C. elegans srGAP is an α-catenin M domain-binding protein that strengthens cadherin-dependent adhesion during morphogenesis

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MS TITLE: A C. elegans srGAP is a novel a-catenin M domain-binding protein that strengthens cadherin-dependent adhesion during morphogenesis

AUTHORS: Joel M. Serre, Bethany Lucas, Sterling C.T. Martin, Xiangqiang Shao, and Jeff Hardin

I apologize for the delay before being able to come back to you. I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express strong interest in your work, but have some significant criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and 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. 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

This manuscript builds on earlier work from the Hardin lab, that identified C. elegans SRGP-1 (the nematode homolog of vertebrate SRGAPs) at adherens junctions and showed that it contributes to junctional integrity (as revealed on backgrounds that were sensitized by weak catenin alleles). The analysis of SRGP-1 is complex because it possesses a) multiple membrane binding domains (including an F-BAR domain); and b) multiple potential downstream effector pathways. The present manuscript thus extends our current knowledge to show that i) the C-terminal of SRGP-1 can bind directly to the M-domain of HMP-1, which promotes its localization to junctions; and ii) SRGP-1 may stabilize HMP-1 to mediate its effects on junctional integrity. The authors further extend their functional analysis to report that the C-terminus, but not the GAP activity of SRGP-1 is necessary for the molecule's function in the embryo. Thus, the paper reports valuable insights into a poorly-understood regulator of adherens junctions in development.

Comments for the author

Specific points
1) In Fig 3 C-D the authors present evidence to suggest that SRGP-1 may stabilize HMP-1/a-catenin. The implication is that this might reflect feedback from SRGP-1 via its direct interaction with HMP-1/a-catenin. But an alternative interpretation is that SRGP-1 is working to stabilize E-cadherin itself. It would be helpful to characterize whether E-cadherin levels and/or stability are affected in the SRGP-1 mutant.
2) Pull-down assays. This is the important evidence that HMP-1 and SRGP-1 can interact directly, but I felt it could have been documented in greater detail:
a) Can the authors quantitate the data or confirm its reproducibility in independent experiments.
b) I missed its description in the Methods. (I did see an "Actin Cosedimentation assay", but this data isn't in the figures. Perhaps this should have been the pull-down assay?)
c) If there is space, I think that the characterization of mutants could go in Fig 1, as it establishes the domain requirements.
3) The authors present rather surprising data to suggest that conformational "opening" of the HMP-1 M-domain actually antagonizes the recruitment of SRGP-1.
This is puzzling because at face value it seems at odds with the observation that SRGP-1 recruits to junctions at a time when AJ appear to be under increased tension (Fig 4H, line 185) – a mechanical effect that would be predicted to open the M-domain. This conclusion would be strengthened if the authors could confirm that the salt-bridge mutants used to "open" the M-domain do indeed alter the conformation of the molecule. Does the Nagafuchi a-18 mAb work in C. elegans embryos?

Minor points
1. If space permits, it would help the reader to introduce a description of the HMP-1/a-catenin mutants into the narrative and also describe the results of the pull-down assays. If there isn't enough space in the main text, it could be added to the caption of Fig S2.

Reviewer 2

Advance summary and potential significance to field

The authors had previously identified the srGAP protein SRGP-1 in an enhancer screen made in a weak C. elegans alpha-catenin/ HMP-1 mutant. Here they have examined in further details the basis for the functional interaction between both proteins. They found using several approaches (yeast 2-hybrid, co-IP, in vivo subcellular localization, genetic interaction studies) that SRGP-1 interacts with HMP-1 at least in part through domain its C-term region (residues 685-1059) on the HMP-1 middle region. They further show that mutating two Arg residues of the HMP-1 middle region in Ala compromises SRGP-1 junctional recruitment, and conversely that an SRGP-1 null allele very slightly reduces HMP-1 mobility (it is marginally significant).

Their model is that SRGP-1 helps stabilize the cadherin-catenin complex at membranes when tension builds up. They further compare HMP-1 to mammalian alpha-catenin, which recruits vinculin when its middle region unfolds under tension; however, unlike that case, they argue that SRGP-1 would preferably bind to the middle region still folded and stabilized by the two Arg residues that they mutated.

The data are solid. On the other hand I was left slightly frustrated with their final model, which I think could be easily put to a functional test.

Comments for the author

1/ Their model predicts that "tension regulates the partially closed conformation of the HMP-1 M domain to support SRG-1 recruitment" (orange box in Fig. 4H). If so, Should we expect that loss of tension will affect SRGP-1 recruitment? What happens in hypo-tension mutants?
2/ Their model also predicts that "SRGP-1 stabilizes the CCC via scaffolding to the plasma membrane" (violet box in Fig. 4H), a conclusion that relies on their previous observation that the F-BAR domain is essential for SRGP-1 function. In their previous paper they had concluded that the 200 residues immediately following the F-BAR domain were sufficient for junctional targeting, hence I was not entirely convinced by the current model. Is it that SRGP-1 can bind to junctions and HMP-1 through two domains, or is it that the SRGP-1 stabilizing function is through another factor rather than through the plasma membrane? Could they test so?
3/ The notion that SRGP-1 stabilizes HMP-1 relies on FRAP data with a significance at P= 0.04, so quite weak. I noticed that they had examined 11 embryos (it is actually written n=1 in the main text and n=11 in the legends). Would it change with additional samples, or with samples at a later stage? Actually the stage at which the FRAP has been performed is not described and should be indicated.

Minor:
1/ Fonts are too small in Fig. 1D, and the deletion symbol Δ is missing in panel C for SUMO-HMP-1(ΔVH2).
2/ I was surprised that they could not test the effects of SRGP-1 deletion constructs in SRGP-1; HMP-1(fe4) double mutants, as I imagine that it should be straightforward to balance one of the mutations.
3/ The Methods section mentions "Actin cosedimentation assays" but I could not find the results of such assays. Do you really need that section?

Reviewer 3

Advance summary and potential significance to field

Maintaining connections between cadherin- based cell junctions and the cytoskeleton is important for both embryonic development and tissue homeostasis. The nature of the connection and the molecules involved have been revealed to be increasingly complex. The Hardin lab has previously used clever genetic screens in C. elegans to identify proteins that enhance the phenotype of weak mutations in alpha-catenin, thus identifying candidate molecules involved in connecting junctions and the cytoskeleton. In a lovely paper in 2010 they identified a Bar domain containing Rho GTPase GAP called SRGP-1 as an interactor. While SRGP-1 mutants have no overt phenotype, they enhance weak mutations in alpha-catenin. Mutants alter catenin dynamics, and the C-terminus acts as a negative regulator. Here they extend this work. Using biochemical approaches, they find that the SRGP-1 C-terminal region binds alpha-catenin VH2. They then explore whether the C-terminus is important for junctional localization, and how different alpha-catenin mutational alterations affect SRGP-1 junctional localization. Finally, they test what domains of SRGP-are important for its genetic interaction with weak beta-catenin mutants or lack thereof. Some of these data are not as clear as one would expect, and some effects observed are fairly modest. I detail these issues below. Altogether, it is important to examine modulators of junctional function, andthis work is a solid addition to our understanding of alpha-catenin/ SRGP-1 interactions, but the breadth of understanding is somewhat limited.

Comments for the author

Things clearly demonstrated versus Major issues
1. The authors clearly demonstrate that SRGP-1 can bind alpha-catenin VH2.
2. Figure 1E explores the role of the C-terminus in junctional localization. They conclude “These data indicate that the SRGP-1 C-terminus is necessary for restricting it to junctions and for normal association with HMP-1. Because some SRGP-1DC can localize to junctions, we infer that additional interactions outside the C terminus also promote accumulation of SRGP-1 at junctions.” However, I found the image used to demonstrate this unconvincing. Outside boxed area the localization seems seem very similar, and in the images in the bottom row the channels seem poorly aligned.” Even in the closeup image used the conclusion that the effect is on SRGP-1 localization was not totally not accurate—if there is an effect it is on alpha-catenin, as they note: “In contrast, HMP-1 frequently no longer followed SRGP- 1(ΔC)::GFP into membrane bends (Figure 1E, insets), as reflected in a change in overlap at junction (Manders overlap coefficient M2 with thresholding, GFP overlap with mScarlet-I = 0.82 for SRGP-1::GFP, n = 57 junctions; M2 = 0.46 for SRGP-1(DC)::GFP, n = 36).” Finally given what follows they should tell us the stage examined here—since they next show us it localizes poorly to junctions!
3. The authors clearly demonstrate that SRGP-1 localization to junctions is weak early and better later.
4. In Figure 2 the authors examine the role of alpha-catenin’s VH2 domain on SRGP-1 localization. They conclude “srgp-1::mScarlet-I; hmp-1(jc48); Ex[hmp-1(ΔVH2)::gfp] embryos retain detectable membrane localized SRGP-1::mScarlet-I in lateral epidermal cells (seam cells) during elongation but atgreatly reduced levels (Figure 2F, H).” However, I found the degree of reduction not very clear, as the mScarlet tagged protein was not as robustly junctional
5. In Figure 3 the authors examine the ability of a fully open alpha-catenin mutant to recruit SRGP-1. They conclude: “Whereas full-length HMP-1::mScarlet-I colocalized precisely with full-length SRGP-1::GFP (Figure 3A, top), HMP-1(R551/554A) did not (Fig. 3A, second row), reflected in a statistically significant decrease in colocalization at junctions”. While there was clearly an effect on co-localization, I thought the data was more complex. Unlike other manipulations, the effect was not simply reduction or loss of membrane localization.
Instead, it was Interesting but potentially more complicated. The two proteins now align “in parallel”—is this an apical/basal localization difference? In contrast, the loss of junctional localization of the Cterm construct was much more straightforward.
6. Later in Figure 3 they examine the effect of SRGP-1 loss on alpha-catenin localization, something they also examined in different ways in 2010. They first look at “Junctional intensity”, concluding “We did find however, that in srgp-1(gk441841) mutants average junctional intensity of an endogenous HMP-1::GFP knock in (Marston et al., 2016) was measurably decreased compared to wildtype (Figure 3C).” How was this measured?—calculating relative junctional intensity between different mutants requires careful controls. They also measure mobility by FRAP: “Loss of SRGP-1 led to a statistically significant decrease in recovery half life (t1/2 for wildtype = 25.0 sec; t1/2 for srgp-1 = 30.5 sec” Both effects, especially that on dynamics, were pretty subtle.
7. The authors clearly demonstrate the importance of different protein domains of SRGP-1 needed for genetic interaction with beta-catenin, extending work in the 2010 paper.
8. The 2010 paper argued that the C-terminal region was a negative regulator of SRGP-1 function—here they argue for much different roles. The discussion does not clearly integrate these views.
Minor issues Introduction. What do we know about roles of SRGP in other systems and contexts? =What is the relevance of the Slit/Robo connection?
Fig 1 Add the diagrams in Fig S1B to Fig 1 Fig. 1A. Make it clear that the IP is of SRGP-1:GFP (i.e., don’t just label it GFP!)
Fig. 1C. It’s tough to determine what was done here….. what was pulled down and then what was done with the pulldowns. Fig S2 is even more cryptic

First revision

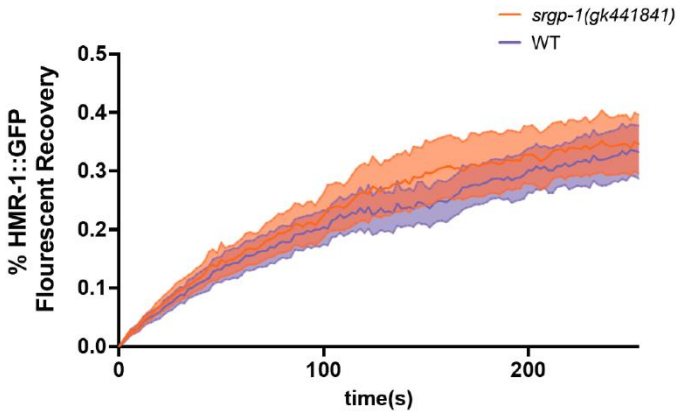
Author response to reviewers' comments

Response to Reviewers
We thank the Reviewers for many helpful comments on our original manuscript. We believe our responses have substantially improved the manuscript. We note several changes to the manuscript:

(1) We have **bolded in red** substantive additions to the manuscript in response to the Reviewers' comments. We detail our responses to the Reviewers in an attached PDF.
(2) *Domain maps:* Reviewers 1 and 2 both requested that we add the domain maps and deletion construct schematics from Supplemental Figure 1 into the main figures. We have done so in revised Figure 1 (new Fig. 1C).
(3) *Citation of previous work:* We realized that we inadvertently omitted explicit discussion of the single precedent for a role for an srGAP in epithelial cells besides our own, work from Alpha Yap's group (Liang *et al.*, 2017. *Nat Commun* **8**, 790). We have rectified that omission in the revision (lines 194-198). This adds slightly to the length of main text, but we hope that you will allow this addition.
(4) *Salt bridge mutant analysis:* We have added new data using limited proteolysis to show that the salt bridge mutations we introduced into HMP-1 in fact do open the HMP-1 M domain. This data is now Supplemental Fig. 2A.
(5) *Additional author*: Jon Heier performed the limited proteolysis and was added as an author.

Reviewer 1
1) In Fig 3 C-D the authors present evidence to suggest that SRGP-1 may stabilize HMP-1/a-catenin. The implication is that this might reflect feedback from SRGP-1 via its direct interaction with HMP-1/a-catenin. But an alternative interpretation is that SRGP-1 is working to stabilize E-cadherin itself. It would be helpful to characterize whether E-cadherin levels and/or stability are affected in the SRGP-1 mutant.

- *This was a sensible suggestion. We performed FRAP with an endogenous HMR-1::GFP knock-in in otherwise wild-type backgrounds and in the srgp-1(gk441841) background and found that in homozygotes for the* srgp-1 *nonsense allele there was a slight (but not statistically significant) increase in half-life of recovery, but no difference in percent mobile fraction:*



We did not include this result in the revised Supplemental Materials, since it is below statistical significance. It is, however, consistent with the HMP-1 FRAP data we have included in the main paper.

2) Pull-down assays. This is the important evidence that HMP-1 and SRGP-1 can interact directly, but I felt it could have been documented in greater detail:
a) Can the authors quantitate the data or confirm its reproducibility in independent experiments.
b) I missed its description in the Methods. (I did see an "Actin Cosedimentation assay", but this

data isn't in the figures. Perhaps this should have been the pull-down assay?)
c) If there is space, I think that the characterization of mutants could go in Fig 1, as it establishes the domain requirements.

- *The pull-down was performed twice (a), and the Materials and Methods have been updated to reflect the correct technique (b). We included the domain maps with mutants and deletion constructs as Fig. 1C in the revision.*

"3) The authors present rather surprising data to suggest that conformational "opening" of the HMP-1 M-domain actually antagonizes the recruitment of SRGP-1. This is puzzling because at face value it seems at odds with the observation that SRGP-1 recruits to junctions at a time when AJ appear to be under increased tension (Fig 4H, line 185) – a mechanical effect that would be predicted to open the M-domain. This conclusion would be strengthened if the authors could confirm that the salt-bridge mutants used to "open" the M-domain do indeed alter the conformation of the molecule. Does the Nagafuchi a-18 mAb work in C. elegans embryos?"

- *Unfortunately, in our experience monoclonals raised against vertebrate proteins fail to cross-react with worm proteins, and this would be expected to be even less likely for such a specific conformation-dependent epitope. HMP-1 and mouse aE-catenin are only 60% similar and 36% identical at the amino acid level, making this unsurprising. Similar results have been obtained in flies. Ulli Tepass's group at the University of Toronto tried the a-18 antibody in* Drosophila *a number of years ago without success (UT, pers. comm.; details are available upon request).*

  To address the issue of conformational opening of the M domain in another way, we performed limited proteolysis of both full-length HMP-1 and the HMP-1 M domain, which confirms that the two salt bridge mutations introduced in this paper result in the M domain adopting a more open conformation. We have added these results to Supplementary Figure 2 as Supp. Fig. 2A. Additionally, we also analyzed the junctional recruitment of the C-terminus of SRGP-1 to find that it is primarily recruited to anterior seam-seam junctions as opposed to anterior seam-ventral or seam-dorsal junctions, which could indicate that the anterior seam-seam junctions are under less tension than are the anterior seam-non-seam junctions (Suppl. Fig. 2C).

Minor
1. If space permits, it would help the reader to introduce a description of the HMP-1/a-catenin mutants into the narrative and also describe the results of the pull-down assays. If there isn't enough space in the main text, it could be added to the caption of Fig S2.

- *We have added some description of the hmp-1[R551/554A] mutant pertaining to how these mutations are predicted to disrupt salt bridges within the HMP-1 M domain. We have also indicated that in* hmp-1(fe4) *mutants S823 is mutated to F.*

Reviewer 2
1/ Their model predicts that "tension regulates the partially closed conformation of the HMP-1 M domain to support SRG-1 recruitment" (orange box in Fig. 4H). If so, Should we expect that loss of tension will affect SRGP-1 recruitment? What happens in hypo-tension mutants?"

- *We agree completely with Reviewer 2 on this point. We have attempted experiments to test this over a two-year period. We have attempted both* mel-11*/myosin phosphatase loss of function (predicted to increase contractility) and* let-502*/ROCK loss of function experiments (predicted to reduce contractility). We initially tried these experiments using temperature-sensitive alleles carrying* srgp-1 *C term constructs. In both cases the embryos either downregulated expression of the SRGP-1 C term fragments or did not survive. This indicates a deleterious genetic interaction. Weak RNAi also tends to have this same effect, but we have found conditions in which very weak RNAi against* let-502 *succeeds but allows embryos to survive until elongation. We found that under these weak feeding conditions, a small, but statistically significant, increase in recruitment of the SRGP-1 C-terminus to seam-non-seam junctions occurs, which we include as*

Supplementary Figure 2D in the revision. We rationalize these results in lines 144-148 in the revised main text. Reduction of tension is prediction to increase the fraction of HMP- 1 that is not in a fully extended confirmation, i.e., it is experiencing moderate tension. In fact, we see increased recruitment of SRGP-1 under these weak let-502 knockdown conditions.

“2/ Their model also predicts that "SRGP-1 stabilizes the CCC via scaffolding to the plasma membrane" (violet box in Fig. 4H), a conclusion that relies on their previous observation that the F-BAR domain is essential for SRGP-1 function. In their previous paper they had concluded that the 200 residues immediately following the F-BAR domain were sufficient for junctional targeting, hence I was not entirely convinced by the current model. Is it that SRGP-1 can bind to junctions and HMP-1 through two domains, or is it that the SRGP-1 stabilizing function is through another factor rather than through the plasma membrane? Could they test so?”

- *We believe that the C-terminus of SRGP-1 interacts with the M domain of HMP-1 in a partially closed conformation. However, based on our data in this and the previous work (Zaidel-Bar et al, 2010), we believe that full-length SRGP-1 and SRGP-1ΔC both have means of being recruited to junctions via another mechanism that nevertheless depends on the presence of the HMP-1 M domain. In this case, however, such recruitment presumably occurs regardless of the conformation of the HMP-1 M domain. We retained a brief discussion of this point in the revision (lines 216-221). We agree that this possibility is very interesting, but we believe that elucidating this second mechanism is beyond the scope of the present paper.*

“3/ The notion that SRGP-1 stabilizes HMP-1 relies on FRAP data with a significance at P= 0.04, so quite weak. I noticed that they had examined 11 embryos (it is actually written n=1 in the main text and n=11 in the legends). Would it change with additional samples, or with samples at a later stage? Actually the stage at which the FRAP has been performed is not described and should be indicated.”

- *We have added text to indicate that that the stage at which the HMP-1::GFP FRAP was conducted was between 1.5 and 1.75 fold. While the p value might decrease with additional replicates, we hesitate to increase n values just to increase statistical significance. The* srgp-1(gk441841) *allele, which is a putative null, has minimal phenotypes by itself, but that* srgp-1 RNAi *in a sensitized background, e.g.,* hmp-1(fe4) *(Lynch et al., 2012) or* hmp-2(qm39) *(the present work) causes strong enhancement of phenotypes supports the notion that SRGP-1 modulation of HMP-1 is part of a combinatorial set of effects on adherens junctions that are only revealed in hypomorphic backgrounds.*

“Minor:
1/ Fonts are too small in Fig. 1D, and the deletion symbol Δ is missing in panel C for SUMO-HMP-1(ΔVH2).
2/ I was surprised that they could not test the effects of SRGP-1 deletion constructs in SRGP-1; HMP-1(fe4) double mutants, as I imagine that it should be straightforward to balance one of the mutations.
3/ The Methods section mentions "Actin cosedimentation assays" but I could not find the results of such assays. Do you really need that section?”

- *We have adjusted the fonts in the relevant panel (now Fig-1E) to increase legibility and made sure to add the Δ symbol.*
- *While balancers exist for the* hmp-1 *and* srgp-1 *loci, they are difficult to use with embryos for the following reasons:*
  - *In numerous situations, including in this case, we have not been able to perform certain crosses to obtain cross progeny. We believe this is because the* fe4 *allele is a maternal effect allele. We have added a phrase indicating this to the text (lines 171-172).*
  - *Balancers which are homozygous embryonic lethal can be difficult to discern from other embryonic lethal backgrounds, especially since* fe4 *worms do not die at a single developmental stage.*

- *The markers in many balancers do not become apparent until after embryos have hatched, which is problematic when trying to measure embryonic rescue.*
- *Although we are examining a stage after the maternal-zygotic transition, it is not clear whether maternally deposited mRNA or protein for either of these genes still has an effect during elongation.*

- *We apologize for the carryover of extraneous text regarding actin cosedimentation from other work. This has been deleted.*

Reviewer 3

Figure 1E explores the role of the C-terminus in junctional localization. They conclude "These data indicate that the SRGP-1 C-terminus is necessary for restricting it to junctions and for normal association with HMP-1. Because some SRGP-1DC can localize to junctions, we infer that additional interactions outside the C terminus also promote accumulation of SRGP-1 at junctions." However, I found the image used to demonstrate this unconvincing. Outside boxed area the localization seems seem very similar, and in the images in the bottom row the channels seem poorly aligned." Even in the closeup image used the conclusion that the effect is on SRGP- 1 localization was not totally not accurate—if there is an effect it is on alpha- catenin, as they note: "In contrast, HMP-1 frequently no longer followed SRGP- 1(ΔC)::GFP into membrane bends (Figure 1E, insets), as reflected in a change in overlap at junction (Manders overlap coefficient M2 with thresholding, GFP overlap with mScarlet-I = 0.82 for SRGP-1::GFP, n = 57 junctions; M2 = 0.46 for SRGP- 1(DC)::GFP, n = 36)." Finally given what follows they should tell us the stage examined here—since they next show us it localizes poorly to junctions!"

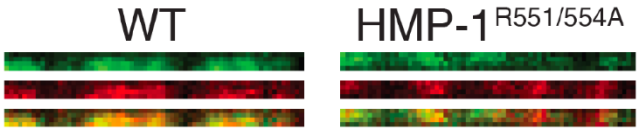
- *We have added text to indicate that we examined 1.5-1.75 fold embryos for this analysis.*
- *All image analysis was performed on image files that were generated by cropping the original two-channel acquisition file. Representative images were also cropped from the original two-channel image file. We believe any observed lack of colocalization is true to expression and localization patterns in vivo.*

4. In Figure 2 the authors examine the role of alpha-catenin's VH2 domain on SRGP-1 localization. They conclude "srgp-1::mScarlet-I; hmp-1(jc48); Ex[hmp-1(ΔVH2)::gfp] embryos retain detectable membrane localized SRGP-1::mScarlet-I in lateral epidermal cells (seam cells) during elongation but at greatly reduced levels (Figure 2F, H)." However, I found the degree of reduction not very clear, as the mScarlet tagged protein was not as robustly junctional.

- *We completely agree that adding line scans for the HMP-1ΔVH2 strain would have helped here. We have added a graph of line scans measuring SRGP-1::mScarlet-I in WT and HMP-1ΔVH2 backgrounds to more clearly indicate the SRGP-1::mScarlet-I recruitment to junctions. This is now Fig. 2I.*

5. In Figure 3 the authors examine the ability of a fully open alpha-catenin mutant to recruit SRGP-1. They conclude: "Whereas full-length HMP-1::mScarlet-I colocalized precisely with full-length SRGP-1::GFP (Figure 3A, top), HMP-1(R551/554A) did not (Fig. 3A, second row), reflected in a statistically significant decrease in colocalization at junctions". While there was clearly an effect on co-localization, I thought the data was more complex. Unlike other manipulations, the effect was not simply reduction or loss of membrane localization. Instead, it was interesting but potentially more complicated. The two proteins now align "in parallel"—is this an apical/basal localization difference? In contrast, the loss of junctional localization of the Cterm construct was much more straightforward."

- *We do not believe that the "in parallel" distribution is a consistent feature of the HMP-1 salt bridge mutant protein. We did, as the Reviewer suggested, perform Z reslices of images of SRGP-1(Full length)::GFP in both HMP-1::mScarlet-I and HMP-1$^{R551/554A}$::mScarlet-I backgrounds, but did not see an apparent mislocalization in Z. We include representative images here:*



There may be subtle effects beyond our ability to detect, but these data indicate there is not a major defect in apicobasal localization of SRGP-1 in the salt bridge mutant.

6. Later in Figure 3 they examine the effect of SRGP-1 loss on alpha-catenin localization, something they also examined in different ways in 2010. They first look at "Junctional intensity", concluding "We did find, however, that in srgp-1(gk441841) mutants average junctional intensity of an endogenous HMP-1::GFP knock in (Marston et al., 2016) was measurably decreased compared to wildtype (Figure 3C)." How was this measured?— calculating relative junctional intensity between different mutants requires careful controls. They also measure mobility by FRAP: "Loss of SRGP-1 led to a statistically significant decrease in recovery half life (t1/2 for wildtype = 25.0 sec; t1/2 for srgp-1 = 30.5 sec" Both effects, especially that on dynamics, were pretty subtle."

- *In the Materials and Methods, we indicate that we traced junctions and measured the mean intensity of signal within these aggregate junctional regions of interest. All imaging was conducted with the same laser settings and exposure time on the same microscope. The HMP-1::GFP was a homozygous endogenous knock-in so we do not expect any changes in intensity to be due to copy number issues that are inherent with transgenic lines.*

8. The 2010 paper argued that the C-terminal region was a negative regulator of SRGP-1 function—here they argue for much different roles. The discussion does not clearly integrate these views."

- *This is a good point that should be addressed. We showed two things in the 2010 paper (Zaidel-Bar et al, 2010): (1) loss of the C-terminus of SRGP-1 results in substantially more tubulated membranes compared to full-length SRGP-1; and (2) the C-terminus and N-terminus of SRGP-1 are able to interact when expressed separately and then recombined in vitro. This suggests that the N terminus is normally inhibited from runaway multimerization, which is what produces the tubules.*

  We did not actually demonstrate that binding of the SRGP-1 C-terminus to its N-terminus is what accounts for the less tubulated membranes in vivo when the C-terminus is present. It is equally possible that SRGP-1 undergoes autoinhibition in the cytosol via a head-to-tail interaction, but that other inhibitory interactions limit multimerization when SRGP-1 is spatially localized by docking with binding partners.

  *Our present results suggest such a model, in which binding at junctions (via HMP-1 and/or other proteins) limits the ability of SRGP-1 to multimerize indiscriminately when bound at the membrane. This may activate SRGP-1 via relieving cytosolic autoinhibition. This model is similar to current thinking about vinculin and talin, which are thought to be autoinhibited in the cytosol via internal, head-to-tail binding, but appear to be activated once bound to binding partners (e.g., Bakolitsa et al 2004. Nature 430, 583-586; Dedden, D. et al., 2019. Cell **179**, 120-131 e113). One possible prediction consistent with this idea would be that when overexpressed, full-length SRGP-1 could undergo unrestrained multimerization at the membrane in the absence of HMP-1, if its N terminus binds the membrane to relieve cytosolic autoinhibition. We have observed this is some embryos, as the image below demonstrates. The embryo is weakly knocked down for hmp-1, so it has not manifested the Humpback phenotype. We hesitate to add images like this to the paper, since overexpression can be difficult to interpret, but they are consistent with such a model.*

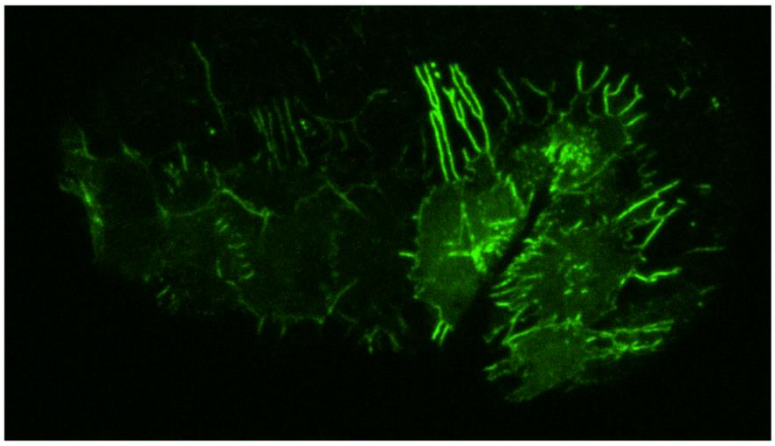


***Figure description**: Membrane tubulations generated by SRGP-1::GFP in a weak* hmp-1 *knockdown (*hmp-1(RNAi)*) embryo. The arrows point to ~ 5 µm tubulations . Such massive tubulations are not observed in otherwise wild-type embryos expressing full- length SRGP-1. Anterior is to the left, dorsal at the top.*

In the revised manuscript we have added a short discussion of the general autoinhibition issue, within the constraints of the short format (lines 222-231).

Minor issues

Introduction. What do we know about roles of SRGP in other systems and contexts? =What is the relevance of the Slit/Robo connection?
Fig 1 Add the diagrams in Fig S1B to Fig 1
Fig. 1A. Make it clear that the IP is of SRGP-1:GFP (i.e., don't just label it GFP!)
Fig. 1C. It's tough to determine what was done here….. what was pulled down and then what was done with the pulldowns. Fig S2 is even more cryptic"

- *We agree that more discussion of the wider context would be valuable. However, we were heavily constrained by the length limits, which preclude extensive discussion of Slit, Robo, etc. We have referred readers to a previous review (Lucas and Hardin, 2017; line 193), and we have added discussion of the work of the Yap lab on srGAP1 (lines 194-198). There is no evidence that Slits or Robo/SAX-3 are involved in the events we describe here during elongation based on genetic analysis and the expression pattern of SAX-3/Robo and SLITs (our unpublished observations; also see Hao et al, 2001. Neuron. 32(1):25-38; Shah et al, 2017. Dev Cell. 41(2):195-203). Loss of function phenotypes are very different for sax-3, which results in disruption of neuroblast organization and other defects. In the srGAP1 work cited above, the events may be downstream of HGF-1/scatter factor signaling, rather than Slit/Robo, indicating that although srGAPs derive their name from their original discovery in association with Robo receptors, there may be many other upstream regulators depending on context. While we absolutely agree that identifying the upstream regulators of SRGP-1 is an important topic for the future, we feel that the current emphasis of the manuscript on SRGP-1 and its binding partner HMP-1 is a sensible focus.*
- *We have added the requested text label to indicate that this is SRGP-1::GFP.*
- *We have updated the Materials and Methods to indicate the pulldown procedure more clearly.*

Second decision letter

MS ID#: DEVELOP/2022/200775

MS TITLE: A C. elegans srGAP is a novel a-catenin M domain-binding protein that strengthens cadherin-dependent adhesion during morphogenesis

AUTHORS: Joel M. Serre, Bethany Lucas, Sterling C.T. Martin, Jonathon A Heier, Xiangqiang Shao, and Jeff Hardin
ARTICLE TYPE: Research Report

I sincerely apologize for the long delay before coming back to you with a decision.
In light of the available reviewers' comments and looking at the revision, I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have reasonably addressed the comments I raised in my earlier review.
I think that this is an informative contribution to the field, and support its publication.

Comments for the author

As above.

Reviewer 3

Advance summary and potential significance to field

As I noted in my previous review, the authors address an important issue: Maintaining connections between cadherin- based cell junctions and the cytoskeleton. The nature of the connection and the molecules involved have been revealed to be increasingly complex. The Hardin lab has previously used clever genetic screens in C. elegans to identify proteins that enhance the phenotype of weak mutations in alpha-catenin, thus identifying candidate molecules involved in connecting junctions and the cytoskeleton. In a lovely paper in 2010 they identified a Bar domain containing Rho GTPase GAP called SRGP-1 as an interactor. While SRGP-1 mutants have no overt phenotype, they enhance weak mutations in alpha-catenin. Mutants alter catenin dynamics, and the C-terminus acts as a negative regulator. Here they extend this work. Using biochemical approaches, they find that the SRGP-1 C-terminal region binds alpha-catenin VH2. They then explore whether the C-terminus is important for junctional localization, and how different alpha-catenin mutational alterations affect SRGP-1 junctional localization. Finally, they test what domains of SRGP-are important for its genetic interaction with weak beta-catenin mutants or lack thereof. I felt that some of these data were not as clear as one would expect, and some effects observed were fairly modest. I detailed these issues in my initial review. I felt the authors did not substantively address some of the issues I raised, and thus, while I agree it is important to examine modulators of junctional function, and that this work is a solid addition to our understanding of alpha-catenin/ SRGP-1 interactions, I remain convinced that the breadth of understanding is somewhat limited. However, if the other reviewers are convinced, I will co along with the consensus.

Comments for the author

See above