A cellular model of albumin endocytosis uncovers a link between membrane and nuclear proteins

Seiya Urae, Yutaka Harita, Tomohiro Udagawa, Koji L. Ode, Masami Nagahama, Yuko Kajiho, Shoichiro Kanda, Akihiko Saito, Hiroki R. Ueda, Masaomi Nangaku and Akira Oka
DOI: 10.1242/jcs.242859

Editor: Jennifer Lippincott-Schwartz

Review timeline
Original submission: 13 December 2019
Editorial decision: 3 February 2020
First revision received: 30 April 2020
Accepted: 20 May 2020

Original submission

First decision letter

MS ID#: JOCES/2019/242859

MS TITLE: The cellular model of albumin endocytosis uncovers link between membrane and nuclear proteins

AUTHORS: Seiya Urae, Yutaka Harita, Tomohiro Udagawa, Koji L. Ode, Masami Nagahama, Yuko Kajiho, Shoichiro Kanda, Akihiko Saito, Hiroki R. Ueda, Masaomi Nangaku, and Akira Oka
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

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

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

In this manuscript Urae et al investigate the mechanisms of albumin endocytosis mediated through the CUBAM ligand receptor complex. The CUBAM complex is made up of the CUBN receptor, which contains numerous CUB repeats, and AMN a transmembrane protein with two endocytosis signal motifs. Through co-IPs, localization studies, and flow cytometry the authors determined that full length CUBN and both signal motifs of AMN are required for albumin endocytosis. Next the authors used a SILAC approach to look for new interacting partners of the CUBAM complex and identified the gene NVL. The interaction with NVL was confirmed through co-IPs with endogenous and overexpressed NVL. Moreover, the authors found that overexpression of AMN leads to the relocalization of endogenous NVL to the extranuclear compartment. Knockdown of NVL reduces CUBN internalization leading to the conclusion that NVL is required for CUBAM-mediated albumin endocytosis.

The identification of NVL as a new binding partner of the CUBAM ligand receptor complex is novel information, however the authors fail to provide any mechanistic insight into why NVL associates with this complex. NVL is closely related to the well-studied protein unfoldase Cdc48/p97 and recent work with the yeast NVL2 homologue Rix7 (Lo Nature Communications 2019) confirmed that Rix7 functions as a molecular unfoldase. More work is needed to shed light on what role the ATPase activity of NVL is playing in albumin endocytosis. What function would an unfoldase have in this process? Could CUBN or AMN be putative substrates for unfolding?

Comments for the author

1. There are two forms of NVL – NVL1 and NVL2 (Nagahama Mol Biol Cell 2004), which differ based on an alternative start codon within the N-terminus yet there is no mention of the two forms of NVL in this manuscript. The authors must determine if NVL1 or NVL2 (or both) associate with CUBAM.
2. The majority of NVL2's interaction partners associate with NVL2 in a nucleotide dependent manner. The authors must test whether or not the nucleotide bound state of NVL influences it ability to associate with CUBAM.
3. The authors determined that the ICD region of AML is sufficient for binding NVL. The authors should carry out the reciprocal experiment and determine which region of NVL (NTD, NBD1, NBD2) associates with AML.
4. NVL is an essential gene because of its role in mediating production of the large ribosomal subunit. Knockdown of NVL should thus inhibit cell cycle progression and induce "nucleolar stress". The knockdown experiments in Figure 7 show a reduction in FITC-albumin update but it is impossible to tell if this is a direct or indirect effect. The authors should look at changes in FITC-albumin update following other inducers of nucleolar stress or knockdown of another gene required for ribosome assembly.

Reviewer 2

Advance summary and potential significance to field

In this study, Dr. Oka et al. focused on the mechanism of albumin endocytosis mediated by Cubilin (CUBN) and amnionless (AMN). Using a quantitative assay to evaluate the albumin uptake by CUBAM, the authors demonstrated efficient albumin uptake by cells expressing full-length CUBN as compared to those expressing mini-CUBN, thus revealing the crucial role of C-terminal part of CUBN. Similarly, endocytosis signal motifs of AMN are also shown to be essential for albumin endocytosis since mutation of these signal motifs abrogated albumin endocytosis. Using SILAC to search for AMN-interacting proteins, the authors further demonstrated nuclear valosin-containing protein like (NVL) as an interacting protein of AMN that is involved in albumin endocytosis. In the presence of AMN, NVL was shown to translocate to the extranuclear compartment to facilitate the internalization of the CUBN-albumin complex.
This manuscript is well written, and these findings have the potential to advance our understanding of the endocytosis process of albumin mediated by CUBN and AMN.

Comments for the author

Some critical details will be needed to support the proposed mechanism:
AMN interacts with CUBN at the cell surface to mediate its endocytosis. Figure 4 shows that AMN and NVL colocalize in the endoplasmic reticulum. How does this ER-localized interaction participate in endocytosis of albumin? Related to this, what is the nature of the dots of NVL staining in Figure 4A? Are they part of ER or endosomes? Does albumin access to these compartments after endocytosis?

Reviewer 3

Advance summary and potential significance to field

Previous studies have established that the AAA ATPase NVL localizes to the nucleoli and functions for the assembly of ribosomal subunits. In this study the authors find a new role of NVL, i.e., its possible involvement in CUBAM-mediated albumin endocytosis. This is an interesting, unexpected finding and surely advances our understanding of the mechanism for CUBAM-mediated albumin endocytosis.

Comments for the author

The limitation of this study is the lack of mechanistic insight into the NVL-mediated endocytosis. Although the authors clearly demonstrated that NVL can be translocated from the nucleoli to the cytosol in an AMN-dependent manner, translocated NVL was found to be predominantly localize to the ER, not the plasma membrane. Only one piece of evidence linking NVL to endocytosis is that in conjunction with AMN it associates with the endocytic adaptor ARH. There are several possible experiments that can strengthen the authors' conclusion and provide mechanistic insight into NVL-mediated endocytosis.

i) Is NVL-mediated endocytosis specific for albumin? What about transferrin or LDL endocytosis? Transferrin is also a ligand for CUBN, but its endocytosis is dependent on megalin. The authors show that megalin is not required for albumin endocytosis in the system used in the present study. Therefore, it may be possible to determine whether NVL depletion does or does not affect transferrin uptake.

ii) Given that NVL principally localizes to the ER, it is possible that CUBAM-mediated albumin endocytosis occurs at ER-plasma membrane contact sites, as is the case of reticulon 3-mediated endocytosis (Caldieri et al., Science: 2017). This possibility can be explored by electron microscopic analysis of NVL-depleted cells. At least, the authors should demonstrate the AMN-dependent proximity between NVL and ARH at the plasma membrane by PLA.

iii) As NVL is a member of the AAA-ATPase family, its interaction with AMN and perhaps ARH may be regulated in a manner of ATP binding and hydrolysis. It is also important to investigate whether ATPase-deficient mutants interact with these proteins. The dominant-negative mutants may also be used for the analysis to determine where CUBAM-mediated endocytosis occurs.

Minor points:
i) Figures 1G, 4C, and 7A: Do the lower panels represent 3D images?
ii) Figure 4A: Does expressed AMN have a myc tag?
iii) Figure 4C: There is no description about the anti-calnexin antibody. As antibodies against DDK and NVL are mouse and rabbit antibodies, respectively,
for triple staining the anti-calnexin must be an antibody from goat, sheep, or other organism.
iv) For what purpose Figure S1B is shown? This should be explained in the text.
v) What are green fluorescent materials in the left panel of Figure S1C?
vi) Figure S4: Nuclear or other co-staining would help to identify the cells.
vii) Throughout the supplementary figures, mm (the unit of scar bars) should read μm.
viii) The reference of Hogan, J. et al. is insufficiently described.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

1. There are two forms of NVL – NVL1 and NVL2 (Nagahama Mol Biol Cell 2004), which differ based on an alternative start codon within the N-terminus yet there is no mention of the two forms of NVL in this manuscript. The authors must determine if NVL1 or NVL2 (or both) associate with CUBAM.

Response: We appreciate your insightful comments. Immunoprecipitation assay demonstrated that NVL2 but not NVL1 interacts with AMN (Fig. 3E). Furthermore, the N-terminus of NVL2 was sufficient for the interaction with AMN (Fig. 5H). We have added these results in the Results section (page 12, line 8–9). We have also revised the text to indicate NVL2 instead of NVL where applicable throughout the manuscript.

2. The majority of NVL2's interaction partners associate with NVL2 in a nucleotide dependent manner. The authors must test whether or not the nucleotide bound state of NVL influences it ability to associate with CUBAM.

Response: To explore the role of the ATPase ability of NVL2 in its interaction with CUBAM, we examined the effect of mutations known to alter ATP-binding capacity of NVL2 (K311M and K628M) (Lo et al., 2019). Immunoprecipitation assays showed that the K311M mutation or the combination of K311M and K628M inhibited the interaction between NVL2 and AMN (Fig. 5I). These mutants also abrogated the trimeric complex formation of NVL2, AMN, and ARH (Fig. S6B). Therefore, the ATP-binding state of NVL2 has a critical role in the interaction between NVL2, AMN, and ARH. These results were added in the Results section (page 16, line 1–13). We appreciate your comments, which have helped us to improve our manuscript.

3. The authors determined that the ICD region of AML is sufficient for binding NVL. The authors should carry out the reciprocal experiment and determine which region of NVL (NTD, NBD1, NBD2) associates with AML.

Response: Immunoprecipitation and pull-down assays demonstrated that the N- terminal domain of NVL2 was necessary and sufficient for its association with AMN (Fig. 5G and 5H). In addition, as mentioned above, the ATP-binding capacity of NVL2 also affects the interaction between NVL2 and AMN (Fig. 5I). The results of these experiments were added in the Results section (page 15, line 13 to page 16, line 13).

4. NVL is an essential gene because of its role in mediating production of the large ribosomal subunit. Knockdown of NVL should thus inhibit cell cycle progression and induce "nucleolar stress". The knockdown experiments in Figure 7 show a reduction in FITC-albumin update but it is impossible to tell if this is a direct or indirect effect. The authors should look at changes in FITC-albumin update following other inducers of nucleolar stress or knockdown of another gene required for ribosome assembly.

Response: We appreciate these helpful comments and insights into highlighting the role of potential nucleolar stress caused by NVL knockdown. To determine whether the endocytosis suppression was caused by nucleolar stress, we induced nucleolar stress by actinomycin D and CX-5461 (Carmo-Fonseca et al., 1992; Drygin et al., 2011). Neither treatment of actinomycin D or CX-5461 impaired CUBAM-mediated albumin uptake (Fig. S8B), while these treatments induced relocalisation of nucleolin (NCL) (Fig. S8A), demonstrating that the endocytosis inhibition by NVL depletion was not mediated by nucleolar stress. We added these results in Fig. S8 and text in the Results section (page 19, line 3–11).

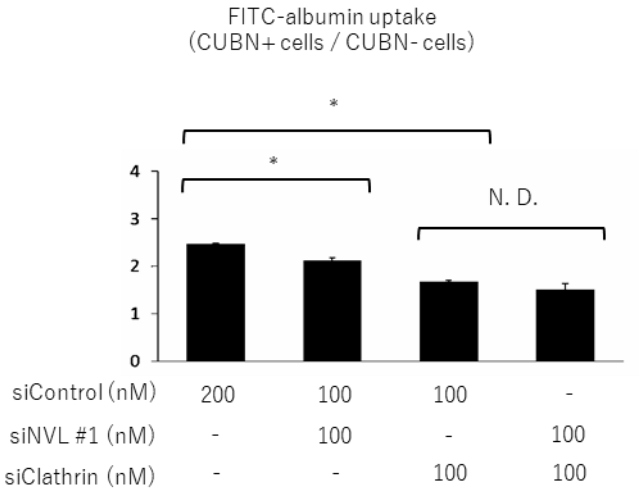
Reviewer 2 Comments for the Author:

Some critical details will be needed to support the proposed mechanism: AMN interacts with CUBN at the cell surface to mediate its endocytosis. Figure 4 shows that AMN and NVL colocalize in the endoplasmic reticulum. How does this ER-localized interaction participate in endocytosis of albumin? Related to this, what is the nature of the dots of NVL staining in Figure 4A? Are they part of ER or endosomes? Does albumin access to these compartments after endocytosis?

Response: We completely agree with reviewer #2. To explore the possibility of localization of NVL2 at the cell surface, its subcellular localisation and association with membrane-targeted CUBN were analyzed using cells expressing both AMN and CUBN, because CUBAM is transported to plasma membrane only when these two molecules are co-expressed (Fyfe et al., 2004; Udagawa et al., 2018). NVL2 was partly colocalized with β-catenin along the plasma membrane in cells expressing AMN and CUBN (Fig. 4D). PLA assays demonstrated the proximity of NVL2 and AMN not only in ER but also along the plasma membrane in CUBAM-expressing cells (Fig. 4E and 4F). We also confirmed the interaction between CUBN at the cell surface and NVL2 by immunoprecipitation (Fig. S3C). These data indicate that in cells expressing AMN and CUBN, NVL2 is associated with the CUBAM complex on the plasma membrane. These results were added in the Results section (page 13, line 2 to page 14, line 2), and we modified Fig. 8.

CUBAM and its ligand are internalized through clathrin-mediated endocytosis (Pedersen et al., 2010). Indeed, clathrin depletion caused a significant reduction in CUBAM-mediated albumin uptake in our system (Fig. R1). Depletion of both clathrin and NVL did not cause further reduction in albumin uptake (Fig. R1), suggesting that NVL has effects on the clathrin-mediated endocytosis process of CUBAM. After endocytosis, albumin is colocalized with CUBN in endosomes (Fig. 1G). In cells expressing AMN or CUBAM, NVL2 is localized in the ER (Fig. 4C and 4E) or at least in part on the cell membrane (Fig. 4D and 4F). In endocytosis assays, we could not obtain data that NVL2 signals colocalize with an endosome marker (EEA1) or endocytosed albumin (data not shown). Based on the results so far, our current presumption is that NVL2 may have a role in clathrin-mediated endocytosis but not in the process after fusion of the endocytosed vesicle to endosome. More detailed mechanisms of NVL2 in the endocytosis pathway need to be clarified in our future study.

Figure R1



Reviewer 3 Comments for the Author:

i) Is NVL-mediated endocytosis specific for albumin? What about transferrin or LDL endocytosis? Transferrin is also a ligand for CUBN, but its endocytosis is dependent on megalin. The authors show

that megalin is not required for albumin endocytosis in the system used in the present study. Therefore, it may be possible to determine whether NVL depletion does or does not affect transferrin uptake.

Response: Thank you for your insightful comments. We explored the possibility that NVL depletion would affect the endocytosis of another CUBN ligand, transferrin, by incubating cells in medium containing fluorescence-conjugated transferrin. Unlike albumin, transferrin was easily endocytosed even by HEK293T cells, which does not express CUBN or AMN (Fig. R2 demonstrates that cells without CUBN (shown in red) endocytosed transferrin (green)). Furthermore, expression of neither mini-CUBN (1–4) nor full-length CUBN increased transferrin uptake (Fig. R2). Quantitative analysis using flow cytometry did not show any difference of transferrin uptake between mini- CUBN (1–4)-expressing cells or full-length CUBN-expressing cells (Fig. R3). We also examined whether NVL knockdown would have any influence on transferrin endocytosis. NVL depletion did not affect cellular transferrin uptake (Fig. R4 and R5). These results indicated that the endocytosis of transferrin may be mediated by another receptor(s) such as TfR1 in our cell model (Smith CP et al, **J** Biol Chem. 2019), and our assay is not suitable for the analysis of endocytosis of transferrin mediated by CUBAM. Further analysis on the mechanism of ligand specificity mediated by CUBAM and other receptors will be included in our future studies.

Figure R2

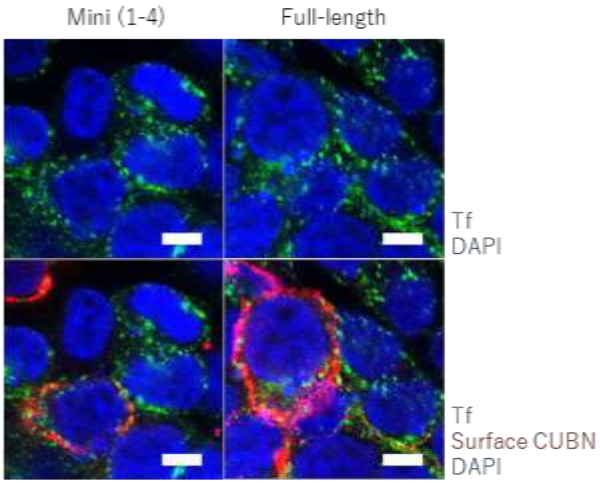


Figure R3

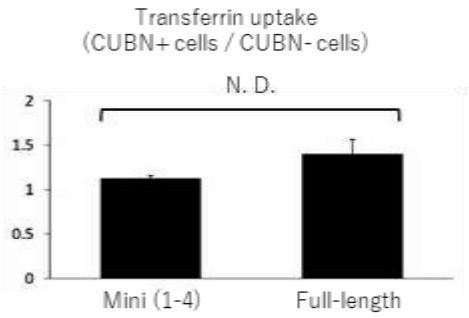


Figure R4

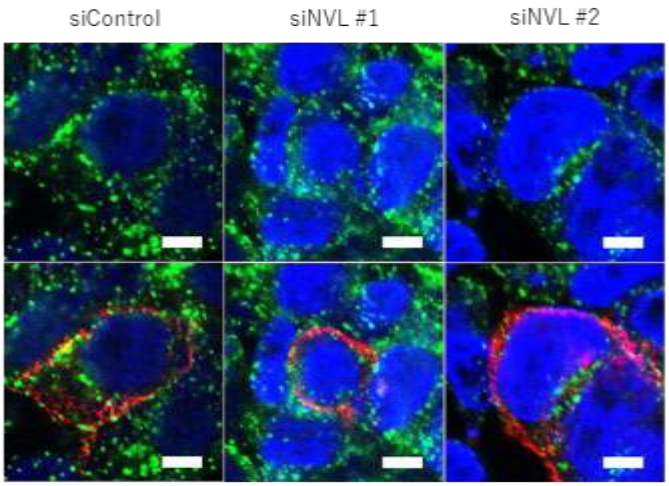
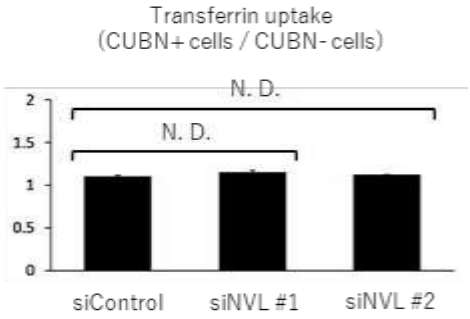


Figure R5

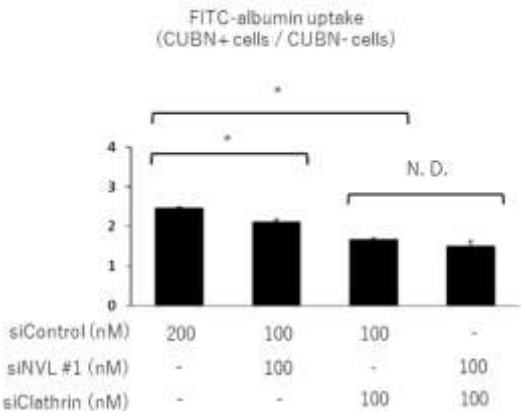


ii) Given that NVL principally localizes to the ER, it is possible that CUBAM-mediated albumin endocytosis occurs at ER-plasma membrane contact sites, as is the case of reticulon 3-mediated endocytosis (Caldieri et al., Science: 2017). This possibility can be explored by electron microscopic analysis of NVL-depleted cells. At least, the authors should demonstrate the AMN-dependent proximity between NVL and ARH at the plasma membrane by PLA.

Response: The extranuclear localization of NVL especially in ER in the original manuscript was demonstrated using cells expressing AMN. Because CUBN and AMN are transported to the plasma membrane in an interdependent manner (Fyfe et al., 2004; Udagawa et al., 2018), we analyzed the localisation of NVL at the cell surface in cells expressing both AMN and CUBN. NVL2 was partly colocalized with β-catenin along the plasma membrane in cells expressing CUBAM (Fig. 4D). PLA assays demonstrated the proximity of NVL2 and AMN not only in the ER but also along the plasma membrane in these cells (Fig. 4E and 4F). PLA assays detected the proximity of NVL2 and ARH in cells expressing AMN (Fig. S6C), and the proximity signals were partly colocaliszed at the plasma membrane in cells expressing AMN and CUBN (Fig. S6D). We also confirmed the interaction between cell surface CUBN and NVL2 by immunoprecipitating CUBN expressed on the cell surface (Fig. S3C). All these data suggested that in cells expressing AMN and CUBN, at least some of the NVL2 molecules associate with the CUBAM complex on the plasma membrane.

CUBAM and its ligand are internalized through clathrin-mediated endocytosis (Pedersen et al., 2010) (Fig. 7–9). As pointed out by reviewer #3, ER-plasma membrane contact sites control the non-clathrin endocytic pathway that is sensitive to reticulon 3 (RTN3) knockdown. In our system, depletion of both clathrin and NVL did not cause further reduction in albumin uptake (Fig. R1). These results did not actively support the possibility that NVL2 was involved in non-clathrin endocytosis regulated by molecules such as RTN3. A more detailed investigation regarding the association of NVL2 and endocytotic machinery will be an issue for future studies.

Figure R1



iii) As NVL is a member of the AAA-ATPase family, its interaction with AMN and perhaps ARH may be regulated in a manner of ATP binding and hydrolysis. It is also important to investigate whether ATPase-deficient mutants interact with these proteins. The dominant-negative mutants may also be used for the analysis to determine where CUBAM-mediated endocytosis occurs.

Response: We appreciate the comments, which have helped us to improve our manuscript. To explore the role of the ATP binding or hydrolysis capacity of NVL2 in its interaction with AMN or ARH, we performed experiments using mutations known to abrogate its ATP-binding capacity (K311M and K628M) or ATP-hydrolysis capacity (E365Q and E682Q) (Lo et al., 2019). Immunoprecipitation assays showed that while NVL2 with ATP-hydrolysis deficient mutations (E365Q, E682Q, or E365Q/E682Q) still interacted with AMN, the K311M mutation clearly abrogated the interaction (Fig. 5I). The interaction between NVL2 and ARH was also impaired by K311M mutation (Fig. S6B). Furthermore, albumin uptake by Dox-induced E365Q/E682Q NVL2-expressing cells was significantly impaired compared with wild-type NVL2-expressing cells (Fig. 7E and 7F). These results suggested that the ATP-binding capacity of NVL2 is essential for the interaction of NVL2 with CUBAM, and ATP-hydrolysis of NVL2 is required for its endocytosis regulation. These results were added in the Results section (page 16, line 1– 13, and page 18, line 14 to page 19, line 2).

Minor points:

i) Figures 1G, 4C, and 7A: Do the lower panels represent 3D images?

Response: The lower panels of Figure 1G, 4C, 4D, and 7Awere 3D surface plots generated using ImageJ to help visualisation of colocalisation of stains. We have added explanation for these 3D surface plots in the end of the “Immunofluorescence” section in “Materials & methods.”

ii) Figure 4A: Does expressed AMN have a myc tag?

Response: The AMN expressed in Figure 4A and 5E are myc-DDK-tagged. We have added this information in the figure legends.

iii) Figure 4C: There is no description about the anti-calnexin antibody. As antibodies against DDK and NVL are mouse and rabbit antibodies, respectively, for triple staining the anti-calnexin must be an antibody from goat, sheep, or other organism.

Response: The anti-calnexin antibody used in the present study is a mouse-derived monoclonal IgG2a antibody, and the mouse anti-DDK antibody belongs to IgG2b. Therefore, we were able to stain FLAG-tagged NVL2 apart from endogenous calnexin using Alexa Fluor 488 conjugated anti-mouse IgG2b antibody and Alexa Fluor 488 conjugated anti-mouse IgG2a antibody as secondary antibodies. We included detailed information (with product numbers) of antibodies in the Methods section.

iv) For what purpose Figure S1B is shown? This should be explained in the text.

Response: In Figure S1B, we demonstrated the expression of mini-CUBN (1–4), mini- CUBN (1–8) and full-length CUBN in transfected cells by immunofluorescence. We added the explanation for this figure in the second paragraph of "Full-length CUBN facilitates albumin endocytosis" in the "Results" (page 9, line 14-15).

v) What are green fluorescent materials in the left panel of Figure S1C?

Response: We apologize for the signals located outside of cells that were assumed to be contaminants such as self-conjugated FITC-BSA. We have replaced Figure S1C with a new image.

vi) Figure S4: Nuclear or other co-staining would help to identify the cells.

Response: We apologize for the lack of clarity in the original manuscript. We have added images of nuclear staining with DAPI to identify the cells (new Figure S5).

vii) Throughout the supplementary figures, mm (the unit of scar bars) should read μm.

Response: Thank you for catching the typographical error. We have corrected it in the Figure Legends.

viii) The reference of Hogan, J. et al. is insufficiently described.

Response: Thank you for pointing out the reference error. We have corrected it in the revised manuscript.

Second decision letter

MS ID#: JOCES/2019/242859

MS TITLE: The cellular model of albumin endocytosis uncovers link between membrane and nuclear proteins

AUTHORS: Seiya Urae, Yutaka Harita, Tomohiro Udagawa, Koji L. Ode, Masami Nagahama, Yuko Kajiho, Shoichiro Kanda, Akihiko Saito, Hiroki R. Ueda, Masaomi Nangaku, and Akira Oka
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.