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Peer Review Report for 10.1105/tpc.16.00720

Targeting of *A. thaliana* KNL2 to Centromeres Depends on the Conserved CENPC-k Motif in its C-terminus

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Review timeline:

TPC2016-00720-RA	Submission received:	Sept. 14, 2016
	1st Decision:	Oct. 19, 2016 *revision requested*
TPC2016-00720-RAR1	1st Revision received:	Dec. 12, 2016
	2nd Decision:	Dec. 17, 2016 *acceptance pending, sent to science editor*
	Final acceptance:	Jan. 4, 2017
	Advance publication:	Jan. 6, 2017

REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016-00720-RA 1st Editorial decision – *revision requested* Oct. 19, 2016

We have received reviews of your manuscript entitled "Targeting of *A. thaliana* KNL2 to centromeres depends on the conserved CENPC-k motif of its C-terminus." Thank you for submitting your best work to *The Plant Cell*. The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

We ask you to pay attention to the following points in preparing your revision.

The reviewers all feel that the study is valuable to the centromere field in general extending beyond plant biology. They do, however, note some aspects of the paper that need attention. Of most importance is the nature of the experiments leading to Figure 2B. The images in the figure support the claims of the paper but there does not appear to be a description of the number of replicates or if these results are repeatable. Further, it was suggested that a confirmation be made that the punctate signals be tested via immune-FISH (or alternatives mentioned in the review) to confirm that they do indeed coincide with the centromere positions. Also, multiple reviewers note that the Discussion could be more focused and one reviewer suggested that adding an additional figure illustrating the take home message would be clarifying for readers. This latter point we suggest be left to your discretion but we agree that the Discussion needs to be better crafted. Lastly, the reviewers have some comments about adjusting the language to conform more accurately with the results; these should be easily fixed.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

---------- Reviewer comments:

Reviewer #1:

This is a beautifully written manuscript with several clear messages that will advance the field of plant centromere biology. I also enjoyed the comprehensive but succinct literature summary in the introduction.

Through a series of bioinformatic analyses and mutagenesis experiments the authors show convincingly that *A. thaliana* KNL2 contains a conserved CENPC-like motif (CENPC-k) at its C-terminus that is required for centromere

localization of the protein KNL2 *in vivo*. KNL2's importance derives from its apparent role in CENH3 loading. This manuscript thus represents and important advance in our understanding of the mechanism that causes persistence of the epigenetic CENH3 signal in plants.

The finding that proteins CENP-C and KNL2 share interchangeable motifs that impart centromere localization represents a significant step forward in untangling centromere protein-protein and protein-DNA interactions. Discovery and verification of DNA binding activity (via EMSA), likely imparted by KNL-2 protein regions flanking the CENPC-k domain, ties DNA- and protein-binding activity together in this molecule.

This work is sure to be noticed outside the plant community as well.

Minor revisions….

Reviewer #2:

This manuscript describes a novel feature of plant KNL2: the CENPC-k motif. It demonstrates the role of this motif in localization to the centromere by testing multiple altered KNL2 versions. A related motif in CENPC can substitute it. In conclusion, the characterization of this motif is well done. The C-terminus of KNL2 is further demonstrated to interact with DNA, although not through the CENPC-k motif. The implications are discussed. Overall, the data support the conclusions and are presented clearly. The manuscript is written clearly and findings are significant enough to constitute a good contribution to *The Plant Cell.*

1) Fig. 2-B. This figure panel is central to the conclusions being made. The images are consistent with the authors' conclusion but they are not compelling in quality and clarity. Could some numbers be provided? Was this experiment repeated? Could a close up of a nucleus be added? The signals for KNL2(C) are consistent with CEN, but it would be nice to demonstrate it with a FISH probe, or with a CENH3/CENPC-RFP. The result is provided in *N. benthamiana* transient expression in the supplemental. Has this been attempted in *A. thaliana*?

2) The C-terminal part of *A. thaliana* KNL2 binds the centromeric repeat *pAL1* DNA *in vitro*: the sentence is factual, but as shown by the authors the binding is essentially non-specific and the heading is thus potentially misleading. The title of Fig. 4 is more informative. The suggestion that repetitive sequences interact better with KNL2 is based on the single euchromatic TUA4 region. Variation in binding may have nothing to do with repetitiveness or other inherent property of cen DNA. The analysis of DNA binding is not complete as the essential features are not identified, except by sequence comparison in the supplemental.

3) The Discussion could be tightened. The role of RNA in scaffolding KNL2 and CENPC is not based on KNL2 data shown here. The "Taking all facts, obtained for animals and plants, together we assume that the specific localization...." this is followed by a laundry list of plausible interactions leading to localization. It may sound better if rephrased as testable hypotheses. The authors could address the non-essentiality of this protein in plants compared to other systems.

Reviewer #3:

Sandman *et al.* identify a CENPC motif in KNL2 and show that it is required for centromere targeting but not for DNA binding. The work is carried out at a high level, including some elegant touches like mutating key residues on the CENPC motif and showing that these residues are required for targeting. There are also some very nice gel shift assays showing that the protein binds DNA non-specifically similar to CENPC. It's a fine paper.

1) Fig. 2. There is no statement of how many transgenics, individuals, or images were analyzed to conclude that the mutated KNL proteins failed to localize to centromeres. These images have a lot of background, some basic quantitation is in order.

2) Pg. 7. Although I see the appeal of replacing the KNL CENPC motif with the CENPC motif from CENPC, it should be noted in the paper that this motif may target any protein to the centromere. For instance the motif alone may be sufficient to target YFP, and it may not be actually complementing the missing portion on KNL2.

3) Suppl. Fig. 2, 3. The authors need to add clarification to the statement that "some cells" the chimeric KNL2(C)-CENPC protein colocalized with CENH3. The images shown are very convincing but is that all there was?

4) The discussion is a bit rambling and the writing can be improved. The authors are making the point that KNL2 functions during loading, while CENPC functions during maintenance. I think this makes sense - and it should be possible to represent this idea in a summary cartoon figure so that readers can better visualize their interpretation. Writing with this sort of language " (KNL2, CENPC, ...)" and "KNL2, CENPC, etc" in the last paragraph should be removed and instead focused on a model to help the readers understand the next steps in understanding how centromeres are initiated and maintained by this important motif.

TPC2016-00720-RAR1 1st Revision received Dec. 12, 2016

Reviewer comments and author responses:

Reviewer #1:

Minor revisions….

RESPONSE: Done

Reviewer #2:

Point 1. Fig. 2-B. This figure panel is central to the conclusions being made. The images are consistent with the authors conclusion but they are not compelling in quality and clarity. Could some numbers be provided? Was this experiment repeated? Could a close up of a nucleus be added? The signals for KNL2(C) are consistent with CEN, but it would be nice to demonstrate it with a FISH probe, or with a CENH3/CENPC-RFP. The result is provided in *N. benthamiana* transient expression in the supplemental. Has this been attempted in *A. thaliana*?

RESPONSE: We added the sentence "For each transformation at least 30 independent transgenic lines were generated and at least five independent lines were used for the in vivo localization studies." To the Materials and Methods section (see p. 13) and explained in Results that for each transformation at least five independent transgenic lines were analyzed and all gave the same result. (see p. 6)

We showed previously that KNL(C) fused with EYFP (containing CENPC-k) colocalizes with cenH3 at bright DAPI stained chromocenters (Lermontova et al. 2013). Now using double immunostaining with anti-GFP (detecting the KNL2(C)CENPC EYFP fusion) and anti-cenH3 antibodies we showed that KNL2(C)CENPC also localizes at chromocenters and colocalizes with cenH3 (Fig. 2C), see p. 6.

We added higher magnification images of the representative nuclei of plants expressing the KNL2(C)CENPC-k(R-A) and KNL2(C)CENPC variants to the Supplemental Figure 1.

Point 2. The C-terminal part of *A. thaliana* KNL2 binds the centromeric repeat pAL1 DNA in vitro: the sentence is factual, but as shown by the authors the binding is essentially non-specific and the heading is thus potentially misleading. The title of fig 4 is more informative.

RESPONSE: Now two paragraphs of the Results: "The C-terminal part of A. thaliana KNL2 binds the centromeric repeat pAL1 DNA *in vitro*" and "KNL2 binds non-centromeric sequences *in vitro*, but *in vivo* it associates preferentially with the centromeric repeat pAL1" are combined together under the new subheading: "KNL2 binds DNA sequence independently *in vitro*, but *in vivo* it is associated with centromeric repeats".

Point 3. The suggestion that repetitive sequences interact better with KNL2 is based on the single euchromatic *TUA4* region. Variation in binding may have nothing to do with repetitiveness or other inherent property of cen DNA.

RESPONSE: The statement "…albeit in a competition assay the euchromatic tubulin sequence TUA4 was bound with lower affinity than repetitive sequences…" and Figure 3D were deleted.

Point 4. The analysis of DNA binding is not complete as the essential features are not identified, except by sequence comparison in the supplemental.

RESPONSE: KNL2 was first identified in 2007, yet no study so far has addressed whether it binds DNA. In this study we showed for the first time that the C-terminus of Arabidopsis KNL2 binds DNA non-specifically, but binds primarily Arabidopsis centromeric repeats *in vivo*. The Arabidopsis KNL2 protein, similar to the CENP-C protein of different organisms, does not contain a recognized DNA-binding family motif.

Using *in silico* analysis we predicted some putative DNA-interacting regions (Supplemental Figure 6). Given the time constraints of this manuscript review, in our next study we will apply a mutagenesis approach to verify whether these regions are involved in interaction of KNL2 with DNA *in vitro* and *in vivo*.

Point 5. Discussion could be tightened. The role of RNA in scaffolding KNL2 and CENPC is not based on KNL2 data shown here. The "Taking all facts, obtained for animals and plants, together we assume that the specific localization...." this is followed by a laundry list of plausible interactions leading to localization. It may sound better if rephrased as testable hypotheses.

RESPONSE: We added additional data for the role of RNA (Figure 6) and rewrote the discussion accordingly to streamline and focus it. We have added a model to help conceptualize the role of RNA binding.

Point 6. The authors could address the non-essentiality of this protein in plants compared to other systems.

RESPONSE: The KNL2 protein is essential for plant growth and development: Knock-out of *A. thaliana* KNL2 leads to anaphase bridges, reduced fertility, reduced levels of DNA methylation and reduced transcription of cenH3 and histone modification proteins such as SUVH4 and SUVH9 (Lermontova *et al.* 2013). (See Introduction, p. 3).

The similarities and differences in function of KNL2 between plants and other systems were discussed in our previous publication (Lermontova et al. 2013).

Reviewer #3:

Point 1. Fig. 2. There is no statement of how many transgenics, individuals, or images were analyzed to conclude that the mutated KNL proteins failed to localize to centromeres. These images have a lot of background, some basic quantitation is in order.

RESPONSE: We added the sentence “For each transformation at least 30 independent transgenic lines were generated and at least five independent lines were used for the *in vivo* localization studies.” To the Materials and Methods section (see p.13) and explained in Results that for each transformation at least five independent transgenic lines were analyzed and all gave the same result. (See p. 6)

We added higher magnification images of the representative nuclei of plants expressing the KNL2(C)CENPC-k(R-A) and KNL2(C)CENPC variants to the Supplemental Figure 1.

Point 2. Pg. 7. Although I see the appeal of replacing the KNL CENPC motif with the CENPC motif from CENPC, it should be noted in the paper that this motif may target any protein to the centromere. For instance the motif alone may be sufficient to target YFP, and it may not be actually complementing the missing portion on KNL2.

RESPONSE: We included the following sentence in the Discussion: “It is possible that the CENPC-k motif of KNL2 or CENPC motif of CENP-C fused with any desired protein may lead to centromeric localization. Although this remains to be tested in further studies, this approach may provide an interesting tool for centromere research.” (See p.10).

Point 3. Suppl. Fig. 2, 3. The authors need to add clarification to the statement that "some cells" the chimeric KNL2(C)-CENPC protein colocalized with CENH3. The images shown are very convincing, but is that all there was?

RESPONSE: We assume that centromeric localization of the KNL2 variants and cenH3 can be seen only in a mitotically active cells, since cenH3 (Lermontova et al. 2006) and very likely KNL2 can be loaded to centromeres only during the mitotic cell cycle. Therefore, only some cells out of 50 cells analyzed in each transiently transformed plant show centromeric localization of both KNL2 and cenH3 proteins. The proportion of such cells varies depending on plant age and efficiency of double transformation. We now point this out (see page 6).

Point 4. The discussion is a bit rambling and the writing can be improved. The authors are making the point that KNL2 functions during loading, while CENPC functions during maintenance. I think this makes sense - and it should be possible to represent this idea in a summary cartoon figure so that readers can better visualize their interpretation. Writing with this sort of language " (KNL2, CENPC, ...)" and "KNL2, CENPC, etc" in the last paragraph should be removed and instead focused on a model to help the readers understand the next steps in understanding how centromeres are initiated and maintained by this important motif.

RESPONSE: We re-structured the Discussion and included a model for the localization of KNL2 and CENPC and their functions during cenH3 loading and kinetochore assembly.

TPC2016-720-RAR1 2nd Editorial decision – *acceptance pending*	Dec. 17, 2016

We are pleased to inform you that your paper entitled "Targeting of *A. thaliana* KNL2 to centromeres depends on the conserved CENPC-k motif of its C-terminus" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership. *The Plant Cell* has appointed several Ph.D. Plant Scientists to serve as Science Editors in this capacity, and you will receive further information on this process very shortly.

Final acceptance from Science Editor	Jan. 4, 2017