THE EMBO JOURNAL

Manuscript EMBO-2012-81192

Oligomers of the ATPase EHD2 confine caveolae to the plasma membrane through association with actin

Miriam Stoeber, Ina Karen Stoeck, Christine Hänni, Christopher Karl Ernst Bleck, Giuseppe Balistreri and Ari Helenius

Corresponding author: Ari Helenius, ETH Zurich

Review timeline:

Submission date:	29 November 2011
Editorial decision	4 January 2012
Resubmission	24 February 2012
Editorial decision	19 March 2012
Revision	22 March 2012
Accepted:	23 March 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 4 January 2012

Thank you for transferring your manuscript to The EMBO Journal together with the referee reports from another journal and a point-by-point response. Based on the earlier referee reports, two major caveats were expressed at that point. First, concerns with respect to the experimental set-up relying strongly on overexpressed tagged EHD2 and CAV1 as well as with respect to using cholera toxin B as a cargo for caveolae-dependent endocytosis were raised. Second, referees 1 and 3 felt strongly that deeper mechanistic understanding of how EHD2 acts at the molecular level to affect CAV1 dynamics would be needed. We can see from your point-by-point response that you are working on addressing the first concern. Looking into the second concern, we needed to take into consideration that the referees' assessment in this point may have been journal-specific. This is why we decided to have the manuscript seen afresh by two referees who did not see the original referees' reports and your response.

In the meantime, and after some delay due to difficulties in finding suitable and willing referees at the time of submission in combination with the past Christmas holiday break, we have now received the reports of two referees who evaluated the manuscript for The EMBO Journal (please see below). As you will see, referee 1 raises similar issues as referee 1 of the other journal and overall thinks that the role of EHD2 would need to be analysed in more depth, albeit without making concrete suggestions. Referee 2 thinks that the study is potentially very interesting and well done as far as it goes, but thinks that the exact role and mode of action of EHD2 would need to be analysed in more depth - again without making specific suggestions. I have had a chance to consult with referee 2 once more, and he/she now put forward a number of more specific points that should be addressed

to increase the mechanistic depth of the study. He/she thinks that deeper understanding of how EHD2 protein acts to provide caveolae stability, i.e. by potentially binding to another protein or via potential cytoskeletal interaction would be helpful in this respect. Other points are a more in-depth characterisation (biochemical and morphological) of EHD2 as a bona fide caveolae protein and an expanded set of FRAP experiments using a larger panel of EHD2 mutants. Now, part of these suggestions have already been put forward by the referees of the other journal, including a potential role of the actin cytoskeleton in the confinement of EHD2-containing CAV1 vesicles. The issue of specific targeting of EHD2 to caveolae (original referee 3) has certainly been - in part - addressed by your point-by-point response and potential further discussion, but would still need to be taken seriously. More experimental support would certainly strengthen this point as well.

Overall, and looking into all the input we have, it becomes clear that the referees feel strongly that even though the study is potentially very interesting, it is too premature and too little developed mechanistically that they can support publication in The EMBO Journal at this point. Also, it is clear (and also mentioned more specifically by referee 2 in his/her additional cross-referee comments) that the amount of work required would be substantial.

We have now also discussed the case within our editorial team in depth. In the light of the new referee reports and all the additional input, we have come to the conclusion that we would need to insist on developing the study further mechanistically along the lines put forward by the referees and outlined above. As the amount of work required is rather substantial and goes well beyond the scope and the time frame of a revision, we have decided not to offer publication of the manuscript at this stage of analysis. Still given the interest expressed by the referees in principle, we would certainly be able to consider a new submission on the same topic should future studies allow you to strengthen the study considerably along the lines suggested in your point-by-point response and to develop the study further mechanistically as explained above. I need to stress, however, that if you wish to send a new manuscript this will need to be treated as a new submission rather than a revision and will be evaluated afresh using our original referees again, also with respect to the literature and the novelty of your findings at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive at this point, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This interesting paper from Stoeber and colleagues presents data arguing that the ATPase EHD2 is recruited to caveolae, and that it has an unsuspected function in that ATPase activity actually stabilizes caveolae at the plasma membrane, rather than modifying or releasing them as one might have predicted. The paper is well written and the data appear to be of high quality. The area of caveolar endocytosis is, however, at least for me becoming very muddled and confused. The interpretation of some of the data in the paper under review is questionable. I am sure that the authors can address these issues, and it may be that I do not fully follow the nuances of their argument.

The first point that I would make is that actually the finding that EHD2 is present in caveolae does not appear so very novel after all - in the results section we discover that figure 1, the fundamental observation that EHD2 is present in caveolae, basically recapitulates the findings from another lab, the Hansen et al paper cited. One would not guess this from the abstract, introduction or discussion of the paper under review.

I am confused as to whether caveolae ever really bud from the plasma membrane. The Helenius lab

seem to have made a bit of an about turn on this issue, with the paper of Hayer et al (JCB 2010) at least implying that apparent caveolar budding may be an artifact of over-expressed caveolin 1-GFP. This undermines the interpretation of the data in figure 2 of the paper under review. I do not think that we really understand fully how cavin recruitment to caveolae is triggered and regulated, nor the dynamics of cavin complexes, so the co-localization of cavin and caveolin is not sufficient to justify the conclusion that the mobile caveolin dots seen when EHD2 function is lost are derived from the plasma membrane. Neither is the dynamin 2 K44A experiment - this mutant may have direct or indirect effects on intracellular trafficking pathways. In the paper of Hayer et al mobile dots of caveolin 1-GFP are interpreted as representing partially assembled caveolin oligomers en route for ubiquitination and degradation. How then can the authors of the current paper interpret the mobile caveolin 1-GFP dots as representing caveolae budded from the plasma membrane? One experiment that might help here is to rely less on caveolin 1-GFP, and instead to quantify the number of caveolar membrane profiles in cells where EHD2 function is impaired - if I follow the authors argument correctly there should be less caveolae at the plasma membrane in this instance.

Another area where the literature is hard to reconcile with the appealingly simple interpretations made in the paper under review is the use of cholera toxin B subunit as a marker for caveolae. According to a collaboration between Helenius and Johannes (Nature Cell Biology 2010) GM1-binding ligands like SV40 virus and cholera toxin do not actually co-localize very much with caveolae. How can this be true, as well as the nice co-localization between CTB and caveolin shown in the paper under review? If CTB is indeed taken up in caveolae then surely it should be possible to use some internalization assay with low pH washing or fluorophore quenching outside the cell to see CTB in demonstrably intracellular caveolar vesicles? This would strengthen the authors' interpretations considerably, as EHD2 perturbations should, if they are right, alter the abundance of such vesicles. I find it hard to see how in 2010 the Helenius lab can publish a paper arguing that CTB induces membrane tubulation for its own uptake, yet in the paper under review they use CTB as a marker for caveolar endocytosis. What is going on?

Much of the rest of the data, characterizing EHD2 mutants and so on, is perfectly interesting and uncontentious. I just think that there are many inconsistencies in the literature, and that alternative models for EHD2 function as well as directly in regulating budding of caveolae from the plasma membrane may also be valid. The idea of ATPase activity being required to tether or stabilize caveolae is certainly somewhat counter-intuitive and probably needs more support.

Referee #2

This is a well constructed manuscript focused on EHD2, an interesting protein containing Eps-15 homology domains (thereby likely playing a role in vesicular transport), it oligomerizes and can tubulate liposomes in vitro and has an intrinsic ATPase property. It was identified as a component of caveolae in a proteomic study but its functional role has not been determined. Overall the manuscript is well done but while the study clearly shows that EHD2 plays some type of role in stabilizing caveolae to the PM few insights are provided as to how this may occur. This expansion would certainly make the study more interesting and complete. Other than this issue of breadth I have only minor suggestions.

The first figure demonstrates a co-localization of EHD2 and caveolae markers including Cav1 and Cavin with supportive data including IEM and FRET based analysis. The resolution on the pdf version of the panels in 1b,c was not ideal so the endogenous protein co-localization was not as convincing in 1c. This problem is not a big issue however, as the authors provide IEM and FRET based co-localization.

EHD2 apparently associates dynamically within stable pools of caveolae on the PM. The authors came to this conclusion after doing FRAP studies in comparison to that of Cavin proteins (Fig4). There also appear to be multiple domains of EHD2 required for caveolae targeting (Fig 5), including functionally distinct domains (Lipid binding, protein association and ATP binding domains) which curiously all seem to affect oligomerization of EHD2 (Fig 5c,d). The authors then find that ATP loading and hydrolysis are required for EHD2 recruitment and exchange/release at caveolae (Fig 6).

Overall, this is a very clear paper with clean results that provide some functional insights for EHD2 while dissecting the dynamic association of this oligomeric protein complex in altering fluidity and function of caveolae.

Resubmission 24 February 2012

Point-by-point rebuttal

1. Editor's comments

"I have had a chance to consult with referee 2 once more, and he/she now put forward a number of more specific points that should be addressed to increase the mechanistic depth of the study. He/she thinks that deeper understanding of how EHD2 protein acts to provide caveolae stability, i.e. by potentially binding to another protein or via potential cytoskeletal interaction would be helpful in this respect. Other points are a more in-depth characterisation (biochemical and morphological) of EHD2 as a bona fide caveolae protein and an expanded set of FRAP experiments using a larger panel of EHD2 mutants."

In a new chapter, in Figure 7, and in movies S5-S8, we provide mechanistic depth by showing that the reason why caveolar dynamics is constrained in cells is that EHD2 oligomers mediate an interaction between caveolae and actin filaments. Our mutant experiments indicate that the interaction requires ATP hydrolysis and that EHD2 itself is part of the physical link.

We have, in addition, complemented the information regarding the role of the ATPase cycle by demonstrating that, unlike wild-type EHD2, a mutant with accelerated ATPase activity forms large, PM-bound complexes in the absence of caveolae, and that these are associated with actin filaments (Fig. 8 & 7, movie S8). This is consistent with our hypothesis that association of wild-type EHD2 with caveolae accelerates ATPase activity and thus triggers stable association of EHD2 complexes with caveolae. In addition, we have added EM data that supports a localization of EHD2 with indented caveolae and caveolar clusters. EM also shows that EHD2 is located closer to the rim of caveolar indentations than CAV1 (Figure 1).

REVIEWER 1

"This interesting paper from Stoeber and colleagues presents data arguing that the ATPase EHD2 is recruited to caveolae, and that it has an unsuspected function in that ATPase activity actually stabilizes caveolae at the plasma membrane, rather than modifying or releasing them as one might have predicted. The paper is well written and the data appear to be of high quality. The area of caveolar endocytosis is, however, at least for me becoming very muddled and confused. The interpretation of some of the data in the paper under review is questionable. I am sure that the authors can address these issues, and it may be that I do not fully follow the nuances of their argument.

The first point that I would make is that actually the finding that EHD2 is present in caveolae does not appear so very novel after all - in the results section we discover that figure 1, the fundamental observation that EHD2 is present in caveolae, basically recapitulates the findings from another lab, the Hansen et al paper cited. One would not guess this from the abstract, introduction or discussion of the paper under review."

Yes, Hansen et al. do show colocalization of over-expressed EHD2-GFP with CAV1 in a supplemental figure of their paper, but they leave this observation unexplored. In Fig. 1 and Supp. Fig. 1 of our paper, we show not only that EHD2-GFP but also the endogenous EHD2 associates with CAV1 and cavins in characteristic spots. The close proximity of EHD2 and CAV1 is confirmed with FRET-analysis. It is also shown that in cells that do not have CAV1, and therefore lack caveolae, the spot-like binding of EHD2 to the cell surface can be restored when CAV1 is expressed. This rescue experiment shows that formation of EHD2 spots depends on caveolae. We have given the Hansen et al. observations more visibility in the introduction of the revised manuscript but are convinced that our data goes far beyond published findings.

I am confused as to whether caveolae ever really bud from the plasma membrane. The Helenius lab seem to have made a bit of an about turn on this issue, with the paper of Hayer et al (JCB 2010) at least implying that apparent caveolar budding may be an artifact of over-expressed caveolin 1-GFP. This undermines the interpretation of the data in figure 2 of the paper under review.

There is no doubt that caveolae can bud off the PM and form vesicles. Below are some of the references in the literature. Regarding Hayer et al. (2010), no such implications were intended. Hence, this must be a misunderstanding.

Lajoie, P. & Nabi, I.R. Lipid rafts, caveolae, and their endocytosis.
International review of cell and molecular biology 282, 135-63 (2010).
Botos, E. et al. Caveolin-1 is transported to multi-vesicular bodies after albumin-induced endocytosis of caveolae in HepG2 cells. Journal of cellular and molecular medicine 12, 1632-9 (2008).
Oh, P., McIntosh, D.P. & Schnitzer, J.E. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. The Journal of cell biology 141, 101-14 (1998).
Sverdlov, M., Shajahan, A.N. & Minshall, R.D. Tyrosine phosphorylation- dependence of caveolae-mediated endocytosis. Journal of cellular and molecular medicine 11, 1239-50 (2007).
Pelkmans, L. et al. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. Nature 436, 78-86 (2005).
Tagawa, A. et al. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. The Journal of cell biology 170, 769-79 (2005).

I do not think that we really understand fully how cavin recruitment to caveolae is triggered and regulated, nor the dynamics of cavin complexes, so the co-localization of cavin and caveolin is not sufficient to justify the conclusion that the mobile caveolin dots seen when EHD2 function is lost are derived from the plasma membrane. Neither is the dynamin 2 K44A experiment - this mutant may have direct or indirect effects on intracellular trafficking pathways.

While it is yet unknown how recruitment of cavin-1 to caveolae is triggered and regulated, the compartments where cavin-1 and caveolae co-localize are known. Independent studies listed below show that cavin-1 is recruited to caveolae at the PM, and that cavin-1 traffics with CAV1 to endosomes. We propose here that the vesicles that we see are derived from the PM because: 1) they contain cavin-1 which means that they have been in the plasma membrane, and 2) because their existence depends on active dynamin2, a factor needed for release of caveolar vesicles from the PM. We do not see anything wrong with this logic.

Hayer, A. et al. Caveolin-1 is ubiquitinated and targeted to intralumenal vesicles in endolysosomes for degradation. The Journal of cell biology 191, 615-29 (2010).
Hill, M.M. et al. PTRF-Cavin, a conserved cytoplasmic protein required for caveola formation and function. *Cell* **132**, 113-24 (2008).
Boucrot, E., Howes, M.T., Kirchhausen, T. & Parton, R.G. Redistribution of caveolae during mitosis. *Journal of cell science* **124**, 1965-72 (2011).

In the paper of Hayer et al mobile dots of caveolin 1-GFP are interpreted as representing partially assembled caveolin oligomers en route for ubiquitination and degradation.

This interpretation is not made in the Hayer paper. What is shown is that high over-expression of CAV1 can lead to expression of disassembled CAV1 in the PM followed by ubiquitination and lysosomal degradation. This is not happening in our stable cell lines which have a moderate (1-fold) over-expression over endogenous CAV1. For clarification, we added a Western-blot of our stable cell lines to Supplementary Figure S3.

How then can the authors of the current paper interpret the mobile caveolin 1-GFP dots as representing caveolae budded from the plasma membrane?

We are confident that our CAV1-GFP spots represent assembled caveolae because: 1) They are

stationary in the plasma membrane in control cells and become mobile upon EHD2 silencing. 2) Moving vesicles are positive for cavin-1, a marker for assembled caveolae coming from the PM and not deriving from the Golgi complex, and 3) the dynamin requirement.

One experiment that might help here is to rely less on CAV-1-GFP, and instead to quantify the number of caveolar membrane profiles in cells where EHD2 function is impaired - if I follow the authors argument correctly there should be less caveolae at the plasma membrane in this instance.

As previously shown, changes in the extent of mobile versus stationary caveolae does not necessarily alter the over-all number of caveolae in the PM. Caveolae can re-fuse with the plasma membrane and this dynamic process is unlikely to be captured with static images of cells. Therefore our live-cell studies on the dynamics of caveolae are more revealing.

Pelkmans, L. & Zerial, M. Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. *Nature* **436**, 128-33 (2005).
Tagawa, A. et al. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. The Journal of cell biology 170, 769-79 (2005).

Another area where the literature is hard to reconcile with the appealingly simple interpretations made in the paper under review is the use of cholera toxin B subunit as a marker for caveolae. According to a collaboration between Helenius and Johannes (Nature Cell Biology 2010) GM1-binding ligands like SV40 virus and cholera toxin do not actually co-localize very much with caveolae. How can this be true, as well as the nice co-localization between CTB and caveolin shown in the paper under review?

The cited paper does not investigate CTB binding in respect to caveolae. As shown in the references below, many groups found that CTB localizes to caveolae and that CTB partly internalizes via caveolae especially when the toxin concentrations are low. In our manuscript, we now emphasize the existing literature on multiple entry pathways of CTB.

Parton, R.G. Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 42, 155-66 (1994).
Pelkmans, L., Burli, T., Zerial, M. & Helenius, A. Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell* **118**, 767-80 (2004).
Boucrot, E., Howes, M.T., Kirchhausen, T., and Parton, R.G. (2011). Redistribution of caveolae during mitosis. Journal of cell science *124*, 1965-1972.
Pelkmans, L. & Zerial, M. Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. *Nature* **436**, 128-33 (2005).

If CTB is indeed taken up in caveolae then surely it should be possible to use some internalization assay with low pH washing or fluorophore quenching outside the cell to see CTB in demonstrably intracellular caveolar vesicles? This would strengthen the authors' interpretations considerably, as EHD2 perturbations should, if they are right, alter the abundance of such vesicles.

In the past, other groups have performed the suggested experiment and indeed detected CTB in caveolae. While it would be interesting to test for an increase in CTB-CAV1-positive vesicles in EHD2 depleted cells, we refrained from establishing this assay, as it would not lead to more mechanistic understanding.

Pelkmans, L. & Zerial, M. Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. *Nature* **436**, 128-33 (2005).
Boucrot, E., Howes, M.T., Kirchhausen, T., and Parton, R.G. (2011). Redistribution of caveolae during mitosis. Journal of cell science *124*, 1965-1972.

I find it hard to see how in 2010 the Helenius lab can publish a paper arguing that CTB induces membrane tubulation for its own uptake, yet in the paper under review they use CTB as a marker for

caveolar endocytosis. What is going on?

As clearly shown by the data, CTB does bind to caveolae under the conditions used, and when EHD2 is deleted it does not enter as efficiently. This does not mean that caveolae are the only mechanism of entry.

Much of the rest of the data, characterizing EHD2 mutants and so on, is perfectly interesting and uncontentious. I just think that there are many inconsistencies in the literature, and that alternative models for EHD2 function as well as directly in regulating budding of caveolae from the plasma membrane may also be valid. The idea of ATPase activity being required to tether or stabilize caveolae is certainly somewhat counter-intuitive and probably needs more support.

We have added more support by including experimental details on the EHD2 mutant I157Q with elevated ATP hydrolysis (Figure 6 and 7). Unlike wild-type EHD2, this mutant forms large, actin-associated complexes even in the absence of caveolae. This is consistent with our hypothesis that association of wild-type EHD2 with caveolae accelerates ATPase activity and thus triggers lateral association of EHD2 complexes with each other and with caveolae.

Referee #2

This is a well constructed manuscript focused on EHD2, an interesting protein containing Eps-15 homology domains (thereby likely playing a role in vesicular transport), it oligomerizes and can tubulate liposomes in vitro and has an intrinsic ATPase property. It was identified as a component of caveolae in a proteomic study but its functional role has not been determined. Overall the manuscript is well done but while the study clearly shows that EHD2 plays some type of role in stabilizing caveolae to the PM few insights are provided as to how this may occur. This expansion would certainly make the study more interesting and complete. Other than this issue of breadth I have only minor suggestions.

The first figure demonstrates a co-localization of EHD2 and caveolae markers including Cav1 and Cavin with supportive data including IEM and FRET based analysis. The resolution on the pdf version of the panels in 1b,c was not ideal so the endogenous protein co-localization was not as convincing in 1c. This problem is not a big issue however, as the authors provide IEM and FRET based co-localization.

EHD2 apparently associates dynamically within stable pools of caveolae on the PM. The authors came to this conclusion after doing FRAP studies in comparison to that of Cavin proteins (Fig4). There also appear to be multiple domains of EHD2 required for caveolae targeting (Fig 5), including functionally distinct domains (Lipid binding, protein association and ATP binding domains) which curiously all seem to affect oligomerization of EHD2 (Fig 5c,d). The authors then find that ATP loading and hydrolysis are required for EHD2 recruitment and exchange/release at caveolae (Fig 6).

Overall, this is a very clear paper with clean results that provide some functional insights for EHD2 while dissecting the dynamic association of this oligomeric protein complex in altering fluidity and function of caveolae.

2nd Editorial Decision 19 March 2012

Thank you for sending us your revised manuscript as a new submission. In the meantime, referee 2 has seen it again and is now in favour of publication of the study here. He/she raises one minor point that should still be addressed. Furthermore, I need to ask you to include a conflict of interest statement to the main body of the manuscript text after the author contributions section. Finally, in the meantime, a similar study has been published by another lab (PMID: 22323287), and it should be discussed briefly in the discussion section and included into the references list. I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

Most of this Reviewer's comments, and I believe that of Reviewer One, have been addressed in the revised manuscript.

The authors have added an entirely new figure (Figure 7), further implicating the involvement of actin in the EHD2 mediated stabilization of caveolin at the PM. They demonstrate an EHD2 sensitive localization of Cav1 with actin and also provide some TIRF microscopy based time-lapse studies looking at the mobility of Cav1-GFP +/- the presence of EHD2. These findings add some more depth to the story.

Other additions include the image in 6E, which together with the data in Fig S5-D indicate the presence of EHD2 protein on the PM even in the absence of Cav1.
An expansion of figure 4 (4B) demonstrates a co-localization of EHD2 and Cav1 on the PM and not in the early endosome/endomembrane compartment, further characterizing the context of this protein association.

The authors expanded Fig 1 with a more extensive look at the IEM of EHD2 and caveolin. They demonstrate the discrete localization of EHD2 closer at the rim of caveolar invaginations, or at least more proximal to the PM than caveolin. All of the panels in Fig. 1D look fairly convincing and the graph summarizes their finding, however, the particular upper right panel of 1D looks as if the 10nm gold particle labeling actually is outside the surface of the cell? This detracts from the overall figure and other IEM panels- replace it?

This study provides a nice balance of biochemical and imaging based approaches along with some mechanistic insights into the stabilization of caveolin at the cell surface by EHD2 proteins.

1st Revision 22 March 2012

Thank you for guiding us through the publishing process and giving us the chance to resubmit our manuscript “Oligomers of EHD2 ATPase confine caveolae to the plasma membrane through association with actin“ by Ina Karen Stoeck, Christine Hänni, Christopher Karl Ernst Bleck, Giuseppe Balistreri, and ourselves.

In the revised version, we exchanged the upper right panel of Figure 1D, following the suggestions of referee 1.

In the last paragraph of the discussion, we now briefly discuss the concomitant study of Moren and colleagues, which supports our finding that EHD2 confines caveolae to the plasma membrane. Furthermore we added a conflict of interest statement.

We thank you and the referees for careful reading and helpful comments.

