Regulation of caveolae through cholesterol-depletion-dependent tubulation mediated by PACSIN2

Aini Gusmira, Kazuhiro Takemura, Shin Yong Lee, Takehiko Inaba, Kyoko Hanawa-Suetsugu, Kayoko Oono-Yakura, Kazuma Yasuhara, Akio Kitao and Shiro Suetsugu
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Original submission

First decision letter

MS ID#: JOCES/2020/246785

MS TITLE: Regulation of caveolae through cholesterol-depletion dependent tubulation by PACSIN2/Syndapin II

AUTHORS: Aini Gusmira Amir, Kazuhiro Takemura, Kyoko Hanawa-Suetsugu, Kayoko Oono-Yakura, Kazuma Yasuhara, Akio Kitao, and Shiro Suetsugu
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We have now reached a decision on the above manuscript.

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

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

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

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

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The effect of cholesterol on PACSIN2-induced model membrane tubulation is very interesting. However, the results presented do not support the conclusion in Fig. 5.

Comments for the author

1. Fig. 1B has to be further explained. What is the difference between Concentration of liposomes 0.000 and no lipo? Why the protein is recovered in P when liposomes are 0.000?

2. Fig. 1C. Experimental data of liposome size and lamellality are necessary.
Fig. 1E shows that the liposomes are heterogeneous and different between POPC/POPS and POPC/POPS/Chol

3. Fig. 1E. Quantitative analysis of liposome shape is necessary.

4. Figure 3 suggests that small decrease of cholesterol is sufficient for tubulation. The effect of cholesterol concentration in model membranes on membrane tubulation has to be examined.

5. Does PACSIN2 induce CF leakage in POPC/POPS and POPC/POPS/Chol liposomes?

6. Fig. 2A. CF leakage at no osmolarity difference has to be shown.

7. What is the effect of osmolarity difference on PACSIN2-induced shape change of liposomes

8. Fig. 3 and 4. Cellular cholesterol content has to be measured biochemically.

9. Fig. 3 and 4. Shapes of caveolae and distribution of caveolin have to be examined by immune electron microscopy (cf. J Cell Sci 124, 2777 (2011)).

10. Fig. 4. Huge difference of the morphology of cells after MβCD suggests that the effect is pleiotropic or secondary effect. Lower concentration of MβCD or shorter incubation time should be examined.

11. Discussion. “because the neck region at the boundary of caveolae was supposed to have smaller amounts of cholesterol than the caveolar bulb”.
Reference has to be shown.

12. The molecular mechanisms of proposed cholesterol removal during caveolae endocytosis have to be discussed.

Minor comments

Fig. 1B and 2C. Use “sup” “ppt” or “S” “P” in both figures.

Page 8. “to obtain an osmolarity difference of 135 mOsmol” Fig. 2B shows the difference is 160 mOsmol.

Page 9. “After MβCD treatment, the amount of caveolin-1 relative to GAPDH was found to be lower in the PACSIN2 knockout cells compared with that in the parental HeLa cells” Fig. 4B shows that the value is higher in the PACSIN 2 knockout.

Reviewer 2

Advance summary and potential significance to field

Amir et al studied PACSIN2, an F-BAR protein localizing to the neck portion of caveolae. They used an in vitro liposome set-up and showed that PACSIN2 has a weaker affinity to membranes containing cholesterol than membranes without cholesterol and concluded that this difference is caused by a decrease in charge density. Then they studied cells and found that acute cholesterol depletion induced PACSIN2-coated tubules with caveolin-1 at their tips. They concluded that the observed phenomenon represents caveolar internalization, which may be regulated by cholesterol level in the plasma membrane.

Comments for the author

The hypothesis proposed by this study: a cholesterol decrease recruits more PACSIN2 thereby inducing caveolar internalization, is attractive but the data supporting the idea are weak.
1) The in vitro assay using liposomes needs appropriate controls to show its validity.
1. Liposomes made of POPC/POPS and POPC/POPS/cholesterol may be different in size. Actually, in Figure 1E, POPC/POPS liposomes appear larger than POPC/POPS/cholesterol liposomes. The difference in diameter and curvature is very likely to affect binding of F-BAR proteins like PACSIN2.
2. Liposomes are supposed to be unilamellar, but a preparation using a membrane filter of 2 μm is likely to contain non-negligible amount of multi-lamellar liposomes. Here again, the proportion of multi-lamellar/unilamellar membranes may be different in the two liposomal preparations.
3. The amount of PACSIN2 to liposomal membranes was estimated from the band intensity of Coomassie Brilliant Blue-stained SDS-gels. It is not clear whether this method of estimation is accurate enough. If recombinant PACSIN2 is the only protein in the preparation microBCA or other protein assays should be more accurate and reproducible.
2) Tubular formation in acute cholesterol depletion is clear for PACSIN2-mCherry in Figure 3A, but not in Figure 3B, although cells were treated similarly. Thus, it is ambiguous whether caveolin-1-GFP dots are located at the end of PACSIN2-mCherry-positive tubules or not. It is also not clear how caveolin-1 cap forms at the end of the PACSIN2 tubule; is it directly derived from caveolin-1 in caveolar bulbs? Live imaging should help answer these questions.
3) The text discussing Figures 4A, B (page 9) is different from the actual result. In the figures, the amount of caveolin-1 relative to GAPDH is larger in the PACSIN2 knockout cells than that in the parental HeLa cells. Whatever the result is, the difference between wild-type and PACSIN2-KO cells appears very trivial. How many samples were analyzed and how were the data analyzed statistically? The author also needs to address how caveolin-1 internalized by the PACSIN2-dependent mechanism is degraded.
4) The method in Figure 4C is not sufficient to evaluate whether caveolin-1 is internalized or not. Quantitative data obtained by appropriate methods are needed.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The effect of cholesterol on PACSIN2-induced model membrane tubulation is very interesting. However, the results presented do not support the conclusion in Fig. 5.

A) We would like to appreciate your supportive comments. We will omit the illustration in Fig 5.

Reviewer 1 Comments for the Authors

1. Fig. 1B has to be further explained. What is the difference between Concentration of liposomes 0.000 and no lipo? Why the protein is recovered in P when liposomes are 0.000?

A) We apologies for the inconsistency of the labeling. 0.000 was a result of accidental truncation of 0.0002.

2. Fig. 1C. Experimental data of liposome size and lamellarity are necessary. Fig. 1E shows that the liposomes are heterogeneous and different between POPC/POPS and POPC/POPS/Chol

A) We measured the lamellarity with fluorescent intensity of NBD-labelled lipids, which can be quenched by the membrane impermeable quencher (revised Figure S1). Most of the liposomes were unilamellar because approximately half reduction of NBD fluorescence was observed with the quencher.

We also measured the size distribution of liposomes by TEM. We found the distributions of liposomes composed of POPC/POPS and that of POPC/POPS/Chol were similar each other (revised Figure 1G). We also replaced the images in Figure 1E with more appropriate ones.

3. Fig. 1E. Quantitative analysis of liposome shape is necessary.

A) We counted the numbers of liposomes with and without tubulation by the images, as well as the diameters of the spherical part of the liposomes, which are shown in revised Figure 1F and G.

4. Figure 3 suggests that small decrease of cholesterol is sufficient for tubulation. The effect of cholesterol concentration in model membranes on membrane tubulation has to be examined.

A) We examined the liposome with tubules with liposomes of POPC/POPS/Cholesterol ratio of 60/40/0, 54/36/10, 42/28/30, and 33/22/45, and found that the increase of cholesterol inhibited the tubulation (revised Figure S2). To overcome the effect of the reduction in PS amount, the amount of liposomes were adjusted to have the same amount of PS in the reaction in this revised manuscript. This result was thought to be consistent with almost absence of the tubule formation by the endogenous level of PACSIN2 (Figure 3B). In this revised manuscript, we also tested various MβCD concentrations, and found that the cellular cholesterol started to decrease at 2 mM MβCD treatment, which was consistent with the caveolar behavior, which also started to decrease from 2 mM MβCD (Figure 3C and 4). The MβCD in 2 mM is higher than the cholesterol concentration in liposomes, and the 2 mM MβCD treatment of liposomes also potentiated the tubule formation by PACSIN2 (Figure S2). Therefore, the cholesterol of plasma membrane was thought to be easily removed by MβCD, which would result in the tubule formation by PACSIN2.

5. Does PACSIN2 induce CF leakage in POPC/POPS and POPC/POPS/Chol liposomes?

A) The overexpression of PACSIN2 did not induce the incorporation of the exogenous dye added to the medium (Figure S3 in Senju et al JCS, 2011). In this manuscript, the ability of PACSIN2 to induce the leakage is not the subject of study. The editor agreed with this idea, and we did not perform this experiment to minimize the stay in the lab for the prevention of infection.

6. Fig. 2A. CF leakage at no osmolarity difference has to be shown.

A) We included the data at no osmolarity difference (revised Figure 2A).

7. What is the effect of osmolarity difference on PACSIN2-induced shape change of liposomes

A) We observed the osmolarity difference at 160 mOsmol did not induce significant difference membrane deformation by PACSIN2 F-BAR domain, as attached, as had been suggested from the no difference in the binding by co-sedimentation assay (Figure 2D). However, this finding makes the story more complex, and therefore, we would like to omit this data from the paper. (Figure is attached in PDF version of response letter)

"NOTE: We have removed unpublished data that had been provided for the referees in confidence."

8. Fig. 3 and 4. Cellular cholesterol content has to be measured biochemically.

A) We measured the amount of cholesterol, which was shown in the ratio to the amount of the abundant lipid, phosphatidylcholine. The presence of MβCD above 2 mM effectively removed cellular cholesterol, which was consistent with the effect on caveolae (revised Figure 4).

9. Fig. 3 and 4. Shapes of caveolae and distribution of caveolin have to be examined by immune electron microscopy (cf. J Cell Sci 124, 2777 (2011)).
A) We think the immuno-electron micrograph is to demonstrate the residence of caveolin-1 on the plasma membrane. Therefore, we would like to substitute this immuno-electron micrographic analysis with the quantification of the cells with plasma membrane caveolin-1 under MβCD treatment under confocal microscope (revised Figure 4E).

We understand that it is much better to do the immuno-electron micrographic analysis. The reduction of PACSIN2 amount by siRNA was resulted by the increase of caveolin-1 at the plasma membrane, which was assessed by the TIRF analysis that illuminated the plasma membrane caveolin-1 (J Cell Sci 124, 2777 (2011) (Hansen et al., 2011). Moreover, the knockout mice of the muscle specific protein PACSIN3/Syndapin III resulted in the loss of caveolae, without removal of plasma membrane caveolin-3, a homologue of caveolin-1 in muscle (Seemann et al., 2017). The caveolin-1 localization in PACSIN2 knockout cells would be expected to be similar to the PACSIN3 knockout cells. We showed that the MβCD treatment internalized caveolin-1 from the plasma membrane. Therefore, the expected results of the electron-micrographic analysis are the plasma membrane localization of caveolin-1 in MβCD-treated PACSIN2 knockout cells, which can be visualized by the confocal microscopy as shown in revised Figure 4D. We agree this data are of worth exploring, however, our electron microscopic facility is not in full activity for the analysis of the cells. Therefore, we would like to omit this experiment from revision, and would like to substitute with the quantification of plasma membrane caveolin-1 upon MβCD treatment. The editor agreed with this exemption of the EM analysis.

10. Fig. 4. Huge difference of the morphology of cells after M¤CD suggests that the effect is pleiotropic or secondary effect. Lower concentration of M¤CD or shorter incubation time should be examined.
A) In the previous manuscript, the MβCD treatment was performed only at 10 mM of MβCD. We performed the treatment of the lower concentration of MβCD and found that the MβCD treatment above 2 mM was effective, and the cells were not significantly deformed at 2 mM of MβCD. Interestingly, the internalization of caveolae started at 2 mM of MβCD, suggesting that the internalization of caveolae was not resulted from the overall deformation of the cells (revised Figure 4). In addition, the cells were treated with cumulative concentration of MβCD, 2 mM for each treatment and the tubule was observed when the MβCD concentration reached 2 mM (revised Figure 3C).

11. Discussion. “because the neck region at the boundary of caveolae was supposed to have smaller amounts of cholesterol than the caveolar bulb”. Reference has to be shown.
A) As we introduced in introduction.
Cholesterol is highly enriched in caveolae (Ortegren et al., 2004; Razani et al., 2002; Smart et al., 1999). Up to 41% of membrane lipids of caveolae in adipocytes reported as cholesterol, which is higher than the typical amount of cholesterol outside caveolae (approximately 22%) (Ortegren et al., 2004). Furthermore, cholesterol is an essential component of caveolae, because cholesterol depletion impairs the morphology of caveolae (Breen et al., 2012; Dreja et al., 2002; Murata et al., 1995; Parpal et al., 2001; Razani et al., 2002; Smart et al., 1999).

Therefore, the boundary should have smaller amount of cholesterol. However, there is no direct evidence and we modified the text as follows: line 283-

Although the concentration of cholesterol at the neck of caveolae is unknown, the neck region as the boundary of caveolae was supposed to have smaller amounts of cholesterol than the caveolar bulb, because caveolae have higher concentration of cholesterol than plasma membrane (Ortegren et al., 2004; Razani et al., 2002; Smart et al., 1999).

12. The molecular mechanisms of proposed cholesterol removal during caveolae endocytosis have to be discussed.
A) We discussed as follows (line 318-):
The tubule formation will recruit more dynamin to the neck of caveolae, which is thought to induce the scission of caveolae for their internalization, because PACSIN2-mediated tubules were increased by the expression of inactive dynamin (Senju et al., 2011). It is also possible that the

longer tubules would be more susceptible to fission by mechanical forces, which also might induce the scission of caveolae for their internalization, as has been reported for the endophilin-mediated tubules (Simunovic et al., 2017). Caveolae are thought to be transported to endosomes after internalization, where the degradation takes place (Kiss and Botos, 2009; Parton, 2004).

Minor comments
Fig. 1B and 2C. Use “sup” “ppt” or “S” “P” in both figures.
A) We modified these accordingly. We used “S” “P” in both figures.

Page 8. “to obtain an osmolarity difference of 135 mOsmol” Fig. 2B shows the difference is 160 mOsmol.
A) The experiments with PACSIN2 were performed with 160 mOsmol, which is of below the significant leakage. We apologize for error in writing, and we modified the text accordingly:

Page 9. “After MβCD treatment, the amount of caveolin-1 relative to GAPDH was found to be lower in the PACSIN2 knockout cells compared with that in the parental HeLa cells” Fig. 4B shows that the value is higher in the PACSIN 2 knockout.
A) We would like to appreciate this comment. The statement was inappropriate. Fig 4B shows the amount of caveolin-1 relative to GAPDH upon MβCD treatment. We modified the label of the figure and the text. It is now (line 254-):
After MβCD treatment, the amount of caveolin-1 relative to GAPDH was found to be lower in the parental HeLa cells, however, the amount was increased in PACSIN2 knockout cells as compared with that in the parental HeLa cells (Figure 4A, B).

Reviewer 2 Advance Summary and Potential Significance to Field:
Amir et al studied PACSIN2, an F-BAR protein localizing to the neck portion of caveolae. They used an in vitro liposome set-up and showed that PACSIN2 has a weaker affinity to membranes containing cholesterol than membranes without cholesterol and concluded that this difference is caused by a decrease in charge density. Then they studied cells and found that acute cholesterol depletion induced PACSIN2-coated tubules with caveolin-1 at their tips. They concluded that the observed phenomenon represents caveolar internalization, which may be regulated by cholesterol level in the plasma membrane.

A) We would like to appreciate your supportive comments.

Reviewer 2 Comments for the Author:
The hypothesis proposed by this study: a cholesterol decrease recruits more PACSIN2, thereby inducing caveolar internalization, is attractive but the data supporting the idea are weak.
1) The in vitro assay using liposomes needs appropriate controls to show its validity. 1.Liposomes made of POPC/POPS and POPC/POPS/cholesterol may be different in size. Actually, in Figure 1E, POPC/POPS liposomes appear larger than POPC/POPS/cholesterol liposomes. The difference in diameter and curvature is very likely to affect binding of F-BAR proteins like PACSIN2.
A) We measured the size distribution of liposomes from the TEM images and found the size distribution of liposomes composed of POPC/POPS and that of POPC/POPS/Chol were similar each other (revised Figure 1G). We also replaced the images of Figure 1E with more appropriate images.

2. Liposomes are supposed to be unilamellar, but a preparation using a membrane filter of 2 μm is likely to contain non-negligible amount of multi-lamellar liposomes. Here again, the proportion of multi-lamellar/unilamellar membranes may be different in the two liposomal preparations.
A) We measured the lamellarity by the fluorescent intensity of the NBD-labeled lipid, which could be quenched by the membrane impermeable quencher (revised Figure S1). The result clearly showed most of the liposomes were unilamellar because approximately half reduction of NBD fluorescence was observed with the quencher.

3. The amount of PACSIN2 to liposomal membranes was estimated from the band intensity of Coomassie Brilliant Blue-stained SDS-gels. It is not clear whether this method of estimation is accurate enough. If recombinant PACSIN2 is the only protein in the preparation, microBCA or other protein assays should be more accurate and reproducible.
A) If we perform the quantification by microBCA, we need to do all the experiments again. We think that CBB staining of the gel has enough accuracy and linearity to the amount of the proteins

as used in many papers, including the paper that directly examined the linearly of the staining (Vincent et al., 1997). Moreover, our CBB staining is to determine the ratio of the proteins in supernatant and the pellet in the same gel, and therefore necessity for the accuracy and reproducibility in the same gel is enough. Therefore, we would like to omit these experiments to minimize the stay in the lab for the prevention of infection. The editor agreed with this idea and we did not replace the data in the revised manuscript.

2) Tubular formation in acute cholesterol depletion is clear for PACSIN2-mCherry in Figure 3A, but not in Figure 3B, although cells were treated similarly. Thus, it is ambiguous whether caveolin-1-GFP dots are located at the end of PACSIN2-mCherry-positive tubules or not. It is also not clear how caveolin-1 cap forms at the end of the PACSIN2 tubule; is it directly derived from caveolin-1 in caveolar bulbs? Live imaging should help answer these questions.

A) First, we apologize for inappropriate text for referring expressed PACSIN2. The PACSIN2 F-BAR domain was overexpressed but it was not overexpressed to the level of the tubules formation. Then we modified the text to (line 227-: the HeLa cells were expressed with GFP-PACSIN2 F-BAR domain to the level without tubulation). The observation for the full- length PACSIN2-mCherry is of the level similar to that of endogenous PACSIN2. We included the time course of the tubules of caveolin-1 with PACSIN2. As shown in revised Figure 3C, the tubules of PACSIN2 appeared from the pre-existing cavelin-1 spots. Interestingly, the dots disappeared after PACSIN2 appearance. We also found that some PACSIN2 spots without apparent caveolin-1, which might be the other subcellular structures.

3) The text discussing Figures 4A, B (page 9) is different from the actual result. In the figures, the amount of caveolin-1 relative to GAPDH is larger in the PACSIN2 knockout cells than that in the parental HeLa cells. Whatever the result is, the difference between wild-type and PACSIN2-KO cells appears very trivial. How many samples were analyzed and how were the data analyzed statistically? The author also needs to address how caveolin-1 internalized by the PACSIN2-dependent mechanism is degraded.
A) We apologize that the text was inappropriate. After MβCD treatment, the amount of caveolin-1 per cell was larger in PACSIN2 knockout cells than in the wild-type cells, and the reduction of caveolin-1 was observed in the wild-type cells. We re-labeled the revised Figure 4B, which shows the time course of the amount of caveolin-1 relative to GAPDH. We also revised the text for clarity. Moreover, the amount of caveolin-1 per cell, not the surface localized caveolae, is reported to reduce in MDCK cells and other cells upon MβCD treatment (Ao et al., 2016; Hailstones et al., 1998). The experiments were repeated in triplicates, and the statistical significance was analyzed by two-tailed t-test in the revised Figure 4B.
Caveolae are thought to be transported to endosomes after internalization, where the degradation takes place (Kiss and Botos, 2009; Parton, 2004). We think the elucidation of the degradation mechanisms is outside of the scope of this paper. Therefore, we would like to add the discussion on possible degradation of caveolin-1 after internalization as follows: (line 318-):
The tubule formation will recruit more dynamin to the neck of caveolae, which is thought to induce the scission of caveolae for their internalization, because PACSIN2- mediated tubules were increased by the expression of inactive dynamin (Senju et al., 2011). It is also possible that the longer tubules would be more susceptible to fission by mechanical forces, which also might induce the scission of caveolae for their internalization, as has been reported for the endophilin-mediated tubules (Simunovic et al., 2017). Caveolae are thought to be transported to endosomes after internalization, where the degradation takes place (Kiss and Botos, 2009; Parton, 2004), which would result in the cholesterol-dependent down-regulation of caveolae.

4) The method in Figure 4C is not sufficient to evaluate whether caveolin-1 is internalized or not. Quantitative data obtained by appropriate methods are needed.
A) We quantified the caveolin-1 internalization by counting the cells with more caveolin-1 in the cytoplasm than at the plasma membrane, of which dependency of MβCD was shown in Figure 4E.

Second decision letter

MS ID#: JOCES/2020/246785

MS TITLE: Regulation of caveolae through cholesterol-depletion dependent tubulation by PACSIN2/Syndapin II

AUTHORS: Aini Gusmira, Kazuhiro Takemura, Shin Yong Lee, Takehiko Inaba, Kyoko Hanawa-Suetsugu, Kayoko Oono-Yakura, Kazuma Yasuhara, Akio Kitao, and Shiro Suetsugu
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

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

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

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

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper showed that PCSIN2 regulates the number of cell surface caveolae by facilitating tubulation of caveolae in the absence of cholesterol. Thus this paper clarified the mechanism of how choelsterol regulates caveolae.

Comments for the author

In this revision, the authors have adequately addressed my concerns. I have a few minor comments:

Page 9 line 228
“to the level without tubulation”
Please explain more precisely.

Page 10 line 240
some caveolin-1 dots did not have PACSIN2
Indeed there are many caveolin dots that do not colocalize PACSIN2.
Quantitation may be necessary for “some caveolin” and “some tubules”

Line 12 line 289-291
“a “beads on a string” morphology, which resembles the curvature of the flask shaped caveolae”

Not clear

Page 12 line 302-303
Due to the presence of the hydrophobic loops thought to be inserted into the packing defects of membrane (Shimada et al., 2010), the increase in packing defects was first hypothesized to lead to an increase in the binding of PACSIN2 to the cell membrane.
Not clear. Maybe separate to two sentences.

Page 10 line 243
suggested -> suggest

line 246
was -> is

252
determine -> determined

page 12 line 277
suggested -> suggests

Reviewer 2

Advance summary and potential significance to field

Amir et al studied PACSIN2, an F-BAR protein localizing to the neck portion of caveolae. They used an in vitro liposome set-up and showed that PACSIN2 has a weaker affinity to membranes containing cholesterol than membranes without cholesterol and concluded that this difference is caused by a decrease in charge density. Then they studied cells and found that acute cholesterol depletion induced PACSIN2-coated tubules with caveolin-1 at their tips. They concluded that the observed phenomenon represents caveolar internalization which may be regulated by cholesterol level in the plasma membrane.

Comments for the author

The authors have addressed every single point I have raised with new experiments and new data. The manuscript has been significantly strengthened.

I have two remaining concerns, though. One is the use of Student's t-test for statistical analysis in several figures, in which the sample number is three and thus the normality of distribution cannot be judged. The other is the lack of statement concerning how many times the experiment was replicated in the laboratory (this is required for figure legends by the journal guideline). I would like to leave these points, especially the second one, to a judgement of the editor.

Second revision

Author response to reviewers' comments

Q) Reviewer 1 Advance summary and potential significance to field
This paper showed that PCSIN2 regulates the number of cell surface caveolae by facilitating tubulation of caveolae in the absence of cholesterol. Thus this paper clarified the mechanism of how cholesterol regulates caveolae.

Reviewer 1 Comments for the author
In this revision, the authors have adequately addressed my concerns. I have a few minor comments:
A) We appreciate your supportive comments on our work.

Q) Page 9 line 228
“to the level without tubulation”
Please explain more precisely.
A) The cells with the expression of the PACSIN2 F-BAR domain but without the tubulation of F-BAR was selected. We modified the text to (Line 228-):
Then, the HeLa cells were expressed with GFP-PACSIN2 F-BAR domain, and the cells with the PACSIN2 F-BAR domain expression without the tubulation were selected. These cells were observed using a microscope during the addition of MβCD to deplete cholesterol from the plasma membrane.

Q) Page 10 line 240
some caveolin-1 dots did not have PACSIN2
Indeed there are many caveolin dots that do not colocalize PACSIN2. Quantitation may be necessary for “some caveolin” and “some tubules”
A) We quantified the colocalization from 7 cells manually and modified the text (line 241-):
Consistent with the previous reports, showing that approximately half of the caveolin-1 has PACSIN2 (Senju et al., 2011), a proportion of caveolin-1 dots (43± 4% from 7 cells) had PACSIN2 (Figure 3C). Furthermore, a proportion of PACSIN2 tubules (33 ± 14%) were observed without detectable levels of caveolin-1 (Figure 3C), which might suggest the PACSIN2 functions in the structures other than caveolae.

Q) Line 12 line 289-291
“a “beads on a string” morphology, which resembles the curvature of the flask shaped caveolae”
Not clear
A) We appreciate this comment. We removed a description “which resembles the curvature of the flask-shaped caveolae”. But we kept the “beads on string” as a description to describe the cholesterol-containing liposomal shape in the presence of the PACSIN2 F-BAR domain.

Q) Page 12 line 302-303
Due to the presence of the hydrophobic loops thought to be inserted into the packing defects of membrane (Shimada et al., 2010), the increase in packing defects was first hypothesized to lead to an increase in the binding of PACSIN2 to the cell membrane.
Not clear. Maybe separate to two sentences.
A) We modified the text to the following (Line 304-):
The hydrophobic loops of PACSIN2 F-BAR domain were thought to be inserted into the packing defects of membrane (Shimada et al., 2010). Therefore, the increase in packing defects was first hypothesized to lead to an increase in the binding of PACSIN2 to the cell membrane.

Q) Page 10 line 243
suggested -> suggest
line 246
was -> is
252
determine -> determined
page 12 line 277
suggested -> suggests
A) We thank the reviewer for pointing out these grammatical errors. We corrected these errors accordingly.

Q) Reviewer 2 Advance summary and potential significance to field
Amir et al studied PACSIN2, an F-BAR protein localizing to the neck portion of caveolae. They used an in vitro liposome set-up and showed that PACSIN2 has a weaker affinity to membranes containing cholesterol than membranes without cholesterol and concluded that this difference is caused by a decrease in charge density. Then they studied cells and found that acute cholesterol depletion induced PACSIN2-coated tubules with caveolin-1 at their tips. They concluded that the observed phenomenon represents caveolar internalization, which may be regulated by cholesterol level in the plasma membrane.
Reviewer 2 Comments for the author
The authors have addressed every single point I have raised with new experiments and new data. The manuscript has been significantly strengthened.

A) We appreciate your supportive comments on our work.

Q) I have two remaining concerns, though. One is the use of Student's t-test for statistical analysis in several figures, in which the sample number is three and thus the normality of distribution cannot be judged. The other is the lack of statement concerning how many times the experiment was replicated in the laboratory (this is required for figure legends by the journal guideline). I would like to leave these points, especially the second one, to a judgement of the editor.
A) We included the number of liposomes that we measured in the legends for Figure 1G, which was not described before. We also redo the statistical analysis for the samples with N=3 (Figure 1F, 4B, 4C, 4E, S2D), by using a one-way ANOVA followed by Tukey's post hoc test, as had been used in the recent papers in JCS including Journal of Cell Science (2020) 133, jcs243709. doi:10.1242/jcs.243709. The statistical significance was mostly the same as before, but there was a change. The cholesterol-dependent decrease of caveolin-1 and the cholesterol depletion was not significant at 2 mM MβCD, but significant at 4 mM MβCD; while internalization by image analysis was significant at 2 mM MβCD. This difference might represent the more sensitivity of the image analysis than the biochemical assays. However, at this moment, we could not precisely discuss the concentration dependency of MβCD. Therefore, we modified the text to (Line 260-):
The decrease of caveolin-1 in the parental HeLa cells occurred by the increase of MβCD, which was consistent with the tubule formation of PACSIN2 by the increase of MβCD (Figure 4A, B). Furthermore, the decrease of cellular cholesterol by MβCD treatment by the increase of MβCD was confirmed by measuring the amount of cholesterol in the cells (Figure 4C).

Third decision letter

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MS TITLE: Regulation of caveolae through cholesterol-depletion dependent tubulation by PACSIN2/Syndapin II

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ARTICLE TYPE: Research Article

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