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Activation of meiotic recombination by nuclear import of the DNA break hotspot-determining complex in fission yeast

Mélody Wintrebert, Mai-Chi Nguyen and Gerald R. Smith
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Original submission

First decision letter

MS ID#: JOCES/2020/253518

MS TITLE: Activation of meiotic recombination by nuclear import of the DNA break hotspot-determining complex

AUTHORS: Melody Wintrebert, Mai-Chi Nguyen, and Gerald R. Smith
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

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all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this paper, Wintrebert et al. have characterized the roles of a set of predicted nuclear localization signal (nls) which are associated with Rec10. The report show that the predicted “nls” are important for nuclear import of Rec10 and other linear element proteins including Rec25 and Rec27. In case, when nls are absent or mutated, cells show residual nuclear signal of LinE proteins and poor recombination. As the linear elements of S pombe are related to the synaptonemal complex proteins of other species, such exploration would be a valuable addition to the meiosis field. The study is reasonably well executed, however below are some suggestions to improve the manuscript.

Comments for the author

-Figure 2 and other microscopy images-
Data presented in different panels of fig. 2 and 3 shows different stages/timing of the horse-tailing. Are these images captured at similar timings during the horse-tailing stage of meiotic progression? In mutant panels except Δrec10, there are some foci, present in the nucleus. I am wondering if these images were taken at the late horse-tail stage when cells start showing elimination of these linear elements.

-Fig. 3: In the panel showing double mutant (ΔA ΔB), size of the nucleus (red signal) looks smaller and fragmented in comparison to the others. What is the reason behind this? Are these mutants cause any form of genomic stress or are sensitive to any genotoxic drugs?

-Page 4 para 2nd-
“….Rec25-GFP is degraded in absence of rec10….”
Have you tried estimating the actual rec25-GFP protein content in nuclear and cytoplasmic fractions separately, using western blotting? To be sure and to justify the statement, it would be better to do a western and see the actual protein contents. This will also rule-out the possibility of signal loss due to any potential conformation change.

-Page 4, last paragraph—
What are the key regulators for cytoplasmic aggregation of Rec10 and other LinE proteins? Are the “nls” self-sufficient to do this job? Have you tried putting these nls sequences with other LinE proteins and estimating their nuclear localization or effect on the recombination?

-In discussion part, page 7, second para-
“In the NLS mutants, nuclear Rec10 levels may be below or near the level of detection by microscopy but still be sufficient……”. To be sure, it would be great to show the level of these protein contents in nuclear and cytoplasmic fraction separately, using western blotting.

-In the introduction part, Page 3, para 1-
“The LinE complexes also form elongated structures mid-way through meiosis…” The study cited here (Chuang and Smith, submitted), tested this hypothesis in a synchronized azygotic meiosis. What happens in normal, asynchronous zygotic meiosis is not mentioned in the paper. Recently, there was a preprint posted on the BioRxiv “Escorcia et. al. 2020 (https://doi.org/10.1101/2020.06.15.153338)” , who did extensive timing and nuclear dynamics analysis of bunch of meiotic proteins including rec27-GFP. I think that would also be an appropriate reference for the line.

-In materials and method section, there is no mention of the reproducibility. It would be great to mention how many biological/technical replicates ran to draw the conclusions. Have you used any statistical tests to check the level of significance?

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- In figure legends, please include the strain numbers (GP.....) to correlate better.

-Please add a scale bar in microscopic image panels.

Reviewer 2

Advance summary and potential significance to field

The subject of this manuscript is the nuclear localization signal of the Rec10 protein, which is one component of the meiotic linear elements (LinE). An important conclusion that is supported by the data is that the Rec10 NLS allows coordinated nuclear entry of the whole complex. Figures 2-4 all show that NLS mutations block entry of protein like Rec25, Rec27 and Mug20 to the nucleus. My assessment is that, although the data is solid, it is not a full-length research article. It has one major finding that is not explored in much detail, examples of which are given below. Therefore, a short format paper would be more appropriate.

Comments for the author

Figures 2-4 show essentially the same result using different forms of the rec10 NLS mutants and assaying for nuclear entry of the LinE proteins. One set of experiments is based on a rec10 plasmid and the other set is based on chromosomal rec10 variants. Rec25 appears to be degraded in the absence of Rec10 (rec10delta). It would be useful to have localization data for the other LinE proteins in a rec10 null. It would also be useful to know the effect of rec25, rec27 or mug20 deletion mutants on the localization of other LinE proteins including Rec10. The authors should discuss the possibility that cytoplasmic Rec10 sequesters the other LinE proteins. Wild-type Rec10 may not interact with the other LinE proteins until it enters the nucleus. In the NLS mutants, which do not enter the nucleus, Rec10 binds to the other LinE proteins and prevents them from entering the nucleus. A prediction of this model is that the rec10 NLS mutants would have a dominant phenotype.

There is not enough discussion about the GFP variant and why it does not fully rescue the mutant phenotype. Is this because of the GFP, which could be tested if there was a plasmid form that had the GFP. Table 1 is a little confusing, although it appears that all the mutants were in the GFP backbone. It is little strange that none of the mutants are tested in the most natural background, the chromosome backbone without GFP.

The weaker mutants have strong cytoplasmic localization but support relatively high levels of recombination. In some images, they also appear to have some nuclear localization. This would be consistent with the phenotype but it does not come out clear in the manuscript whether mutants like AlaB have more nuclear localization than mutants with more severe recombination phenotypes. It would be helpful to see the GFP channels without DNA in the figures.

First revision

Author response to reviewers' comments

Author's response, in red, to reviews, in black

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In this paper, Wintrebert et al. have characterized the roles of a set of predicted nuclear localization signal (nls) which are associated with Rec10. The report show that the predicted "nls" are important for nuclear import of Rec10 and other linear element proteins including Rec25 and Rec27. In case, when nls are absent or mutated, cells show residual nuclear signal of LinE proteins and poor recombination. As the linear elements of S pombe are related to the synaptonemal complex proteins of other species, such exploration would be a valuable addition

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to the meiosis field. The study is reasonably well executed, however below are some suggestions to improve the manuscript.

Reviewer 1 Comments for the Author:
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Data presented in different panels of fig. 2 and 3 shows different stages/timing of the horse-tailing. Are these images captured at similar timings during the horse-tailing stage of meiotic progression? In mutant panels except Δrec10, there are some foci, present in the nucleus. I am wondering if these images were taken at the late horse-tail stage when cells start showing elimination of these linear elements.

Figures 2, 3, and 4 show homothallic (*h*^{90}) cells in asynchronous meiosis at an incubation time (16 hr) when cells at all stages of meiosis are present. We illustrate cells with elongated (horsetail) nuclei indicative of mid-meiosis. We show cells similar to many cells present at the time of examination and thus representative. It is unlikely that only very late meiotic cells would be present in these preparations. We repeated the experiments on 2 or 3 different days, further making it unlikely that only late stage cells are illustrated. The low recombinant frequency of the *nls* mutants (Table 1) indicates that some LinE protein, but not wt levels, must be present in the nucleus during these meioses, as the images show; the exception is *rec10Δ*, which has essentially no recombination and no visible nuclear LinE foci, further supporting our conclusions.

-Fig. 3: In the panel showing double mutant (ΔA ΔB), size of the nucleus (red signal) looks smaller and fragmented in comparison to the others. What is the reason behind this? Are these mutants cause any form of genomic stress or are sensitive to any genotoxic drugs?

In a random sample of meiotic cells, as we use, some horsetail nuclei are turning the corner at the end of the cell and appear small, sometimes nearly round. The seeming fragmentation may result from aberrant chromosome conformations in the absence of LinEs, which are normal chromosome components in wt. Since LinEs are expressed only in meiosis, we do not expect any sensitivity to genotoxic drugs in standard (mitotic) tests. They might be sensitive in meiosis, which could well reflect their recombination deficiency that we report (Table 1).

-Page 4 para 2nd-
"….Rec25-GFP is degraded in absence of rec10…."
Have you tried estimating the actual rec25-GFP protein content in nuclear and cytoplasmic fractions separately, using western blotting? To be sure and to justify the statement, it would be better to do a western and see the actual protein contents. This will also rule-out the possibility of signal loss due to any potential conformation change.

We regret our error here. Davis *et al.* (*Current Genetics*, 2008) showed by Western blot analyses that Rec25 and Rec27 are as abundant in *rec10Δ* as in wt. We have changed the wording on page 4 to correct our oversight, and we have added as Fig. S2 in the Supplemental Information data showing that Rec25-GFP fluorescence increases during meiosis in the nucleus strongly in *rec10*$^{+}$ and less, but still significantly, in *rec10Δ*. This increase, without visible foci, of Rec25-GFP in *rec10Δ* indicates that this small protein can enter the nucleus but remains uniformly distributed in *rec10Δ* and thus difficult to see. Cytoplasmic fluorescence of Rec25-GFP also increases in *rec10Δ* but not in *rec10*$^{+}$, as expected from our interpretations.

-Page 4, last paragraph—
What are the key regulators for cytoplasmic aggregation of Rec10 and other LinE proteins? Are the "nls" self-sufficient to do this job? Have you tried putting these nls sequences with other LinE proteins and estimating their nuclear localization or effect on the recombination?

Our results show that the NLS on Rec10 is necessary for nuclear localization of all four LinE proteins. Other research has shown that NLSs of other proteins in other species are sufficient for nuclear localization. We have not moved the Rec10 NLS to other LinE proteins. If this construction led to nuclear localization of the LinEs, as we expect, we think we would have learned little new; if it did not, that could well be because the conformation, complex-formation, or activity of the small (14 – 17 kDa) LinEs could not accommodate an inserted NLS.

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-In discussion part, page 7, second para-
"In the NLS mutants, nuclear Rec10 levels may be below or near the level of detection by microscopy but still be sufficient……". To be sure, it would be great to show the level of these protein contents in nuclear and cytoplasmic fraction separately, using western blotting.

Separating nuclear and cytoplasmic proteins in *S. pombe* meiotic cells for differential Western blots is difficult; we have unsuccessfully tried this in the past. We see large amounts of Rec10-GFP in the cytoplasm (Figure 3) and nearly full (wt) levels of total Rec10 in these mutants (Figure 6). Thus, there must be some Rec10 still in the nucleus, because Rec10 binds to DNA (Fowler et al., 2013) and is essential for formation of DSBs and recombinants (Ellermeier and Smith, 2005); there is no evidence that cytoplasmic Rec10 can promote recombination.

-In the introduction part, Page 3, para 1-
"The LinE complexes also form elongated structures mid-way through meiosis…" The study cited here (Chuang and Smith, submitted), tested this hypothesis in a synchronized azygotic meiosis. What happens in normal, asynchronous zygotic meiosis is not mentioned in the paper. Recently, there was a preprint posted on the BioRxiv "Escorcia et. al. 2020 (https://doi.org/10.1101/2020.06.15.153338)" , who did extensive timing and nuclear dynamics analysis of bunch of meiotic proteins including rec27- GFP. I think that would also be an appropriate reference for the line.

Chuang and Smith also studied matings between haploid cells of opposite mating-type, which give "normal, asynchronous zygotic meiosis" as we use here. Escorcia et al. followed individual cells, whereas the other papers examined populations of many cells. We do not think it proper to cite work from other labs that is not published in a refereed journal.

-In materials and method section, there is no mention of the reproducibility. It would be great to mention, how many biological/technical replicates ran to draw the conclusions. Have you used any statistical tests to check the level of significance?

Table 1 gives the number of crosses and progeny analyzed to assay recombination, as well as SEM; we have added *p* value upper limits for each mutant *vs* wt. We have added to the legends of Figures 2, 3, and 4 the number of cultures and approximate number of cells examined by microscopy.

- In figure legends, please include the strain numbers (GP.....) to correlate better.

Our study reported here used about 70 strains, all listed in Tables S2 and S5. We indicate there the experiments for which each strain was used. We think that adding strain numbers to the figures and tables would add confusion, since they are not necessary to understand the experiments and their results. Those wishing to get strains from us can readily find the strain numbers or ask us.

-Please add a scale bar in microscopic image panels. We have added scale bars to Figures 2, 3, 4, and S1.

Reviewer 2 Advance Summary and Potential Significance to Field:
The subject of this manuscript is the nuclear localization signal of the Rec10 protein, which is one component of the meiotic linear elements (LinE). An important conclusion that is supported by the data is that the Rec10 NLS allows coordinated nuclear entry of the whole complex. Figures 2-4 all show that NLS mutations block entry of protein like Rec25, Rec27 and Mug20 to the nucleus. My assessment is that, although the data is solid, it is not a full-length research article. It has one major finding that is not explored in much detail, examples of which are given below. Therefore, a short format paper would be more appropriate.

Our report here explores the Rec10 NLS in multiple ways, by mutating the NLS in six different ways in three different genetic contexts (plasmid and chromosomal with and without GFP fusion) and assaying four meiotic features (protein abundance, cellular localization, DSB formation, and recombination).

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We think our extensive data are most readily understood in the manner we have presented them. Shortening the paper would require, for example, removing three of the seven displays; a shorter text would have to omit important information and conclusions. We prefer to leave it in the present format.

Reviewer 2 Comments for the Author:
Figures 2-4 show essentially the same result using different forms of the rec10 NLS mutants and assaying for nuclear entry of the LinE proteins. One set of experiments is based on a rec10 plasmid and the other set is based on chromosomal rec10 variants. Rec25 appears to be degraded in the absence of Rec10 (rec10delta). It would be useful to have localization data for the other LinE proteins in a rec10 null. It would also be useful to know the effect of rec25, rec27 or mug20 deletion mutants on the localization of other LinE proteins including Rec10. The authors should discuss the possibility that cytoplasmic Rec10 sequesters the other LinE proteins. Wild-type Rec10 may not interact with the other LinE proteins until it enters the nucleus. In the NLS mutants, which do not enter the nucleus, Rec10 binds to the other LinE proteins and prevents them from entering the nucleus. A prediction of this model is that the rec10 NLS mutants would have a dominant phenotype.

Localization of each LinE-GFP in wt and in each LinE deletion mutant has been published before in references we cited (Davis et al., 2008; Estreicher et al., 2012). We show one (Rec25-GFP in *rec10Δ*; Figure 2A) as an example. We show that Rec10-NLS mutants have Rec10 and the other LinE proteins in the cytoplasm, which we suppose is what the reviewer means by “sequesters.” As we say on page 7, it is thus simplest to infer that Rec10 binds the other LinEs before entering the nucleus. Otherwise, the Rec10-NLS mutant would have to enter the nucleus, bind the other LinEs, and then exit the nucleus carrying the other LinEs with it. It is not clear why the Rec10-NLS mutants would enter the nucleus without an active NLS and why they would then leave the nucleus. We have now added on page 7 that alternatives have not been excluded. We have not tested the *rec10-nls* mutants for dominance.

There is not enough discussion about the GFP variant and why it does not fully rescue the mutant phenotype. Is this because of the GFP, which could be tested if there was a plasmid form that had the GFP. Table 1 is a little confusing, although it appears that all the mutants were in the GFP backbone. It is little strange that none of the mutants are tested in the most natural background, the chromosome backbone without GFP.

The top line of Table 1 indicates whether the Rec10-NLS used for the experiments in each column was fused to GFP or not and whether it was encoded by the chromosome or a plasmid. As requested by this reviewer, we have added new data to Table 1 – assays of recombination with chromosomal *rec10-nls* without the GFP fusion – for direct comparison of chromosomal *rec10* with and without the GFP fusion in each of the six NLS mutant (and wt) states. The results show that, in most cases, the recombinant frequency with the GFP fusion was about 1/2 that without the fusion. We mention the possibility that the GFP fusion slightly lowers the activity, and presumably the conformation, of the LinE complex.

The weaker mutants have strong cytoplasmic localization but support relatively high levels of recombination. In some images, they also appear to have some nuclear localization. This would be consistent with the phenotype but it does not come out clear in the manuscript whether mutants like AlaB have more nuclear localization than mutants with more severe recombination phenotypes. It would be helpful to see the GFP channels without DNA in the figures.

In Figure S1, we have added separate views with just GFP or just DNA for each of the images in Figures 2, 4B, and 4C. We thank the reviewer for this suggestion. The low levels of LinE proteins in the nuclei of *rec10-nls* mutants makes it difficult to reliably assay the amount of LinE in each mutant. Thus, we cannot make a valid comparison of protein level and recombination proficiency.

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Second decision letter

MS ID#: JOCES/2020/253518

MS TITLE: Activation of meiotic recombination by nuclear import of the DNA break hotspot-determining complex

AUTHORS: Melody Wintrebert, Mai-Chi Nguyen, and Gerald R. Smith
ARTICLE TYPE: Research Article

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As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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Reviewer 1

Advance summary and potential significance to field

Here, authors have characterized the role of a nuclear localization signal (NLS), associated with a linear element protein, Rec10. The linear element proteins in S pombe are related to the synaptonemal complex (SC) proteins of other species. This will be a useful information for researchers working on SC complexes and meiosis in general.

Comments for the author

The authors have satisfactorily answered all the questions raised by me. I am happy with their explanations and would recommend this manuscript for the publication.

Reviewer 2

Advance summary and potential significance to field

The authors show that the Rec10 NLS is important for nuclear important and normal levels of meiotic recombination. Failure of Rec10 to enter the nucleus seems to cause other LinE proteins like Rec25, Rec27 and Mug20 to form complexes in the cytoplasm.

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Comments for the author

I have only minor comments on the revision and feel the paper is suitable for publication.

The idea about “sequesters” was to ask if the proposed cytoplasmic interaction between Rec10 and other LinE proteins only occurs in the mutant. This was not to suggest that the Rec10-NLS interacts with other LinEs in the nuclear and then leave again. But does the wild-type protein enter the nuclear and then interact with other LinEs. One can infer from the mutant that they can interact in the cytoplasm, but it is possible this only occurs in the mutant because, for example, there is an abnormally high concentrations of Rec10 in the cytoplasm.

Indeed, the speckles in the cytoplasm are quite large and may represent an abnormally forming structure. Could these be analogous to SC-related polycomplexes in other species? A caveat that should be mentioned (eg. on pg 7) is that there is no experiment that shows colocalization of two LinE proteins. There is no direct evidence that Rec10-NLS colocalizes with other LinEs.

There is also no measurement of how much LinE protein actually enters the nucleus but fails to form a nuclear structure. It appears this could be difficult to measure. But the authors do mention the low level of LinE proteins in the nuclei of rec10-nls mutants, therefore, can this be measured by fluorescence intensity to show that less enters the nucleus. This could be difficult and indeed the other reviewer asked a similar question but with western blots.

I think the authors have room to put the GFP channels in Figures 2-4. It can be a little annoying to go to the supp figures every time you want to see the GFP channel.

Second revision

<u>Author response to reviewers' comments</u>

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[We are grateful to both Reviewers for their positive comments.]

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[In the Discussion on page 7, we have added, "An alternative is that the small LinE proteins enter the nucleus on their own and associate with Rec10 if Rec10 has entered the nucleus (with wild-type

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NLS); otherwise, the small LinE proteins remain in the cytoplasm if Rec10 remains in the cytoplasm (with mutant NLS)."]

Indeed, the speckles in the cytoplasm are quite large and may represent an abnormally forming structure. Could these be analogous to SC-related polycomplexes in other species? A caveat that should be mentioned (eg. on pg 7) is that there is no experiment that shows colocalization of two LinE proteins. There is no direct evidence that Rec10-NLS colocalizes with other LinEs.
[In the Discussion on page 7, we referred to polycomplexes: "These speckles appear qualitatively larger and less numerous than the nuclear foci in wild-type NLS strains. We suppose that the cytoplasmic NLS complex forms aggregates when it cannot enter the nucleus, much as SC proteins of other species form aggregates ("polycomplexes") when they cannot form proper nuclear structures (Sym and Roeder, 1995)." The Reviewer may have overlooked this statement.

On pages 5 and 7, we cited previous work (Davis et al., 2008; Estreicher et al., 2012; Fowler et al., 2013) indicating co-localization of the LinE proteins. We attempted additional experiments using two proteins with fluorescent labels. Using Rec10-GFP and Mug20-tdTomato, we confirmed the nuclear colocalization in Rec10-NLS+ published by Fowler et al., 2013. But other combinations, such as Rec10-GFP and Rec25-mCherry, gave confusing results. In Rec10-NLS ΔA ΔB with Mug20-tdTomato or Rec25-mCherry, small amounts of Rec10-GFP and the other labeled protein were in the nucleus and colocalized, as for the large amounts seen in Rec10-NLS+ with Mug20-tdTomato (Fowler et al., 2013); Rec10-GFP made many small speckles in the cytoplasm but the other labeled protein was not visible. We think it may have been degraded in this triple mutant situation. We suppose that two fluorescent proteins (~28 kDa each) sometimes interfere with each other when fused to any of the three small LinE proteins (15 – 17 kDa each). We think the simplest interpretation of our current and previous observations is that the LinE proteins form a complex and colocalize. We noted on page 2, e.g., that "deletion of any one gene reduces or eliminates foci of the others," which supports this view.]

There is also no measurement of how much LinE protein actually enters the nucleus but fails to form a nuclear structure. It appears this could be difficult to measure. But the authors do mention the low level of LinE proteins in the nuclei of rec10-nls mutants, therefore, can this be measured by fluorescence intensity to show that less enters the nucleus. This could be difficult and indeed the other reviewer asked a similar question but with western blots.
[The total amount of Rec10-GFP is about the same in wt or each NLS mutant, by direct Western blot analysis for Rec10-GFP (Figure 6) and by fluorescence for all four LinE-GFPs (Figures 2 – 4). Essentially all of it is in the nucleus in wt or in the cytoplasm in each NLS mutant. Thus, there must be less Rec10 in the nucleus in the NLS mutants. The total fluorescence of the other LinE proteins also is about the same in Rec10-NLS+ and Rec10-NLS mutants; again, the fraction in the nucleus is much less in the Rec10-NLS mutants, and we infer the amount is also less in the Rec10-NLS mutants.]

I think the authors have room to put the GFP channels in Figures 2-4. It can be a little annoying to go to the supp figures every time you want to see the GFP channel.
[As requested, we have moved Figure S1, with separate images for the GFP and chromatin channels for Rec25-GFP, Rec27-GFP, and Mug20-GFP in wt and Rec10-NLS mutants, to the main text (Figures 2 and 4). We had put them in the supplemental information because there are no unexpected results here: the GFP channel is readily seen as green, or as yellow if overlapping the chromatin, in each of the figures. In our view, the merged images are the most important, for only in those can one be sure of co-localization, or not. We hope this will satisfy the Reviewer's request.]

Third decision letter

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AUTHORS: Melody Wintrebert, Mai-Chi Nguyen, and Gerald R. Smith
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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