# The voltage-gated sodium channel β2 subunit associates with lipid rafts by S-palmitoylation

Eric Cortada, Robert Serradesanferm, Ramon Brugada and Marcel Verges
DOI: 10.1242/jcs.252189

**Editor**: John Heath

**Review timeline**
Original submission: 24 July 2020
Editorial decision: 27 August 2020
First revision received: 25 January 2021
Accepted: 8 February 2021

---

**Original submission**

First decision letter

MS ID#: JOCES/2020/252189

MS TITLE: The voltage-gated sodium channel β2 subunit associates with lipid rafts by S-palmitoylation

AUTHORS: Eric Cortada Almar, Robert Serradesanferm, Ramon Brugada, and Marcel Verges
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 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*

This paper describes a wide range of experiments that seek to identify the role of Palmitoylation of the juxta-cytoplasmic membrane Cys182 in the beta-2 subunit of the voltage-gated Na+ channel, which they argue is palmitoylated, thereby conferring selective biosynthetic trafficking of the subunit to the apical and not basolateral surface of polarized MDCK cells, a process reliant upon the protein subunit being incorporated into lipid rafts, a step mediated by palmitoylation of Cys182.

*Comments for the author*

Overall, there is enough proof from the variety of approaches used that palmitoylation of Cys182 controls biosynthetic trafficking of β2. I have, however, found this paper very hard to read, in part because the data is often over-interpreted, or precautiously interpreted.

To start with Fig 1A, which the authors state "This experiment suggests that β2 is palmitoylated". In fact it shows that the subunit has a reacted Cys that can be hydrolysed by hydroxylamine, to enable it then to be biotinylated. Fig. 1B shows that mutation of Cys182 to Ser vastly reduces biotinylation of the β
2 band on a gel. Down in Fig. 1D, it is shown that an inhibitor of palmitoylation partially inhibits biotinylation of β2. There is a considerable literature on the modification of juxta-cytoplasmic membrane Cys residues by palmitoylation, and the effect of this in giving the protein access to 'lipid rafts, so there is good reason to think the result is correct. Proof would be the incorporation of radioactive palmitate; but I will accept 'highly probable' as a result of this evidence.
If Fig 1C & E, the relevant gel bands were scanned, converted to means±SEM, and plotted with significance of the undefined "normalized palmitoylation signal" indicated. The blots were visualized using peroxidase-labelled secondary antibodies. The peroxidase reaction produces active O that attacks the enzyme, so the kinetics of a peroxidase reaction are only linear at its very early stages. Fortunately, labelling of b2 is sufficiently weak for their label to be linear and reproducible (and hence significant), but labelling of the very strong Ig heavy chain bands would be very non-linear.

And so to Fig 2, Detergent Resistant Membranes. Some palmitoylated β2 is in the DRM fraction. But how much? If the bands on the gel shown were scanned and quantitated, I suspect about 10%. And I would be very surprised if the non-DRM fraction is substantially 'burnt out'. The DRM proportion of β2 is probably 1-5%. The vast majority of β2 is not in DRMs, and so possibly not in Lo domains.
It could be that passage through an Lo/DRM is essential for apical trafficking, which happens quickly and so only a small proportion of the total is in there at any one time.
What are the levels of expression of β2 on the MDCK cells compared to their normal physiological abundance? Perhaps there is just too much β2 being made to fit into DRMs?
And, of course, lipid rafts are at physiological temperature, DRMs at 4°C; the phase of lipids is determined by their transition (melting) temperatures, and the subset of Lo domains you have selected may be the tip of the lipid iceberg at 4°C.

The cholesterol depletion experiments are well done, as are the (non)-endoctyosis experiments. The FRAP experiments look good (and show an interesting result for the role of the cytoplasmic domain of 4°C (although I have no personal experience here). The co-localization of fluorescence of Cholera Toxin and β2 shows the two localize in the same compartments. The fluorescence used could not possibly resolve individual 'rafts'. There are well developed methods using immunoaffinity purification of DRMs to identity whether the raft components are in the same vesicles. Or, of course, since you frequently quote his recent Traffic review, there is single molecule trafficking.

Overall, this work is, with comprehensive revision, publishable, but I think you should be more logical in your analysis of the meaning of your results. I would prefer it if your Figure legends described their Figures; at the moment you squeeze in a distracting amount of experimental detail that should be in the Methods; and I think you would benefit with a more detailed Discussion of your results, which are genuinely interesting.

And one very little thing: living cells are called ‘live cells’ in English

Reviewer 2

*Advance summary and potential significance to field*

Cortada et al have provided a comprehensive study demonstrating palmitoylation of the voltage gated sodium channel β2 subunit (SCN2B) and its role in trafficking to the apical membrane of Madin-Darby Canine Kidney (MDCK) cells.
Using a palmitoylation deficient mutant (β2-C182S) and a palmitate analog in biotinylation, immunoprecipitation and sucrose density gradient experiments they demonstrated that beta 2 is palmitoylated at this cysteine residue thereby enhancing the fraction associated with lipid rafts, specifically cholesterol rich detergent resistant membranes. However, expression is still predominantly within the remaining solubilised fractions for both beta 2 and beta 2-C182S. Application of cholesterol depleting agents (MBCD and lovastatin) resulted in a redistribution of beta 2 to the basolateral membrane further corroborating the role of beta 2 association with lipid rafts and appropriate membrane targeting.
Similarly, loss of cholesterol and the palmitoylation deficient mutant are both associated with a decreased mobility of beta2 within the range associated with diffusion inside lipid rafts, therefore suggesting a reduction in association with cholesterol rich lipid rafts. Confirmation of beta 2 expression within lipid raft domains was established by co-localisation of the beta 2 signal with the lipid raft specific CTX. These findings identify the importance of beta 2 palmitoylation for its appropriate localisation within the apical membrane of MDCK cells, from which it is inferred that this would also likely influence the Nav alpha binding partner(s), to specific plasma membrane localities in human tissues such as cardiac myocytes. However, this study lacks any direct demonstration of functional consequences on beta 2 and Nav function, although alluded to in the discussion. That said, it does provide a thorough assessment of the mechanisms of the relatively unknown process of palmitoylation and how it regulates the cellular distribution of beta 2 which is widely applicable and informative for membrane targeting of both related and unrelated transmembrane proteins.

*Comments for the author*

The manuscript is generally well written and presented to a high standard. The conclusions on the whole are well justified, however I have some suggestions/comments to enhance the completeness of the findings.

Major
1. Some key experiments were not performed with the palmitoylation deficient mutant. Most notably, the experiments that clearly demonstrate beta 2 expression within lipid raft domains by virtue of their association with CTX.
Performing these with beta 2-C182S would help clarify if the fraction of beta 2-C182S that reaches the apical membrane does so via lipid rafts or another potential mechanism. Similarly, if palmitoylation is relatively important to the apical targeting of beta2 it would be good to see the subcellular distribution of beta2-C182S in confocal microscopy and the effect of MBCD and lovastatin, albeit the authors do demonstrate a mixed apical and basolateral distribution in supplementary western blots, but there is no evidence of the effect of cholesterol depletion.

2. Did the authors notice any differences in expression level of the beta 2-C182S that might influence the results (particularly normalization) given the apparent difference in band density of beta 2 in fig 1D and supp fig 3 (unclear if this is also true for fig 1b due to saturation of bands) where the beta 2-C182S band is particularly weak in the lysate fraction compared with the WT beta 2.

3. In figure 2, I agree with the authors rationale and conclusions on the general distribution of beta 2 through the different fractions in the Western blot. However, I am confused by how these results tally with what is shown graphically in panels B and C. WT beta2 for example shows a higher

density in the first fraction relative to the second and third, yet in panel B the peak for the DRM portion is shown in fraction 3. How do these two data sets relate to each other? Presumably there has been some normalization that explains the mismatch?

4. In figure 5 it is not clear to me that similar subcellular/membrane areas have been exposed to bleaching. For example in panel D the WT beta2 expressing cells have clearly been targeted at a cell-cell junction, it is not apparent that this is a similar position in the beta2-181X expressing cells. Unless this is a reflection of a differential distribution of beta2 here? Similarly, in panel B cell-cell junctions have been targeted but not in panels A and C. Could this be clarified as presumably the argument is that cholesterol rich apical membranes are being targeted?

5. Given the importance of appropriate Nav1.5 surface expression to channel function and the role of beta 2 in trafficking, as commented on by the authors and their previous publications in this area, it is a shame that the effect of beta 2 palmitoylation on Nav1.5 distribution has not been assessed.

Minor:
1. In the first paragraph of Results, it is stated "HAM exposes thiol groups that can then be biotinylated". Is it not more accurate that HAM selectively reduces thiols attached to palmitate, hence the use of this reagent to measure palmitoylation. There are other thiol groups in the beta2 Ig domain, but these would be blocked by the NEM

2. Fig 1D arrow indicators are included to denote the beta 2 band at 0, 100 and 200 uM 2BP treatment, this has been omitted from 50 uM.

3. Live cells has been written as life cells in a few places including in the abstract, first line of p5 and p7 and again in the penultimate paragraph of p7 and the legend for fig 6.

4. In some western blots a skewed atypical marker has been used, presumably due to the narrow gap between blots. Perhaps some blots could be enlarged to accommodate for the markers (eg. Fig 4a, the gp114 is particularly narrow extending beyond 100 kDa marker would leave room for a more typical labelling of 55 kDa in the Vps26 blot)

5. On p5 there is a sentence "....overnight incubation with a discrete amount of lovastatin". Please clarify.

6. Fig 4 is not labelled for the IP (as has been done in previous figures). In the legend the sentence "... remaining protected the endocytosed biotinylated proteins" does not quite make sense.

---

**First revision**

Author response to reviewers' comments

Sunday, January 24, 2021

Dr. John Heath
Editor – Journal of Cell Science

Dear Dr. Heath,

In response to the comments and suggestions from the reviewers on our manuscript JOCES/2020/252189 submitted for publication in the Journal of Cell Science, we want to thank the reviewers for their positive comments and constructive input and for having given us the opportunity to respond to the issues raised in a revised manuscript. We have conducted a number of experiments, analyzing the new data thoroughly, and now resubmit our manuscript – which we believe has improved considerably – to be reconsidered for publication.

As you will see, our results give support to the idea that a portion of ß2 is present within DRM/Lo membrane/rafts domains in MDCK cells. While short-lived and proceeding rapidly through these, the presence in rafts of ß2 is possibly quite important for its apical deliver. We have performed the additional experiments requested, in particular, to assess the role of cholesterol in trafficking and localization of the non-palmitoylable ß2 mutant, C182S, which might help understanding how ß2 reaches the apical membrane domain. Finally, we have made an effort to make the manuscript more easily readable, giving a more realistic rationale behind the evidence provided.

Major modifications include the following. [1] We conclusively confirm that the non- palmitoylable mutant, ß2 C182S, maintains apical polarity, behaving similarly as the WT upon cholesterol depletion, thus becoming mislocalized to the basolateral domain. [2] We show that, as ß2 WT, C182S also overlaps with CTX-labeled raft domains at the apical surface. And [3], we found no evidence that palmitoylation of ß2 may be required to promote surface localization of Naγ1.5. While ß2 palmitoylation – which we state as highly probable – does not determine, by itself, its apical localization, our data suggest that it may contribute to ß2 partition into apical raft subdomains potentially important to establish its polarized distribution.

Next, the reviewers' comments appear in *italics* and our point-by-point responses are in **bold**. Changes made in the original text are highlighted in yellow in the new manuscript. We also uploaded a clean, unmarked version of the manuscript.

Yours sincerely,
Marcel Vergés

*Reviewer #1:*

*This paper describes a wide range of experiments that seek to identify the role of Palmitoylation of the juxta-cytoplasmic membrane Cys182 in the beta-2 subunit of the voltage- gated $Na^+$ channel, which they argue is palmitoylated, thereby conferring selective biosynthetic trafficking of the subunit to the apical and not basolateral surface of polarized MDCK cells, a process reliant upon the protein subunit being incorporated into lipid rafts, a step mediated by palmitoylation of Cys182.*

*Overall, there is enough proof from the variety of approaches used that palmitoylation of Cys182 controls biosynthetic trafficking of ß2. I have, however, found this paper very hard to read, in part because the data is often over-interpreted, or precoutiously interpreted.*

**We appreciate the reviewer's comment about our paper and, in particular, on how the results have been explained. In this revised version, we have made an effort to provide a more realistic rationale behind the evidence provided. An example of that would be when we replaced the statement that ß2 palmitoylation is necessary (or required) for its association with (localization to) lipid rafts with that its palmitoylation increases ß2 affinity for (or association with) raft domains.**

*To start with Fig 1A, which the authors state "This experiment suggests that ß2 is palmitoylated". In fact it shows that the subunit has a reacted Cys that can be hydrolysed by hydroxylamine, to enable it then to be biotinylated.*

**We have modified the seantence accordingly.**

*Fig. 1B shows that mutation of Cys182 to Ser vastly reduces biotinylation of the ß2 band on a gel.*

**We have also modified this sentence.**

*Down in Fig. 1D, it is shown that an inhibitor of palmitoylation partially inhibits biotinylation of ß2. There is a considerable literature on the modification of juxta-cytoplasmic membrane Cys residues by palmitoylation, and the effect of this in giving the protein access to 'lipid rafts, so there is good reason to think the result is correct. Proof would be the incorporation of radioactive palmitate; but*

*I will accept 'highly probable' as a result of this evidence.*

**As suggested by the reviewer, we have pointed this out at the end of this section of the *Results*. We have indeed considered metabolic labeling with radioactive palmitate. Unfortunately, our institution does not have a facility to work with radioactive isotopes nor can provide authorization for personnel to carry out experiments with radioactive samples. Nevertheless, we may also have to keep in mind that metabolic labeling with the palmitate analog 2-BP inhibited palmitoylation in cells. This suggests that this irreversible acyltransferase inhibitor effectively blocked incorporation of cellular palmitoyl-CoA to ß2 Cys-182. Thus, as the reviewer indicates, we have emphasized throughout the text that our data provide substantial evidence of ß2 palmitoylation, emphasizing that it takes place with high probability.**

*If Fig 1C & E, the relevant gel bands were scanned, converted to mean ± SEM, and plotted with significance of the undefined "normalized palmitoylation signal" indicated.*

**In the *Methods* section, we have now defined the normalized palmitoylation (Palm.) signal.**

*The blots were visualized using peroxidase-labelled secondary antibodies. The peroxidase reaction produces active O that attacks the enzyme, so the kinetic of a peroxidase reaction are only linear at its very early stages. Fortunately, labelling of b2 is sufficiently weak for their label to be linear and reproducible (and hence significant), but labelling of the very strong Ig heavy chain bands would be very non-linear.*

**When describing how we have normalized the palmitoylation level (or signal), we now indicate that intensity of each band detected with the antibody to biotin has been adjusted with the corresponding ß2 band in each lane.**

**The Ig heavy chain bands in Fig. 1 are indeed highly overexposed in comparison to the band of biotinylated ß2. This is caused by the considerable abundance of the rabbit polyclonal antibody against GFP used to immunoprecipitate ß2, which is recognized by the 2º antibody used to detect biotin for Western blot. Unfortunately, we do not have a monoclonal antibody to biotin. Although we did try detecting biotinylated ß2 by streptavidin-HRP, the reaction was always dimmer and harder to interpret. Therefore, in all experiments we decided to go for an anti-biotin blot. On the other hand, we overexposed a bit on purpose the blot anti-biotin to make sure that no relevant band would show up in the absence of HAM (vs. "with HAM"), in the mutant, and also in immunoprecipitations with the irrelevant antibody.**

*And so to Fig 2, Detergent Resistant Membranes: Some palmitoylated ß2 is in the DRM fraction. – But how much? If the bands on the gel shown were scanned and quantitated, I suspect about 10%. And I would be very surprised if the non-DRM fraction is substantially 'burnt out'. The DRM proportion of ß2 is probably 1-5%. The vast majority of ß2 is not in DRMs, and so possibly not in Lo domains.*

**As the reviewer anticipates, a considerable portion of ß2 is not in DRM fractions and, in consequence, not in Lo domains. Indeed, we have quantified the total signal from the bands within the DRMs – that is, the upper 2-3 fractions, as indicated in *Methods* and shown in Fig. 2A – obtaining an avg. portion of 12% over the total signal coming from ß2 in all fractions collected. In comparison, the portion of the mutant within DRMs actually came down to ~ 4%.**

**We have added these estimates in the manuscript, with the important remark that there may be somewhat overestimated, as the reviewer suspects, due to the comparatively much higher intensity that the ß2 bands display in the detergent-soluble fractions found toward the bottom of the gradient. Nevertheless, virtually in all experiments in which we have performed band quantitation we detected the chemiluminescence reaction by a *ChemiDoc MP* unit, instead of using X-ray film. Since the former uses a CCD image sensor for light detection, it has a much greater linear range than film does. Therefore, we reckoned that the digital data generated would be relatively quantitative. We have indicated this at the subsection describing western blotting.**

*It could be that passage through a Lo/DRM is essential for apical trafficking, which happens quickly*

*and so only a small proportion of the total is in there at any one time.*

**We fully agree with this hypothesis, which indeed may partially explain why we measured such a small portion of ß2 within rafts in MDCK cells. We have therefore added this comment in the *Discussion*. Classical studies, such as that by Matlin & Simons (J Cell Biol. 1984), already showed that direct transport to the apical surface is manifested as early as 25 min from pulse-labeling, becoming almost complete at 45 min (PMID: 6501415). It should be taken also into account the dynamic nature of raft domains, along with the implication of lipid modification on proteins as determinants of raft association – please, see for instance Levental *et al*. (PNAS 2010, and the review in Cold Spring Harbor, 2011; PMID: 21131568 and PMID: 21628426), where quantitation of the portion of plasma membrane proteins partitioned into rafts shows that nonraft outnumber raft proteins by ~ 2:1, in spite that > 50% of integral raft proteins appear to be palmitoylated.**

*What are the levels of expression of ß2 on the MDCK cells compared to their normal physiological abundance?*

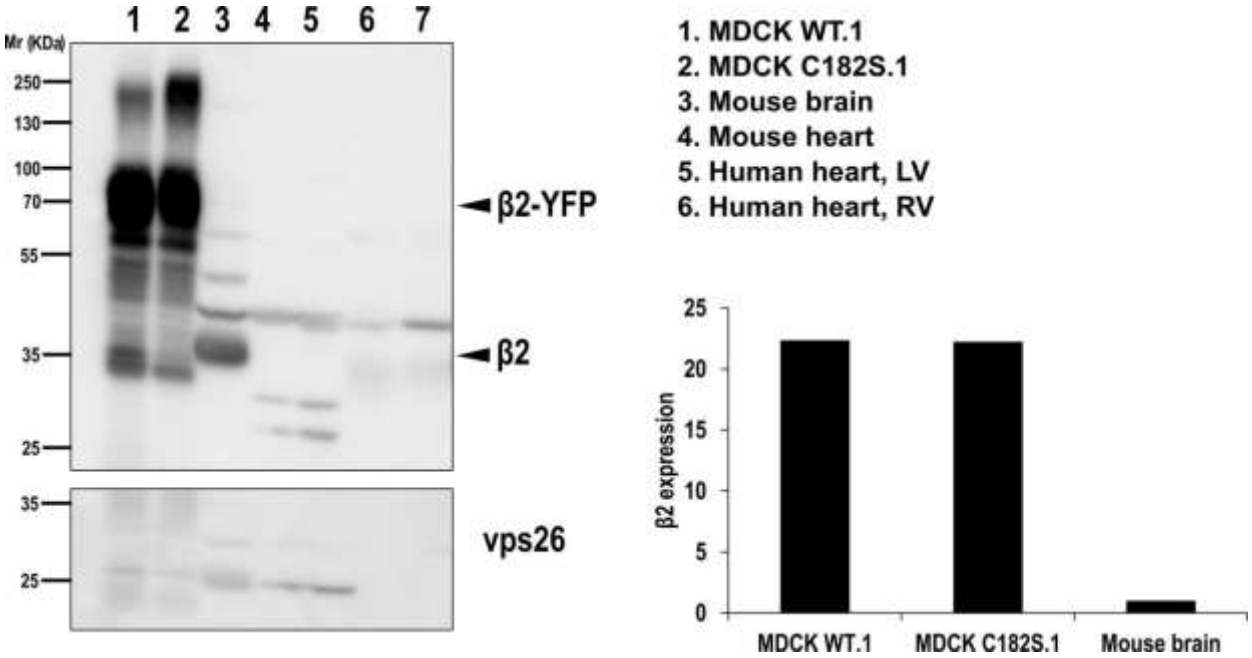
**Attending to the reviewer's request, we have attempted to perform this estimation. To this end, we got hold of tissues with reported endogenous expression of ß2, either from own sources or from colleagues. These included mouse and human heart ventricle, and total brain mouse.**

**The image below shows that stable MDCK cells have a considerable higher abundance of overexpressed ß2, either WT or C182S, compared with mouse brain, where we detected a reliable band; 5 µg protein was loaded of MDCK lysates, and 50 µg from tissue after TCA precipitation. Upon quantitation over a loading control (endosomal Vps26), we estimate that it may be at least in the order of 20-fold more. To note, judging by the aspect of the endogenous ß2 band, it is also likely glycosylated, running at an apparent molecular weight of 40-45 kDa. A doublet at 25/35 kDa in mouse heart may correspond to unglycosylated ß2, and faint shadows can also be perceived in human heart samples.**

**Protein from the four heart samples and whole mouse brain was extracted following the same procedure, that is, by means of the Thermo M-PER Mammalian Protein Extraction Reagent, and using the Qiagen TissueLyser LT, suitable in principle to extract protein from various tissues. Thus, while it seems puzzling that the loading control (Vps26) was not detected in human heart ventricle, protein concentration in all heart fractions was in the similar order, i.e., of 0.6-0.9 µg/µl, just slightly lower than in mouse brain.**

**We also run samples from various mouse brain regions, all of which, except for an extract of olfactory bulb, displayed comparable levels to those in total brain extract (image not included).**

**We reckon that having found much higher expression levels in our stable cells was quite expected. Although to some extent it may have an influence when studying subcellular distribution of the protein, as done here, we think that approaches such as fractionation to isolate DRMs, if performed from tissue, where there is often large cellular heterogeneity, compared to cells growing in culture, may lead to results more difficult to interpret.**




*Perhaps there is just too much β2 being made to fit into DRMs?*

**We also share the reviewer's view, and indeed this is an obvious limitation of our study. Thus, along the lines of the comment above, regarding the likely traffic of β2 through DRM/Lo membrane domains, it is quite possible that these may harbor only a small portion of β2 at any given instant, while all remaining protein has already reached its location at the cell surface, or it is still on its way, in the endoplasmic reticulum or along the exocytic pathway. On the other hand, to achieve sufficient protein abundance, DRM isolation was performed from cells growing partially polarized in plates; 4 x 10-cm dishes with confluent cells were needed per condition. In the *Discussion*, we have now commented on these limitations from our approach.**

*And, of course, lipid rafts are at physiological temperature, DRMs at 4˚C; the phase of lipids is determined by their transition (melting) temperatures, and the subset of Lo domains you have selected may be the tip of the lipid iceberg at 4˚C.*

**Keeping in mind this important issue, we have also added a comment on this in the *Discussion*, once more to emphasize that we may be underestimating the presence of β2 in DRM/Lo membrane domains.**

*The cholesterol depletion experiments are well done, as are the (non)-endocytosis experiments. The FRAP experiments look good and show an interesting result for the role of the cytoplasmic domain of 4˚C (although I have no personal experience here).*

**We fully appreciate the reviewer's judgment that our experiments are well performed.**

**Regarding the FRAP experiments, we must point out that these were done with live cells at 37ºC and 5% CO2, as described in the *Methods* section. On the role of β2 cytoplasmic domain, since we found that the tail-minus mutant displayed complete fluorescence recovery and moved twice as fast as the WT, we propose that the immobile fraction of β2 is anchored to the submembrane cytoskeleton by its cytoplasmic domain.**

*The co-localization of fluorescence of Cholera Toxin and β2 shows the two localize in the same compartments. The fluorescence used could not possibly resolve individual 'rafts'. There are well developed methods using immunoaffinity purification of DRMs to identity whether the raft components are in the same vesicles. Or, of course, since you frequently quote his recent Traffic review, there is single molecule trafficking.*

**We have indeed considered the criticism that the approach and equipment that we used could**

**not resolve individual 'rafts', and seriously pondered complementary strategies to reinforce the idea that a fraction of β2 is within raft domains. First, concerning immunoaffinity isolation to purify DRMs, we reckoned that including another subcellular fractionation technique to our work would have not contributed substantially to make the current data stronger, and perhaps would even have added some confusion.**

**Regarding single-molecule tracking of membrane molecules, to our understanding, it was developed by Kusumi and collaborators about 10 years ago, or earlier, and is based on fluorescence detection and tracking of molecules by means of individual colloidal gold nanoparticles in live cells. Upon debating with scientists with certain experience on related matters, we realized that it would require a setup with high technology, along with special training. Unfortunately, lacking at the moment the necessary resources – including access to super-resolution imaging – expertise, and time, we had to discard this option.**

**As alternative strategies, we also thought on labeling with the ANEP fluorescence dye, di- 4-ANEPPDHQ. Like Laurdan, it also displays a 60 nm spectral blue shift between disordered and ordered bilayer phases. Unlike Laurdan, it does not require multiphoton excitation (equipment that we do not have access to), since it can be excited with the 488 nm argon-ion laser. Thus, as far as we understand, it would emit fluorescence with peak at 560 nm if residing in rafts vs. 620 nm if not in rafts. However, the emission peak of YFP is approx. 525 nm. Thus, we could not come up with a way to pick up the 3 emissions separately to avoid spectra overlap, therefore preventing bleed-through.**

**Regarding more sensitive and photostable Laurdan derivatives, such as C-Laurdan, C- Laurdan2, or SL2, the dyes fluoresce with an emission peak at 450 nm when in the ordered phase and at 500 nm in the disordered phase, so for the latter we would encounter the same problem as above with the YFP emission peak.**

**Finally, we considered the widely used cholesterol marker filipin, for which we would not have issues with wavelength interference, since it naturally fluoresces upon UV excitation at 360 nm and emits at 480 nm. However, our understanding is that it has poor fluorescence properties, toxicity, and can disrupt rafts (please see for instance Orlandi & Fishman, JCB 1998; PMID: 9585410).**

**Therefore, to find support to the observation of β2 presence in raft domains, we proceeded as follows. First, we tested whether colocalization of β2 with CTX still takes place without forcing crosslinking of the CTX-labeled lipid rafts with the anti-CTB antibody. To this end, we labeled fixed cells with CTX and subsequently performed the immunofluorescence. The procedure has been added in the *Methods* section, and we have added these data, which are in Supplementary Fig. S6 (Partial overlap of β2 WT and C182S with CTX-labeled raft domains).**

**As in live cells, CTX labeled strongly the apical surface, although a weaker staining at the lateral region was also detected. Yet, as by CTX labeling of live cells – please, see the immunofluorescence data also added in Fig. 6 – we could not distinguish obvious differences between β2 WT and C182S.**

**Secondly, we attempted to detect a potential interaction of β2 with proteins present in DRMs by coimmunoprecipitation. Although our idea was to try it from isolated DRM fractions, we discarded it due to the inability to compare between β2 WT and C182S, since the latter is nearly undetected in these. Therefore, we decided to immunoprecipitate β2-YFP from whole lysate. However, neither flotillin-1 nor caveolin-1 was detected interacting with β2. In spite of this negative data, we still think that our study provides substantial evidence to sustain that a fraction of β2 associates with cholesterol-rich lipid rafts by means of palmitoylation.**

*Overall, this work is, with comprehensive revision, publishable, but I think you should be more logical in your analysis of the meaning of your results. I would prefer it if your Figure legends described their Figures; at the moment, you squeeze in a distracting amount of experimental detail that should be in the Methods; and I think you would benefit with a more detailed Discussion of your results, which are genuinely interesting.*

**We fully appreciate that the reviewer considers our results interesting. In preparing the revised manuscript, we have looked critically at our data and carried out – as best we could – a logical approach in order to ensure a comprehensive and rigorous analysis of our data.**

**Following on this suggestion, we have removed, insofar as it has been possible, experimental detail from the *Figure* legends, which are now simplified. On the other hand, we have tried to get into more detail about the implications of our data in the *Discussion* section, in particular taking into account new dada added in the revised version.**

*And one very little thing: living cells are called 'live cells' in English*

**We thank the reviewer and sincerely apologize for our distraction, which we have now amended.**

*Reviewer #2*

*Cortada et al have provided a comprehensive study demonstrating palmitoylation of the voltage gated sodium channel β2 subunit (SCN2B) and its role in trafficking to the apical membrane of Madin-Darby Canine Kidney (MDCK) cells. Using a palmitoylation deficient mutant (β2- C182S) and a palmitate analog in biotinylation, immunoprecipitation and sucrose density gradient experiments they demonstrated that beta 2 is palmitoylated at this cysteine residue, thereby enhancing the fraction associated with lipid rafts, specifically cholesterol-rich detergent resistant membranes.*

**We appreciate the reviewer's judgment that we provide a comprehensive study concluding that β2 is palmitoylated.**

*However, expression is still predominantly within the remaining solubilised fractions for both beta2 and beta2-C182S.*

**As the reviewer states, a considerable portion of β2, either WT or C182S, is recovered in the solubilized fractions, therefore not in DRMs and, in consequence, not in Lo domains. In order to get an approximate value, we have quantified the total signal from the bands within the DRM fractions – that is, the upper 2-3 fractions, as indicated in *Methods* and shown in Fig. 2A – obtaining an avg. portion of 12% over the total signal coming from β2 in all fractions collected. In comparison, the portion of the mutant in DRM fractions actually came down to ~ 4%.**

**We have added this estimation in the text, with the important remark that it may indeed be overestimated due to the comparatively much higher intensity that the β2 band displays in the detergent-soluble fractions found toward the bottom of the gradient. Nevertheless, virtually in all experiments in which we have performed band quantitation we have detected the chemiluminescence reaction by a ChemiDoc MP unit, instead of using X-ray film. Since the former uses a CCD image sensor for light detection, it has a much greater linear range than film does. Therefore, we reckoned that the digital data generated would be relatively quantitative. We have indicated this at the subsection describing western blotting.**

*Application of cholesterol depleting agents (MBCD and lovastatin) resulted in a redistribution of beta 2 to the basolateral membrane, further corroborating the role of beta 2 association with lipid rafts and appropriate membrane targeting. Similarly, loss of cholesterol and the palmitoylation deficient mutant are both associated with a decreased mobility of beta2 within the range associated with diffusion inside lipid rafts, therefore suggesting a reduction in association with cholesterol rich lipid rafts. Confirmation of beta 2 expression within lipid raft domains was established by co-localisation of the beta2 signal with the lipid raft specific CTX. These findings identify the importance of beta 2 palmitoylation for its appropriate localisation within the apical membrane of MDCK cells, from which it is inferred that this would also likely influence the NaV alpha binding partner(s), to specific plasma membrane localities in human tissues such as cardiac myocytes.*

*However, this study lacks any direct demonstration of functional consequences on beta 2 and NaV function, although alluded to in the discussion.*

**In this revised version, we have seriously considered this possibility. Our previous data support**

**the model that associated ß2 subunits fulfill their role within the Naγ channel mainly by promoting cell surface expression of the α subunit.**

**In spite of our expectations, we have found this role to be relatively "easy" for ß2 to perform, as long as the subunit itself can reach effectively the cell surface, which would be the apical domain in our cellular model. A remarkable example of this would be that a single glycosylation site in ß2 is sufficient to allow its trafficking to the apical surface, and also to promote surface localization of Naγ1.5 (please see Cortada et al., JBC 2019; PMID: 31614896), although it has not always been the case, as in the mutant associated with Brugada syndrome, D211G (please see Dulsat et al., Biol. Cell 2017; PMID: 28597987).**

**As discussed however in a recent review (please see Salvage et al., Biomolecules 2020; PMID: 32630316), it may be more complex than that. First of all, gating effects of ß2 on Naγ1.5 were demonstrated several years ago and found due to ß2 sialylation, potentially by influence of its negative charged sialic acids on *N*-linked glycans of the α subunit (Johnson & Bennett, JBC 2006; PMID: 16847056). On the other hand, there is increasing evidence that ß subunits – perhaps including ß2 – participate in trans-heterophilic interactions to promote cellular adhesion in the adjacent perinexal membranes, which may influence local clustering, and thereby functioning, of Naγ1.5.**

**Our preliminary data (not included in the first submission) pointed to a mild contribution, if at all, of ß2 palmitoylation to Naγ1.5 trafficking and localization. Indeed, we have now corroborated our suspicions, and found non-palmitoylated ß2 to be similarly effective as ß2 WT in promoting surface localization of Naγ1.5. We have added a new figure showing these data (new Fig. 7), both by microscopy and by cell surface biotinylation. Although Naγ1.5 distributes more widespread along the cells' *z*-axis in the presence of ß2 C182S, with a tendency toward the nuclear level, differences with the WT were found nonsignificant. Biotinylation indeed confirmed lack of an obvious defect in the presence of ß2 C182S. Interestingly, this result agrees with what has been recently reported for the palmitoylation-deficient ß1 subunit, found as effective as the WT in modulating sodium current density (please see Bouza et al., JBC 2020; PMID: 32503841). Of course, additional elements, perhaps only present in cardiomyocytes and/or other excitable cells, may participate in interactions between ß2 and Nav1.5, thereby revealing potential effects, as well as the consequent defects in mutants of ß subunits.**

**Therefore, palmitoylation of ß2 does not appear required to promote surface localization of Naγ1.5; nevertheless, it may still have important implications in regulating ß2 association with lipid raft domains and in its polarized localization to the cell surface, aspects that we have emphasized throughout the text.**

*That said, it does provide a thorough assessment of the mechanisms of the relatively unknown process of palmitoylation and how it regulates the cellular distribution of beta 2 which is widely applicable and informative for membrane targeting of both related and unrelated transmembrane proteins.*

**We appreciate the reviewer's judgment that we have performed a detailed analysis in our study.**

*The manuscript is generally well written and presented to a high standard. The conclusions on the whole are well justified, however I have some suggestions/comments to enhance the completeness of the findings.*

*Major*

*1. Some key experiments were not performed with the palmitoylation deficient mutant. Most notably, the experiments that clearly demonstrate beta 2 expression within lipid raft domains by virtue of their association with CTX. Performing these with beta 2-C182S would help clarify if the fraction of beta 2-C182S that reaches the apical membrane does so via lipid rafts or another potential mechanism.*

**We have now included these experiments with the palmitoylation deficient mutant. As requested, we have analyzed colocalization by confocal immunofluorescence microscopy of ß2 C182S with CTX-labeled raft domains. The overlap that we found was also considerable, comparable with that found in the WT, either by using an anti-CTB antibody to force crosslinking of the CTX-labeled lipid rafts in live cells, or by CTX labeling of previously fixed cells. Respectively, these data are shown in Fig. 6 and in the new Supplementary Fig. S6.**

**Probably, at least a couple of considerations must be made about these data. First of all, the endogenous expression of GM1 in MDCK cells, to which CTX specifically binds, is said to be very low (please see for instance Fig. 1 in Crespo et al., FEBS J. 2008; PMID: 19021775), yet the ganglioside localizes apically upon transfection of the right galactosyltransferase (Fig. 2 of the same article). In fact, we have observed that CTX binds to the apical surface very unevenly, with some strongly positive regions of the monolayer amidst large negative areas. A second important limitation is that the conventional confocal microscope that we used cannot resolve discrete raft domains, and therefore the images that we present are the best possible approximation that we could achieve with our instrument. We have pointed out these limitations in the *Discussion*.**

**Therefore, we cannot exclude that lipid raft association contribute to the localization of ß2 C182S to the apical domain. We have also addressed this in the *Discussion*, linked to the fact of having found such a relatively small fraction of ß2 WT in DRM fractions. Thus, we hypothesize that ß2 traffic through DRM/Lo/raft membrane domains, while possibly important for its apical deliver, may be short-lived and proceed rapidly, so that only a small portion of total ß2 can be detected there at any given time. Moreover, it is possible that these domains can accommodate only a small portion of total ß2, which our data suggest not to be the C182S mutant.**

*Similarly, if palmitoylation is relatively important to the apical targeting of beta2 it would be good to see the subcellular distribution of beta2-C182S in confocal microscopy and the effect of MBCD and lovastatin, albeit the authors do demonstrate a mixed apical and basolateral distribution in supplementary western blots, but there is no evidence of the effect of cholesterol depletion.*

**We have now performed these experiments with the palmitoylation deficient mutant. Thus, we have analyzed subcellular distribution of ß2 C182S by confocal immunofluorescence microscopy upon treatment with MßCD plus lovastatin. These data are shown in the Suppl. material. Specifically, Suppl. Fig. S4 shows that ß2 C182S is mislocalized to the basolateral surface due to cholesterol depletion using MßCD/lovastatin. Besides this evidence by microscopy, we also confirmed that it behaves similarly as the WT by cell surface biotinylation of MßCD-treated cells (Suppl. Fig. S3). Moreover, blocking intracellular cholesterol transport with U18666A, along with lovastatin, also led to basolateral surface mislocalization of the C182S mutant to a similar extent as the WT (Suppl. Fig. S2).**

**These data do not exclude that palmitoylation can be important for ß2 apical localization. In this regard, as we had originally mentioned at the end of the *Discussion*, ß2 C182S properly localizes to the apical domain in a manner indistinguishable to the WT. Thus, palmitoylation by itself does not determine apical localization of ß2, although it may contribute to its partition into raft subdomains at the apical membrane. Along these lines, we hypothesize that ß2 localization within membrane subdomains may also be affected by the nature and length of its transmembrane domain, aspects that we reckoned are beyond the scope of the present study.**

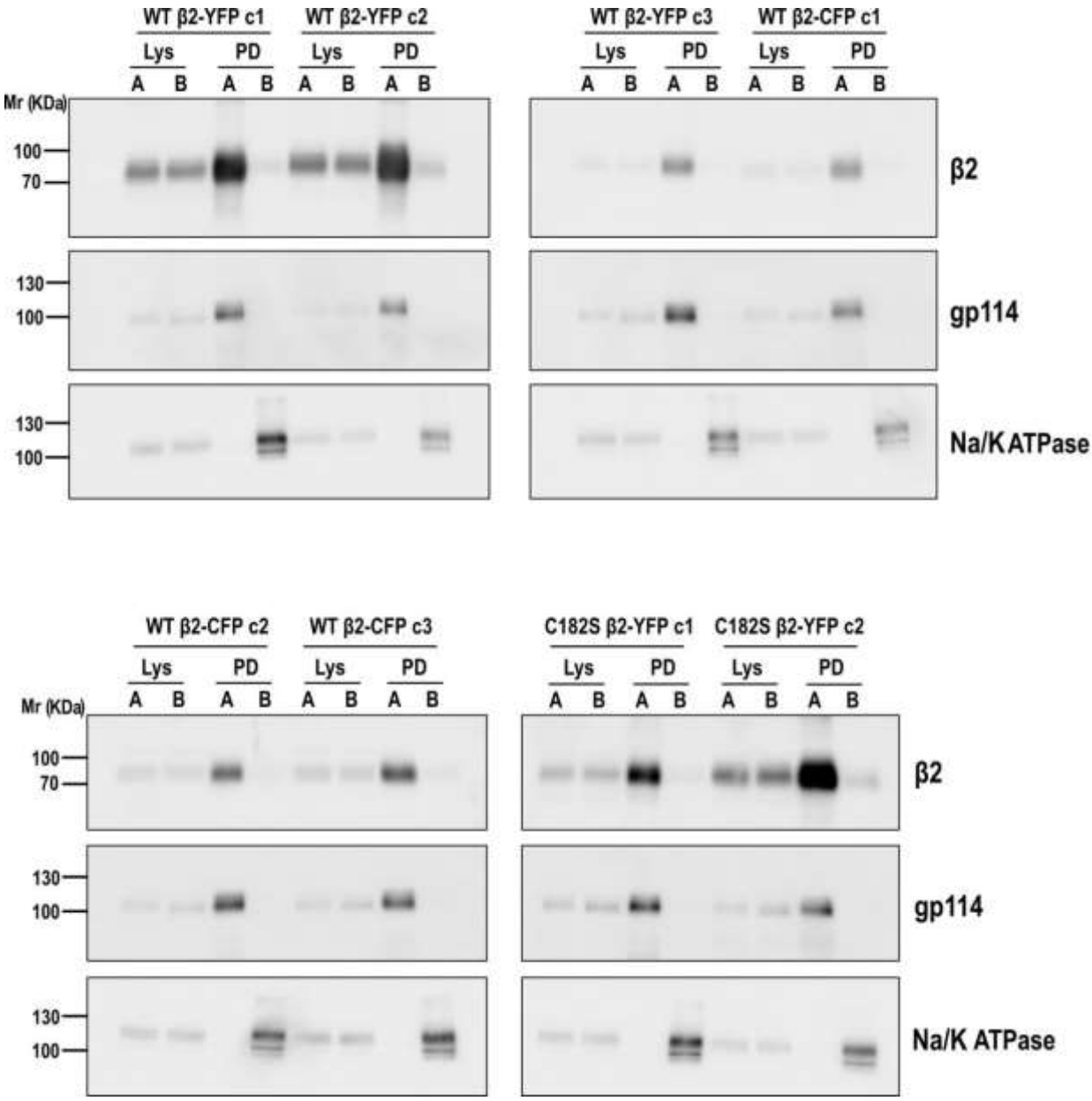
*2. Did the authors notice any differences in expression level of the beta 2- C182S that might influence the results (particularly normalization) given the apparent difference in band density of beta 2 in fig 1D and supp fig 3 (unclear if this is also true for fig 1b due to saturation of bands) where the beta 2-C182S band is particularly weak in the lysate fraction compared with the WT beta 2.*

**We have noticed some differences in expression levels, which we have attributed to clonal variation, since they may appear within clones in general, including the WT and C182S clones used in this work and, as mentioned, when comparing a WT with a mutant clone. This is indeed apparent in the previous Suppl. Fig. S3 (ß2-YFP C182S localizes to the apical surface.). However, it is not so obvious in Fig. 1B, although the ß2 bands do look saturated here.**

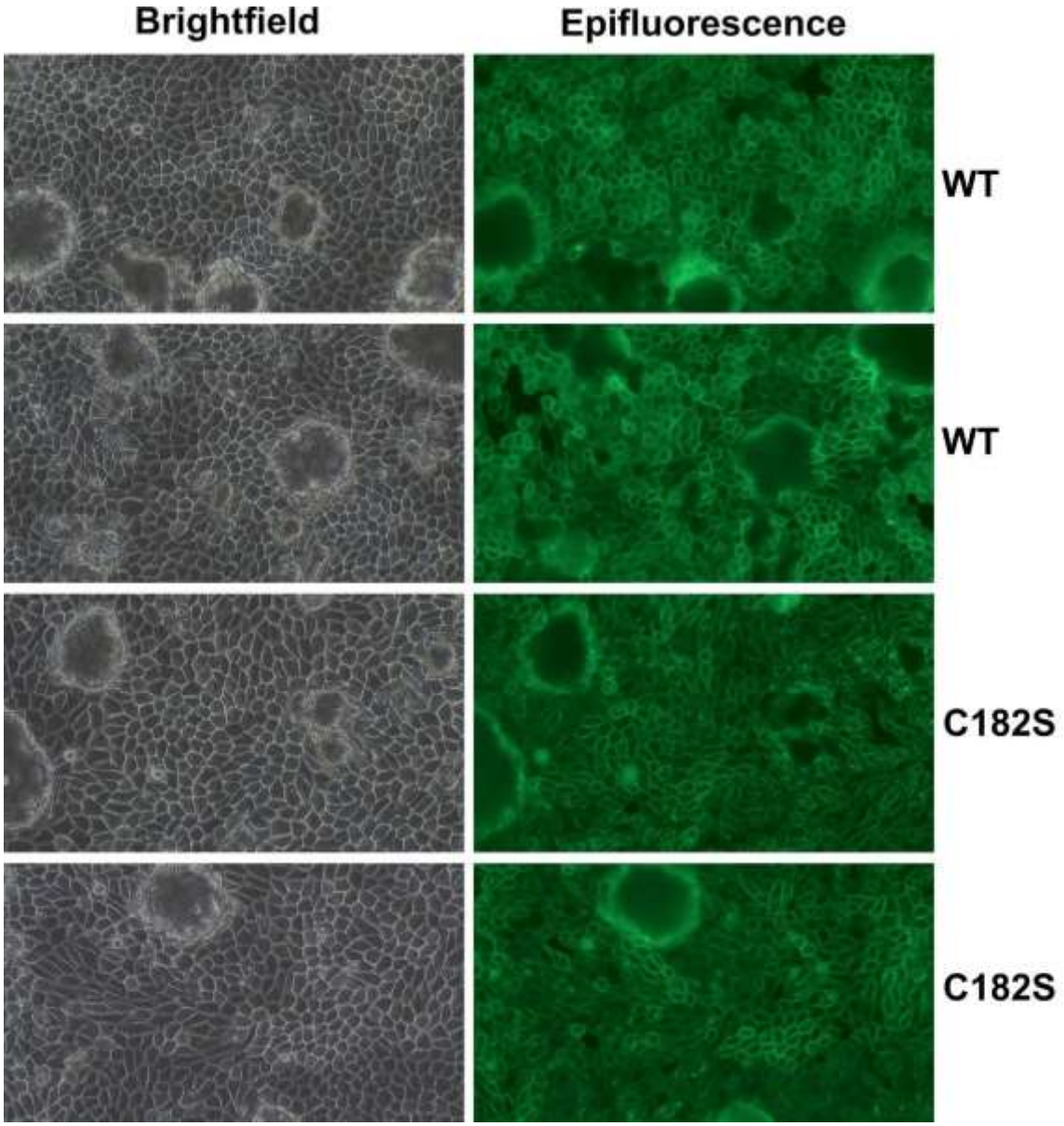
**This is probably a classical problem – if we may consider it such – seen in studies using stable MDCK cells. As an example taken from the literature (please see Fig. 2B of Capell et al., JBC 2002; PMID: 11741885), cell surface targeting of ß-secretase (BACE) was also analyzed by biotinylation. The authors showed three individual MDCK cell clones that, while expressing different levels of BACE, the protein in all mainly goes apical. Yet, it is obvious from that image that clones with high expression levels – even in stable cells – may lead to saturation of the polarized sorting machinery. Therefore, apical delivery of ß2 C182S in clones with comparatively low expression levels may even look "cleaner" (well- defined) than what ß2 WT displays.**

**Nevertheless, we have conducted a number of tests to address this aspect.**

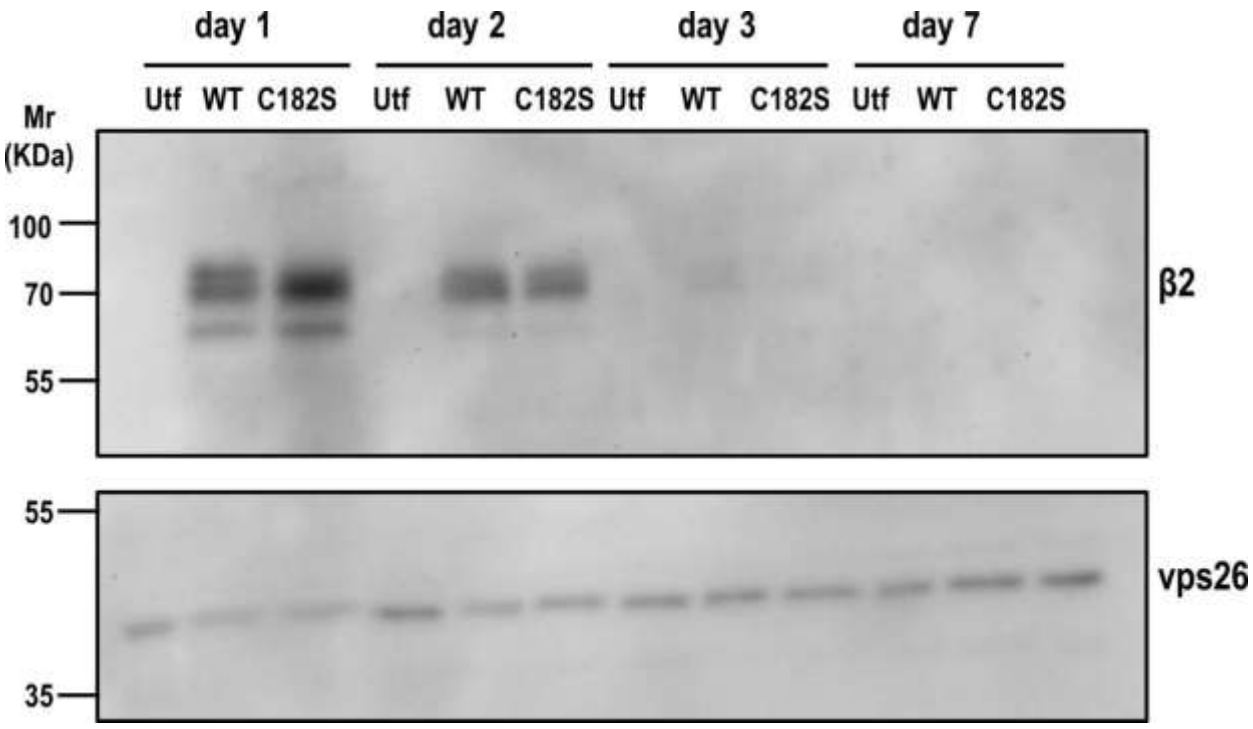
**First, we verified proper apical distribution in various WT and mutant clones (in the former, either YFP- or CFP-tagged). From the following image, we can conclude that the exclusive apical localization of both WT and mutant ß2 is remarkably comparable among different clones (c; Lys, refers to lysate; PD, to pulldown; A, apical; and B, basolateral).**



**Secondly, we took pictures of 10-cm confluent plates of the clones used in the experiments. The following images confirm, by epifluorescence, that the intensity derived from β2-YFP is comparable between the WT and the mutant (2 images of each, with their corresponding bright-field picture). Note also that the protein traces the cell membrane in these confluent monolayers and only may be perceived its preferred apical localization in cells around domes.**




**Finally, also upon transient transfection, the expression throughout time did not seem to lead to variations between the WT and the mutant that could be reason of concern (Utf, represents untransfected cells; and Vps26, a major endosomal protein, was chosen as a loading control).**




*3. In figure 2, I agree with the authors rationale and conclusions on the general distribution of beta 2 through the different fractions in the Western blot. However, I am confused by how these results tally with what is shown graphically in panels B and C. WT beta2 for example shows a higher density in the first fraction relative to the second and third, yet in panel B the peak for the DRM portion is shown in fraction 3. How do these two data sets relate to each other? Presumably there has been some normalization that explains the mismatch?*

**As suspected by the reviewer, the graphs in panels B and C show data expressed as "normalized abundance", which we have now explained in the *Methods*, and it is also indicated in the legend. Thus, for each protein, we quantified the intensity of blotted protein bands from each fraction and plotted relative band intensity, i.e., over 1, giving the value of "1" to that obtained in the fraction with the maximum band intensity. The relative portion of ß2 within DRMs was then estimated by determining the ratio between the bands' signal within the DRM fractions over the total signal coming from ß2 in all fractions collected. Therefore, apparent differences in levels seen between the representative blots and the curves are due to the fact that the plot shows the avg. ± SD from 3 experiments analyzed.**

*4. In figure 5 it is not clear to me that similar subcellular/membrane areas have been exposed to bleaching. For example, in panel D the WT beta2 expressing cells have clearly been targeted at a cell-cell junction, it is not apparent that this is a similar position in the beta2-181X expressing cells. Unless this is a reflection of a differential distribution of beta2 here?*

**To clarify how we proceeded for bleaching in Fig. 5D, we have added Suppl. Fig. S5, which shows that the ß2 mutant lacking the intracellular domain (181X), while it can localize to the apical surface, it mostly remains in the endoplasmic reticulum. We show this by Endo H deglycosylation of immature, but not complex, *N*-glycans on ß2 present in the ER, which is the faster-migrating band of the WT and the single band of the 181X mutant (we have added an explanation of the procedure used in *Methods*). Therefore, intracellular ß2 181X mostly accumulates in the ER (as seen also in panel D of Fig. 5). Yet, a small portion reaches the cell surface – please look at the comparatively higher enrichment of the WT at the apical domain over lysate than what we found in the mutant in panel A of Suppl. Fig. S5 – and this is the location that we attempted to target in the FRAP experiments in order to emulate the location of ß2 WT. Please, bear in mind however that the analysis was not done in polarized but in subconfluent cells, as indicated in the legend and in *Methods*. We have also added an explanatory note in the legend of Fig. 5.**

*Similarly, in panel B, cell-cell junctions have been targeted but not in panels A and C. Could this be*

*clarified as presumably the argument is that cholesterol rich apical membranes are being targeted?*

**Here, we must point out that the experiment treating with MβCD (panel B) was done in cells only 1 day after plating, as indicated, since we tried to ensure the effect of the treatment on β2 mobility. These cells were very flat and their lateral membrane, i.e., between adjacent cells, was the only available region to target. In contrast, in panel C, where we compared β2 WT with the C182S mutant, as well as in the control performed to simply check for reduction of MF by increasing the bleached radius (A), cells had been grown for 2 days and it appeared possible to bleach the protein which we assumed that, at least in part, will be found in clusters already at the surface; if you wish, please see a more detailed explanation on these aspects in our previous work (Cortada et al., JBC 2019; PMID: 31511323).**

*5. Given the importance of appropriate Nav1.5 surface expression to channel function and the role of beta 2 in trafficking, as commented on by the authors and their previous publications in this area, it is a shame that the effect of beta 2 palmitoylation on Nav1.5 distribution has not been assessed.*

**Since this important point was raised earlier in the reviewer's report to the authors, we refer this query to the beginning of our response to the reviewer, specifically, in the point "However, this study lacks any direct demonstration of functional consequences on beta 2 and NaV function".**

*Minor:*

*1. In the first paragraph of Results, it is stated "HAM exposes thiol groups that can then be biotinylated". Is it not more accurate that HAM selectively reduces thiols attached to palmitate, hence the use of this reagent to measure palmitoylation. There are other thiol groups in the beta2 Ig domain, but these would be blocked by the NEM*

**We have corrected this sentence.**

*2. Fig 1D arrow indicators are included to denote the beta 2 band at 0, 100 and 200 uM 2BP treatment, this has been omitted from 50 uM.*

**We apologize for the omission, and have replaced the figure, indicating in the legend that, in panel D, red arrowheads actually point to the position of biotinylated β2.**

*3. Live cells has been written as life cells in a few places including in the abstract, first line of p5 and p7 and again in the penultimate paragraph of p7 and the legend for fig 6.*

**We thank the reviewer for bringing this up, and sincerely apologize. We have corrected this mistake in all instances.**

*4. In some western blots a skewed atypical marker has been used, presumably due to the narrow gap between blots. Perhaps some blots could be enlarged to accommodate for the markers (e.g. Fig 4a, the gp114 is particularly narrow, extending beyond 100 kDa marker would leave room for a more typical labelling of 55 kDa in the Vps26 blot)*

**We have substituted all lanes from Fig. 4 with enlarged ones, so labels for the molecular weight markers are now more clearly displayed.**

*5. On p5 there is a sentence ".... overnight incubation with a discrete amount of lovastatin". Please clarify.*

**As requested, we have now specified the concentration of lovastatin in the text.**

*6. Fig 4 is not labelled for the IP (as has been done in previous figures). In the legend the sentence "... remaining protected the endocytosed biotinylated proteins" does not quite make sense.*

**We apologize for the confusion; however, Fig. 4 does not show an immunoprecipitation experiment. Instead, it shows an experiment of cell surface biotinylation, at 4ºC, with subsequent endocytosis, at 37ºC, and finally stripping with reduced glutathione, which cleaves (also at 4ºC) the disulfide bond within the biotin reagent only of those labeled proteins remaining at the plasma membrane, leaving all those previously labeled, and already endocytosed, protected from the extracellular reducing agent.**

**Upon request by Reviewer 1, we have simplified the *Figure* legends by removing experimental detail – including the sentence mentioned – which we believe remains now sufficiently explanatory in the *Methods*. We also refer there to our previous work (see for instance Fig. 9 of Cuartero et al., Traffic 2012; PMID: 22758778), in which the procedure used is explained in somehow greater detail.**

---

Second decision letter

MS ID#: JOCES/2020/252189

MS TITLE: The voltage-gated sodium channel β2 subunit associates with lipid rafts by S-palmitoylation

AUTHORS: Eric Cortada Almar, Robert Serradesanferm, Ramon Brugada, and Marcel Verges
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.

Reviewer 1

*Advance summary and potential significance to field*

This paper builds upon extensive work by this group and others, seeking to identify how the cardiac/neuronal voltage-gated Na+ channel is assembled into functional, topographically correct units, a quest that is informed by the analysis of human channelopathies. The core channel is provided by a large, multi-pass transmembrane protein, Nav1.5, to which 4 smaller beta subunits bind. Previous work from this lab has shown the bets2 subunit is required to transport Nav1.5 to the apical and not basolateral surfaces of polarized MDCK cells.

This paper examines whether palmitoylation of beta 2 at Cys182 plays a role in directing beta2/Nav1.5 complexes to the apical surface of MDCK cells, and in particular drives the Nav1.5/beta2 complex into 'lipid rafts' on the cell surface. Using an appropriate combination of biochemical and light microscopic techniques, they find beta2 is palmitoylated on Cys182, and this results in ~12% of beta2 being recovered in the Detergent Resistant Membrane fraction. However, trafficking of Nav1.5 to the apical surface chaperoned by beta2 is not dependent upon whether the beta2 is palmitoylated or not.
This is a negative result, but an important one, reflecting the complexity of the mechanisms controlling the trafficking and function of voltage-gated ion channels.

*Comments for the author*

The authors have fully addressed, both experimentally and in analysis of their data, my comments on their first submission.
This much revised version includes significant additional experiments/data, including extensive Supplementary Figures. The wide ranging controls are impressive, and truly test the hypotheses. And there is a more critical approach to the text.

The authors, in common with much of the literature, treat DRMs as a single entity. Where people have separated DRMs, usually by immunopurification, find they differ substantially in their lipid and protein composition, presumably dictated by the dominant protein(s) selecting specific mixes of lipids. Were you to purify the DRM enclosing beta2/Nav1.5, you may find it enables functions you have not yet tested.

Reviewer 2

*Advance summary and potential significance to field*

In this resubmission, Cortada et al. have convincingly demonstrated that β2 is likely palmitoylated, a property which tends to enhance its association with lipid rafts and thereby influence its function within the cell. The authors have adequately addressed my previous comments, further clarifying some misunderstandings. In particular some key extra experiments have now been performed, including those pertaining to the impact (or lack thereof) of β2-palmitoylation induced membrane distribution on corresponding distributions of Nav1.5. I am inclined to agree with the authors' comments suggesting that if palmitoylation of β2 does influence Nav1.5 function, then it is likely to be more subtle and complex than chaperoning to the cell surface.

*Comments for the author*

I only have very minor comments, as follows:

In the discussion, second sentence: change ‘increases’ to increase

On p21, penultimate paragraph, 3rd sentence. ‘According to our results, one could thus assume that, as long as the associated β subunit reaches efficiently the PM which would be the apical domain in MDCK cells, there would be no detectable defect on bringing NaV1.5 along’
The words ‘reaches’ and ‘efficiently’ should be swapped around ‘…..the associated β subunit efficiently reaches….’