

We have discussed the poor response of R loops to RNAseH at the rDNA locus with experts in the field including the Gromak and Proudfoot groups and they also observed the same and they think R-loops are very concentrated in rDNA sites and the enzyme may not be able to access/process these regions.

- Figure 5. Showing that R-loops per se trigger ATRX recruitment to telomeric repeats is of foremost importance for this study. Authors were, however, not able to show this through RNAseH1 overexpression. They state that "it is commonly accepted that this approach (RNAseH1 overexpression or depletion) is not always successful". However, based on the work by Arora et al (Nat Comm, 2014), it seems that RNAseH1 overexpression only affects telomeric R-loops in the context of ALT cells, while telomerase-positive cells are insensitive. And same holds true for

 

siRNA-mediated depletion of RNAseH1. Surely, authors should refer to that study to comment on their failure to modulate R-loops in their ectopic context of telomeric repeats.
We thank the referee for pointing that out. We have cited the work by Arora et al. in our revised manuscript. We have also talked to many groups who work on R-loops and they all agreed that manipulating R-loop enrichment by RNaseH1 overexpression/deletion is only successful in certain circumstances.

- To circumvent that problem, authors made use of CPT, an inhibitor of topoisomerase I, believed to transiently increase R-loop formation but to eventually lead to their abolition. Agreeing with this dual consequence of CPT treatment, while rDNA R-loops were increased, 16p tel R-loops were unaffected and R-loops at ectopic telomeric repeats were decreased.. Although R-loop data were consistent with corresponding changes in ATRX recruitment at these various loci, the CPT approach is somehow unconvincing. It would also have been useful to have data showing R-loops and ATRX recruitment at endogenous telomeres of CPT-treated cells using the dot-blot assay (see above).

We thank the referee for that suggestion. We have done this slot blot for the endogenous telomeres and submitted the data in the revised manuscript.

- Figure 6. The shATRX experiment suggests that R-loops are increased at ectopic telomeric repeats. Again, R-loops at endogenous telomeres should be analyzed using the dot-blot assay. Authors also show that transcription of the ectopic locus decreases upon ATRX knock-down. Here too, authors should refer to previously published papers that showed reduced endogenous telomere transcription (TERRA) upon ATRX knock-down (Episkopou et al, Nucl Acids Res, 2014 and Eid et al, Mol Cell Biol, 2015) as information is not new but consistent.

We are grateful for the referee's advice and have included these references. We have also added data for R-loops at endogenous telomeres in our U-2 OS system where ATRX expression can be turned on and off.

Finally, the authors speculate a lot on the possible impact of their findings on ALT cells. Why has this hypothesis not been tested? Two studies reported that ATRX overexpression represses ALT mechanism (only one is mentioned however and authors should definitely cite Napier et al, Oncotarget, 2015). Using the same ATRX overexpression system in, for instance, U-2 OS ALT cells, authors should look at the impact on R-loop formation at endogenous telomeres of these cells. The experiment is not complicated and would add a lot to the study.

We agree this would strengthen the paper; we have included the data in the revised manuscript.

Minor comments:

-Using deep-sequencing approaches, authors show that ATRX recruitment is increased at endogenous telomeric repeats of mouse Suv39h dn cells. Would this be a consequence of mouse telomere transcription being increased as previously reported in human cells (Arnoult et al, Nat Struct Mol Biol, 2012)? This should be discussed.

We will discuss this possibility in the revised manuscript.

-Page 11: problem in the sentence related to H3K4me3.

We have amended this in the revised manuscript

-Page 12: writing that reduced nucleosome density could be a consequence of transcription is, in my opinion, over-stated as histone deposition following RNA PolII transcription-dependent eviction, occurs extremely rapidly.

Thank you to the reviewer for pointing this out; we have amended the manuscript.

 

2nd Editorial Decision 25 January 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed referee reports as well as cross-comments.

As you will see, while referees 2 and 3 are more positive, referee 1 raises several concerns. However, upon cross-commenting on each others' reports, it turns out that referees 2 and 3, while agreeing that more control experiments should be performed to quantify transcription, point out that in their opinion, not all concerns of referee 1 would need to be addressed. Specifically, as mentioned in points 1, 2, 4 and 5 of referee 1, the qRT-PCR control experiments should be performed. On the other hand, the 5 independent clones mentioned in point 1 are not necessary, and neither point 3 has to be addressed.

Given these constructive comments, I would like to invite you to address all referee concerns and submit a revised manuscript to EMBO reports as soon as possible, including a detailed point-by-point response. Please let me know when you anticipate submitting the final manuscript.

Note that Appendix figure S3, Figure 1C,D, EV2, and EV5 state that n=2, in which case no error bars can be calculated. Please either repeat the experiments one more time or remove the error bars. You can show the single data points along with their mean if n=2. Please also make sure that all error bars are specified in the figure legends.

Table EV3 lists GEO accession numbers, however, these must be part of the main manuscript file. Please add the accession numbers to the materials and methods section and delete table EV3. Table EV1A and B must be combined into one table, this can be done by adding one more column to the table, for example.

I look forward to seeing a revised version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

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REFEREE COMMENTS

Referee #1:

I have carefully read the revised version of the study by Nguyen et al. Based on a series of concerns that I list below, in my opinion this manuscript is not suitable for a publication in EMBO Reports.

Major concerns:

1) The link between transcription and ATRX presence at chromatin is still unclear. Although correlations appear to exist between transcription and ATRX presence using the ectopic system with 71 TTAGGG repeats (42 repeats does not show convincing ATRX binding upon transcriptional induction), there are a series of controls that are missing to be fully convinced. First, throughout the manuscript, qRT-PCR experiments are missing to control for the level of transcription (GFP-exon 1 AND GFP-exon 2 should be monitored by qRT-PCR). And, because the experiment of Fig. 1 is important, I suggested previously to have analyses done in 5 independent (TTAGGG)71 clones shown separately. This has not been done.
2) In Figure 2, we observe, as expected, R-loops directly upstream and downstream of the TTAGGG repeats. This localization of R-loops does not correspond to ATRX binding and there is no explanation as why this is the case. In the experiment in which orientation of the repeats is changed, ATRX binding drops, but, again, there is no qRT-PCR control.. Expression level of GFP-exon 1 and exon 2 should be compared in both cell lines and upon induction. It is also surprising to see that induction of R-loops is still quite high with (TTAGGG)71 template (Fig EV4).
3) I also previously suggested to analyze the impact of expression modulation on the recruitment of ATRX at endogenous loci (telomeres). I suggested to use DNMT KO cell lines but it appeared not to be possible. There are other published ways to modulate telomere transcriptional activity that authors could have tried.

 

4) In Fig 3, the CPT experiment is very confusing with sometimes increased and sometimes decreased R-loops. Is there any correlation with transcriptional activity of the loci in CPT-treated cells? ChIP data for telomere-bound ATRX in DMSO and CPT-treated cells are not convincing as input amounts were very different. The experiment in which RNAseH1 is overexpressed to remove R-loops appeared not to have worked (response to reviewers). This is a pity as this prevents testing hypotheses further.
5) In Fig 4, because of the lack of control for expression (qRT-PCR), we cannot conclude that ATRX directly impacts on R-loops by binding to these structures to unfold them as it may well be that ATRX impacts on transcription that, itself, impacts on R-loops. The ChIP experiment for R-loops at endogenous telomeres is not very convincing either.
Altogether, this study mostly brings correlative data that do not allow to build any mechanism about the interplay between transcription, ATRX and R-loops.

Minor concerns:

1) Napier et al reference is still missing in this revised manuscript.
2) Page 16, stating that "A similar reduction in transcription upon ATRX knock-down was observed at endogenous telomeres (TERRA)." does not make any sense in the context of the results presented. Authors' results suggest that ATRX rescue in U2OS cells reduces R-loops. How does the above sentence fit in that context? All this is extremely confusing.

Referee #2:

This paper shows that ATRX is recruited to telomeric repeats and this recruitment is dependent on repeat length, orientation and transcription. ATRX loss is associated with increased R-loop formation at telomeres providing evidence that ATRX can help suppress the formation of problematic secondary structures. As I reviewed this manuscript on its original submission to the EMBO Journal, I will [comment] on the revisions made to the paper. I raised two substantive points.

The first concerned the issue of whether R-loops are actually necessary to recruit ATRX, which is currently a correlation due to the technical difficulty, in this instance, of removing them with RNAseHI overexpression. The authors have strengthened their approach of using camptothecin and the correlation is now much more persuasive. My only minor issue is that the R-loop slot blots do not have loading controls (e.g. blotting with an anti-DNA antibody).

The second point concerns the chicken and egg problem of whether ATRX is being recruited by R-loops directly, or via the previously demonstrated interaction with G quadruplexes. The authors haven't directly addressed whether ATRX binds R-loops with an in vitro experiment, but I think the authors' argument in response to Reviewer 1 is correct.

The authors have also satisfactorily addressed the other points that I raised. Overall, I think the paper is significantly improved over the original submission and I think the paper represents an interesting step forward in the ATRX story.

Referee #3:

In the revised version of this paper, the authors have addressed several of the points I had raised or they explain why they cannot do the experiment. I think the Discussion could be shortened a bit. On the other hand, the authors should introduce TERRA for the general readership and cite the relevant papers.

Cross-comments from referee 3 on referee 1's report:

Major concerns:

1) The link between transcription and ATRX presence at chromatin is still unclear. Although correlations appear to exist between transcription and ATRX presence using the ectopic system with 71 TTAGGG repeats (42 repeats does not show convincing ATRX binding upon transcriptional induction), there are a series of controls that are missing to be fully convinced. First, throughout the

 

manuscript, qRT-PCR experiments are missing to control for the level of transcription (GFP-exon 1 AND GFP-exon 2 should be monitored by qRT-PCR). And, because the experiment of Fig. 1 is important, I suggested previously to have analyses done in 5 independent (TTAGGG)71 clones shown separately. This has not been done.

Comment: For the first point I agree that induction of the reporter must be checked, but usually the Tet-inducible promoters work well. Thus I would not expect a problem here. I find it exaggerated to request the experiment to be repeated with 5 independent clones. As the recruitment occurs specifically upon induction of the promoter (+dox) the effect should not be a clonal artifact.

2) In Figure 2, we observe, as expected, R-loops directly upstream and downstream of the TTAGGG repeats. This localization of R-loops does not correspond to ATRX binding and there is no explanation as why this is the case. In the experiment in which orientation of the repeats is changed, ATRX binding drops, but, again, there is no qRT-PCR control.. Expression level of GFP-exon 1 and exon 2 should be compared in both cell lines and upon induction. It is also surprising to see that induction of R-loops is still quite high with (TTAGGG)71 template (Fig EV4).

Comment: I agree that induction of the reporter can be checked by qRT-PCR. It should not pose a problem for the authors to carry out this control.

3) I also previously suggested to analyze the impact of expression modulation on the recruitment of ATRX at endogenous loci (telomeres). I suggested to use DNMT KO cell lines but it appeared not to be possible. There are other published ways to modulate telomere transcriptional activity that authors could have tried.

Comment: I agree this experiment could be carried out using an inducible system to promote TERRA transcription at a telomere. Such a system was described in (PLoS One. 2012;7(4):e35714. doi: 10.1371/journal.pone.0035714.) and the corresponding cell line could be requested. Is it essential to carry out this experiment? It would be a nice addition for sure but I'm not sure this is essential for a Report.

4) In Fig 3, the CPT experiment is very confusing with sometimes increased and sometimes decreased R-loops. Is there any correlation with transcriptional activity of the loci in CPT-treated cells?

Comment: Valuable point; the authors could check this by RT-qPCR.

ChIP data for telomere-bound ATRX in DMSO and CPT-treated cells are not convincing as input amounts were very different.

Comment: Indeed the inputs should be the same. Nevertheless I think the ChIP is OK.

The experiment in which RNAseH1 is overexpressed to remove R-loops appeared not to have worked (response to reviewers). This is a pity as this prevents testing hypotheses further.

5) In Fig 4, because of the lack of control for expression (qRT-PCR), we cannot conclude that ATRX directly impacts on R-loops by binding to these structures to unfold them as it may well be that ATRX impacts on transcription that, itself, impacts on R-loops.

Comment: Valid point. The authors should quantify transcription by RT-qPCR

The ChIP experiment for R-loops at endogenous telomeres is not very convincing either.

Comment: This ChIP data may be OK and according to the Figure legend it was repeated 3 times.

Altogether, this study mostly brings correlative data that do not allow to build any mechanism about the interplay between transcription, ATRX and R-loops.

Minor concerns:

 

1) Napier et al reference is still missing in this revised manuscript.
2) Page 16, stating that "A similar reduction in transcription upon ATRX knock-down was observed at endogenous telomeres (TERRA)." does not make any sense in the context of the results presented. Authors' results suggest that ATRX rescue in U2OS cells reduces R-loops. How does the above sentence fit in that context? All this is extremely confusing.

2nd Revision - authors' response 10 March 2017

RESPONSE TO REFEREES

Referee #1:

I have carefully read the revised version of the study by Nguyen et al. Based on a series of concerns that I list below, in my opinion this manuscript is not suitable for a publication in EMBO Reports.

Major concerns:

1) The link between transcription and ATRX presence at chromatin is still unclear. Although correlations appear to exist between transcription and ATRX presence using the ectopic system with 71 TTAGGG repeats (42 repeats does not show convincing ATRX binding upon transcriptional induction), there are a series of controls that are missing to be fully convinced. First, throughout the manuscript, qRT-PCR experiments are missing to control for the level of transcription (GFP-exon 1 AND GFP-exon 2 should be monitored by qRT-PCR). And, because the experiment of Fig. 1 is important, I suggested previously to have analyses done in 5 independent (TTAGGG)71 clones shown separately. This has not been done.

The level of transcription (GFP-exon 1 AND GFP-exon 2) showing induction of transcription by doxycycline is now included in Figure EV2A. As agreed with the Editor, data from 5 independent clones is not required.

2) In Figure 2, we observe, as expected, R-loops directly upstream and downstream of the TTAGGG repeats. This localization of R-loops does not correspond to ATRX binding and there is no explanation as why this is the case. In the experiment in which orientation of the repeats is changed, ATRX binding drops, but, again, there is no qRT-PCR control.. Expression level of GFP-exon 1 and exon 2 should be compared in both cell lines and upon induction. It is also surprising to see that induction of R-loops is still quite high with (TTAGGG)71 template (Fig EV4).

The level of transcription (GFP-exon 1 AND GFP-exon 2) showing induction of transcription by doxycycline is now included in Figure EV2B. R loops can form in both orientations of the repeat but it is known that they are more stable when the RNA is G-rich and the DNA C-rich. This is explained in the text and relevant papers referenced.

3) I also previously suggested to analyze the impact of expression modulation on the recruitment of ATRX at endogenous loci (telomeres). I suggested to use DNMT KO cell lines but it appeared not to be possible. There are other published ways to modulate telomere transcriptional activity that authors could have tried.

We have previously explained why we took the approach we used.

4) In Fig 3, the CPT experiment is very confusing with sometimes increased and sometimes decreased R-loops. Is there any correlation with transcriptional activity of the loci in CPT-treated cells? ChIP data for telomere-bound ATRX in DMSO and CPT-treated cells are not convincing as input amounts were very different. The experiment in which RNAseH1 is overexpressed to remove R-loops appeared not to have worked (response to reviewers). This is a pity as this prevents testing hypotheses further.

We provided data showing that camptothecin treatment leads to a reduction in transcription at the loci examined (Figure S3 in the appendix) and explain that changes in ATRX binding are not correlated with transcription on p15.

 

5) In Fig 4, because of the lack of control for expression (qRT-PCR), we cannot conclude that ATRX directly impacts on R-loops by binding to these structures to unfold them as it may well be that ATRX impacts on transcription that, itself, impacts on R-loops. The ChIP experiment for R-loops at endogenous telomeres is not very convincing either.
Altogether, this study mostly brings correlative data that do not allow to build any mechanism about the interplay between transcription, ATRX and R-loops.

We have shown that the increase in R-loops associated with ATRX knock-down was not due to increased transcription; GFP expression, by FACS analysis, was reduced after ATRX knockdown (Figure S4 in the appendix). This is discussed on p16.

Minor concerns:

1) Napier et al reference is still missing in this revised manuscript.

This reference has been added.

2) Page 16, stating that "A similar reduction in transcription upon ATRX knock-down was observed at endogenous telomeres (TERRA)." does not make any sense in the context of the results presented. Authors' results suggest that ATRX rescue in U2OS cells reduces R-loops. How does the above sentence fit in that context? All this is extremely confusing.

We have removed this sentence.

Referee #2:

This paper shows that ATRX is recruited to telomeric repeats and this recruitment is dependent on repeat length, orientation and transcription. ATRX loss is associated with increased R-loop formation at telomeres providing evidence that ATRX can help suppress the formation of problematic secondary structures.

As I reviewed this manuscript on its original submission to the EMBO Journal, I will [comment] on the revisions made to the paper. I raised two substantive points.

The first concerned the issue of whether R-loops are actually necessary to recruit ATRX, which is currently a correlation due to the technical difficulty, in this instance, of removing them with RNAseHI overexpression. The authors have strengthened their approach of using camptothecin and the correlation is now much more persuasive. My only minor issue is that the R-loop slot blots do not have loading controls (e.g. blotting with an anti-DNA antibody).

Thank you for your comment. We have normalised all of our R-loop DIP data to the level of Input to give a percentage Input as is the standard presentation for all ChIP/DIP data. Any variability in the initial starting amount of DNA is therefore always internally controlled within each experiment, negating the requirement for a further loading control. This method is widely accepted in the literature for analysing ChIP data by slot blot (see Clynes et. al., 2015 Nature Comms 6:7538).

The second point concerns the chicken and egg problem of whether ATRX is being recruited by R-loops directly, or via the previously demonstrated interaction with G quadruplexes. The authors haven't directly addressed whether ATRX binds R-loops with an in vitro experiment, but I think the authors' argument in response to Reviewer 1 is correct.

We are grateful that the referee accepts our argument

The authors have also satisfactorily addressed the other points that I raised.

Overall, I think the paper is significantly improved over the original submission and I think the paper represents an interesting step forward in the ATRX story.

 

Referee #3:

In the revised version of this paper, the authors have addressed several of the points I had raised or they explain why they cannot do the experiment. I think the Discussion could be shortened a bit. On the other hand, the authors should introduce TERRA for the general readership and cite the relevant papers.

We have added an additional sentence introducing TERRA with the relevant reference.

Cross-comments from referee 3 on referee 1's report:

Major concerns:

1) The link between transcription and ATRX presence at chromatin is still unclear. Although correlations appear to exist between transcription and ATRX presence using the ectopic system with 71 TTAGGG repeats (42 repeats does not show convincing ATRX binding upon transcriptional induction), there are a series of controls that are missing to be fully convinced. First, throughout the manuscript, qRT-PCR experiments are missing to control for the level of transcription (GFP-exon 1 AND GFP-exon 2 should be monitored by qRT-PCR). And, because the experiment of Fig. 1 is important, I suggested previously to have analyses done in 5 independent (TTAGGG)71 clones shown separately. This has not been done.

Comment: For the first point I agree that induction of the reporter must be checked, but usually the Tet-inducible promoters work well. Thus I would not expect a problem here. I find it exaggerated to request the experiment to be repeated with 5 independent clones. As the recruitment occurs specifically upon induction of the promoter (+dox) the effect should not be a clonal artifact.

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Comment: Valuable point; the authors could check this by RT-qPCR.

ChIP data for telomere-bound ATRX in DMSO and CPT-treated cells are not convincing as input amounts were very different.

Comment: Indeed the inputs should be the same. Nevertheless I think the ChIP is OK.

 

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Comment: Valid point. The authors should quantify transcription by RT-qPCR

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Comment: This ChIP data may be OK and according to the Figure legend it was repeated 3 times.

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2) Page 16, stating that "A similar reduction in transcription upon ATRX knock-down was observed at endogenous telomeres (TERRA)." does not make any sense in the context of the results presented. Authors' results suggest that ATRX rescue in U2OS cells reduces R-loops. How does the above sentence fit in that context? All this is extremely confusing.

3rd Editorial Decision 20 March 2017

Thank you for the submission of your revised manuscript. We have now received the enclosed report from referee 2 who was asked to assess it and who overall supports the publication of your study. Only a few changes are necessary before we can proceed with the official acceptance of your manuscript.

Please explain whether the specification of the error bars and "n" refers to all or a single figure panel only. It is currently not clear for Fig 2, 4, 5 and EV5. In Fig S4 "n" still needs to be specified.

The legend for Fig 4 is not in the correct order (it has "e, g, f, h"), please correct.

Some Figs are submitted at insufficient resolution, please upload all figures at a minimum of 300 dpi. Please also change all landscape figures to portrait format. Please also change the "experimental procedures" header to "Materials & Methods". Please add a table of content page to the Appendix.

Please add a running title and up to 4 keywords.

The EV table legends should be deleted from manuscript page 46.

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

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REFEREE COMMENTS

Referee #2:

Although a number of issues remain unresolved in this work, stemming largely from the difficulty with ectopic RNAseHI expression, the findings are interesting and I feel the authors have gone far enough to address the original concerns raised.

 

3rd Revision - authors' response 21 March 2017

Authors made requested changes.

4th Editorial Decision 27 March 2017

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

 

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| Corresponding Author Name: Richard Gibbons |
| Journal Submitted to: EMBO Reports |
| Manuscript Number: EMBOR-2016-43078V2-Q |

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

**A- Figures**

**1. Data**

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

**2. Captions**

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

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**B- Statistics and general methods**

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

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| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | Not applicable |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | Not applicable |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | Not applicable |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | Not applicable |
| For animal studies, include a statement about randomization even if no randomization was used. | Not applicable |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | No subjective measurements were made. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | Not applicable |
| 5. For every figure, are statistical tests justified as appropriate? | Yes |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Yes. The statement was included in Statistical analysis in Materials and Methods section, p30. |
| Is there an estimate of variation within each group of data? | Yes. The statement was included in Statistical analysis in Materials and Methods section, p30. |
| Is the variance similar between the groups that are being statistically compared? | The statement is included in Statistical analysis in Materials and Methods section, p30 |

**C- Reagents**

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|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | All the antibodies used in the study were cited with catalog numbers and companies provided in the materials and methods section of the manuscript, p24-25. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | 293T-Rex cell line was purchased from Invitrogen (p22) and U2OS 22/3 was derived from U2OS in our laboratory. They were all tested for mycoplasma contamination. |

* for all hyperlinks, please see the table at the top right of the document

**D- Animal Models**

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | Not applicable |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | Not applicable |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | Not applicable |

**E- Human Subjects**

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | NA |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | NA |

## F- Data Accessibility

| | |
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| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | GEO Accession Numbers of published data are provided in the Materials and Methods section, p27. Other datasets that were generated in this study are pending for deposition. |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | We are in the process of depositing the data. |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | Not applicable |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | Yes, p30-31. |
| 22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | Not applicable |

## G- Dual use research of concern

| | |
|---|---|
| 23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | No |