Disruption of a ~23-24 nucleotide small RNA pathway elevates DNA damage responses in *Tetrahymena thermophila*

Suzanne Lee, Daniel Pollard, Domenico Galati, Megan Kelly, Brian Miller, Christina Mong, Megan Morris, Kerry Roberts-Nygren, Geoffrey Kapler, Matthew Zinkgraf, Hung Dang, Erica Branham, Jason Sasser, Erin Tessier, Courtney Yoshiyama, Maya Matsumoto, and Gaea Turman

*Corresponding author(s): Suzanne Lee, Western Washington University*

**Review Timeline:**

- Submission Date: 2020-10-13
- Editorial Decision: 2020-11-05
- Revision Received: 2021-04-11
- Editorial Decision: 2021-05-03
- Revision Received: 2021-05-09
- Accepted: 2021-05-10

*Editor-in-Chief: Matthew Welch*

**Transaction Report:**

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Dr. Lee:

As you will see from their comments, both reviewers found your study of interest and we are interested in looking at a revised manuscript. The one point both referees raise is that they would like to see some more evidence regarding the role of the RdRP complex components in genome integrity and DNA damage. We would not expect a full delineation of the relevant mechanisms but some additional information on the type of DNA damage or the nature of the affected DNA damage processes would greatly strengthen the paper.

Sincerely,

Tom Misteli
Monitoring Editor
Molecular Biology of the Cell

------------------------------------------------------------------------

Dear Dr. Lee,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors
Reviewer #1 (Remarks to the Author):

I found this is a good study that carefully describing cytological defects and gene expression alternations caused by loss of the components of RdRP complexes (RDRC) in Tetrahymena. This study shows that an RNAi pathway is required for somatic genome integrity in this organism, which provides us important insights about evolution of the relationship between RNAi and chromatin regulations.

A negative aspect I found in this study is that it remains unclear in which process the RDRC components are involved. Although the phenotypes caused by the loss of RDRC components (slower cell growth, accumulation of DSBs, larger and more frequent appearance of Extranuclear chromatin bodies, up-regulations of DNA replication stress, damage response and repair genes) are clearly described, their causal relationships are not investigated. While this could mostly be a topic of the authors’ future research, the authors may consider to add a few experiments which may provide some hints for the role of the RDRC components in protecting the genome integrity.

For example, because the defects caused by the loss of Rsp1 and Rdn2 are similar to those in cells treated with hydroxy urea (HU), I think it is very interesting to ask if RDRC co-localizes with Rad51/gammaH2A.X foci upon HU treatment. If this is the case, one can argue that RDRC acts in DSB repair under DNA replication stress.

Otherwise, I just have several minor comments:

1) Abstract, ~line 28: I think it is better to avoid using gene names in the abstract without explaining what they are.

2) Because several RDRC components with similar names are studied in this paper, it would be helpful for readers to show a schematic drawing presenting the compositions of different RDRC (like the one shown in Figure 1A of Talsky and Collins 2012) at the beginning of this paper.
3) line 106: If S phase is just slowed, more EdU positive MACs (with fainter signals) would be expected by EdU pulse labeling. I believe less EdU positive cells means either relative duration of the S-phase in cell cycle is shorter or cell cycle is stalled at some step (not necessarily at the onset of S-phase). I think the interpretation of the results here are better to be reconsidered.

4) line 119 and Figure 2A: Although I can see lagging chromosomes (or chromosomal pieces), it is not clear if there are more of them in RSP1Δ/RDN2Δ than in WT. Some quantification is necessary.

5) Figure S3: Only the legend is visible in the pdf file of Figure S3. If the Excel file is the supplemental material that the authors intend to show, it is better to be uploaded as a table or other supplemental material.

6) Figure 5C: It looks like Rad51/gammaH2A.X are outside of the DAPI positive area. Are they really on chromatin?

7) Because Rdn2 is the component of one of the three RDRC, I think the milder phenotypes in RDN2 KO than in RDR1 KO is quite reasonable. On the other hand, I am puzzled by the phenotype of RSP1 KO. Although Rdr1, Dcr2 and Rsp1 are all required for the biogenesis of 23-24-nt sRNAs, Rdr1 and Dcr2 are essential while Rsp1 is only involved in maintaining genome integrity. Do these mean Rdr1 and Dcr2 have a sRNA-independent function other than (or in addition to) maintaining genome integrity? Although this might not be directly related to this study, it would be nice if the authors consider to add some comment/discussion.

8) I found some points in the manuscript that do not meet the requirements listed in a section of the MBoC Author Submission.
   Section I: It is not described which statistical tests were used for the experiments shown in Figure 1C.
   Section IV: BioProject: PRJNA669066 seems not available yet in Sequence Read Archive (SRA) database.
   Section IV: Information on programming language(s) for the scripts used for RNA-seq and image analysis are not provided.

Reviewer #2 (Remarks to the Author):

Review for "Disruption of a ~23-24 nucleotide small RNA biogenesis pathway leads to DNA damage in Tetrahymena thermophila" by SR Lee et al.

This manuscript analyzes the effect of important, but non-lethal sRNA biogenesis mutants on gene expression in T. thermophila. These sRNA biogenesis mutants exhibit slowed growth, see delayed or slowed DNA replication, and enlarged chromatin extrusion bodies. Through RNA-seq they find that genes involved in the cell cycle, DNA metabolism and chromosome organization are differentially expressed in sRNA biogenesis mutants. In particular, they find that RAD51 levels are increased in these mutants and that RAD51 localized to areas of DSBs in macronuclear foci. Altogether these findings fit together nicely to define a new, important role for RNAi-dependent mechanisms in protecting the genome in T. thermophila. Given the similarities between T. thermophila and other organisms, these findings will likely generate a high level of interest.

However, there are some points that could be strengthened as outlined below:
1 - The data certainly suggests that the sRNA biogenesis pathway leads to DNA damage, but the authors don't specifically show that DNA damage occurs. Thus, the title could be softened to more accurately reflect this.

2 - The data is well presented as described in Section I. In addition, the methodology and statistics are well described in the Figure Legends and in the Results/Discussion section. However, it could be further helpful to have a separate Statistical Analysis section in the Materials and Methods section of the paper. Additionally, there are many instances when counting or digital analysis of microscopic images is done (e.g. Fig 1c, Fig 2b,c and Fig sup 4). However, it is unclear if blinded analysis was done on these samples to minimize bias. This should be clearly described.

3 - With the delayed growth of rsp1Δ and rdn2Δ strains it is important to describe the timing of cells collected for study, since the time of collection could impact results. Were the cells staged to be collected at the same time in the cell cycle or were samples collected from mixed cell cycle stages (i.e. asynchronous)? The authors' proposed study of the relative timing of DNA replication, DSBs and CEB formation and fate will be important to determine if such effects exist.

4 - In Fig 2A it is unclear what the yellow arrows in the DIC pictures refer to. This is particularly confusing because these DIC arrows point to a different region of the image than is pointed to in the DAPI, -pH3S10 and merge images. Please clarify. It might also be helpful to enlarge the area of the DIC that is pointed to, if it really points to something important because it's hard to see otherwise.

5 - There is some inconsistency throughout the paper in which strains have data shown. For example, in Fig 2 the RDF2Δ data is included in B, but not A or C. Additionally, RDF1Δ is included in most figures but not Fig 2. Consistency, or explanations associated with data inclusion would be helpful.

6 - The results/discussion text notes that there were significantly over-enriched GO terms in the RSP1Δ, RDN2Δ, and RDF2Δ strains. Were there no over-enriched GO terms in the RDF1Δ strain? Is this why there is no data associated with the RDF1Δ strain in Sup. Fig 3, even though the legend mentions this strain? If there is no data associated with the RDF1Δ strain, why is it included in Fig 3? Importantly in Sup. Fig. 3C, color intensity associates with more genes, but the few genes impacted in the RDF1Δ strain means that you can't tell which classes those genes fall into. Is there a way to show where those genes fall without compromising the clear depiction of GO analysis in the other strains?

7 - On first glance (fig 3) RDF2Δ GO enrichment genes matched RSP1Δ genes more than RDF2Δ particularly in the cell cycle and division category. Based on their functions why do you think this is? Why isn't the phenotype of RDF2Δ more similar to RSP1Δ and RDN2Δ?

8 - The authors mention that there is a significant number of downregulated genes in the RNA-seq analysis. Do you think the downregulated genes are direct targets of the sRNA biogenesis genes, or downstream targets of RNAi pathways?

9 - The RNAseq data is strong and it is great that the authors showed that RAD51 protein levels were altered. It would also be nice to further confirm some of the RNAseq data through qRT-PCR.
Minor Points
Line 195 refers to Rad51 localization in both RSP1 and RDN2, but sup fig 4A only labels and refers to RDN2 lines. Are one of these mislabeled?

Line 628 lists RDF2 twice, when one should refer to RDF1
Dr Tom Misteli  
Monitoring Editor, Molecular Biology of the Cell  

Dear Dr. Misteli,

I am pleased to submit our revised manuscript for consideration as a Brief Report in *Molecular Biology of the Cell*. This study represents research conducted primarily by several undergraduate student researchers and collaborators located at Western Washington University in Bellingham, WA, with contributions from collaborators at Texas A & M University.

We greatly appreciated the Monitoring Editor’s and Reviewers’ comments, and hope that we have adequately addressed them below in our point-by-point rebuttal.

Sincerely and with thanks for your continued consideration,

Suzanne R Lee (Corresponding and Senior Author)
As you will see from their comments, both reviewers found your study of interest and we are interested in looking at a revised manuscript. The one point both referees raise is that they would like to see some more evidence regarding the role of the RdRP complex components in genome integrity and DNA damage. We would not expect a full delineation of the relevant mechanisms but some additional information on the type of DNA damage or the nature of the affected DNA damage processes would greatly strengthen the paper.

A major addition to our revised manuscript is evidence for the involvement of Twi8 in repressing accumulation of markers of macronuclear (somatic) DNA damage (ie. elevated Rad51 protein levels and Rad51 and gammaH2A.x nuclear foci). Please see Figure 5, lines 257-260 in the revised manuscript. Previous studies found that Twi8 both binds to ~23-24 nt sRNAs and localizes to the macronucleus under normal growth conditions (Couvillion et al 2009, Farley and Collins 2017). Thus, our new findings suggest that the role of RdRP complex (RDRC) components in genome integrity is to generate sRNAs that Twi8 binds to for the purpose of maintaining genome integrity, perhaps similar to the sRNA-directed role that RNAi pathways play in other organisms in influencing chromatin structure, DNA repair, or other emerging functions for RNAi in DNA metabolism, though it is also still possible that the RNAi machinery is operating in a sRNA-independent fashion as we mention in the revised Perspectives section. We appreciated recognition by the monitoring editor and Reviewer #1 that a full delineation of mechanisms is not expected for publication in MBoC at this time.

In addition to the major update described above, we have also made the edits described below in our point-by-point reply to the original Reviewers and added a reference published in February that describes a newly identified role for an endogenous RNAi pathway in DNA repair at transcription termination sites in human cells (see Hatchi et al 2021, lines 283, 287, 299, 530).

Reviewer #1 (Remarks to the Author):

I found this is a good study that carefully describing cytological defects and gene expression alternations caused by loss of the components of RdRP complexes (RDRC) in Tetrahymena. This study shows that an RNAi pathway is required for somatic genome integrity in this organism, which provides us important insights about evolution of the relationship between RNAi and chromatin regulations.

A negative aspect I found in this study is that it remains unclear in which process the RDRC components are involved. Although the phenotypes caused by the loss of RDRC components (slower cell growth, accumulation of DSBs, larger and more frequent appearance of Extranuclear chromatin bodies, up-regulations of DNA replication stress, damage response and repair genes) are clearly described, their causal relationships are not investigated. While this could mostly be a topic of the authors’ future research, the authors may consider to add a few experiments which may provide some hints for the role of the RDRC components in protecting the genome integrity.

For example, because the defects caused by the loss of Rsp1 and Rdn2 are similar to those in cells treated with hydroxy urea (HU), I think it is very interesting to ask if RDRC co-localizes with Rad51/gammaH2A.X foci upon HU treatment. If this is the case, one can argue that RDRC acts in DSB repair under DNA replication stress.

As indicated above, we have now included evidence that TWI8, which encodes a known ~23-24 nt sRNA binding protein that localizes under normal growth conditions to the macronucleus (Couvillion et al 2009, Farley and Collins 2017), is also required to maintain macronuclear genome stability. The absence of TWI8 leads to an elevation in Rad51 protein levels and Rad51 and gammaH2A.x nuclear foci (Figure 5, lines 257-260). This suggests that the role of RDRC components in genome integrity is to generate sRNAs that Twi8 binds to for its nuclear role in maintaining genome integrity, though it is certainly still possible that the RNAi machinery is operating in an sRNA-independent fashion as we discuss in the Perspectives section.
While our focus in this current study is on the role that the RNAi pathway plays during normal vegetative growth, we appreciate the Reviewer's suggestion of studying the localization of RDRC components in the presence of HU and are eager to pursue experiments in that vein in the future. Localization of endogenously GFP-tagged RDRC components Rdn2, Rdf1 and Rdf2 under normal growth conditions was previously found to be inconclusive, likely due to insufficiently high or concentrated GFP signal (Talsky and Collins 2012). We have not observed an increase in expression levels of endogenously tagged Rdn2 under HU treatment (data not shown), which suggests that our future localization studies of these biogenesis proteins will require an alternative approach to endogenous GFP-tagging.

Otherwise, I just have several minor comments:

1) Abstract, ~line 28: I think it is better to avoid using gene names in the abstract without explaining what they are.

Thank you for the suggestion. We have clarified text in the Abstract to include a brief description that these genes are involved in sRNA biogenesis (line 29).

2) Because several RDRC components with similar names are studied in this paper, it would be helpful for readers to show a schematic drawing presenting the compositions of different RDRC (like the one shown in Figure 1A of Talsky and Collins 2012) at the beginning of this paper.

Thank you for this suggestion as well. We have created a schematic and included it in Figure S1A. Please note that Rsp1 is not an RDRC component, but acts in a yet unknown way to permit the accumulation of all RDRC-dependent sRNAs (see lines 81-82).

3) line 106: If S phase is just slowed, more EdU positive MACs (with fainter signals) would be expected by EdU pulse labeling. I beleive less EdU positive cells means either relative duration of the S-phase in cell cycle is shorter or cell cycle is stalled at some step (not necessarily at the onset of S-phase). I thinks the interpretation of the results here are better to be reconsidered.

We agree and have now clarified the text discussing the EdU labeling (lines 105-112).

4) line 119 and Figure 2A: Although I can see lagging chromosomes (or chromosomal pieces), it is not clear if there are more of them in RSP1Δ/RDN2Δ than in WT. Some quantification is necessary.

Thank you for the suggestion of quantification. Unfortunately, in asynchronous cultures, cells engaged in amitosis (when the MAC genome content is beginning to segregate into prospective daughter cells) represent only a small fraction of cells (see Figure S1D, ~2-3%). In particular, 2 MAC 2 MIC cells which represent cells in late amitosis comprise ~0.2-0.8% of the asynchronous populations we examined. However, of these cells in RDN2Δ and RSP1Δ cultures (11 and 6 cells, respectively, out of all cells examined across all replicates), 100% were found to be in the late stages of cytokinesis with lagging MAC genetic material and a strong cytokinetic furrow. In contrast, in 2 MAC 2 MIC SB210 (parental control) and RDF2Δ cells (9 and 3 cells, respectively), 67% of 2 MAC 2 MIC cells were found to be in the earlier stages of cytokinesis (with a weak or apparently absent cytokinetic furrow) yet with MAC DNA already nearly segregated and only 33% were found to be in the late stages of cytokinesis. We have updated the text to include the % of cells undergoing amitosis in the cultures we analyzed so that readers may put our observation, such as it is, in the proper context (lines 124-128).

5) Figure S3: Only the legend is visible in the pdf file of Figure S3. If the Excel file is the supplemental material that the authors intend to show, it is better to be uploaded as a table or other supplemental material.

The Excel file was included as supplemental material; please let us know if we have uploaded our document incorrectly.

6) Figure 5C: It looks like Rad51/gammaH2A.X are outside of the DAPI positive area. Are they really on
Previous images have now been replaced with new images (presented in Figures 4C and 5C) taken with a higher resolution, confocal microscope that more clearly show that the vast majority of the elevated Rad51/gammaH2A.X signal we observe in mutant strains is present within MAC nuclei and presumably on chromatin. Moreover, in these images, we've co-stained for Rad51 and gammaH2A.X in the same cells to reveal simultaneous elevation of both Rad51/gammaH2A.X in the same nuclei. Although some of the extranuclear signal observed may reflect cytosolic pools of protein, minor punctate signals outside of the nuclei were determined through spectral analysis to be a combination of autofluorescence and non-specific secondary antibody staining rather than specific primary antibody staining. We have updated text in both the Methods and Figure 4 legend to include these updated approaches and analyses (lines 416-426).

7) Because Rdn2 is the component of one of the three RDRC, I think the milder phenotypes in RDN2 KO than in RDR1 KO is quite reasonable. On the other hand, I am puzzled by the phenotype of RSP1 KO. Although Rdr1, Dcr2 and Rsp1 are all required for the biogenesis of 23-24-nt sRNAs, Rdr1 and Dcr2 are essential while Rsp1 is only involved in maintaining genome integrity. Do these mean Rdr1 and Dcr2 have a sRNA-independent function other than (or in addition to) maintaining genome integrity? Although this might not be directly related to this study, it would be nice if the authors consider to add some comment/discussion.

We share the reviewer’s curiosity as to why Rdr1 and Dcr2 are essential, while Rsp1 is not. As discussed in lines 81-84, Rsp1 is required for the accumulation of ~23-24 nt sRNAs that are known to be RDRC-dependent, but not the ~23-24 nt sRNAs that are apparently RDRC-independent. It is possible that the basal level of RDRC-independent sRNA production by Dcr2 (perhaps influenced by physically associated Rdr1) is important for cell viability; however, whether the biogenesis pathway for the RDRC-independent sRNAs actually depends on Dcr2 has not yet been determined. Alternatively, as the reviewer suggests, Rdr1 and Dcr2 could have sRNA-independent roles. Since it is yet unknown what the essential functions of Rdr1 and Dcr2 are, we agree that they are not directly related to this study. However, we have added language in the Introduction that we hope clarifies that the essential functions of Rdr1 and Dcr2 are yet unknown (lines 88-90).

8) I found some points in the manuscript that do not meet the requirements listed in a section of the MBoC Author Submission. 
Section I: It is not described which statistical tests were used for the experiments shown in Figure 1C. Logistic regression was used and this is included in the figure legend for Figure 1C (lines 642-643).

Section IV: BioProject: PRJNA669066 seems not available yet in Sequence Read Archive (SRA) database. We apologize for this oversight; this BioProject is now accessible to reviewers using this link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA669066?reviewer=t5pf9sa0sfv8ptgospnt4mppe.

Section IV: Information on programming language(s) for the scripts used for RNA-seq and image analysis are not provided. This information has now been added in the Materials and Methods section under “Data deposition and code availability” (lines 463-466).

Reviewer #2 (Remarks to the Author):

Review for "Disruption of a ~23-24 nucleotide small RNA biogenesis pathway leads to DNA damage in Tetrahymena thermophila" by SR Lee et al.

This manuscript analyzes the effect of important, but non-lethal sRNA biogenesis mutants on gene expression in T. thermophila. These sRNA biogenesis mutants exhibit slowed growth, see delayed or slowed DNA replication, and enlarged chromatin extrusion bodies. Through RNA-seq they find that
genes involved in the cell cycle, DNA metabolism and chromosome organization are differentially expressed in sRNA biogenesis mutants. In particular, they find that RAD51 levels are increased in these mutants and that RAD51 localized to areas of DSBs in macronuclear foci. Altogether these finds fit together nicely to define a new, important role for RNAi-dependent mechanisms in protecting the genome in T. thermophila. Given the similarities between T. thermophila and other organisms, these findings will likely generate a high level of interest.

However, there are some points that could be strengthened as outlined below:

1 - The data certainly suggests that the sRNA biogenesis pathway leads to DNA damage, but the authors don't specifically show that DNA damage occurs. Thus, the title could be softened to more accurately reflect this.

To address this comment, as well as accommodate the addition of the TWi8Δ knockout data, our title has been updated to: “Disruption of a ~23-24 nucleotide small RNA pathway elevates markers of DNA damage in Tetrahymena thermophila”.

2 - The data is well presented as described in Section I. In addition, the methodology and statistics are well described in the Figure Legends and in the Results/Discussion section. However, it could be further helpful to have a separate Statistical Analysis section in the Materials and Methods section of the paper. Additionally, there are many instances when counting or digital analysis of microscopic images is done (e.g. Fig 1c, Fig 2b,c and Fig sup 4). However, it is unclear if blinded analysis was done on these samples to minimize bias. This should be clearly described.

Thank you for the suggestion for a separate section in the Materials and Methods section on Statistical analysis. We feel that in-context explanations of the statistical analysis performed better serves the Materials and Methods than a separate section and have accordingly added sentences about the analysis to each relevant section within the existing Materials and Methods (for example, lines 391-392 and 403-404).

We have also added text in the Materials and Methods that clarifies how bias was minimized in our analysis of our microscopy images (lines 401-402, 429, 439-441, and 458-459).

3 - With the delayed growth of rsp1Δ and rdn2Δ strains it is important to describe the timing of cells collected for study, since the time of collection could impact results. Were the cells staged to be collected at the same time in the cell cycle or were samples collected from mixed cell cycle stages (i.e. asynchronous)? The authors’ proposed study of the relative timing of DNA replication, DSBs and CEB formation and fate will be important to determine if such effects exist.

All cells, with the exception of HU-treated samples used as a positive control for Rad51 signal on Western blots (Figure 3B), were collected from asynchronous, mid-log cultures. We have added clarifying text to the Methods section in the revised manuscript (lines 331, 385, 395, and 407). We are thrilled that the Reviewer considers our proposed future study of timing to be of interest.

4 - In Fig 2A it is unclear what the yellow arrows in the DIC pictures refer to. This is particularly confusing because these DIC arrows point to a different region of the image then is pointed to in the DAPI, α-pH3S10 and merge images. Please clarify. It might also be helpful to enlarge the area of the DIC that is pointed to, if it really points to something important because it's hard to see otherwise.

Thank you for pointing out the confusing nature of our initial presentation of these data. We have now replaced the DIC arrows with a dotted outline of the dividing cells we wish to highlight.

5 - There is some inconsistency throughout the paper in which strains have data shown. For example, in Fig 2 the RDF2Δ data is included in B, but not A or C. Additionally, RDF1Δ is included in most figures but not Fig 2. Consistency, or explanations associated with data inclusion would be helpful.
We have updated the manuscript and data figures in several places throughout the manuscript to provide greater consistency and clarity for the data shown. Most notably, this included adding RDF2Δ into Figs 2C (the original omission of which was accidental) and removing RDF1Δ from figures where there was either no difference from parental control Sb210 (ex. CEB accumulation) or no results to report (ie. GO enrichment analysis), while preserving text that describes the negative results we observed for RDF1Δ where appropriate (lines 118, 178, 191-192). In Figure 2A, RDF2Δ was omitted due to space constraints, but our observations are quantified in Figures 2B and C.

6 - The results/discussion text notes that there were significantly over-enriched GO terms in the RSP1Δ, RDN2Δ, and RDF2Δ strains. Were there no over-enriched GO terms in the RDF1Δ strain? Is this why there is no data associated with the RDF1Δ strain in Sup. Fig3, even though the legend mentions this strain? If there is no data associated with the RDF1Δ strain, why is it included in Fig. 3? Importantly in Sup. Fig. 3C, color intensity associates with more genes, but the few genes impacted in the RDF1Δ strain means that you can't tell which classes those genes fall into. Is there a way to show where those genes fall without compromising the clear depiction of GO analysis in the other strains?

Please see clarifications in response to Comment 5 above. Indeed, there were no enriched GO terms in the differentially expressed gene set in RDF1Δ strain.

7 - On first glance (fig 3) RDF2Δ GO enrichment genes matched RSP1Δ genes more than RDF2Δ, particularly in the cell cycle and division category. Based on their functions why do you think this is? Why isn't the phenotype of RDF2Δ more similar to RSP1Δ and RDN2Δ?

Greater overlap in certain enriched GO term categories was indeed seen between RSP1Δ and either RDN2Δ or RDF2Δ than was observed of RDN2Δ and RDF2Δ. This is consistent with the requirement of RDN2 and RDF2 in the accumulation of distinct, though partially overlapping, subsets of sRNA classes while both RDN2- and RDF2- dependent sRNAs require RSP1 for accumulation (Couvillion et al., 2009; Talsky and Collins, 2012). These differences likely reflect the fact that Rdn2 and Rdf2 reside in different RDRCs (Supplemental Figure S1A) which likely have distinct RNA targets and may differ in biological functions. In addition, Rdf2 and Rdf1 both reside in distinct Rdn1-containing RDRCs, and it may be that Rdf1 in RDF2Δ cells suppresses the phenotypes more readily observed in RSP1Δ and RDN2Δ.

We have now added text to clarify the above in the Results and Discussion section (lines 184-188, 308-312). In addition, in our revised manuscript, the original Figure 3 can be found in Supplemental Figure S2C and a full listing of enriched GO term categories and subcategories is in Supplemental Figure S3.

8 - The authors mention that there is a significant number of downregulated genes in the RNA-seq analysis. Do you think the downregulated genes are direct targets of the sRNA biogenesis genes, or downstream targets of RNAi pathways?

We think the downregulated genes are not likely to be direct targets of the repressive functions of canonical RNA (see lines 163-168), though the mechanisms leading to the downregulated gene set will require future investigation.

9 - The RNAseq data is strong and it is great that the authors showed that RAD51 protein levels were altered. It would also be nice to further confirm some of the RNAseq data through qRT-PCR.

We appreciate this suggestion, but are not set up at the moment to pursue confirmation of our RNAseq data by qRT-PCR. However, we hope that the strength of the existing RNAseq data, the confirmation of RAD51 elevation at the protein level, and other corroborating evidence outside of changes in gene expression support our primary conclusions in such a way that what qRT-PCR of select genes would add may be modest (and may be more necessary in future studies that dissect the nature of DNA damage in RNAi pathway mutants).
Minor Points
Line 195 refers to Rad51 localization in both RSP1 and RDN2, but sup fig 4A only labels and refers to RDN2 lines. Are one of these mislabeled?

We limited the representative images of Rad51 and gamma-H2A.X localization data to SB210 and RDN2Δ in Supplemental Figure 4A for space considerations; however, analysis of Rad51 localization in RSP1Δ is included in the quantification presented in Figure 4A and B. We have clarified the text reference to Supplemental Figure S4A and updated the Supplemental Figure 4 legend as well (lines 214-215, 753-756).

Line 628 lists RDF2 twice, when one should refer to RDF1

Thank you for pointing this typo out; this issue has been resolved in the revised text.
Dear Dr. Lee-

We returned your manuscript to the original two reviewers and as you will see they are both supportive of publication. They point out a few minor corrections that should be made and I ask you to resolve these in a final version of the manuscript, which, I assure you, we will handle as expeditiously as possible toward formal acceptance.

Sincerely,
Tom Misteli
Monitoring Editor
Molecular Biology of the Cell

-------------------------------------------------------------------------------------------------

Dear Dr. Lee,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available
Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

I think the authors responded to my previous comments properly and the new data showing the involvement of nuclear Argonaute/Piwi (Twi8) in suppressing accumulation of DNA damage have strengthened the authors conclusion that ~23-24 nt sRNAs produced by RDRPs are important for maintaining genome integrity in Tetrahymena.

I just have a few minor comments.

1) In the data shown in Figure 5B, TWI2∆ seems to have a weak effect on Rad51 accumulation. Though this might be statistically significant, the authors may consider to mention about this effect.

2) In the legend for Figure 5B, it is written as p<0.008 first then p<0.009 later. If both are correct p<0.008 should be used.

3) I understand the title has been changed according to the reviewer 2's suggestion. Although it is very accurate, it may be less attractive to readers. I think the title can also be "Disruption of a ~23-24 nucleotide small RNA pathway elevates DNA damage response in Tetrahymena thermophila"

Reviewer #2 (Remarks to the Author):

This manuscript is nicely improved from the previous submission and the authors have made significant effort in addressing my concerns and the concerns of the other reviewer. In particular, the addition of the Twi8 data nicely adds to the idea that the RdRP complex components contribute to genome integrity.
Minor Point:
- Figure 2C is never referred to in the text beyond being described in the figure legend. There are clearly areas where this data is referred to in the text and it should be cited accordingly.
Dr Tom Misteli
Monitoring Editor, Molecular Biology of the Cell

May 8th, 2021

Dear Dr. Misteli,

I am pleased to submit our revised manuscript in response to the minor corrections kindly suggested by the reviewers for publication in Molecular Biology of the Cell.

We hope that we have adequately addressed the corrections outlined below in our point-by-point rebuttal.

Sincerely,

Suzanne R Lee (Corresponding and Senior Author)

Reviewer #1 (Remarks to the Author):

1) In the data shown in Figure 5B, TWI2Δ seems to have a weak effect on Rad51 accumulation. Though this might be statistically significant, the authors may consider to mention about this effect. To clarify, the effect of TWI2Δ on Rad51 levels is statistically insignificant, while TWI8Δ cells exhibited elevated Rad51 to levels that are similar to that observed in RDN2Δ. Assuming that the reviewer is indeed commenting on Rad51 levels in TWI2Δ, we have expanded on the text at lines 256-257 in the revised manuscript, as per the reviewer's suggestion.

2) In the legend for Figure 5B, it is written as p<0.008 first then p<0.009 later. If both are correct p<0.008 should be used. Our apologies for the typo. We have corrected the text to p<0.008 in line 706 in the revised manuscript.

3) I understand the title has been changed according to the reviewer 2's suggestion. Although it is very accurate, it may be less attractive to readers. I think the title can also be "Disruption of a ~23-24 nucleotide small RNA pathway elevates DNA damage response in Tetrahymena thermophila" Thank you so much for this suggestion. We have updated the title accordingly.

Reviewer #2 (Remarks to the Author):

Minor Point:
- Figure 2C is never referred to in the text beyond being described in the figure legend. There are clearly areas where this data is referred to in the text and it should be cited accordingly. We have revised our references of "Figure 2" to "Figure 2A-C" at lines 117 and 238 in the revised manuscript.
Dear Dr. Lee:

Thank you for addressing these remaining points. I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Tom Misteli
Monitoring Editor
Molecular Biology of the Cell

------------------------------------------------------------------------

Dear Dr. Lee:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
