# Interplay of septin amphipathic helices in sensing membrane-curvature and filament bundling

Benjamin Woods, Kevin Cannon, Ellysa Vogt, John Crutchley, and Amy Gladfelter

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## 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.)

RE: Manuscript #E20-05-0303
TITLE: Interplay of septin amphipathic helices in sensing membrane-curvature and filament bundling

Dear Amy,

Your manuscript has now been reviewed by two experts in the field. While the data that Shs1 septin exhibits curvature sensing like Cdc12 is interesting, both reviewers have major concerns about the strength of the data presented to support this conclusion and the possible role of the amphipathic helix in regulating septin bundling.

Because of the interesting initial observation, I encourage you to revise this manuscript with additional experiments to strengthen the conclusions and address the reviewers' concerns thoroughly. A number of points can likely be addressed by careful re-writing and additional explanations, but other points will need substantial additional experiments, in particular experiments from reviewer 1 to test the binding of Cdc12 AH domain swap protein in vitro, and from reviewer 2 to i) convince that Shs1-septin complexes are stable and not aggregating, ii) test Shs1ΔAH membrane binding in vitro, and iii) consolidate the data presented in figure 2 if required.

Additional notes from me:
- RitC is not a S. pombe protein, it is the C-terminal membrane-binding domain of the mammalian Rit GTPase.
- Please make sure you indicate the number of experimental replicates performed and generally follow the submission checklist.

Sincerely,

Sophie Martin
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Gladfelter,

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.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
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Reviewer #1 (Remarks to the Author):

Woods et al describe studies on the amphipathic helices (AH) in the non-essential S. cerevisiae septin Shs1 and the essential septin Cdc12. The authors show that an AH is predicted in Shs1 from S. cerevisiae and that its hydrophobicity profile differs from that of Cdc12. In experiments mixing constructs containing AH domains or octamers with membrane-coated beads of various diameters they show that the Shs1 AH domain and purified Shs1 capped octamers sense the same membrane curvature as the Cdc12 AH and Cdc11 capped octamers (which contain the Cdc12 AH). Using a series of SHS1 truncation constructs in the cdc12-6 mutant, the authors show that the Shs1 AH is needed for proper S. cerevisiae morphology and that the CTE is needed for viability. They create a series of chimeras in which heterologous AH domains are fused to the C-terminus of Cdc12 or Cdc12-6 and show that all are inviable. The authors show that S. cerevisiae cdc12-6 mutants treated w pheromones and A. gossypii cdc12-6 mutants make needle like septin structures with greater persistence lengths and that these "bundled" filaments do not form on stabilized membranes. The authors suggest this shows a role for the Cdc12 AH in preventing bundling and suggest that Cdc3 and Cdc12 coiled coils interact to form a togglable switch that controls bundling.

General points:
This study builds on previous work from the Gladfelter lab focused on the AH in Cdc12 (Cannon et al 2019). There are some interesting ideas in this manuscript, but in many places I found it difficult to follow and had to refer to referenced literature to make sense of it. I suspect part of the problem might be that the needed background from Cannon et al 2019 was not always included in enough detail.

The conclusions about Shs1 from the first section of the paper are well-supported by the data: Shs1 has an AH that recognizes the same curvature as the AH in Cdc12. In the cdc12-6 mutant, the Shs 1 AH restores curvature sensing and is needed for proper morphology. Something in the Shs1 CTE other than the AH is needed for viability.

The nature of the cdc12-6 mutation is not made clear and could be very relevant. The cdc12-6 K391N, E392stop mutations truncate the predicted AH, but do not eliminate it, 3 of the hydrophobic residues are still present. It seems possible that some function remains, especially in the context of septin octamers or higher order structures which might help orient it. Alternately perhaps this mutation disrupts the structure in something like a dominant negative.

It is not clear what the authors think the Shs1 AH function is and how it relates to the Cdc12 AH.

The conclusions about a role for the Cdc12 AH in preventing bundling, while very interesting, do not seem supported by the data. This might be partly because the authors don't describe what they mean by "needles" or "bundling." Are they just longer, more persistent filaments or parallel associations of filaments? How does the persistence data (flexibility) fit into this? Perhaps a better explanation of needles/bundles would make the logic behind the conclusions more clear.

Specifics:

1) In the Abstract: "In mutants lacking a fully functional Cdc12 AH domain, the Shs1 AH domain becomes essential."

On P. 5, paragraph 2 and in Fig 3 B the elimination of the Shs1 AH in cdc12-6 is not lethal, it is 90% viable, so the AH isn't essential. The elimination of the entire CTE is lethal though. This suggests that something else in the CTE, outside of the AH is essential.

2) On P. 2 more detail on the cdc12-6 mutation would be helpful here and elsewhere. It would also be helpful to add Cdc12-6 to Fig 1 C to aid the reader.

3) On p. 5, paragraph 2 and in Fig 3B, truncations of Shs1 in cdc12-6 are described as synthetic lethal based on tetrad analysis. According to the methods these were constructed using PCR based C terminal modification. I assume this was followed by transformation into a diploid to create the het diploid which was then induced to sporulate. Is this right? If so please add to methods.

4) P. 5, paragraph 2 and Fig 3B, there is some disagreement between the text, methods, and Fig 3B on strains. The text and methods refer to shs1ΔAH but it is not in 3B (though the GFP version is there).

Similarly, P. 6 first paragraph: "Moreover, we could discount the possibility that the 3xHA tag adjacent to the AH domain was responsible for the lethality since the 3xHA tag adjacent to the Cdc12 AH domain(Cdc12 400-407) had no effect on viability (Figure 3B)."
The 400-407 deletion is not listed in Fig 3B and it is not clear which strains have the HA tag.

5) In many cases Fig 3B indicates that the GFP fusion phenotype was more severe than the corresponding non GFP strain (not clear if these have HA though). Why might this be?

6) p. 5 last paragraph: "The increased cytoplasmic distribution of Shs1[illegible]-551-GFP provides an explanation for the observed synthetic lethality with cdc12-6 mutants."

Meaning is not clear. Are the authors suggesting that lack of membrane localization causes lethality?

7) P. 6, paragraph 1: "These data indicate that the Cdc12 AH domain cannot be simply swapped for another AH domain, even if chimeric AH domain recognizes similar curvatures."

Based on the methods, the chimeras appear to have been constructed by fusing the heterologous AH domains to the C-terminus of Cdc12 or Cdc12-6. This is not a "swap." The extra 44 amino acids C-terminal to the AH domain might be required for proper spacing or anchoring. Though the chimeras are stable by Western blot, it is not clear if these heterologous AH domains are functional in sensing the same curvatures as the Cdc12 and Shs1 AH domains. Adherence to membrane-coated beads would give more confidence that these heterologous AH domains are functional in S. cerevisiae, though the potential spacing issue without the final portion of the CTE would still remain.

8) P. 6, last paragraph: "The existence of naturally occurring bundled septin structures in cells suggest that cdc12-6 bundles are not necessarily only a gain-of-function mutation but that the C-terminus of Cdc12 could be relevant for bundling septins under certain contexts (DeMay et al., 2009; Liu et al., 2019)."

This was confusing in context. After looking more closely at the references, it seems the authors are referring to the existence of "needles" in WT S. cerevisiae under certain conditions and in S. pombe. Perhaps they could explain that needles are also found in some WT situations in S. cerevisiae and other fungi? There are many published cases of septin rods and bars in filamentous fungi that resemble the "needles" shown here.

9) P7, paragraph 1: "This transition is dependent on the kinase Gin4,which is predicted to interact with the coiled-coil element of Cdc3 and phosphorylates Shs1 in S.cerevisiae (Longtine et al., 1998; Mortensen et al., 2002). The Cdc3/Cdc12 coiled-coil may act like togglable switch, whose disassembly - either through phosphorylation or Cdc12 AH domain sequestration - could promote septin bundling through Cdc3 coiled-coil oligomerization".
Conclusion: "cdc12-6 induces filament bundling into non-physiological needle-like structures, possibly exacerbating its phenotype".
This is confusing. The authors suggest that cdc12-6 might not fold properly and so allows Cdc3 to oligomerize leading to bundling. But the bundles visualized are of cdc12-6 (6B)? Perhaps the authors could clarify?

10) In Fig 4 it would be useful to show the WT controls. Also in Figure 4, it is not clear what the coil drawings represent relative to the septin octamer. If the yellow balls are Cdc 11 or Shs1 caps, where are the AH domains? Please add detail to the legend, especially on color schemes.

11) In Figures 3 and 4 more detail is needed in the legends (or the methods). Number of replicates performed and number of cells observed are not shown.

12) The authors might be interested in a set of experiments reported by Lindsey et al in 2010 (doi:10.1371/journal.pone.0009858). In this work A. nidulans AspC (the Cdc12 ortholog) was shown to rescue the S. cerevisiae cdc12-6 mutant at restrictive temperature. AspC incorporated into the septin ring at the S. cerevisiae neck, replacing Cdc12 and causing elongated buds. So, AspC restored viability, but resulted in abnormal morphology in cdc12-6, consistent with the results presented in this manuscript for Shs1.

Reviewer #2 (Remarks to the Author):

This manuscript by Woods et al., entitled "Interplay of septin amphipatic helices in sensing membrane curvature and filament bundling" describes the role of amphipatic helices from septin proteins Shs1 in sensing curvature. This article is the follow up of a report by Cannon et al. (2019) which demonstrated that Cdc12 was carrying an amphipatic helix as well, able to sense curvatures. This current report relies on in vitro and cell biology experiments to show that Shs1 carries an amphipatic helix able

to sense curvatures. Even though the results are interesting, some of the in vitro versus in vivo experiments are contradictory and misleading for the reader. Curvature sensing is thus not obvious and not unequivocally demonstrated. Hence, I would recommend undertaking major revisions and additional experiments before the manuscript can be published in Molecular biology of the Cell.
You will find my comments below:
Major comments:
1. It is not known how the amphipatic helix was identified. Was it identified "in silico"? Using which software? The authors should describe the procedure used either in the results section or in the methods section.
2. In addition, with the primary sequence indicating the presence of an amphipatic helix, the authors should make sure those are indeed amphipatic helices using alternative experimental methods (for instance: Circular Dichroïsm, binding assays to liposomes, Structural methods: molecular dynamics, NMR).
3. It is difficult to appreciate how molecular cues at nanometer scale like amphipatic helices can induce curvature sensing at micrometer scale. Usually, much higher curvatures (at nanometer scales) are sensed by amphipatic helices. For instance, ALPS proteins would interact with 50 nm diameter liposomes (as an example see: Mesmin et al., 2007, Biochemistry). What would be the mechanisms inducing micrometer curvature sensing by a 20 residues long helix? Can the authors discuss this?
4. The authors suggest that curvature sensing is the only and primary factor responsible for septin recruitment. They point out that Cdc12-6 mutants localize to the bud neck at permissive temperatures but are expelled at restrictive temperatures because of curvature sensitivity deficiency. However, other factors are known to be crucial for septin recruitment as well (Bni5 for instance, see lee at al. 2002, Mol. Cell. Biol.). The manuscript should discuss this.
5. It is not known whether the experiments generating figure 2 have been repeated and how many times. The error bars (highest curvatures, figure 2.A) seem extremely large. More data should be added to improve the statistics.
6. The protein-membrane interaction visualized in Figures 2.A and 2.B does not look homogeneous. In most of the displayed images, septins or polypeptides bind to silica beads as clusters of proteins. Hence, we might wonder whether these are not aggregated proteins and whether the protein membrane interaction results from unspecific aggregation. The main conclusions are thereby altered and suffer from the quality of the experimental data.
Hence, I would suggest the following:
- The fitness of the purified polypeptides and septin complexes in solution should be tested for solubility, proper refolding and integrity of complexes. For instance, elution profiles from size exclusion, electron microscopy images could be displayed. It is known that Shs1 complexes are not as robust as Cdc11 complexes (see Garcia et al. JCB, 2011 and Weems et al., Genetics, 2014).
- The fluorescence intensity of the protein signal should be calibrated to give some insights in the actual protein density covering the beads.
- The quality of the lipid bilayer covering the silica bead should be examined. Quantitative evaluation of the lipid fluorescence signal could be performed to check whether it is fully homogeneous. SEM imaging, similarly to the data shown in Cannon et al 2019, could also be carried out to visualize both the membrane and the septins.
- Besides, the proportion of charged lipids (PI at 25 %) seems quite high to be fully incorporated within a bilayer. It is most likely probable that the actual PI concentration is lower, within the bilayer. A range of PI concentrations as well as other charged lipids in the lipid mixture should be tested to check for protein membrane interaction variability.
7. It is not mentioned why, in Figure 2.B, data at curvatures higher than 2 µm-1 is not shown. This should be added otherwise one does not understand why it is not shown, in comparison with Cannon et al. 2019 and Figure 2.A.
8. Testing membrane curvature sensitivity with a polypeptide might not be an optimal choice since it might behave completely differently from a protein within a much larger complex. The authors do not comment or explain this choice.
9. A significant (about 5 times) lower affinity is observed for Shs1 complex with Cdc12-6 mutant than for Shs1 wild type complex (Figure 2.B, right). The stability of this complex in solution should be analyzed since an altered refolding or stability might be responsible for this observation.
10. Why were the mutations performed in situ not tested as well in vitro for curvature sensitivity? Testing truncated Shs1 mutants in vitro as well would bring essential knowledge to understand the phenotypes observed in Figure 3. Without those analyses, essential evidences are missing to demonstrate that the AH domain of shs1 is indeed able to sense curvature rather than only localizing septin complexes at the bud neck.
- At least, the ShS1 (delta AH) mutant capping the septin complex should be tested for its interaction with silica beads.
- That would be beneficial to test point mutations that alter the electrostatic properties of the AH helix, both in vitro and in situ.
11. It is puzzling that the chimeric strains are not viable. It is possible that those constructs induce significant misfolding and unstability of the septins complexes and thus exclude the proteins by quality control mechanisms (see Johnson et al. 2015, Elife).This suggest that the delta AH domain and the CTE might have additional functions in addition to curvature sensing: protein folding, proper integration into the complex.
Minor comments
1. Figure 1 should be described properly. The colors code used is not described. The residues should be numbered. Figure 3A should be displaced within Figure 1.
2. When introducing the AH domain, the authors should describe, in the text, how the AH sequence compares with the sequence of the coiled coil domain and which are the residues involved. A scheme would also be beneficial.
3. Statistics issues: It is not mentioned whether the experiments from figure 2, figure 3 and figure 4 have been repeated and how many times. Besides, the exact number of beads where a fluorescence intensity measurement has been performed is not specified. The plots from figure 2A and 2B would benefit greatly from being drown as clouds of dots to visualize better how the data is spread.

4. The last paragraph describing the needles in asshby appears slightly unrelated to the rest of the report. Partial truncation within the coiled coil domain of Cdc12 might impair the pairing ability of septin complexes and induce the aggregated bundled structures visualized. The needles would require some quantification in terms of dimensions and proportion of needles versus "standard" structures.

We appreciate thorough and constructive critiques on our work. In response, we have addressed each of the issues and concerns raised by the reviewers. This includes 1) testing a wider range of membrane curvatures, 2) including additional replicates to strengthen the interpretations of the data, 3) examining membrane curvature sensitivity on different lipid compositions, 4) providing a more thorough explanation of the statistics, 5) including analysis assessing the quality of the septin complexes, and 6) a descriptive analysis including quantification of the bundled needle septin filament morphology observed in *cdc12-6 Ashbya* mutants. We hope you agree that we have sufficiently addressed the critiques and suggestions provided by the Reviewers. Responses to specific criticisms are below.

Dear Amy,

Your manuscript has now been reviewed by two experts in the field. While the data that Shs1 septin exhibits curvature sensing like Cdc12 is interesting, both reviewers have major concerns about the strength of the data presented to support this conclusion and the possible role of the amphipathic helix in regulating septin bundling.

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Additional notes from me:
- RitC is not a S. pombe protein, it is the C-terminal membrane-binding domain of the mammalian Rit GTPase.
We apologize for this oversight and have made textual edits to fix this mischaracterization.
- Please make sure you indicate the number of experimental replicates performed and generally follow the submission checklist.
Thank you for this reminder. We have now included experimental replicate number for each experiment either in the text or figure legend.

Sincerely,

Sophie Martin
Monitoring Editor
Molecular Biology of the Cell

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Reviewer #1 (Remarks to the Author):

Woods et al describe studies on the amphipathic helices (AH) in the non-essential S. cerevisiae septin Shs1 and the essential septin Cdc12. The authors show that an AH is predicted in Shs1 from S. cerevisiae and that its hydrophobicity profile differs from that of Cdc12. In experiments mixing constructs containing AH domains or octamers with membrane-coated beads of various diameters they show that the Shs1 AH domain and purified Shs1 capped octamers sense the same membrane curvature as the Cdc12 AH and Cdc11 capped octamers (which contain the Cdc12 AH). Using a series of SHS1 truncation constructs in the cdc12-6 mutant, the authors show that the Shs1 AH is needed for proper S. cerevisiae morphology and that the CTE is needed for viability. They create a series of chimeras in which heterologous AH domains are fused to the C-terminus of Cdc12 or Cdc12-6 and show that all are inviable. The authors show that S. cerevisiae cdc12-6 mutants treated w pheromones and A. gossypii cdc12-6 mutants make needle like septin structures with greater persistence lengths and that these "bundled" filaments do not form on stabilized membranes. The authors suggest this shows a role for the Cdc12 AH in preventing bundling and suggest that Cdc3 and Cdc12 coiled coils interact to form a togglable switch that controls bundling.

General points:
This study builds on previous work from the Gladfelter lab focused on the AH in Cdc12 (Cannon et al 2019). There are some interesting ideas in this manuscript, but in many places I found it difficult to follow and had to refer to referenced literature to make sense of it. I suspect part of the problem might be that the needed background from Cannon et al 2019 was not always included in enough detail.

Thanks for pointing out the insufficient background information. We have now added more information in the background and elaborate on cdc12-6 mutant to the text to help the reader make sense of the data presented here. See lines 41-52:

> "When purified septins are mixed with membranes at a range of different curvatures, septins polymerize into aligned filaments wrapped at an optimal curvature (Beber et al., 2019b; Cannon et al., 2019; Tanaka-Takiguchi et al., 2009). However, even individual septin complexes can distinguish membranes with micron-scale curvature by displaying different kinetics of septin-membrane binding depending on the curvature (Cannon et al., 2019). Septins, like other curvature sensitive proteins, possess an amphipathic helix (AH) domain (Cannon et al., 2019; Drin and Antonny, 2010; Kim et al., 2017). AH domains detect and bind to lipid packing defects within curved membranes. In the budding yeast *Saccharomyces cerevisiae*, the amphipathic helix at the C-terminus of Cdc12 is necessary and sufficient for septins to distinguish between different curvatures of the plasma membrane (Cannon et al., 2019). The mechanisms by which Cdc12-AH membrane binding are coupled to filament polymerization and organization to cause curvature-dependent assembly of septins remains a challenging problem."

We also have made a point of either repeating experiments with Cdc12-AH/Cdc12 complexes for direct comparison within Figure 2 and Supplementary Figure 1, and have more clearly described the data from Cannon 2019 at places throughout the text to aid reader.

The conclusions about Shs1 from the first section of the paper are well-supported by the data: Shs1 has an AH that recognizes the same curvature as the AH in Cdc12. In the cdc12-6 mutant, the Shs 1 AH restores curvature sensing and is needed for proper morphology. Something in the Shs1 CTE other than the AH is needed for viability.

The nature of the cdc12-6 mutation is not made clear and could be very relevant. The cdc12-6 K391N, E392stop mutations truncate the predicted AH, but do not eliminate it, 3 of the hydrophobic residues are still present. It seems possible that some function remains, especially in the context of septin octamers or higher order structures which might help orient it. Alternately perhaps this mutation disrupts the structure in something like a dominant negative.

The reviewer raises an important point about residual hydrophobicity in the original allele. While Cannon et al., 2019 reported that this mutation blocks any curvature sensitivity, it remains plausible that there are functions retained, which are difficult to disentangle, and we have now attempted to address this possibility in the text (lines 199-201;244-253). The allele is recessive in our hands so we do not think a dominant negative phenotype is likely.

It is not clear what the authors think the Shs1 AH function is and how it relates to the Cdc12 AH.

The conclusions about a role for the Cdc12 AH in preventing bundling, while very interesting, do not seem supported by the data. This might be partly because the authors don't describe what they mean by "needles" or "bundling." Are they just longer, more persistent filaments or parallel associations of filaments? How does the persistence data (flexibility) fit into this? Perhaps a better explanation of needles/bundles would make the logic behind the conclusions more clear.

We appreciate that we were not clear enough in presenting a model for the role of the Shs1 AH and apologize for the brevity of our description of the “needles”. It is our interpretation that the Shs1 AH operates in parallel with the Cdc12 AH to promote septin membrane curvature sensitivity, and that lacking both explains (at least partially) why double mutants are inviable (lines 147-152). We have now expanded our analyses by quantifying the needle abundance in *cdc12-6* mutants (updated Figure 4, D). We hypothesize these non-native structures could exacerbate the *cdc12-6* phenotype by sponging up available septin complexes, preventing their localization to functional structures (see lines 226-227).

Specifics:

1) In the Abstract: "In mutants lacking a fully functional Cdc12 AH domain, the Shs1 AH domain becomes essential."

On P. 5, paragraph 2 and in Fig 3 B the elimination of the Shs1 AH in cdc12-6 is not lethal, it is 90% viable, so the AH isn't essential. The elimination of the entire CTE is lethal though. This suggests that something else in the CTE, outside of the AH is essential.

Thank you for pointing out this imprecision in our language as indeed the AH domain in this context is not essential. We modified the text to read "the C-terminal extension of Shs1, containing an AH domain, becomes essential" (line 22-23).

2) On P. 2 more detail on the cdc12-6 mutation would be helpful here and elsewhere. It would also be helpful to add Cdc12-6 to Fig 1 C to aid the reader.

We thank the reviewer for bringing to our attention our abbreviated overview of the *cdc12-6* mutant. We changed the line in the abstract to read: "In a septin mutant which lacks a fully functional Cdc12 AH domain (*cdc12-6*)" (see lines 21-22).

We also added more detail on the nature *cdc12-6* gene product in the text: "cdc12-6 (K390N, and truncation of the AH domain including the C-terminal residues 391 through 407)" (see lines 66-67) with appropriate references (Adams and Pringle, JCB 1984; and Johnson et al, MBoC 2015).

Finally, we also added cdc12-6 to the sequence alignment (now Fig 1D).

3) On p. 5, paragraph 2 and in Fig 3B, truncations of Shs1 in cdc12-6 are described as synthetic lethal based on tetrad analysis.
According to the methods these were constructed using PCR based C terminal modification. I assume this was followed by transformation into a diploid to create the het diploid which was then induced to sporulate. Is this right? If so please add to methods.

Yes, the reviewer is correct. We added a description of the tetrad analysis to the methods (see lines 279-283).

4) P. 5, paragraph 2 and Fig 3B, there is some disagreement between the text, methods, and Fig 3B on strains. The text and methods refer to shs1ΔAH but it is not in 3B (though the GFP version is there). Similarly, P. 6 first paragraph: "Moreover, we could discount the possibility that the 3xHA tag adjacent to the AH domain was responsible for the lethality since the 3xHA tag adjacent to the Cdc12 AH domain(Cdc12 400-407) had no effect on viability (Figure 3B)."
The 400-407 deletion is not listed in Fig 3B and it is not clear which strains have the HA tag.

We apologize for this being unclear. The *shs1*$^{\Delta AH}$ strain should be referenced as *shs1*$^{488\text{-}507}$ which is in the table. That is corrected in the text (see line 160).

As for the Cdc12(Δ400-407) the reviewer is correct that this is erroneously referenced in **Figure 3B**. We have now incorporated viability results a 37°C from tetrad dissections in the text (see lines 193-195).

We also included more detail on which constructs have a 3xHA tag verse which have a GFP tag in the Figure 3 legend.

5) In many cases Fig 3B indicates that the GFP fusion phenotype was more severe than the corresponding non GFP strain (not clear if these have HA though). Why might this be?

We have now clarified in the text that GFP-tagged Shs1 constructs do not have an HA tag (line 165). Our interpretation of the reduced viability of the Shs1 truncations with a GFP-tag in the *cdc12-6* background is that the fusion interferes with functions of the Shs1 CTE (lines 169-170).

6) p. 5 last paragraph: "The increased cytoplasmic distribution of Shs1$^{\Delta 341-551}$-GFP provides an explanation for the observed synthetic lethality with cdc12-6 mutants."

Meaning is not clear. Are the authors suggesting that lack of membrane localization causes lethality?

We apologize for the cryptic interpretation. We have more clearly stated our interpretation in the text as follows: "The reduced neck-localized signal of Shs1$^{\Delta 341-551}$-GFP suggests a reduced capacity of Shs1-complexes to associate with the membrane which may limit their ability to incorporate into septin filaments and sense curvature.  In cdc12-6 mutants, we hypothesize such a deficiency is lethal." (Lines 172-175).

7) P. 6, paragraph 1: "These data indicate that the Cdc12 AH domain cannot be simply swapped for another AH domain, even if chimeric AH domain recognizes similar curvatures."

Based on the methods, the chimeras appear to have been constructed by fusing the heterologous AH domains to the C-terminus of Cdc12 or Cdc12-6. This is not a "swap." The extra 44 amino acids C-terminal to the AH domain might be required for proper spacing or anchoring. Though the chimeras are stable by Western blot, it is not clear if these heterologous AH domains are functional in sensing the same curvatures as the Cdc12 and Shs1 AH domains. Adherence to membrane-coated beads would give more confidence that these heterologous AH domains are functional in S. cerevisiae, though the potential spacing issue without the final portion of the CTE would still remain.

We apologize to the reviewer for the confusion. There are only 8 residues C-terminal to the Cdc12 AH domain. The Cdc12$^{\Delta 400-407}$ construct (in which those 8 residues are deleted with an HA tag after the AH domain) appears to be at least somewhat functional, as we were able to isolate viable strains expressing this construct as the sole source of Cdc12 even at 37°C (see point **4** above). In this context, we characterized the AH domain chimeras as "swaps".

We agree that it is necessary to test whether the heterologous AH domains are functional when fused to Cdc12 as to rule out other possibilities for their non-functionality. This is an excellent point. As such, we tested this with the other best characterized AH domain with micron-scale membrane curvature sensitivity, SpoVM, fused to cdc12-6. We found this chimeric recombinant septin complex could distinguish nanometer and micron scale membrane curvatures (see new Figure 3, D). We interpret the fact that this chimera could *not* rescue temperature sensitivity in budding yeast gives credence to the hypothesis that the Cdc12 AH domain/CTE has additional roles other than binding and distinguishing membrane curvature, which is the basis of Figure 4.

8) P. 6, last paragraph: "The existence of naturally occurring bundled septin structures in cells suggest that cdc12-6 bundles are not necessarily only a gain-of-function mutation but that the C-terminus of

Cdc12 could be relevant for bundling septins under certain contexts (DeMay et al., 2009; Liu et al., 2019)."

This was confusing in context. After looking more closely at the references, it seems the authors are referring to the existence of "needles" in WT S. cerevisiae under certain conditions and in S. pombe. Perhaps they could explain that needles are also found in some WT situations in S. cerevisiae and other fungi? There are many published cases of septin rods and bars in filamentous fungi that resemble the "needles" shown here.

The reviewer raises an interesting point as to the nature of different types of bundles of septins. While we agree that bundled septin filaments have been documented before, we are unaware of septin filaments that look like thosewe have documented here in *Ashbya* with the *cdc12-6* mutant. As such, we have included additional images of a representative *cdc12-6 Ashbya* mutant with wild-type tagged septins in *Ashbya* for comparison in  Supplementary Figure 3. Moreover, we also quantified the proportion of these “needle-like” filaments based on their fluorescence relative to other physiological septin structures to highlight their abundance (new Figure 4, D). These needle-like bundled structures are numerous and can be large, taking up the length of entire hyphae, with little to any contact to the plasma membrane. Our interpretation (now clarified in the text) is that “needles” may act like a sponge, competing septin complexes from the cytoplasm thereby preventing their localization to functional septin structures. We are not aware of structures like these documented before in cells. If septin filaments like this *have been* published, we apologize and would really appreciate if the reviewer shared relevant literature documenting this phenomenon.

9) P7, paragraph 1: "This transition is dependent on the kinase Gin4,which is predicted to interact with the coiled-coil element of Cdc3 and phosphorylates Shs1 in *S.cerevisiae* (Longtine et al., 1998; Mortensen et al., 2002). The Cdc3/Cdc12 coiled-coil may act like togglable switch, whose disassembly - either through phosphorylation or Cdc12 AH domain sequestration - could promote septin bundling through Cdc3 coiled-coil oligomerization".
Conclusion: "cdc12-6 induces filament bundling into non-physiological needle-like structures, possibly exacerbating its phenotype".
This is confusing. The authors suggest that cdc12-6 might not fold properly and so allows Cdc3 to oligomerize leading to bundling. But the bundles visualized are of cdc12-6 (6B)? Perhaps the authors could clarify?

We have worked to clarify this point. First, we emphasize that this is a speculative model as we have no direct data to support the conclusion.  However, the Cdc12 AH truncation is predicted to disrupt the potential coiled-coil between Cdc12 and Cdc3 by MultiCoil, and may “release” the Cdc3 coiled-coil element to oligomerize with other Cdc3 coiled-coil elements (see MultiCoil plot of Cdc12 C-terminus below in response to a comment by Reviewer 2). The reviewer is correct that bundles are visualized by the GFP tag on cdc12-6, however there is no indication that cdc12-6 is *not* incorporated into the octamers (which would presumably be lethal). Thus, we interpret cdc12-6 localization to be reflective of septin complexes, generally. We hypothesize that if cdc12-6 were not incorporating into octamers, then *cdc12-6* mutants would phenocopy *cdc12Δ* which is sick, prone to lysis and has aberrant morphology in *Ashbya*.

10) In Fig 4 it would be useful to show the WT controls. Also in Figure 4, it is not clear what the coil drawings represent relative to the septin octamer. If the yellow balls are Cdc11 or Shs1 caps, where are the AH domains? Please add detail to the legend, especially on color schemes.

We agree that including wild-type controls would be beneficial in underscoring the septin needle phenotype of the *cdc12-6* mutants. We have included images of *Ashbya* cells expressing wild-type septin from the endogenous locus with a GFP-tag as a supplementary figure (see above). We also updated the cartoon model and legend to be more descriptive characterizing the bundled needle-like septin phenotype and described more carefully the color schemes in the legend. Note we omitted the Cdc11 CTE for clarity.

11) In Figures 3 and 4 more detail is needed in the legends (or the methods). Number of replicates performed and number of cells observed are not shown.

We have now included information on the number of replicates in the legends for Figures 2, 3 and 4.

12) The authors might be interested in a set of experiments reported by Lindsey et al in 2010 (doi:10.1371/journal.pone.0009858). In this work A. nidulans AspC (the Cdc12 ortholog) was shown to rescue the S. cerevisiae cdc12-6 mutant at restrictive temperature. AspC incorporated into the septin ring at the S. cerevisiae neck, replacing Cdc12 and causing elongated buds. So, AspC restored viability, but resulted in abnormal morphology in cdc12-6, consistent with the results presented in this manuscript for Shs1.

We thank the reviewer for bringing this interesting work to our attention. AspC restoring viability in *cdc12-6* mutants is consistent with it having a functional AH domain. It is curious how its expression leads to elongated buds. Perhaps this is due to its overexpression behind either the *GAL1* or *ADH1* promoter resulting in septin octamer subunit stoichiometry imbalances or only partial rescue of the septin assembly. As the original authors point out, AspC-GFP localizes not only to the bud neck but also to the tips of elongated buds. Similar mis-localization of septins to bud tips in other mutant contexts has been interpreted to be due to instability and disorganization of the septin ring leading to subsequent Swe1 stabilization (Wee1-kinase homolog), serving to delay the cell-cycle and promote elongated buds (see Gladfelter et al., 2005 J. Cell Sci: PMID: 15784684 DOI: 10.1242/jcs.02286).

Reviewer #2 (Remarks to the Author):

This manuscript by Woods et al., entitled "Interplay of septin amphipatic helices in sensing membrane curvature and filament bundling" describes the role of amphipatic helices from septin proteins Shs1 in sensing curvature. This article is the follow up of a report by Cannon et al. (2019) which demonstrated that Cdc12 was carrying an amphipatic helix as well, able to sense curvatures. This current report relies on in vitro and cell biology experiments to show that Shs1 carries an amphipatic helix able to sense curvatures. Even though the results are interesting, some of the in vitro versus in vivo experiments are contradictory and misleading for the reader. Curvature sensing is thus not obvious and not unequivocally demonstrated. Hence, I would recommend undertaking major revisions and additional

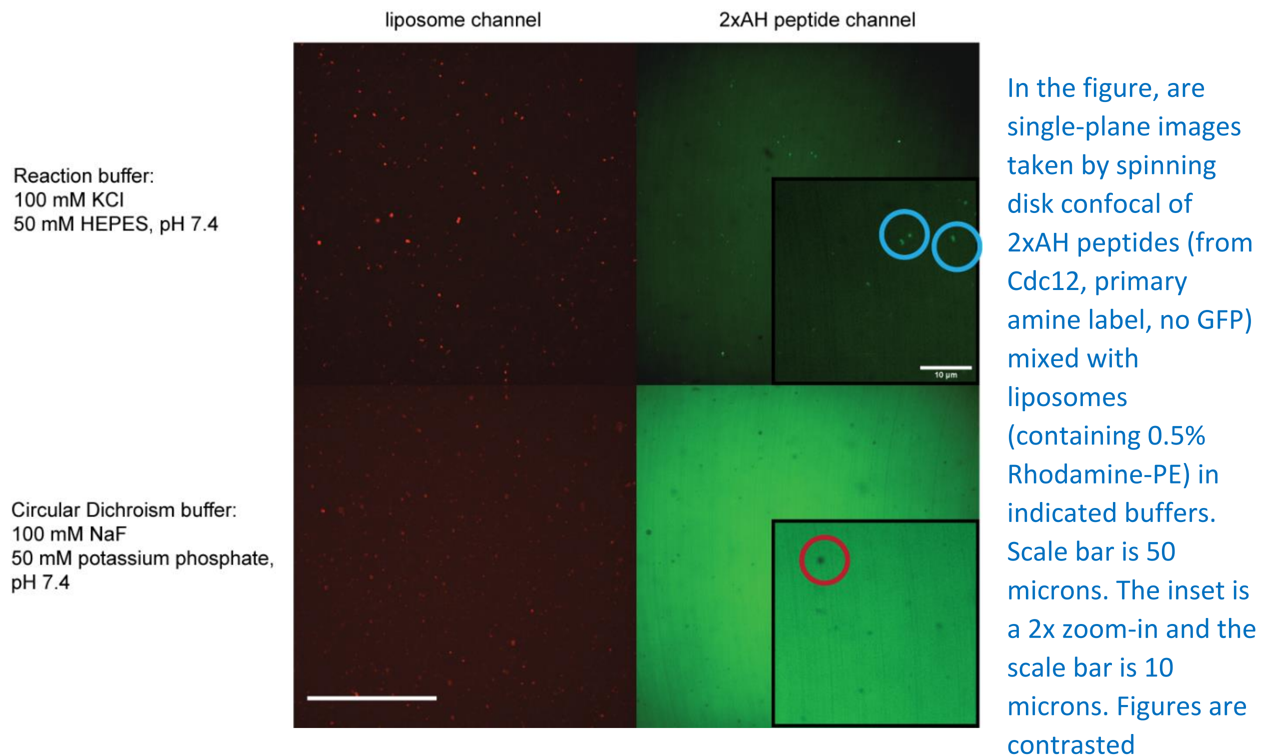
experiments before the manuscript can be published in Molecular biology of the Cell.
You will find my comments below:
Major comments:
1. It is not known how the amphipatic helix was identified. Was it identified "in silico"? Using which software? The authors should describe the procedure used either in the results section or in the methods section.

We apologize to the reviewer for the limited description. We have now included in the Methods a more detailed description of how we bioinformatically screened for amphipathic helix domains within fungal septin protein sequences (lines 354-357).

2. In addition, with the primary sequence indicating the presence of an amphipatic helix, the authors should make sure those are indeed amphipatic helices using alternative experimental methods (for instance: Circular Dichroïsm, binding assays to liposomes, Structural methods: molecular dynamics, NMR).

We have expanded experiments related to Figure 2 (testing a wider range of curvatures and including additional controls) which are the results of binding assays to supported lipid bilayers on silica beads of varying diameters. Preparing lipid bilayers on silica beads, as opposed to using liposomes, affords us more control over the presented membrane curvature in the assays because liposomes can be tubulated by septins, which leads to changes in the curvature. Supported lipid bilayers on beads been successfully used before to demonstrate micron-scale membrane curvature sensitivity. (Please see Gill Jr. et al 2015 PNAS doi: 10.1073/pnas.1423868112, Bridges et al., 2016 JCB DOI: 10.1016/bs.mcb.2016.03.025 and Cannon et al., 2019 JCB DOI: 10.1083/jcb.201807211). We interpret the increased adsorption onto membranes of certain curvatures as evidence of the predicted primary sequence functioning as other amphipathic helices have been shown to do for both nanometer and micron-scale curvature sensors.

We agree with the reviewer it would be ideal to confirm that the primary sequence is indeed an amphipathic helix with additional approaches. Attempts to optimize our binding assay for Circular Dichroism have proven difficult, as we are unable to observe AH peptide binding to liposomes in CD buffer (100 mM NaF and 50 mM potassium phosphate pH 7.4, bottom). This contrasts with our observations where AH peptide binding to liposomes is readily observed in a minimal reaction buffer (100 mM KCl and 50 mM HEPES pH 7.4, top):




In the figure, are single-plane images taken by spinning disk confocal of 2xAH peptides (from Cdc12, primary amine label, no GFP) mixed with liposomes (containing 0.5% Rhodamine-PE) in indicated buffers. Scale bar is 50 microns. The inset is a 2x zoom-in and the scale bar is 10 microns. Figures are contrasted differently to highlight the adsorption discrepancy. Note the liposomes are visible in Reaction buffer on the AH peptide channel as diffracted limited puncta because the AH construct readily adsorbs to the liposomes (blue circles). In contrast, liposomes in CD buffer appear as "ghosts" (red circle) as there is absence of fluorescent signal relative to the background fluorescence, indicating minimal to no binding.

Efforts towards troubleshooting and including CD on the primary sequence of multiple putative AH domains are planned to be part of subsequent manuscript but after significant effort, we could not find conditions compatible for CD.

3. It is difficult to appreciate how molecular cues at nanometer scale like amphipatic helices can induce curvature sensing at micrometer scale. Usually, much higher curvatures (at nanometer scales) are sensed by amphipatic helices. For instance, ALPS proteins would interact with 50 nm diameter liposomes (as an example see: Mesmin et al., 2007, Biochemistry). What would be the mechanisms inducing micrometer curvature sensing by a 20 residues long helix? Can the authors discuss this?

The reviewer raises a wonderful challenge and conundrum in understanding the mechanism of curvature sensing in septins! We hypothesize micrometer curvature sensitivity might emerge from the relative spacing of amphipathic helixes within the octamer and/or a polymerized filament. However, additional factors could also be at play, such as the stoichiometry of amphipathic helices within the complex, as well as nature of the lipid packing defects that are presented at the micron-scale vs nanometer-scale, which could affect the depth of helix insertion within the membrane. These factors, of course, remain difficult to measure and speculative. However, the septins are not alone in this capacity, as SpoVM is a prokaryotic peptide that recognizes micron-scale curvature via a 13-residue AH domain. Molecular dynamics simulations suggest its insertion deeper into the membrane enables it to distinguish

slight vs highly curved membranes (see Gill et al., *Structural basis for the geometry-driven localization of a small protein*. PNAS (2015) doi: 10.1073/pnas.1423868112). Oligomerization either via the septin complex and/or through AH-AH interactions is likely key to help bridge the lengths scales of the single helices to the sensed curvature. We have discussed this in a review (Cannon K.S., Woods B.L., Gladfelter A.S. Trends Biochem Sci. (2017) Dec;42(12):966-976; doi: 10.1016/j.tibs.2017.10.001.) and to a certain degree in Cannon (2019). We are limited on character count here so have limited our elaborations in this manuscript.

4. The authors suggest that curvature sensing is the only and primary factor responsible for septin recruitment. They point out that Cdc12-6 mutants localize to the bud neck at permissive temperatures but are expelled at restrictive temperatures because of curvature sensitivity deficiency. However, other factors are known to be crucial for septin recruitment as well (Bni5 for instance, see lee at al. 2002, Mol. Cell. Biol.). The manuscript should discuss this.

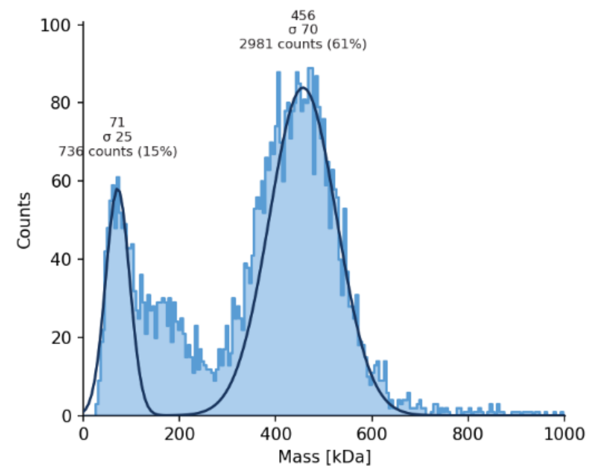
We apologize if the text conveyed that the AH domain(s) are solely responsible for septin localization. This is obviously not the case, as the reviewer points out, as an array of polarity proteins can bind and recruit septins to the nascent bud-site (Gladfelter et al., 2002; Lee et al., 2002; Lai et al., 2018, and many others). As this work does not directly assess initial septin localization and recruitment in cells, we deemphasized this assertion (see line 73).

5. It is not known whether the experiments generating figure 2 have been repeated and how many times. The error bars (highest curvatures, figure 2.A) seem extremely large. More data should be added to improve the statistics.

We agree that repetition of these experiments would strengthen the case presented in Figure 2. As such, we have repeated these experiments on additional curvatures with multiple replicates. Each measured adsorption to a supported lipid bilayer on an individual bead is now included as a point in the figures. We have also included statistical tests comparing relative membrane adsorption.

6. The protein-membrane interaction visualized in Figures 2.A and 2.B does not look homogeneous. In most of the displayed images, septins or polypeptides bind to silica beads as clusters of proteins. Hence, we might wonder whether these are not aggregated proteins and whether the protein membrane interaction results from unspecific aggregation. The main conclusions are thereby altered and suffer from the quality of the experimental data.
Hence, I would suggest the following:
- The fitness of the purified polypeptides and septin complexes in solution should be tested for solubility, proper refolding and integrity of complexes. For instance, elution profiles from size exclusion, electron microscopy images could be displayed. It is known that Shs1 complexes are not as robust as Cdc11 complexes (see Garcia et al. JCB, 2011 and Weems et al., Genetics, 2014).

We have now included a Silver stain or Coomassie blue SDS-PAGE of the Shs1-capped constructs validating their stoichiometry. To further determine the integrity of the Shs1-capped complexes, we assessed their complexed state using interoferometric scatter mass spectrometry (iSCAMS) to determine the mass of the complex based on the degree of light scatter (Young et al., Science 2018, 360 (6387), 423-7; doi: 10.1126/science.aar5839). The majority of the light scatter measurements from the recombinant Shs1-GFP capped complex corresponded to a molecular mass of 456 kDa, within the standard error expected by iSCAMS. (The predicted molecular weight of the Shs1-GFP-capped complex is 467 kDa). Cdc10 is 37 kDa, Cdc3 is 60 kDa, Cdc12 is 47 kDa and Shs1 fused to GFP is 89 kDa. It is conceivable the lower peak (~71 kDa) represents dimerized Cdc10 subunits (74 kDa). Alternatively, the peak corresponding to ~71 kDa could be a bacterial contaminant, potentially a chaperone, and unlikely representative of any septin subunit. For the Shs1-2XAH, aggregation could be a concern. However, we predict that the puncta we see are most likely oligomers because if they were aggregates non-specifically binding to membranes then we would expect to see no curvature specificity. However, we observe that these constructs bind differentially to different curvatures, suggesting they are not aggregating.

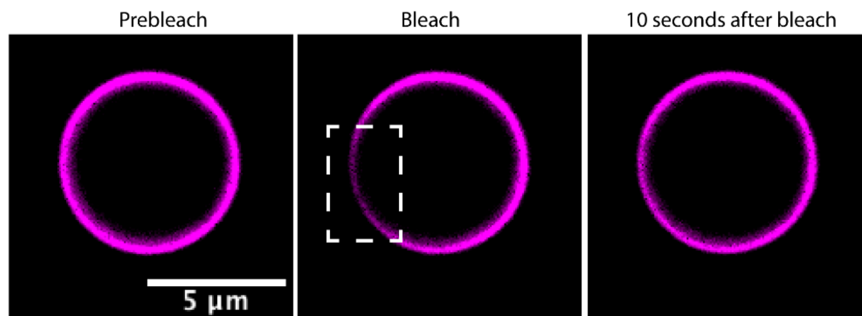



- The fluorescence intensity of the protein signal should be calibrated to give some insights in the actual protein density covering the beads.

We would also really like to have absolute concentrations of septin filaments on beads. Unfortunately, adsorption was measured using spinning disk confocal microscopy and it is not possible to robustly calibrate this experiment using this optical system.

- The quality of the lipid bilayer covering the silica bead should be examined. Quantitative evaluation of the lipid fluorescence signal could be performed to check whether it is fully homogeneous. SEM imaging, similarly to the data shown in Cannon et al 2019, could also be carried out to visualize both the membrane and the septins.

We assessed the fluidity of the bilayers by FRAP, partially bleaching one side of larger beads (> 5 μm) to assess recovery. The half-time to recovery was approximately 7.4 seconds. Information regarding the fluidity of the supported lipid bilayer is included in the Methods (lines 333-335). Unfortunately, we could not test lipid bilayer fluidity on smaller diameter beads due to limits in constricting the photobleaching laser focus. Data for this included here:




- Besides, the proportion of charged lipids (PI at 25 %) seems quite high to be fully incorporated within a bilayer. It is most likely probable that the actual PI concentration is lower, within the bilayer. A range of PI concentrations as well as other charged lipids in the lipid mixture should be tested to check for protein membrane interaction variability.

This is indeed a high concentration of PI however the bud neck is likely highly anionic based on FRET reporters for PIP2 (Garrenton L.S., Stefan C.J., McMurray M.A., Emr S.D., Thorner J. PNAS (2010) 107(26): 11805-11810; https://doi.org/10.1073/pnas.1005817107). It is a good point that different lipid compositions may affect the adsorption and curvature preferences. Therefore, we tested how curvature sensitivity is altered replacing phosphatidylinositol with phosphatidylserine. PS has a bulkier headgroup but has the same charge as PI. The results of these experiments are now included in Figure 2. On PS containing lipid mixtures, we found that the Shs1-capped complexes (whether with wild-type Cdc12 or cdc12-6) had similar curvature preferences to one another, with the greatest affinity to smaller curvatures ($\kappa$ = 6.7, 4, 2 $um^{-1}$) than to larger curvatures ($\kappa$ = 0.67, 0.4$um^{-1}$). Thus, curvature sensitive binding by septins can be seen in the context of two different anionic lipids.

7. It is not mentioned why, in Figure 2.B, data at curvatures higher than 2 µm-1 is not shown. This should be added otherwise one does not understand why it is not shown, in comparison with Cannon et al. 2019 and Figure 2.A.

We have now expanded experiments in Figure 2 to test additional curvatures.

8. Testing membrane curvature sensitivity with a polypeptide might not be an optimal choice since it might behave completely differently from a protein within a much larger complex. The authors do not comment or explain this choice.

This is an experiment to test sufficiency of the AH domain in sensing membrane curvature, which is why for these limited set of experiments the polypeptide isolated from the complex is used. We have modified the text to justify our rationale for testing the polypeptide alone: “We next assessed whether the *S. cerevisiae* Shs1 AH domain … alone is sufficient to distinguish between membrane curvatures.” (See lines 85-86 for context).

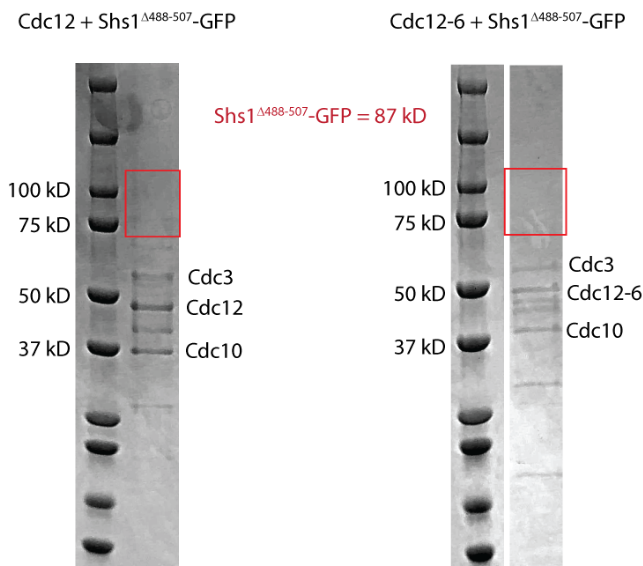
9. A significant (about 5 times) lower affinity is observed for Shs1 complex with Cdc12-6 mutant than for

Shs1 wild type complex (Figure 2.B, right). The stability of this complex in solution should be analyzed since an altered refolding or stability might be responsible for this observation.

The reviewer is correct that the overall adsorption of the cdc12-6 Shs1 complex is less than the Cdc12 complex. However, we found no evidence to suggest that the either of the purified wild-type Shs1-complexes (either with cdc12-6 or Cdc12) presented in this work are not as robust as Cdc11-complexes (see our comments to point 6 and Cannon et al., 2019). Nonetheless, we explicitly point out this lower affinity in the text and offer our interpretation (see lines 137-143).

10. Why were the mutations performed in situ not tested as well in vitro for curvature sensitivity? Testing truncated Shs1 mutants in vitro as well would bring essential knowledge to understand the phenotypes observed in Figure 3. Without those analyses, essential evidences are missing to demonstrate that the AH domain of shs1 is indeed able to sense curvature rather than only localizing septin complexes at the bud neck.
- At least, the ShS1 (delta AH) mutant capping the septin complex should be tested for its interaction with silica beads.

Unfortunately, we were unable to purify the shs1$^{\Delta 488\text{-}507}$ Cdc12-6 complex or shs1$^{\Delta 488\text{-}507}$ Cdc12 complex to test its adsorption on curvatures as suggested. A Coomassie stained gel of our attempt at purifying these complexes is below for the reviewer's benefit (red boxes indicate the absence of the expected Shs1 subunit bands). This of course raises the possibility that that combining these mutations may inhibit complex formation in cells, which could be the basis for lethality. However, given the data that wild-type Shs1 rescues the ability of septin complexes harboring Cdc12-6 to sense curvature, and that the Shs1 AH domain can distinguish similar curvatures on its own are consistent with the notion that Shs1 imparts at least *some* membrane curvature sensitivity. The ability of truncated shs1 to localize to the bud neck in otherwise wild-type cells (*CDC12*) suggests our inability to purify truncated Shs1 complexes may be specific to recombinant protein. Given the myriad of caveats and their permutations, we have more carefully characterized our interpretations of the data under the retitled Results section "**The Shs1 CTE harboring an AH domain is required for normal septin function in the *cdc12-6* mutant**" (line 145).




- That would be beneficial to test point mutations that alter the electrostatic properties of the AH helix, both in vitro and in situ.

This is an excellent point, and unfortunately a series of mutations in the AH that change the electrostatic properties all perturbed filament formation *in vitro*, precluding possible interpretations of adsorption on different curvatures. Instead, we have now included results from Cdc11 Cdc12-6-SpoVM$^{AH}$ complex (see new Figure 3, D), which is the prokaryotic micron-scale curvature sensor. Interestingly, the SpoVM AH

does restore curvature sensitivity of Cdc12-6 in vitro despite being unable to complement in vivo.

11. It is puzzling that the chimeric strains are not viable. It is possible that those constructs induce significant misfolding and unstability of the septins complexes and thus exclude the proteins by quality control mechanisms (see Johnson et al. 2015, Elife). This suggest that the delta AH domain and the CTE might have additional functions in addition to curvature sensing: protein folding, proper integration into the complex.

We think it is unlikely that chimeric constructs induce significant instability considering the normal expression (based on Western blots) of the chimeras relative to the more functional constructs (Cdc12-6 and Cdc12$^{\Delta 400\text{-}407}$), which suggests these constructs are not being degraded (see Nagaraj S, Rajendran A, Jackson CE, Longtine MS (2008) Mol Cell Biol 28, 5120–5137). We wholeheartedly agree that the AH domain and/or the CTE may have additional functions that are important. This hypothesis is what lead us to further investigate this relationship and discover the bundled-needle like structures observed in the Cdc12-6 mutants in yeast and *Ashbya*. Since the Cdc12$^{\Delta AH}$ chimeric strains are inviable, we cannot directly assess whether this phenotype is exacerbated and perhaps therefore the cause of the lethality.

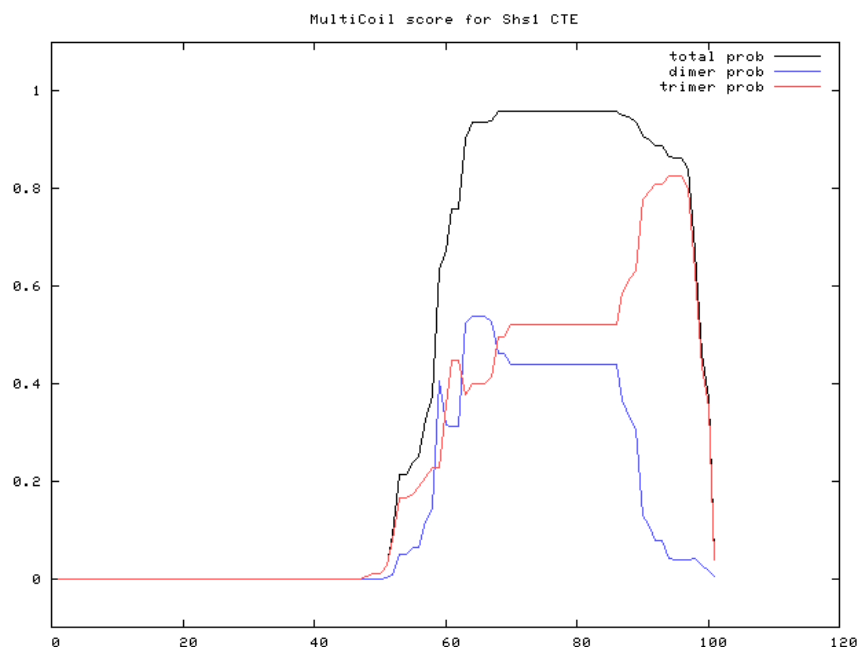
Minor comments

1. Figure 1 should be described properly. The colors code used is not described. The residues should be numbered. Figure 3A should be displaced within Figure 1. We have a more thorough description of Figure 1 in the legend, have displaced Figure 3, A to Figure 1 as suggested.

2. When introducing the AH domain, the authors should describe, in the text, how the AH sequence compares with the sequence of the coiled coil domain and which are the residues involved. A scheme would also be beneficial.

We have included in the text the residue location of the AH domain in Cdc12 and the relevant Cdc12-6 mutant. For the reviewer's benefit here are the last 100 residues of Cdc12 (AH domain highlighted): VRQLGREIKQENENLIRSIKTESSPKFLNSPDLPERTKLRNISETVPYVLRHERILARQQKLEELEAQSAKELQKRIQELERKAHELKLREKLINQNKLNG

This sequence run through MultiCoil predicts a strong coiled coil:




3. Statistics issues: It is not mentioned whether the experiments from figure 2, figure 3 and figure 4 have been repeated and how many times. Besides, the exact number of beads where a fluorescence intensity

measurement has been performed is not specified. The plots from figure 2A and 2B would benefit greatly from being drown as clouds of dots to visualize better how the data is spread. We have now included number of experimental repetitions and bead number in the legends and have adjusted the presentation for Figure 2, A & B as suggested to better represent the data spread. We have also included details to the tetrad dissections in Figure 3, and the observations of needles in Figure 4

4. The last paragraph describing the needles in asshby appears slightly unrelated to the rest of the report. Partial truncation within the coiled coil domain of Cdc12 might impair the pairing ability of septin complexes and induce the aggregated bundled structures visualized. The needles would require some quantification in terms of dimensions and proportion of needles versus "standard" structures. This is an excellent point. We have therefore quantified the abundance of these structures in terms of their relative fluorescence compared to that of other physiological structures, and compared that to that of wild-type *Ashbya*. Please see our response to Reviewer 1 point #8.

RE: Manuscript #E20-05-0303R
TITLE: "Interplay of septin amphipathic helices in sensing membrane-curvature and filament bundling"

Dear Amy,

I thank you for sending your revised manuscript. It has now been evaluated by the same two reviewers, who both appreciate the additional work you have performed and support publication. They have very minor comments, which you should be able to address rapidly with text changes (addition of tag information to the legend of figure 4 and acknowledgment in the text that the curvature preference shown by the Shs1 AH varies depending on context). Upon these modifications, I should be able to accept your manuscript rapidly.

Best wishes,
Sophie

Sophie Martin
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Gladfelter,

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.

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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

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Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office

mbc@ascb.org

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Reviewer #1 (Remarks to the Author):

In this revised submission Woods et al did a good job of clarifying areas that were problematic in the first version, especially around the specifics of the cdc12-6 mutations, truncation constructs, and the nature of the "needles" observed in cdc12-6 in Ashbya.

My only remaining suggestion is to state the tags used in Fig 4, E, F and H. Based on the other panels in this figure I think that the imaging is of cdc12-gfp or cdc12-6-gfp, but it would be helpful to have this stated.

This paper now clearly supports the very interesting ideas that the Shs1 AH alters curvature sensing of the septin complexes carried out by Cdc12 and that the Cdc12 AH has a role in regulating septin filament bundling.
Reviewed by Michelle Momany

Reviewer #2 (Remarks to the Author):

As requested, Woods and collaborators have performed a large number of additional experiments to improve the statistics of their assays and answer to the different comments. I thank them for pursuing their efforts. In particular, the paragraph on the role of the CDC12 AH in septin bundling is convincing. Hence I would recommend the report to be published in Molecular Biology of the cell after they comment on the following point:
- Looking at the data, it is unclear whether SHS1 AH domain within a polypeptide or within a complex has any curvature preference. Instead it seems that the interaction is not correlated with any specific curvature. Hence I would only recommend to rephrase and tame some of the affirmative statements. For instance, the first paragraph subtitle might be too affirmative...
For future publications, I would recommend to try to find alternative methods to prove the curvature sensing nature of these amphipatic helices. In addition, it is still unclear why the septins bind to silica beads as clusters of proteins instead of covering them homogeneously. It would thus be quite revealing to visualize septins on the beads by SEM methodologies as already performed in Cannon et al. (2019).

We appreciate the reviewers' enthusiasm on our work following their suggested revisions. In this most recent draft of the manuscript we have addressed the minor comments brought to our attention by the reviewers, which includes information on the tag in the legend of Figure 4, and tamed the language in reference to Shs1 AH membrane curvature binding. More detailed responses to these reviewer comments are below.

Dear Amy,

I thank you for sending your revised manuscript. It has now been evaluated by the same two reviewers, who both appreciate the additional work you have performed and support publication. They have very minor comments, which you should be able to address rapidly with text changes (addition of tag information to the legend of figure 4 and acknowledgment in the text that the curvature preference shown by the Shs1 AH varies depending on context). Upon these modifications, I should be able to accept your manuscript rapidly.

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Sophie

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Monitoring Editor
Molecular Biology of the Cell

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We apologize for omitting this important information. We have now included the necessary information on the tags in the legend (lines 588, 591, and 595-596). Note that for Figure 4, F and H, recombinant Cdc11 is SNAP tagged, which is conjugated to an Alexafluor488 dye.

This paper now clearly supports the very interesting ideas that the Shs1 AH alters curvature sensing of the septin complexes carried out by Cdc12 and that the Cdc12 AH has a role in regulating septin filament

bundling.
Reviewed by Michelle Momany

Reviewer #2 (Remarks to the Author):

As requested, Woods and collaborators have performed a large number of additional experiments to improve the statistics of their assays and answer to the different comments. I thank them for pursuing their efforts. In particular, the paragraph on the role of the CDC12 AH in septin bundling is convincing. Hence I would recommend the report to be published in Molecular Biology of the cell after they comment on the following point:
- Looking at the data, it is unclear whether SHS1 AH domain within a polypeptide or within a complex has any curvature preference. Instead it seems that the interaction is not correlated with any specific curvature. Hence I would only recommend to rephrase and tame some of the affirmative statements. For instance, the first paragraph subtitle might be too affirmative...

We recognize that the Shs1 AH domain binding to membranes is weaker than that of the Cdc12 AH domain. We have therefore rephrased our interpretation of these results to be less affirmative as suggested by the reviewer. For example, the first subtitle has been changed to "A predicted AH domain of Shs1 differentially binds various membrane curvatures *in vitro*" (line 65). Other modifications that tame the assertions are at lines 58-60, and 144.
For future publications, I would recommend to try to find alternative methods to prove the curvature sensing nature of these amphipatic helices. In addition, it is still unclear why the septins bind to silica beads as clusters of proteins instead of covering them homogeneously. It would thus be quite revealing to visualize septins on the beads by SEM methodologies as already performed in Cannon et al. (2019).

RE: Manuscript #E20-05-0303RR
TITLE: "Interplay of septin amphipathic helices in sensing membrane-curvature and filament bundling"

Dear Amy,

Thank you for the few additional changes in your manuscript. I am pleased to accept it for publication in Molecular Biology of the Cell. It makes a nice addition to our understanding of membrane curvature-sensing by septins.

Best wishes,
Sophie

Sophie Martin
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Gladfelter:

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.

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