Title: **Transport of the alpha subunit of ~~the~~ L-type calcium channel through the sarcoplasmic reticulum occurs prior to localization to triads and requires the beta subunit but not Stac3 in skeletal muscles**

Authors: Jeremy W. Linsley[1,2] , I-Uen Hsu[2] , Wenjai Wang[2] , John Y. Kuwada[1,2,*]

Article Type: Original Research

Monitoring Editor	Michael Marks
Date Submitted	6 February 2017
Date for Decision 1	27 March 2017
Date Resubmitted	12 June 2017
Date for Decision 2	26 June 2017
Accepted	6 July 2017

Decision and Reviews

Dear Dr. Kuwada,

Thank you for submitting your manuscript “Skeletal muscle DHPR is trafficked via sarcoplasmic membrane to triadic junctions” for consideration for publication in Traffic. I asked three colleagues who are experts in the field to review the paper and their verbatim comments are appended below. Although they differ in overall enthusiasm, the referees share the view that the work presented in this paper would, in principle, be of interest to the readers of Traffic. However, all three referees feel that your conclusions are not fully supported by the data presented. In particular, all referees have a number of questions regarding the methodology used in the FRAP experiments. The referees have very clearly outlined their suggestions for additional experiments and/or clarifications of the text that I agree will strengthen the paper and clarify the conclusions that can or cannot be drawn.

Although I cannot accept your manuscript for publication at this point, I suspect that you will be able to address the referees' concerns and I look forward to receiving your revised manuscript. To expedite handling when you resubmit please be sure to include a response detailing how you have addressed each of the referees’ concerns.

Sincerely,

Michael S. Marks, Ph.D.
Co-Editor

Referee's Comments to the Authors

Referee: 1

Comments to the Author
Excitation-contraction coupling is essential to skeletal muscle function and depends on the exquisite spatial organization of two calcium sensors and channels in two different membrane systems: the dihydropyridine receptor (DHPR) in the T-tubules which are invaginations of the plasmalemma, and the Ryanodine Receptor which is in the Sarcoplasmic Reticulum (SR), a system of endomembranes that serves both as ER and as calcium storage compartment. Molecular and spatial defects in the organization of these membranes have been linked to various myopathies. The current manuscript is based on Zebrafish work, which the senior author, Dr. Kuwada, has a long experience with. Here, Dr. Linsley et al propose a mechanism for trafficking of the DHPR to the T-tubule membrane. They use mostly immunofluorescence microscopy and FRAP recordings and make good use of two mutant Zebrafish lines, one lacking Stac3, a new subunit of DHPR, the other one lacking the beta subunit of DHPR. The techniques were used in a recent elegant paper from the same lab (Linsley et al, 2016, PNAS).

Technical remarks/questions:

1. FRAP experiments:
1a. The FRAP recordings went for up to an hour. It is generally recommended to have protein synthesis inhibitors present for long sequences; FRAP should reflect diffusion of unbleached protein, not synthesis of new one. Did the authors inhibit protein synthesis? Were the FRAP recordings done at 37°C? Did they correct the results for photobleaching during the recovery period, by simultaneously quantitating fluorescence of a non-bleached area?
1b. Furthermore, the movie provided in the supplemental material shows that the plane of focus varied during a FRAP sequence; and there is a large green blob (aggregate of overexpressed EGFP-DHPRa?) that disappears during the sequence. Did the authors attempt to mostly use fibers with modest overexpression?
2. Immunofluorescence
2a. Zebrafish microtubules in myotubes and muscle fibers look very much like those in their mammalian counterparts but tubulin staining in Flg 4A does not look like microtubules. The Methods section does not mention an anti-alpha-tubulin but mentions an anti-acetylated alpha-tubulin. The authors need to clarify this.
2b. In Fig. 4D, were the 2 panels imaged with the same parameters? Why would nocodazole cause the total disappearance of the 2-beta-DG?
2c. There is no information in the Methods about what was done to the images (Photoshop or other software). Some panels are a lot noisier than others (for example Fig5A, WT sibling, compared to 5B, WT sibling). Furthermore, many images show saturation which prevents their use for quantification. In contrast, Fig 5G is excellent.
2d. I am surprised to see anti-GM130 staining as dense and continuous lines. Again, saturation may reduce the resolution but I would expect at least some punctate staining. Have the authors verified with expression of Gal-T for example that the antibody does indeed label Golgi? In mouse muscle, Golgi elements are found near the Z line (see Kaisto & Metsikko, 2003), not at T-tubules.
3. Typos etc.
page4, first column, last line: change DHPRa to DHPRb.

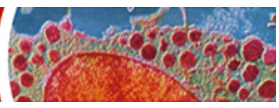
4. Evidence for the proposed model; suggestions for improvement
4a. The model of ERES and Golgi-mediated traffic of DHPR from the SR to the T-tubule is interesting but it will take more work to gather evidence for it. The claim in the introduction "we resolved the trafficking route of the DHPRa..." is too optimistic. The authors have established here that DHPRa is associated with the longitudinal SR. Given the long time-course of FRAP it is possible that the apparent recovery results from new synthesis (see point 1), something that would be interesting to establish. But they do not provide evidence that DHPR transits through ERES or Golgi on its way to the T-tubules; the images of anti-DHPR and anti-sec23 would need much better resolution to show colocalization, let alone co-trafficking.
4b. It would be helpful to be presented in the introduction with a summary of the relevant results in the authors' recent PNAS paper, instead of keeping this for the discussion.
4c. The authors' enthusiasm pushes them a bit far in their claims. In the discussion, the authors claim that "mRNA encoding DHPRa has also been found throughout the SR..." (ref34, Nissinen et al, 2005). The Nissinen et al paper locates DHPRa mRNA to the mouse muscle fiber subsarcolemmal space possibly linked to protein synthesis on ER/SR-linked ribosomes. Neither ribosomes nor mRNA were suggested to be in the SR.

Referee: 2
Comments to the Author
Comments and suggestions as follows:

1. Muscle fiber type: Is there a difference between fast/slow type fiber in zebrafish with regards to the triads function/all proteins investigated in this study? All experiments done on fast or slow type fiber? Some background/introduction to different muscle EC physiology in zebrafish in comparison to mammalian system would be helpful.

2. General information on the mutants (any structural defects? Severe muscle phenotype?) included is lacking. In M&M, more info about the DNA constructs (encoding fused protein? Under which promoter? expression time? etc.) will help to understand the basis of the live imaging results.

3. Did the authors do FRAP of EGFP-DHPRa on a stac3-/- background? To add to Figure 2 results show stac3 does not affect the trafficking of DHPR?



4. Immunolabeling nicely showed SR/ER export machinery and Golgi outposts localize adjacent triads, however, structural proximity does not necessarily relate to function. Is there any method available to monitor the dynamics of SR/ER and Golgi using live imaging? Alternatively, pharmacological blocking of SR-Golgi trafficking would be a direct way to monitor this in a FRAP setting. The strong conclusion on “DHPRa trafficking via ER/SR” in discussion needs more experimental support.

5. Nacodazole treatment- whole animal drug treatment needs further comment. Is the effect highly specific? a well-characterised positive control is needed to show the addition of nacodazole in this context (what is the treatment duration, dose) sufficiently blocked microtubule mediated trafficking without off-target effects. A larger subject number of Nacodazole group should be considered for Stats in Figure 4 Panel C.

6. Is DHPR trafficking a calcium dependent process?

7. How transferrable is the data obtained from an overexpression model in the endogenous context?

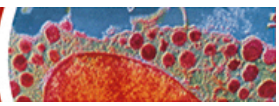
8. Live imaging is a powerful tool to investigate cellular events in real-time, however, the resolution is limited to visualise subcellular dynamics. Did the authors consider adding a few TEM or STED experiments to confirm the results with higher resolution?

Referee: 3

Comments to the Author
This interesting study examines mechanisms responsible for trafficking of DHPRalpha to triadic junctions in skeletal muscle. The experiments are carried out in zebrafish skeletal muscle, providing a well-differentiated model system to address this question. Ultimately, the authors provide evidence that DHPR diffuses along longitudinal sarcoplasmic reticulum (SR)/ER membranes prior to its transport to triad T-tubules sites. This latter mechanism may involve local ER exit sites and Golgi outposts. They also define the role of several proteins in this pathway. Taken together, this is a useful contribution to our understanding of the biogenesis of sites of excitation-contraction coupling in muscle.

In general, I found this manuscript to be well written and the experiments (mostly) convincing and well controlled. For example, the authors provide clear evidence that there are differences in the distribution of DHPRalpha among zebrafish mutants, and also show that not all proteins behave in the same way as DHPRalpha. I do however have some questions about how some of the FRAP analysis was done, as well as the interpretation of some of the results. Some additional controls are also needed.

1. A control experiment should be carried out to show that no FRAP recovery of DHPRalpha occurs in fixed fish. This will help rule out the possibility that photoswitching of the fluorescent protein is occurring (although this seems unlikely given the results shown).
2. It is also important to include a control experiment using cycloheximide (assuming this is possible in zebrafish) to test if the recoveries occur via new protein synthesis as opposed to diffusion.
3. Diffusion coefficients are reported in several figures. However, it is unclear how these values were extracted from the FRAP curves, as no details about the analysis are included at in the Materials and Methods. This should be explained. The authors should also indicate how they benchmarked their FRAP analysis to confirm that the D values it produces are accurate.
4. The authors should also clarify whether the fluorescence recoveries were quantified across the entire bleached region for all of the experiments. This is an important point because in some cases the proteins of interest are primarily found at triads, other times at longitudinal SR, and yet other times both. It would be useful to compare the apples with apples in each case. For example, for an experiment such as the one shown in Figure 6, it would seem appropriate to draw comparisons between the SR-localized pools of protein and consider the triad-associated population separately since it is essentially immobile. This difference could potentially impact the interpretation of these experiments.
5. On a related note, it would be useful if the authors made the point early on in the paper that the "mobile fraction" of DHPRalpha in the triads is very low over the timescale of their experiments. This is implied based on their comment that recovery occurs first in the SR, but from the images shown it is difficult to detect any fluorescence recovery at all in triads.
6. Several places in the text refer to “mobility” of DHPRalpha. In each instance the authors should clarify if they are referring to differences in the kinetics of recovery/D or differences in mobile fraction.



7. The authors should also explicitly state throughout the manuscript that they are proposing that DHPR is undergoing lateral diffusion within the plane of the SR membrane (assuming I am understanding this correctly). Although this is implied, currently the recoveries are typically referred to as “trafficking,” a term which could be easily misinterpreted.
8. The model shown in Figure 7 is confusing as presented. In some panels, it looks like some DHPR is not membrane associated. The orientation of DHPR with respect to the membrane also changes in different parts of the figure.

Other comments
a. I did not follow what was supposed to be different between panels 3B and C.
b. Several panels of Figure 5 are referred to as being in Figure 4 in the text.
c. The units of time post bleach are indicated in some but not all of the figures.

Author Rebuttal

Authors’ Response to Reviewers’ Comments

Referee 1:

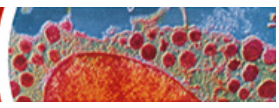
1. FRAP experiments: 1a The FRAP recordings went for up to an hour. It is generally recommended to have protein synthesis inhibitors present for long sequences; FRAP should reflect diffusion of unbleached protein, not synthesis of new one. Did the authors inhibit protein synthesis? Were the FRAP recordings done at 37°C? Did they correct the results for photobleaching during the recovery period, by simultaneously quantitating fluorescence of a non-bleached area?

Thank you for these useful comments. Note all the FRAP recordings in the manuscript were conducted at room temperature (22O-25OC), as zebrafish cannot tolerate 37O. We have made a note of the FRAP temperature in Materials and Methods (lines 546-547).
As pointed out, some of our FRAP recordings were conducted for up to an hour, and we did not inhibit protein synthesis in our original FRAP experiments. However, in Figures 2a and 4b, DHPR-GFP can be seen in longitudinal SR within 10 minutes, and the mean τ1/2 of DHPR-GFP in wild type skeletal muscle is 9.3 minutes (Fig 6b), so time lapse imaging up to an hour is not necessary to see FRAP. Furthermore, the fact that estimates of maturation time for EGFP at 37O C is 25 min (Sarkisyan et al., 2015, Iizuka et al., 2011) and likely even slower at room temperature argues against the notion that the early increase in longitudinal SR fluorescence we observed was due to new EGFP translation. However, as a further control to exclude the possibility of new translation being responsible for fluorescence recovery of EGFP-DHPR in longitudinal SR, we preformed FRAP experiments on live zebrafish incubated in cyclohexamide as suggested. We added the data from these experiments into a new figure (Supplemental Figure 2) and a new table (Supplemental Table 1). First, we used hsp70:egfp transgenic zebrafish to show that 14µM of cyclohexamide inhibited heat induction of GFP thus presumably translation (Supplemental Figure 2a,b). We then performed FRAP on wt zebrafish in the presence of 14µM of cyclohexamide and found recovery in the longitudianal SR (Supplemental Figure 2c). These experiments showed that EGFP-DHPR recovery in longitudinal SR occurred even when translation is inhibited. Additionally, we added description of this data into the results section (lines 179-185) reprinted below.
"An alternative interpretation of fluorescence recovery might be that it represents local translation of new EGFP-DHPRα from translational machinery in the SR. However, trafficking in the SR persisted in the presence of cyclohexamide, which blocks translation of new protein in zebrafish (Supplemental Figure 2)."

The data generated in the manuscript has not been corrected for photobleaching, since we found low levels of photobleaching over the course of our GFP-DHPRα experiments. We quantified photobleaching of unbleached areas of GFP within a FRAP experiment and found less than 10% bleaching (of initial fluorescence) by 30 minutes and less than 15% by 60 minutes. We added this information in Materials and Methods (lines 554-557).
"Low laser intensities were used to minimize bleaching (8% 488nm). Quantification of non-FRAP regions found less than 10% and 15% bleaching, respectively after 30 and 60 min."

1b. Furthermore, the movie provided in the supplemental material shows that the plane of focus varied during a FRAP sequence; and there is a large green blob (aggregate of overexpressed EGFP-DHPRa?) that disappears during the sequence. Did the authors attempt to mostly use fibers with modest overexpression?

We replaced the supplemental movie with a shifting plane of focus and a green blob with a new movie (Supplemental Movie 1) that showed recovery of fluorescence in the longitudinal SR without shifting focal planes and no green blob.



We used mostly fibers with modest expression in all of our experiments since high levels of expression sometimes caused disruption of triads in skeletal muscle. We carefully avoided recording from muscle fibers with this type of appearance or similar characteristics. We did occasionally see green blobs which often disappeared with time and do not know what these blobs are.

2. Immunofluorescence
2a. Zebrafish microtubules in myotubes and muscle fibers look very much like those in their mammalian counterparts but tubulin staining in FIg 4A does not look like microtubules. The Methods section does not mention an anti-alpha-tubulin but mentions an anti-acetylated alpha-tubulin. The authors need to clarify this.

Thank you for this comment. “Anti-acetylated alpha-tubulin” was a typo and should have read “anti-alpha-tubulin” as was written in the caption for Figure 4. We have corrected this in Materials and Methods (line 506).
The original images of muscles in Figure 4a were of fixed wholemount zebrafish at low resolution making it hard to see the lattice of microtubules in control fish. We repeated the nocodazole treatment and got better wholemount immunolabeling of microtubules, which can now be seen in new images for Figure 4a. In the new images a network of microtubules were labeled in DMSO controls but nocodazole treatment disrupted this network.

2b. In Fig. 4D, were the 2 panels imaged with the same parameters? Why would nocodazole cause the total disappearance of the 2-beta-DG?

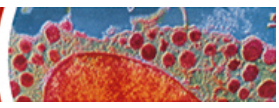
The 2 panels in Figure 4D were imaged with identical parameters, though the expression levels of BDG can vary from muscle fiber to muscle fiber. Since the focus of the paper is on trafficking of DHPR and we have not examined trafficking of BDG besides the nocodazole treatment, we eliminated the BDG experiments and Figure 4D.

2c. There is no information in the Methods about what was done to the images (Photoshop or other software). Some panels are a lot noisier than others (for example Fig5A, WT sibling, compared to 5B, WT sibling). Furthermore, many images show saturation which prevents their use for quantification. In contrast, Fig 5G is excellent.

All images were processed in Fiji (ImageJ) and the contrast in each image adjusted to optimize viewing. Care was taken in all imaging to keep imaging conditions within the dynamic range without saturation. All the quantification was done under these conditions. However, GFP-DHPR in longitudinal SR is often much dimmer than GFP-DHPR in T-tubule triadic striations, and in order to display longitudinal SR in a micrograph, the image contrast was adjusted posthoc and this resulted in T-tubule triadic GFP-DHPR to appear saturated. For example, in Figure 5A, the contrast was enhanced so that longitudinal SR could be observed, but the enhanced contrast also caused triadic puncta to appear saturated. However the quantification shown in Figure 5A was done from measurements taken from images collected entirely within the dynamic range. We have made a note of contrast enhancements and ImageJ processing in the materials and methods section (lines 516-535) reprinted below.
"Quantification of triadic and longitudinal striation fluorescence was performed by measuring the mean fluorescence across vertical lines along or between striations standardized to the mean fluorescence within the surrounding ROI using FIJI (ImageJ) (Figure 5C-J) or when quantifying immunolabeling using the mean fluorescence between striations to along striations (Figure 5A,B). All quantitative comparisons of mutants versus wild type siblings were made between embryos within the same clutch, and all labeling was performed simultaneously and identically between conditions. Confocal imaging settings were carefully calibrated so as not to observe saturated pixels during imaging. Contrast has been enhanced in some micrographs posthoc in order to display longitudinal SR in some instances, but quantification was done within the dynamic range. Identical contrast enhancements were made in qualitative comparisons of nocodazole exposure between drug and DMSO treatments."

The variable noise from image to image is due to different labeling experiments done separately. For example, the labeling experiments shown in Figure 5A and Figure 5B were performed at different times and are not comparable. However, the comparison between WT sibling and relaxed in 5A are from labeling done at the same time and so are comparable. Similarly for comparison between WT sibling and stac3-/- in 5B. Furthermore, the differences in triadic anti-DHPRα labeling in 5A are consistent with Schredelseker et al., 2005, and in 5B are consistent with Linsley et al., 2016.

2d. I am surprised to see anti-GM130 staining as dense and continuous lines. Again, saturation may reduce the resolution but I would expect at least some punctate staining. Have the authors verified with expression of Gal-T for example that the antibody does indeed label Golgi? In mouse muscle, Golgi elements are found near the Z line (see Kaisto & Metsikko, 2003), not at T-tubules.



We redid the GM130 labeling to avoid saturation so that the punctate labeling can be more clearly seen. We had previously found an occasional fiber in which GM130 labeling was seen as a single row of dots (3C) as opposed to a doublet that flanked the triads (3B). We relabeled wholemount muscles with ant-GM130 and found that in nearly all cases a doublet was labeled so now show only that case from the new labeling experiment. In the new Figure 3B GM130 labeled doublets can be seen straddling the triads labeled with anti-DHPR.

3. Typos etc.
page4, first column, last line: change DHPRa to DHPRb.

Thank you for correcting this typo. Ww have corrected the text on line 253 to read:
"Both stac3-/- mutants and relaxed (DHPRβ null) mutants."

4. Evidence for the proposed model; suggestions for improvement
4a. The model of ERES and Golgi-mediated traffic of DHPR from the SR to the T-tubule is interesting but it will take more work to gather evidence for it. The claim in the introduction "we resolved the trafficking route of the DHPRa..." is too optimistic. The authors have established here that DHPRa is associated with the longitudinal SR. Given the long time-course of FRAP it is possible that the apparent recovery results from new synthesis (see point 1), something that would be interesting to establish. But they do not provide evidence that DHPR transits through ERES or Golgi on its way to the T-tubules; the images of anti-DHPR and anti-sec23 would need much better resolution to show colocalization, let alone co-trafficking.

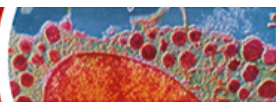
We agree that our model requires more validation. We modified our language in the last paragraph of the introduction to say that our data “suggests a new trafficking route for DHPRα within mature differentiated skeletal muscle to triadic junctions and show how longitudinal SR expression of DHPRα is affected by mutations in EC coupling components” rather than we have “resolved” the trafficking route (lines 117-122). The new FRAP experiments under conditions in which new translation is inhibited, strongly suggest that DHPRs move within the longitudinal SR (Supplemental Figure 2, Supplemental Table 1). Nevertheless, the point still stands that we do not show direct evidence that DHPR transits through ERES or Golgi, and we have not been able to show this trafficking at high enough resolution to resolve this pathway. We hope our hypothesis for DHPR trafficking will motivate future studies with the aid of technological breakthroughs such as super resolution microscopy to address these issues.

4b. It would be helpful to be presented in the introduction with a summary of the relevant results in the authors' recent PNAS paper, instead of keeping this for the discussion.

We added additional text to the Introduction describing relevant results in our recent PNAS paper (lines 75-99) as suggested and describe relevant findings of a new paper on Stac3 that was published during the review period of this paper. The relevant lines are reprinted below.
"More recently, zebrafish stac3 was found to be required for normal DHPR levels, organization, stability and function at triads, and causal for the debilitating Native American myopathy (NAM)11,13. Like muscle fibers from relaxed zebrafish, stac3 null muscle fibers also do not show gross morphological defects, suggesting a specific role for Stac3 in EC coupling13. Furthermore, zebrafish expressing the hypomorphic, missense NAM allele of stac3 were aberrant in levels, organization and function of DHPRs at triads underscoring the importance of DHPR expression and localization in skeletal muscles for human disease11. Yet while Stac3 has been shown to co-immunoprecipitate with DHPR13 and stably interact with the C1 domain of DHPR in skeletal myotubes14, whether Stac3 interacts with DHPR during the process of trafficking DHPR to the triad or instead acts exclusively in stabilizing the DHPR at the triad has been controversial. While heterologous expression of Stac3 in cultured nonmuscle cell lines promotes the trafficking of DHPR to the membrane15, stac3 null zebrafish show only a 30% reduction in DHPR at triads11. Accordingly, a more thorough understanding of the involvement of Stac3 in the trafficking of DHPR is necessary."

4c. The authors' enthusiasm pushes them a bit far in their claims. In the discussion, the authors claim that "mRNA encoding DHPRa has also been found throughout the SR..." (ref34, Nissinen et al, 2005). The Nissinen et al paper locates DHPRa mRNA to the mouse muscle fiber subsarcolemmal space possibly linked to protein synthesis on ER/SR-linked ribosomes. Neither ribosomes nor mRNA were suggested to be in the SR.

Thank you for the clarification. We changed this claim in the discussion (lines 402-406).
“Interestingly, mRNA encoding DHPRα has also been found in the subsarcolemmal space, possibly linked to protein synthesis of ER/SR-linked ribosomes34. Thus mRNA may also be targeted to SR-linked ribosomes to locally translate



DHPRα."

Referee 2:

1. Muscle fiber type: Is there a difference between fast/slow type fiber in zebrafish with regards to the triads function/all proteins investigated in this study? All experiments done on fast or slow type fiber? Some background/introduction to different muscle EC physiology in zebrafish in comparison to mammalian system would be helpful.

All quantifications in this study were performed on fast twitch fibers in zebrafish as they are more prevalent. We also examined slow twitch muscle, though not in much detail, and did not notice any differences with fast twitch. We added text in Materials and Methods to clarify this point (lines 492-496).
"In all cases shown in this report, findings from the analysis of fast twitch muscles are presented. Although not analyzed in as much detail, slow twitch muscles were also examined and found to exhibit similar trafficking of DHPRs."

We also added a short description of EC physiology in zebrafish as suggested (lines 60-64).
"Zebrafish embryos have emerged as a robust and popular model for EC coupling as they rapidly develop mature skeletal muscle fibers, develop externally, have translucent skin, and show highly homologous EC coupling physiology to mammals9."

2. General information on the mutants (any structural defects? Severe muscle phenotype?) included is lacking. In M&M, more info about the DNA constructs (encoding fused protein? Under which promoter? expression time? etc.) will help to understand the basis of the live imaging results.

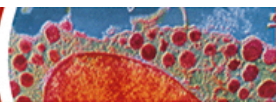
Additional information has been added on zebrafish mutant lines reprinted below (lines 64-74).
"The paralyzed zebrafish relaxed mutant that is null for DHPRβ subunit12, has disrupted tetrad formation and reduced DHPR expression at triads despite normal expression and localization of RyR19. DHPRβ is thought to act as a chaperone to traffic and then allosterically allow conformational folding of the DHPR into tetrads that are anchored to RyR18. Remarkably, muscle fibers from relaxed mutant zebrafish embryos display normal gross morphology, suggesting a specific role for DHPRβ in EC coupling9,12."

(lines 78-82)
"Like muscle fibers from relaxed embryos, stac3 null embryonic fibers also do not show gross morphological defects, suggesting a specific role for Stac3 in EC coupling13."

Additionally, information on DNA constructs used for experiments including promoters, fusion proteins produced, and expression constructs were added to the Materials and Methods section under the "Animal care and phenotypic analysis" subheading (lines 482-492) reprinted below.
"Embryos were injected into the yolk at 1-cell stage with DNA expression constructs using the muscle actin promoter to constitutively express EGFP-DHPRα or Stac3-EGFP fusion proteins in skeletal muscle, and were sorted for fluorescence with a fluorescent dissecting microscope (Leica) as previously described11. Transgenic hsp70:EGFP zebrafish embryos were also used39, and EGFP expression was induced by placing embryos in a 37degree water bath for 1 hour as previously described39."

3. Did the authors do FRAP of EGFP-DHPRa on a stac3-/- background? To add to Figure 2 results show stac3 does not affect the trafficking of DHPR?

The FRAP in Figure 2 is in a wild type background, as is noted in the figure caption (line 623). FRAPs of EGFP-DHPRα in a stac3-/- compared to a stac3 wt sibling were performed in our previous paper, Linsley et al. 2016, PNAS, and the data is summarized in this manuscript in our newly added Supplemental Table 1. In those data, we showed that stac3-/- zebrafish embryos have slightly increased EGFP-DHPRα trafficking rate, but that EGFP-DHPRα trafficking to triads is not lost in zebrafish skeletal muscle with the loss of Stac3. Thus we believe DHPR trafficking in longitudinal SR is independent of Stac3. This is now also referenced in the last sentence of the results section (lines 198-200) reprinted below.
"In fact, the loss of Stac3 does not prevent trafficking of DHPRα to triads11 consistent with a lack of chaperone activity of Stac3 for DHPR."



4. Immunolabeling nicely showed SR/ER export machinery and Golgi outposts localize adjacent triads, however, structural proximity does not necessarily relate to function. Is there any method available to monitor the dynamics of SR/ER and Golgi using live imaging? Alternatively, pharmacological blocking of SR-Golgi trafficking would be a direct way to monitor this in a FRAP setting. The strong conclusion on “DHPRa trafficking via ER/SR” in discussion needs more experimental support.

SR-Golgi trafficking is difficult for us to resolve spatially with confocal microscopy and we have not been able to directly assay trafficking from the SR to Golgi outposts to T tubules. Nevertheless, our evidence suggests that DHPR is trafficking within longitudinal SR membrane. We have generated a hypothesized pathway for DHPR from the triadic region of the SR to local Golgi to the triadic region of the T tubules as one possible reason for DHPR trafficking along the longitudinal SR. To make this clear we modified the text “resolved the trafficking route” of DHPRα via ER/SR through Golgi to triads" to those shown below (lines 117-122).
"Here using dynamic imaging of skeletal muscle in zebrafish mutants, we suggest a novel trafficking route of the DHPRα within mature differentiated skeletal muscle to triadic junctions and show how longitudinal SR expression of DHPRα is affected by mutations in EC coupling components."

5. Nacodazole treatment- whole animal drug treatment needs further comment. Is the effect highly specific? a well-characterised positive control is needed to show the addition of nacodazole in this context (what is the treatment duration, dose) sufficiently blocked microtubule mediated trafficking without off-target effects. A larger subject number of Nacodazole group should be considered for Stats in Figure 4 Panel C.

We added more details to the nocodazole experiments for clarification (lines 236-240)
"48hpf embryos were incubated in 1 µg/ml nocodazole for 24 hours at room temperature that did not cause gross morphological abnormalities to the embryo, but disrupted microtubule formation in myofibers (Figure 4A)."

We believe Figure 4A acts as a well characterized positive control for nocodazole activity. Due to the low resolution of the original micrographs in Figure 4A, we repeated the experiment, and added new micrographs that more clearly illustrate microtubules with and without nocodazole treatment. As for off target effects, it is difficult to fully characterize all the off target effects of drug dosage on a whole animal. However, the nocodazole treatment did not greatly affect the morphology of the embryo nor the skeletal muscles and did not kill the embryo (which is common when dosing embryos with drugs that carry off target effects).

6. Is DHPR trafficking a calcium dependent process?

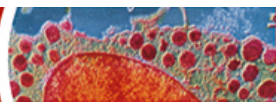
This is an interesting idea. As calcium affects many processes within cells, it is certainly possible that calcium levels could affect trafficking of DHPRα, but we have not directly tested this possibility. Of note, stac3-/- mutants have severe calcium release defects (Horstick et al., 2015, Linsley et al., 2017) thus reducing the calcium within the cytoplasm of the myofiber during EC coupling. Despite this stac3-/- mutants are able to traffic DHPRα to the triad. Thus substantial calcium released during EC coupling may not be required for trafficking of DHPR. Calcium levels within the SR may also potentially regulate DHPR trafficking. Since we (Linsley et al., 2016) found that stac3-/- mutants have similar levels of SR calcium as wt siblings but have slightly increased DHPRα trafficking, trafficking can be regulated by mechanisms not involving SR calcium. This does not rule out that SR calcium may also regulate DHPR trafficking but the role of calcium for trafficking remains to be clarified.

7. How transferrable is the data obtained from an overexpression model in the endogenous context?

This is an important issue. It has not been possible to examine trafficking of endogenous DHPRα so we assayed a tagged exogenous version of the protein as performed in many other trafficking studies. However, the co-labeling of endogenous DHPRα with anti-DHPRα with the longitudinal SR with anti-Serca demonstrates that endogenous DHPRα does reside in the longitudinal SR (Figure 1). This finding establishes that our dynamic findings of movement of EGFP-DHPRα in longitudinal lines maybe representative of the behavior of endogenous DHPRα.

8. Live imaging is a powerful tool to investigate cellular events in real-time, however, the resolution is limited to visualize subcellular dynamics. Did the authors consider adding a few TEM or STED experiments to confirm the results with higher resolution?

We attempted some PALM as well as STED super resolution imaging, yet were unable to get the light and spectral resolution required to do meaningful experiments to contribute to this manuscript.



Referee: 3

1. A control experiment should be carried out to show that no FRAP recovery of DHPRα occurs in fixed fish. This will help rule out the possibility that photoswitching of the fluorescent protein is occurring (although this seems unlikely given the results shown).

We performed the suggested control experiment on fixed wt zebrafish embryos expressing GFP-DHPRα and found that no FRAP recovery occurs ruling out photoswitching of GFP-DHPRα during FRAP. We have added this data in Supplemental Figure 1 and Supplementary Table 1.

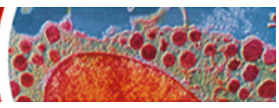
2. It is also important to include a control experiment using cycloheximide (assuming this is possible in zebrafish) to test if the recoveries occur via new protein synthesis as opposed to diffusion.

To exclude the possibility of new translation being responsible for fluorescence recovery of EGFP-DHPR in longitudinal SR, we performed FRAP experiments on live zebrafish incubated in cyclohexamide. These experiments showed that EGFP-DHPR recovery in longitudinal SR persists when translation is inhibited in the zebrafish. We have included this data in a new figure Supplemental Figure 2 and a new table Supplementary Table 1. See answer to same question (1) raised by Referee 1 for a fuller response. We added description of this data into the first paragraph of the results section reprinted below (lines 179-185).
"An alternative interpretation of fluorescence recovery might be that it represents local translation of new EGFP-DHPRα from translational machinery in the SR. However, trafficking in the SR persisted in the presence of cyclohexamide, which blocks translation of new protein in zebrafish (Supplemental Figure 2)."

3. Diffusion coefficients are reported in several figures. However, it is unclear how these values were extracted from the FRAP curves, as no details about the analysis are included at in the Materials and Methods. This should be explained. The authors should also indicate how they benchmarked their FRAP analysis to confirm that the D values it produces are accurate.

We added a more thorough description of how FRAP experiments were performed and quantified, and diffusion coefficients derived in the Materials and Methods section (lines 546-572). Additionally, we performed benchmarking experiments on zebrafish skeletal muscle fibers expressing GFP and derived D values that are consistent with published diffusion rates for cytoplasmic GFP (Lipponcott-Schwartz et al, 2001). An additional description of the benchmarking for the FRAP analysis was included in the Materials and Methods (Lines 572-581) as well as a new figure (Supplemental Figure 1), and a new table (Supplemental Table 1). The entire changes in the Materials and Methods are reprinted below.
"All FRAP assays were conducted at room temperature (22-25C). FRAP within the triad was quantified along striations and the data fit to a non-linear regression to obtain best-fit values for mobile fraction and diffusion rates. For each individual FRAP trace used in the analysis, the mean fluorescence of all post-bleach triads was significantly higher than the mean fluorescence of all triads at T=0 (ANOVA $p<0.05$). Low laser intensities were used to minimize bleaching (8% 488nm). FRAP bleaching was achieved with 10 scans of 100% 488nm laser intensity over the course of 1 minute, and subsequent imaging with 8% 488nm excitation laser (band-pass 493-555 nM). Data for EGFP-DHPRα FRAP assays are displayed in units of normalized percentage of pre-bleach fluorescence as calculated according to the equation: (F(t)-Fpost)/(Fpre-Fpost)*100 where F(t)= fluorescence at time t in minutes after bleaching, where Fpost= fluorescence immediately after bleaching, and Fpre= fluorescence before bleaching. Data was plotted and each time-lapse was fit to a plateau followed by one phase association (EGFP-DHPRα) using Prism6, and a best fit plateau value and rate constant (k) were derived. Rate constants were converted into diffusion rates (D) using previously described equations42. To benchmark our FRAP analysis, additional FRAP was performed on skeletal muscle fibers expressing EGFP (heat shock induced from transgenic hsp70:EGFP zebrafish), and the diffusion rate of cytoplasmic EGFP was found to be comparable to published reports (62.8μm/second)(Supplemental Figure 1, Supplemental Table 1)43. Nocodazole and DMSO treated embryos were fixed with Dent's fixative after imaging and immunolabeled."

4. The authors should also clarify whether the fluorescence recoveries were quantified across the entire bleached region for all of the experiments. This is an important point because in some cases the proteins of interest are primarily found at triads, other times at longitudinal SR, and yet other times both. It would be useful to compare the apples with apples in each case. For example, for an experiment such as the one shown in Figure 6, it would seem appropriate to draw comparisons between the SR-localized pools of protein and consider the triad-associated population separately since it is essentially immobile. This difference could potentially impact the interpretation of



these experiments.

As mentioned in the above comment (#3), we added a description of how the fluorescence was quantified in FRAP experiments in the Materials and Methods explaining that fluorescence was quantified specifically across triad striations in all EGFP-DHPRα FRAP assays. There is a 60% decrease in triadic DHPRs in relaxed mutant muscles (Schedelseker et al, 2005) so there is still a significant amount of DHPR at triads in these mutants for us to make comparisons in Figure 6. Thus our FRAP assays allowed us to get a dynamic sense of how DHPRs were trafficked to triads in the mutants.

5. On a related note, it would be useful if the authors made the point early on in the paper that the "mobile fraction" of DHPRα in the triads is very low over the timescale of their experiments. This is implied based on their comment that recovery occurs first in the SR, but from the images shown it is difficult to detect any fluorescence recovery at all in triads.

We added text to the Results (lines 167-179) to describe the relative stability of DHPRα at the triad and instability of DHPRα between triads at the longitudinal SR as suggested.
"As early as five minutes after photobleaching, EGFP-DHPRα could be seen to migrate along the longitudinal SR within the region that was photobleached, before accumulating at triadic areas in WT embryos (Figure 2, Supplemental Movie 1). The temporal pattern of recovery of longitudinal lines preceding accumulation in triads is consistent with EGFP-DHPRα trafficking to triads via the longitudinal SR. The mobile fraction of DHPR at triads was low (~30%) during the timescale of FRAP recordings, consistent with previous reports11, and indicating most of the triadic fraction of EGFP-DHPRα is stable."

6. Several places in the text refer to “mobility” of DHPRα . In each instance the authors should clarify if they are referring to differences in the kinetics of recovery/D or differences in mobile fraction.

We clarified what we mean by "mobility" as suggested. In the discussion, we changed a sentence referring to DHPRα mobility to (lines 435-438).
"Furthermore, EGFP-DHPRα in triads of relaxed myofibers have a higher mobile fraction, suggesting that chaperone activity of DHPRβ is required to stabilize EGFP-DHPRα in the triad."

We changed "EGFP-DHPR mobility" for clarification in the Results (lines 334-339).
"Thus, EGFP-DHPRα in relaxed myofibers diffuses laterally within the plane of the SR membrane at a normal rate, yet displays increased mobile fraction compared to WT myofibers, consistent with the role of DHPRβ as a chaperone involved in the assembly of DHPR at triads."

The caption of Figure 6 has also been altered for clarification (lines 716-717).
"Figure 6. DHPRα in relaxed mutants has an increased mobile fraction."

7. The authors should also explicitly state throughout the manuscript that they are proposing that DHPR is undergoing lateral diffusion within the plane of the SR membrane (assuming I am understanding this correctly). Although this is implied, currently the recoveries are typically referred to as “trafficking,” a term which could be easily misinterpreted.

Yes we are implying that DHPRα is laterally moving within the longitudinal SR membrane. We made this more explicit by changing all appropriate instances of “trafficking in the SR” to “trafficking in the SR membrane” (lines 18, 58, 144, 182-3, 317, 422-423, 442-443). Additionally, we have added the clarifying description to the results section (lines 153-155)
"This pattern of labeling is consistent with DHPRα being present in the plane of the longitudinal SR membrane throughout the myofiber."

8. The model shown in Figure 7 is confusing as presented. In some panels, it looks like some DHPR is not membrane associated. The orientation of DHPR with respect to the membrane also changes in different parts of the figure.
Thank you for this insightful comment. We have adjusted Figure 7 to maintain the orientation of DHPR and other schematic proteins with respect to the membrane. We also adjusted the schematic on the cover page to reflect these changes. We believe this makes both of these illustrations easier to interpret.

Other comments
a. I did not follow what was supposed to be different between panels 3B and C.

We replaced panels 3B and C with a single panel 3B from a new set of GM130 labeling. We had previously found an occasional fiber in which GM130 labeling was seen as a single row of dots (3C) as opposed to a doublet that flanked the triads (3B). We relabeled wholemount muscles with anti-GM130 and found that in nearly all cases a doublet was labeled so now show only that case from the new labeling experiment.

b. Several panels of Figure 5 are referred to as being in Figure 4 in the text.

Thanks for the correction. We have fixed all incorrect references to Figure 4 in the text (lines 269, 272, 279, 292, 296, 298).

c. The units of time post bleach are indicated in some but not all of the figures.

We have corrected time post bleach in Figure 2 and Figure 6 to match Figure 4.

Decision and Reviews

Dear Dr. Kuwada,

Thank you for submitting your revised manuscript “Skeletal muscle DHPR is trafficked via sarcoplasmic membrane to triadic junctions” to Traffic. Referees 1 and 2 were unavailable to read the revised manuscript. I have attached here the comments from Referee 3 and a new referee (Referee 4). Referee 3 is satisfied that you have addressed his/her previous concerns, and Referee 4 feels that you adequately addressed the technical concerns raised by the original referees. However, Referee 4 notes that some of your conclusions regarding the trafficking of DHPR from the sarcoplasmic reticulum to the triad are overstated, and notes that your data do not actually demonstrate trafficking. Referee 4 thus recommends either including additional experiments to directly address mechanisms that regulate trafficking or substantially toning down your conclusions throughout the manuscript.

As you have noted in your response to the initial reviews, this study provides the foundation for the future studies that will directly address the mechanism by which DHPR is targeted to the triad from the SR. Therefore, in my view the paper would be acceptable if you were to make revision to the text to tone down your conclusions to better reflect the findings within the paper, and the conclusions drawn. This includes revisions to the title, abstract, synopsis, and the text of the Results and Discussion. With those changes, I would be pleased to accept this paper for publication in Traffic. When you resubmit, please clearly mark the changes to the text, preferably using a different colored font, so that I can evaluate them without sending the paper back out for re-review.

Sincerely,

Michael Marks, Ph.D.
Co-Editor, Traffic

Referee's Comments to the Authors

Referee: 4

Comments to the Author
The revised version of the manuscript addresses the technical concerns of the three referees. However, as pointed out by referees 1 and 2, there is no data showing actual "trafficking" of DHPR from the SR to the triad. In the title and throughout the manuscript there is constant referral to DHPR "trafficking", but nowhere is it directly analysed. The text should be adjusted accordingly to tone down the interpretation. This is particularly true of the synopsis, but also applicable elsewhere in the manuscript- see for example the abstract where the text claims "These findings define a novel trafficking route for DHPR", which is simply not the case. Given the lack of trafficking data, I then wonder how much of an advance the current study actually represents. We know DHPR is made in the SR and needs to get to the triads, so DHPRbeta playing a role in this process is not so surprising. The interesting question is how, but this is not addressed at all in the manuscript. Similarly, the role of Stac3 is not so well defined either. I would be more supportive if the authors could tone down their interpretation, without inferring too much from their data, or better, had some data to more directly address trafficking. There are many ways to perturb trafficking out of the ER and through the Golgi, but the obvious experiments were not done.

Referee: 3

Comments to the Author
I am satisfied with the revisions and think the paper has been much improved as a result.

Author Rebuttal

Referee 4:

1. The revised version of the manuscript addresses the technical concerns of the three referees. However, as pointed out by referees 1 and 2, there is no data showing actual "trafficking" of DHPR from the SR to the triad. In the title and throughout the manuscript there is constant referral to DHPR "trafficking", but nowhere is it directly analysed. The text should be adjusted accordingly to tone down the interpretation. This is particularly true of the synopsis, but also applicable elsewhere in the manuscript- see for example the abstract where the text claims "These findings define a novel trafficking route for DHPR", which is simply not the case.

Thank you for the helpful suggestions. While we agree that we do not fully define the complete pathway of DHPR trafficking from the SR to the triad, we disagree with the statement that we do not analyze trafficking within the manuscript. Distribution of DHPR protein by movement through SR membrane of the muscle fiber is an entirely novel phenomenon that, though not canonical, still represents trafficking of a protein towards its target. Thus, we believe in showing, perturbing, and analyzing the flow of DHPR through the SR for the first time, we are indeed analyzing DHPR trafficking. Nevertheless, we are receptive to your criticism that as we only infer a pathway from which DHPR can leave the membrane of the SR, move to the Golgi, and then to the triad, we should air caution in our interpretations. In response, we have changed the following sentences to more clearly state the trafficking pathway that we have resolved from that which we infer.

Title: L-type calcium channels are transported in the SR membrane in skeletal muscles

Running Title: DHPRs are transported in SR

Synopsis: We find that DHPR is trafficked via sarcoplasmic membrane to triads independent of microtubules, where it may be processed at local ER/SR export machinery and golgi outposts.

Abstract (Lines 14-21): Using dynamic imaging of zebrafish muscle fibers we find that DHPR is transported along the longitudinal SR in a microtubule independent mechanism. Furthermore, transport of DHPR in the SR membrane is differentially affected in null mutants of Stac3 or DHPRβ, two essential components of EC coupling. These findings suggest a novel trafficking route for DHPR.

Results
Lines 123-126: These data are consistent with a unique mechanism for trafficking the DHPR asw well as distinctive functions for DHPRβ and Stac3 in the DHPRα pathway.

Lines 242-245: Thus, it appears that trafficking of DHPR along the SR membrane occurs independently of Stac3 in a microtubule independent process.

Line 248-249: DHPR transport along the longitudinal SR is differentially affected by EC coupling mutations

Line 250-253: Since both DHPRβ and Stac3 are required for normal expression of DHPRα at the triad, we investigated the roles of the two proteins for transport of DHPRα through the SR.

Line 306-311: Another measure of the ability of the myofiber to transport EGFP-DHPRα from the longitudinal SR membrane to triads is the ratio of EGFP-DHPRα fluorescence at triads to the fluorescence in the longitudinal SR immediately to the right of the triads (Figure 5C).

Line 317-320: To generate a more dynamic picture of how EGFP-DHPRα moves within the SR membrane without DHPRβ, FRAP imaging was performed on relaxed mutants expressing EGFP-DHPRα.

Discussion

Line 356-368: Thus, expression of EGFP-DHPRα revealed a potential trafficking conduit that includes the ER/SR for membrane bound proteins to achieve wide distribution throughout skeletal muscle. Furthermore, we found that movement of EGFP-DHPRα along the longitudinal SR membrane proceeded independently of microtubules and Stac3, yet is temperature sensitive11. SR resident proteins such as Calsequestrin and secreted viral proteins have previously been reported to traffic throughout the ER/SR network within myofibers23,32, but this report potentially represents the first example of an endogenous non-resident SR protein using the ER/SR network for protein trafficking.

Line 462-465: Taken together, this study provides the first evidence that DHPRα is transported in the SR membrane throughout the skeletal myofiber, and differentiates the roles of DHPRβ and Stac3 in the trafficking of DHPRα.

2. Given the lack of trafficking data, I then wonder how much of an advance the current study actually represents. We know DHPR is made in the SR and needs to get to the triads, so DHPRbeta playing a role in this process is not so surprising. The interesting question is how, but this is not addressed at all in the manuscript.

Understanding the role of how DHPRβ works to chaperone DHPRα to the triad is indeed an interesting question. Our evidence suggests that DHPRα is trafficked along the SR prior presumably to transport to the T tubules, and that DHPRβ is not required for this as indicated by trafficking in the SR in DHPRβ null zebrafish. Thus our findings refined the role of DHPRβ as one primarily for trafficking of DHPRα from the SR membrane to the T tubule at the triad but not transport within the SR membrane.
Our finding for transport of DHPRα within the SR membrane represents a departure from the current thinking of DHPR trafficked along a canonical ER/Golgi vesicular pathway. In fact, previous reports described DHPR as confined to the perinuclear ER when trafficking to the membrane is disrupted by a lack of Stac3 or DHPRβ (Polster et al 2015, Buraei and Yang 2010). In this manuscript, we localized the trafficking defect due to the loss of DHPRβ in mature muscle fibers to regions around the triad.
We disagree with the statement “We know DHPR is made in the SR”. To our knowledge, this manuscript represents the first demonstration of DHPR in the membrane of the SR. In fact, a previous reviewer (referee #1) cautioned against an interpretation of DHPR being translated in the SR we cited in a previous draft:
"The authors' enthusiasm pushes them a bit far in their claims. In the discussion, the authors claim that "mRNA encoding DHPRa has also been found throughout the SR..." (ref34, Nissinen et al, 2005). The Nissinen et al paper locates DHPRa mRNA to the mouse muscle fiber subsarcolemmal space possibly linked to protein synthesis on ER/SR-linked ribosomes. Neither ribosomes nor mRNA were suggested to be in the SR."
We feel that demonstration of movement of DHPR in the SR membrane represents an advance in our understanding of DHPR trafficking in mature skeletal muscles.

3. Similarly, the role of Stac3 is not so well defined either.

We disagree with this statement. Our previous publication (Linsley et al PNAS 2016) showed that Stac3 was required for stabilization of DHPRs in tetrads at triads, and voltage dependency of DHPRs, but not for trafficking to triadic T tubules. However given that other findings suggested that Stac3 acts as a chaperone in trafficking DHPR to triads (Campiglio and Flucher 2017, Polster et al 2016, Polster et al 2015) we further studied a potential chaperone role for Stac3 in this paper. Our findings confirmed that the loss of Stac3 does not prevent trafficking of DHPR to triads including within the SR membrane unlike DHPRβ which we confirmed is clearly required for chaperoning DHPRα. Thus the action of Stac3 is primarily limited to regulation of DHPR once at the triads.

4. I would be more supportive if the authors could tone down their interpretation, without inferring too much from their data, or better, had some data to more directly address trafficking. There are many ways to perturb trafficking out of the ER and through the Golgi, but the obvious experiments were not done.

As explained above we made changes to the text to be less interpretative as suggested.

5. Other changes

We changed the text Lines 217-220 because Figure 3 contained only two panels.
"Furthermore the Golgi marker, anti-GM130, labeled what appears to be Golgi outposts in the triadic regions that flank triads in zebrafish muscles (Figure 3B) as found in some mammalian muscles25-27."

We added scale bars for the figures.