THE EMBO JOURNAL

Manuscript EMBO-2010-73738

α-catulin CTN-1 is required for BK channel subcellular localization in C. elegans body-wall muscle cells

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Review timeline:

Submission date:	21 January 2010
Editorial Decision:	24 February 2010
Revision received:	23 June 2010
Editorial Decision:	19 July 2010
Revision received:	19 July 2010
Accepted:	20 July 2010

Transaction Report:

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

1st Editorial Decision 24 February 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers recognise the potential interest in your work, but also have a large number of concerns with the study in its current state. In particular, all three referees comment on the poor quality of the co-localisation data, and stress the need to provide more convincing evidence that BKIP-2 specifically affects SLO-1 trafficking. In this context, referee 1 also highlights the need to investigate the relationship between BKIP-2 and DAPC in more detail - to be able to conclude definitively whether the two regulators are independent or act together. In addition, both referees 1 and 2 argue that further analysis comparing the bkip-2 loss of function phenotype to that of slo-1 would be important.

Given the interest shown by the referees, we would like to give you the opportunity to revise your manuscript according to their suggestions. I do realise that this will likely entail a lot of work, and I would stress that we would likely only favourably consider a revised version that significantly strengthens the study along the lines mentioned above, as well as responding to the other concerns raised by the referees. I should also add that it is EMBO Journal policy to allow only a single round of revision. Acceptance, or rejection, of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may

be able to grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In a genetic screen for mutations suppressing the phenotype caused by overactivation of the BK channels in C. elegans, the authors identified the alpha catulin homolog as a protein required for SLO-1 localization at dense bodies in body-wall muscle cells. They provide evidence for direct interaction between alpha-catulin with SLO-1 at dense bodies and in heterologous expression systems. Altogether, this work identifies a new partner of the BK channel and also points to a new function of the alpha-catulin, an evolutionarily conserved protein with few known functions. Hence these data are potentially interesting for a broad audience of neurobiologists and cellular biologists. However, severeal points need to be addressed:
- first, I think that "bkip-2" is a confusing nomenclature, especially for non C. elegans specialists. Alpha-catulin was previously named in other species, and I find it inappropriate to rename this gene, previously called ctn-1 in Wormbase. Did the authors get approval for this new gene class? Why bkip-2 while there is no bkip-1 published? The title of this manuscript would be more informative as "alpha-catulin is required...".
- the central point of the paper is provided in figure 4, showing that SLO-1-GFP is delocalized in bkip-2 mutants. However, this low magnification image is poor and even suggests that a significant amount of fluorescence remains at dense bodies. Double staining experiments with a Dense Body marker is required, including high magnification pictures and quantification of the fluorescence in WT and mutant animals.
- is BKIP-2 required for localization at Dense Bodies or trafficking to the plasma membrane in muscle cells ? This point needs to be addressed.
- another question is the role of alpha-catulin for SLO-1 function. If bkip-2 is important for SLO-1 function, then it should have a phenotype similar to slo-1(lf). The authors state that "bkip-2(lf) mutants were grossly similar to slo-1(lf) mutants in locomotion behaviors" (p. 6).This is an important point that needs to be quantified in bkip-2(lf) as well as in Pmyo-3- and Prab-3-bkip-2 transgenic lines.
- more data are required to characterize the role of bkip-2 in neurons. Figure 3 data points to a lack of function for bkip-2 in neurons, but the ctn-1 locus seems to be a complex locus that generates multiple transcripts based on Wormbase. Since this work is the first characterization of ctn-1, the presence of multiple transcripts needs to be tested. If there are multiple transcripts, are there neuron-specific isoforms that do not interact with SLO-1? In addition, neuronal expression seems to be very weak as compared to muscle. The bkip-2 expression pattern is only based on a transcriptional reporter containing 5' regions of the gene. It would be much more convincing to use a full length genomic fragment to control GFP expression, including intronic sequences and potential 3' regulatory sequence. If bkip-2 is really expressed in neurons, then it will be necessary to test if bkip-2 is expressed in all motoneurons or only in a subset of motoneurons: the presence of normal ePSCs at bkip-2 NMJs might reflect the absence of BKIP-2 function on SLO-1 in neurons or restricted expression of BKIP-2 to a subset of motoneurons.
- the authors report that BKIP-2 localizes at Dbodies in dys-1 mutants. Conversely, is the dystrophin-associated protein complex properly assembled in bkip-2 mutants ? Disorganization of DAPC might account for the bkip-2 phenotype.
- the absence of BKIP-2 effect on SLO-1 expressed in Xenopus oocytes is difficult to interpret

because there is no evidence that BKIP-2 is indeed expressed and able to interact with SLO-1 in this system.
- BiFC results are very suggestive. However, it is necessary to show that SLO-1(1-370)::YFPa is expressed and stable in muscle cells before concluding that the lack of fluorescence is due to a lack of SLO-1/BKIP-2 interaction.

- minor point: it does not make complete sense to claim that identification of "12 independent alleles of bkip-2 in the genetic screen" means that "BKIP-2 is a key player in SLO-1 localization" (discussion). There might be more 'important' genes which are essential or give stronger phenotypes when mutated, hence precluding their identification in this specific screen.

Referee #2 (Remarks to the Author):

The calcium-activated Slo potassium channels are conserved regulators of neurotransmission. In this paper, the authors suppress the uncoordinated movement caused by overexpressing a slo-1(gf) mutation in C. elegans, and identify mutations in the catenin-related cytoplasmic protein alpha catulin (bkip-2). Alpha catulin has previously been shown to interact with the kinase NEK1, RhoGEF, and IKK-beta, a regulator of NF-kappa B signaling. Expression of bkip-2 in muscles restores slow locomotion to slo-1(gf) animals. A GFP-tagged SLO-1 protein is reduced in expression in muscles of bkip-2 mutant worms, suggesting an effect either on muscle structure or on channel localization. BKIP-2 and SLO-1 colocalize to the same region of muscles and also associate closely enough to immunoprecipitate together and reconstitute GFP fluorescence from split GFP versions expressed in muscles. However, bkip-2 does not affect the electrophysiological function of slo-1 in Xenopus oocytes or a neuromuscular junction assay. These results suggest that BKIP-2 regulates SLO-1 function, possibly by affecting subcellular localization in muscles.

The work in this paper is generally good and interesting, but there are a few concerns about the interpretation that could be resolved with straightforward additional experiments. Briefly, the biological relationship between bkip-2 and slo-1 should be established more firmly.

1. All of the interpretations of bkip-2/slo-1 are based on interactions with an overexpressed mutated slo-1 transgene. It would greatly strengthen the paper to show a role for bkip-2 in the normal function of slo-1. There are reported phenotypes of slo-1 loss of function mutants in several assays, including locomotion and ethanol sensitivity. The authors should examine bkip-2 mutants and bkip-2 slo-1 double mutants in some of these settings to ask whether bkip-2 is required for normal slo-1 function. An alternative to their model is that bkip-2 has a role in quality control of membrane proteins (for example) rather than a specific role in slo-1 function.

2. The only result the authors obtain with respect to endogenous slo-1 function is negative (Figure 3B). However, the electrophysiological assay used to examine bkip-2 interactions with slo-1 in neurons is not entirely convincing. The evoked potential assay is variable and not the most reliable assay in the C. elegans arsenal. Even the way the authors describe it shows its variability (scoring the "two largest ePSCs in each experiment"). I realize that this group has used this assay successfully in the past, but there are better and more quantitative tools now, such as channelrhodopsin expressed in motor neurons. These could be used here. Again, the more the authors can relate bkip-2 to endogenous slo-1 function, the better this paper will be.

3. The negative result on localization effects in neurons would be much stronger if it were done at a single cell level (eg expressing slo-1 and bkip-2 together in a class of motor neurons such as VD/DD).

4. Almost every membrane-associated protein expressed in muscles appears in the "dense body" puncta - not just the vinculin-associated proteins but potassium channels, calcium channels, dystrophin complex, G proteins, RGS proteins, and so on. Localization to this area is only a little more informative than localization to the plasma membrane. The authors should point out that this is a low-resolution view of muscle.
The authors should look at another membrane protein in this compartment to show that it is not affected by bkip-2. Vinculin, a structural protein in the cytoplasm, is not the best comparison to an

ion channel in the membrane.

5. Do slo-1 mutations affect bkip-2 localization in muscle?

6. The authors say that dys-1 does not affect bkip-2 localization. Can the authors recapitulate the published effect of dys-1 on slo-1 localization with their transgenes?

Minor comments

Figure 3a. Add control showing that Pmyo-3::BKIP-2 does not lead to slow locomotion in WT worms

Page 6. It is unlikely that these "promoter constructs" cover the entire endogenous expression patterns of slo-1 and bkip-2.

Page 7. The description of the electrophysiology here and in the figure legend is unclear. The authors never explain their assumptions about the assay or the cellular sites of actions of phenotypes. I think in part that's because the assay itself is a bit unclear, ie point 2 above.

Page 12. C. elegans has at least four beta-catenins, so a null mutant of bar-1 is only slightly informative.

Referee #3 (Remarks to the Author):

This manuscript reports the putative role of a protein termed BKIP-2 (BK channel interacting protein-2) in determining the subcellular location of the pore-forming alpha subunit of the BK channel (SLO-1) in C. elegans.

I did not think that this manuscript was particularly well written or the data well described. Quite a lot of detail was left to the legends and not mentioned in the main text. Although I did find some figures hard to interpret, the most obvious problem is the sequence alignment in Figure 1. The text is too small when the figure is reproduced at a size that would be published. The most major comment concerning the manuscript is that although the authors demonstrate reasonably well the association and localization of BKIP-2 and SLO-1, the effect on locomotion is not explained. It would be worth determining what effect on the muscle action potential is observed, as well as what happens to muscle cell firing patterns. More specific comments are;

1. Results. Figure 2. I did not find the apparent colocalization of BKIP-2 and SLO-1 to be convincing. I accept that they are broadly in similar cellular locations, but I did not see compelling evidence of colocalization in any tissue.
2. Results. Figure 3. Is there any control for how much BKIP-2 is expressed? Obviously, if the protein is over-expressed then localization will be affected. In addition, the authors report that the inhibition of locomotion by SLO-1(gf) is not prevented by P-myo-3:BKIP-2. Would not this construct of BKIP-2 allow the protein to be expressed only in muscle? Therefore, why does it not reverse the effect of SLO-1(gf) and why do the authors state that it shows that the 'suppression of the SLO-1(gf) phenotype by BKIP-2(lf) result mainly from BKIP-2 deficiency in muscle cells.'?
3. Results. p. 8, para 1. I presume that data studying vinculin is not shown.
4. Results. Figure 5. Why is the antibody heavy chain showing as a band between 80 and 100 kDa, when it should be approximately 50 kDa?
5. Results. Figure 7. The authors should also show the subcellular localization of SLO-1 in the dys-1 mutant.
6. The authors showed that BKIP-2 interacts with the large intracellular portion of the SLO-1 channel, between the two RCK domains. This region also contains the calcium bowl that is critical for gating of the channel by calcium ions. Are the authors surprised that interaction of the two proteins does not affect function?
7. The authors show that BKIP-2 and SLO-1 are present in both nerve and muscle, yet it only appears to affect subcellular location in muscle. Why? Is it possible that the low resolution approach used to show colocalization has missed an effect in neurons?

8. Where does SLO-1 get the calcium from for its activation in muscle? Could BKIP-2 be affecting SLO-1 location to fine-tune this requirement?

1st Revision - Authors' Response 23 June 2010

Many thanks for your consideration of our manuscript (EMBOJ-2010-73738) and for granting us an extension of the revision period. We greatly appreciate the reviewers' careful review of the manuscript. In response to their constructive and thoughtful comments, we performed new experiments and revised the manuscript accordingly. The following are our point-to-point responses to the reviewers' comments:

Referee #1

Comment: *first, I think that "bkip-2" is a confusing nomenclature, especially for non C. elegans specialists. Alpha-catulin was previously named in other species, and I find it inappropriate to rename this gene, previously called ctn-1 in Wormbase. Did the authors get approval for this new gene class? Why bkip-2 while there is no bkip-1 published? The title of this manuscript would be more informative as "alpha-catulin is required...".*

Response: In a mutant screening for *slo-1(gf)* suppressors, we isolated 25 mutants which belong to three distinct genes. We named these genes *bkip-1*, *-2*, and *-3*, respectively, for *BK* channel *i*nteracting *p*roteins. The new gene names were officially approved before submission of the manuscript, and our manuscript on *bkip-1* is currently under review in another journal. Nevertheless, we agree with the reviewer that it is more appropriate to use *ctn-1* instead of *bkip-2*. Therefore, we have replaced *bkip-2* with *ctn-1* in the revised manuscript.

Comment: *the central point of the paper is provided in figure 4, showing that SLO-1-GFP is delocalized in bkip-2 mutants. However, this low magnification image is poor and even suggests that a significant amount of fluorescence remains at dense bodies. Double staining experiments with a Dense Body marker is required, including high magnification pictures and quantification of the fluorescence in WT and mutant animals.*

Response: The picture in the old Figure 4 has been replaced with a new one with higher magnification. In the new figure (Figure 5A), the SLO-1::GFP puncta can be clearly seen in the wild-type but are essentially absent in *ctn-1* mutant. We also quantified the intensity of SLO-1::GFP puncta in the wild-type and *ctn-1* mutant, and found that there was a huge difference between the two groups (Figure 5B). We did not perform the double staining experiments because (1) the new picture and data analyses (Figure 5A, 5B) clearly show that SLO-1 was mislocalized in *ctn-1(lf)*, (2) CTN-1::EGFP was clearly localized to dense bodies (Figure S4A), and (3) CTN-1 colocalized with SLO-1 in body-wall muscle cells (Figure 5F).

Comment: *is BKIP-2 required for localization at Dense Bodies or trafficking to the plasma membrane in muscle cells? This point needs to be addressed.*

Response: We performed surface biotinylation experiment with transfected HEK293 cells, and found that both total and surface levels of SLO-1 protein were comparable between cells expressing SLO-1 alone and those expressing SLO-1 plus CTN-1 (Figure 5E). This result suggests that CTN-1 is not required for SLO-1 trafficking to the plasma membrane. Because *ctn-1* mutant also did not affect *slo-1* transcription or SLO-1 protein synthesis *in vivo* (Figure 5C, 5D), we conclude that CTN-1 is required for SLO-1 subcellular localization.

Comment: *another question is the role of alpha-catulin for SLO-1 function. If bkip-2 is important for SLO-1 function, then it should have a phenotype similar to slo-1(lf). The authors state that "bkip-2(lf) mutants were grossly similar to slo-1(lf) mutants in locomotion behaviors" (p. 6).This is an important point that needs to be quantified in bkip-2(lf) as well as in Pmyo-3- and Prab-3-bkip-2 transgenic lines.*

Response: In response to this thoughtful comment , we developed an automated worm tracking and analyzing system to quantify worm locomotion behaviors. Consistent with an observation of a recent study (Kim et al, PLoS Genet, 5:e1000780, 2009), we found that *slo-1(lf)* mutant had a bigger head bending angle than the wild-type. In addition, we found that this phenotype was shared by *ctn-1(lf)*, and did not show an additive effect in the *ctn-1(lf);slo-1(lf)* double mutant (Figure 3B, 3C), suggesting that CTN-1 and SLO-1 likely function in the same genetic pathway. The head bending phenotype of *ctn-1(lf)* could be rescued by expressing wild-type CTN-1 in muscles but not in neurons (Figure 3B, 3C), suggesting that the mutant phenotype was mainly caused by CTN-1 dysfunction in muscle cells.

Comment: *more data are required to characterize the role of bkip-2 in neurons. Figure 3 data points to a lack of function for bkip-2 in neurons, but the ctn-1 locus seems to be a complex locus that generates multiple transcripts based on Wormbase. Since this work is the first characterization of ctn-1, the presence of multiple transcripts needs to be tested. If there are multiple transcripts, are there neuron-specific isoforms that do not interact with SLO-1? In addition, neuronal expression seems to be very weak as compared to muscle. The bkip-2 expression pattern is only based on a transcriptional reporter containing 5' regions of the gene. It would be much more convincing to use a full length genomic fragment to control GFP expression, including intronic sequences and potential 3' regulatory sequence. If bkip-2 is really expressed in neurons, then it will be necessary to test if bkip-2 is expressed in all motoneurons or only in a subset of motoneurons: the presence of normal ePSCs at bkip-2 NMJs might reflect the absence of BKIP-2 function on SLO-1 in neurons or restricted expression of BKIP-2 to a subset of motoneurons.*

Response: We performed several experiments to address this comment. Based on the Wormbase, the *ctn-1* locus encodes four different splicing variants. To analyze the expression patterns of these splicing variants, we cloned *ctn-1* genomic DNA, including sequences before the initiation site (4.3 kb) and after the last exon (0.6 kb), , and inserted GFP into a unique axon of each splicing variant (Supplementary Figure S1A). Analyses of the transgenic worms revealed strong *ctn-1d* expression in both neurons and muscle cells, good *ctn-1c* expression in muscle cells but weak *ctn-1c* expression in neurons, and no detectable *ctn-1a* or *ctn-1b* expression(Supplementary Figure S1B). Unlike CTN-1d, CTN-1c was unable to reinstate the lethargic phenotype caused by *slo-1(gf)* when it was expressed in the *slo-1(gf);ctn-1(lf)* double mutant, suggesting that CTN-1d is the major isoform with respect to SLO-1 function. Therefore, we focused our analyses on CTN-1d in subsequent experiments.

Comment: *the authors report that BKIP-2 localizes at Dbodies in dys-1 mutants. Conversely, is the dystrophin-associated protein complex properly assembled in bkip-2 mutants? Disorganization of DAPC might account for the bkip-2 phenotype.*

Response: To address this comment, we analyzed the localization pattern of mStrawberry-tagged DYB-1 (dystrobrevin), which is a major component of the DAPC, in body-wall muscle cells of the wild-type and *ctn-1* mutant. The fusion protein showed similar punctate localization in both the wild-type and *ctn-1* mutant animals (Supplementary Figure S6), suggesting that *ctn-1* mutant did not cause disorganization of the DAPC in muscle cells.

Comment: *the absence of BKIP-2 effect on SLO-1 expressed in Xenopus oocytes is difficult to interpret because there is no evidence that BKIP-2 is indeed expressed and able to interact with SLO-1 in this system.*

Response: The expression of CTN-1 in *Xenopus* oocytes was verified by western blot (data not shown). The co-IP experiments with transfected HEK293 cells show that CTN-1 was able to interact with SLO-1 (Figure 6B). It is not surprising that a protein mediating channel subcellular localization lacks an effect on channel functional properties. For example, ISLO-1 is required for SLO-1 localization in *C. elegans* body-wall muscle cells but does not affect SLO-1 functional properties in Xenopus oocytes (Kim et al, PLoS Genet, 5:e1000780, 2009).

Comment: *BiFC results are very suggestive. However, it is necessary to show that SLO-1(1-370)::YFPa is expressed and stable in muscle cells before concluding that the lack of fluorescence is due to a lack of SLO-1/BKIP-2 interaction.*

Response: In our study of BKIP-1, a different SLO-1-interacting protein identified through the same genetic screen, the same transgenic stain expressing SLO-1(1-370)::YFPa was used to determine which region of SLO-1 is responsible for its interaction with BKIP-1. We observed BiFC signals in muscle cells when SLO-1(1-370)::YFPa was co-expressed with BKIP-1::YFPc. This observation indicates that SLO-1(1-370)::YFPa was stably expressed in this strain.

Comment: *minor point: it does not make complete sense to claim that identification of "12 independent alleles of bkip-2 in the genetic screen" means that "BKIP-2 is a key player in SLO-1 localization" (discussion). There might be more 'important' genes which are essential or give stronger phenotypes when mutated, hence precluding their identification in this specific screen.*

Response: We agree with the reviewer that there might be other proteins that are also important to SLO-1 localization. We have revised the sentence accordingly.

Referee #2

Comment: *All of the interpretations of bkip-2/slo-1 are based on interactions with an overexpressed mutated slo-1 transgene. It would greatly strengthen the paper to show a role for bkip-2 in the normal function of slo-1. There are reported phenotypes of slo-1 loss of function mutants in several assays, including locomotion and ethanol sensitivity. The authors should examine bkip-2 mutants and bkip-2 slo-1 double mutants in some of these settings to ask whether bkip-2 is required for normal slo-1 function. An alternative to their model is that bkip-2 has a role in quality control of membrane proteins (for example) rather than a specific role in slo-1 function.*

Response: This comment was similar to one of Reviewer #1's comments. As described above, we addressed this comment by analyzing locomotion behaviors of the wild-type and mutant worms using an automated worm tracking system. The data support our conclusion that CTN-1 is mainly required for SLO-1 function in body-wall muscle cells.

Comment: *The only result the authors obtain with respect to endogenous slo-1 function is negative (Figure 3B). However, the electrophysiological assay used to examine bkip-2 interactions with slo-1 in neurons is not entirely convincing. The evoked potential assay is variable and not the most reliable assay in the C. elegans arsenal. Even the way the authors describe it shows its variability (scoring the "two largest ePSCs in each experiment"). I realize that this group has used this assay*

successfully in the past, but there are better and more quantitative tools now, such as channelrhodopsin expressed in motor neurons. These could be used here. Again, the more the authors can relate bkip-2 to endogenous slo-1 function, the better this paper will be.

Response: Because the amplitude of ePSCs could be affected by the position of the stimulating electrode, it is often necessary to change the position of the stimulating electrode until a maximal evoked response is obtained. Therefore, it is reasonable to use the average of the largest two ePSC peaks from each preparation in statistical analyses. As suggested by the reviewer, the revised manuscript now shows ePSCs induced by photoactivation of channelrhodopsin-2 expressed in cholinergic motoneurons. Consistent with the previous results obtained by electrode stimulation, the new data suggest that CTN-1 is not required for the function of SLO-1 in regulating neurotransmitter release at the neuromuscular junction (Figure 4).

Comment: *The negative result on localization effects in neurons would be much stronger if it were done at a single cell level (eg expressing slo-1 and bkip-2 together in a class of motor neurons such as VD/DD).*

Response: We appreciate this thoughtful suggestion of the reviewer. In fact, we had attempted to express SLO-1 in the RIA interneuron and DA9 motoneuron using the *glr-3* and *mig-13* promoters, respectively, which had been successfully used for analyzing protein subcellular localization in these neurons (Brockie et al, J Neurosci, 21:1510-22, 2001; Klassen et al, Cell, 130:704-16, 2007). However, we were unable to detect SLO-1::GFP signal using these promoters. For unknown reasons, it seems difficult to drive SLO-1 expression in neurons using other neuronal promoters, including the strong pan-neuronal *rab-3* promoter (unpublished). We believe that the lack of this kind of analyses does not have a major impact on our conclusion that CNT-1 is primarily required for SLO-1 subcellular localization in body-wall muscle cells because 1) SLO-1 subcellular localization in the nerve ring was indistinguishable between the wild-type and *ctn-1(lf)* mutant (Figure 5A); 2) *ctn-1(lf)* mutant did not affect synaptic transmission at the NMJs as *slo-1* mutant did (Figure 4); 3) expression of CTN-1 in muscle but not in neurons reinstated the lethargic phenotype of *slo-1(gf)* (Figure 3A); and 4) the head bending phenotype of *ctn-1(lf)* mutant shared by *slo-1(lf)* was rescued by expressing of wild-type CTN-1 in muscles but not in neurons (Figure 3B, 3C).

Comment: *Almost every membrane-associated protein expressed in muscles appears in the "dense body" puncta - not just the vinculin-associated proteins but potassium channels, calcium channels, dystrophin complex, G proteins, RGS proteins, and so on. Localization to this area is only a little more informative than localization to the plasma membrane. The authors should point out that this is a low-resolution view of muscle. The authors should look at another membrane protein in this compartment to show that it is not affected by bkip-2. Vinculin, a structural protein in the cytoplasm, is not the best comparison to an ion channel in the membrane.*

Response: Indeed, a variety of proteins appear to be localized to dense body regions in *C. elegans* body-wall muscle cells. As suggested by the reviewer, we analyzed the subcellular localization of another membrane protein, innexin-11, and found that it was similarly localized at dense body areas in the wild-type and *ctn-1(lf)* mutant (Supplementary Figure S3A). This observation, together with the data from vinculin immunostaining (Supplementary Figure S3B), suggests that the SLO-1 mislocalization observed in *ctn-1(lf)* mutants did not result from a general defect in protein subcellular localization.

Comment: *Do slo-1 mutations affect bkip-2 localization in muscle?*

Response: As suggested, we examined the subcellular localization of CTN-1::EGFP in body-wall muscle cells of the wild-type and *slo-1(lf)* mutant, and found no difference between these two strains (Supplementary Figure S4B), suggesting that *slo-1* mutations do not affect *ctn-1/bkip-2* localization.

Comment: *The authors say that dys-1 does not affect bkip-2 localization. Can the authors recapitulate the published effect of dys-1 on slo-1 localization with their transgenes?*

Response: We expressed SLO-1::GFP in *dys-1(cx18)* mutant, and observed mislocalization of the fusion protein in body-wall muscle cells (Figure 7), which is in agreement with the data of a recent study (Kim et al, PLoS Genet, 5:e1000780, 2009).

Comment: *Figure 3a. Add control showing that Pmyo-3::BKIP-2 does not lead to slow locomotion in WT worms*

Response: We performed the experiment as suggested, and found P*myo-3*::CTN-1 itself did not affect locomotion in wild-type worms. The data have been added to Figure 3A.

Comment: *Page 6. It is unlikely that these "promoter constructs" cover the entire endogenous expression patterns of slo-1 and bkip-2.*

Response: These *promoter*::GFP constructs may or may not cover the entire *in vivo* expression patterns of *slo-1* and *ctn-1*. However, since the mutant phenotype of *slo-1(lf)* and *ctn-1(lf)* could be fully rescued with these promoters, it is likely that the *promoter*::GFP constructs largely recapitulated the endogenous expression patterns of *slo-1* and *ctn-1*.

Comment: *Page 7. The description of the electrophysiology here and in the figure legend is unclear. The authors never explain their assumptions about the assay or the cellular sites of actions of phenotypes. I think in part that's because the assay itself is a bit unclear, ie point 2 above.*

Response: As suggested by the reviewer, the old electrophysiological data have been replaced by new data obtained with channelrhodopsin-2 (Figure 4). In addition, we have tried to make the description clearer.

Comment: *Page 12. C. elegans has at least four beta-catenins, so a null mutant of bar-1 is only slightly informative.*

Response: We have removed the description of this negative observation from the revised manuscript.

Referee #3

Comment: *Quite a lot of detail was left to the legends and not mentioned in the main text.*

Response: The manuscript has been revised accordingly.

Comment: *Although I did find some figures hard to interpret, the most obvious problem is the sequence alignment in Figure 1. The text is too small when the figure is reproduced at a size that would be published.*

Response: The size of the figure has been increased.

Comment: *The most major comment concerning the manuscript is that although the authors demonstrate reasonably well the association and localization of BKIP-2 and SLO-1, the effect on locomotion is not explained. It would be worth determining what effect on the muscle action potential is observed, as well as what happens to muscle cell firing patterns.*

Response: The essence of this comment is similar to that of a comment from the first two reviewers: i. e. whether or not CTN-1 is required for SLO-1 function in body-wall muscle. As described about, quantitative analyses of worm locomotion suggest that *ctn-1(lf)* and *slo-1(lf)* similarly increased the head bending angle, their effects were not additive, and the effect of *ctn-1(lf)* may be rescued by expressing wild-type CTN-1 in muscle. We did not record action potentials from body-wall muscle cells because (1) it is difficult to maintain SLO-1 physiological activities using the classical whole-cell configuration due to dialysis of intracellular [Ca^{2+}] by the pipette solution, and (2) action potentials are difficult to record from body-wall muscle cells. Only one study has reported the observation of action potentials in body-wall muscle cells, and action potentials were observed in only two out of eight cells in that study (Jospin et al, JCB, 159: 337-348, 2002).

Comment: *Results. Figure 2. I did not find the apparent colocalization of BKIP-2 and SLO-1 to be convincing. I accept that they are broadly in similar cellular locations, but I did not see compelling evidence of colocalization in any tissue.*

Response: Figure 2 only shows the similar expression patterns of *slo-1* and *ctn-1*. The colocalization of SLO-1 and CTN-1 was shown in Figure 4D (Please see Figure 5F in the revised manuscript).

Comment: *Results. Figure 3. Is there any control for how much BKIP-2 is expressed? Obviously, if the protein is over-expressed then localization will be affected. In addition, the authors report that the inhibition of locomotion by SLO-1(gf) is not prevented by P-myo-3:BKIP-2. Would not this construct of BKIP-2 allow the protein to be expressed only in muscle? Therefore, why does it not reverse the effect of SLO-1(gf) and why do the authors state that it shows that the 'suppression of the SLO-1(gf) phenotype by BKIP-2(lf) result mainly from BKIP-2 deficiency in muscle cells.'?*

Response: These comments suggest that we did not make ourselves understood, for which we apologize. In Figure 3A, we showed that *slo-1(gf)* inhibited worm locomotion, which was reversed by *ctn-1(lf)*. Expression of wild-type CTN-1 in muscles in *slo-1(gf);ctn-1(lf)* double mutant reinstated the lethargic phenotype, whereas expression of CTN-1 in neurons had no effect. Obviously, suppression of the *slo-1(gf)* phenotype by *ctn-1(lf)* resulted mainly from *ctn-1* deficiency in muscle cells. The P*myo-3* is a commonly used promoter to drive gene expression specifically in *C. elegans* muscle cells. Over-expression of one protein does not necessarily mean that the localization of itself or another protein will be altered.

Comment: *Results. p. 8, para 1. I presume that data studying vinculin is not shown.*

Response: We thank the reviewer for pointing this out. We showed the vinculin data in Supplementary Figure S1 but forgot to cite it in the main body of the original manuscript. We have corrected this error and included the data in Supplementary Figure S3B in the revised manuscript.

Comment: *Results. Figure 5. Why is the antibody heavy chain showing as a band between 80 and 100 kDa, when it should be approximately 50 kDa?*

Response: In co-IP experiments, we incubated the protein samples at 37°C for 5 min before loading the gel, which resulted in dimerization of the antibody heavy chain (appeared as a band at around 90 kDa). If the samples were heated at 95°C for 5 min (standard procedure), the antibody heavy chain would only appear at 50 kDa. However, for unknown reasons, the SLO-1 band would disappear with this treatment.

Comment: *Results. Figure 7. The authors should also show the subcellular localization of SLO-1 in the dys-1 mutant.*

Response: This comment is identical to one from Reviewer #2. As described above, we were able to confirm SLO-1 mislocalization in a *dys-1(lf)* mutant (Figure 7).

Comment: *The authors showed that BKIP-2 interacts with the large intracellular portion of the SLO-1 channel, between the two RCK domains. This region also contains the calcium bowl that is critical for gating of the channel by calcium ions. Are the authors surprised that interaction of the two proteins does not affect function?*

Response: The Ca^{2+} bowl is located in the second RCK domain rather than in the linker region between the two RCK domains (Schreiber M and Salkoff L, Biophys J., 73:1355-63, 1997; Yuan et al., Science 2010, volume and page numbers are not yet available). Both the co-IP and BiFC experiments showed that the intracellular portion of SLO-1 interacted with CTN-1 (Figure 6A, 6B). We did not specify which portion of the SLO-1 carboxy-terminal domain interacted with CTN-1.

Comment: *The authors show that BKIP-2 and SLO-1 are present in both nerve and muscle, yet it only appears to affect subcellular location in muscle. Why? Is it possible that the low resolution approach used to show colocalization has missed an effect in neurons?*

Response: As shown in Figure 5A, the SLO-1 subcellular localization in the nerve ring was indistinguishable between the wild-type and *ctn-1* mutant, suggesting that *ctn-1* mutant does not affect SLO-1 localization in most neurons. This conclusion is consistent with the data of locomotion and head bending analyses. Perhaps neurons use a different mechanism to localize SLO-1, or the function of CTN-1 is redundant in neurons. However, we cannot exclude the possibility that CTN-1 may play an important role in SLO-1 localization in some neurons. A potentially useful approach to investigate the role of CNT-1 in the nervous system is to perform analyses with only one or a few neurons. As described in the response to a comment from Reviewer #2, we had tried to perform this kind of analyses but were unsuccessful.

Comment: *Where does SLO-1 get the calcium from for its activation in muscle? Could BKIP-2 be affecting SLO-1 location to fine-tune this requirement?*

Response: A recent study showed that SLO-1 is colocalized with the L-type calcium channel EGL-19 in the dense body areas (Kim et al, PLoS Genet, 5:e1000780, 2009), which raises the possibility that Ca^{2+} influx from EGL-19 may activate SLO-1 channels. We discussed about the potential physiological significance of CTN-1-mediated SLO-1 localization with regard to Ca^{2+} sources for SLO-1 activationin the revised manuscript (page 13)

2nd Editorial Decision 19 July 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-73738R to the EMBO Journal. It has now been seen again by the original referees 1 and 2, whose comments are enclosed below. As you will see, both referees find your manuscript to be substantially improved and are fully supportive of publication. However, referee 1 does still have a couple of remaining concerns. Most notably, he/she finds that your evidence that ctn-1 is specifically required for dense body localisation rather than plasma membrane trafficking remains somewhat weak. I have to say that I agree with his/her point that over-expression in HEK293 cells does not provide a good model for the system you are studying, and that any conclusions drawn from these experiments may not relate to the C. elegans situation. Minimally, I would therefore ask you to tone down your statements in this regard, and to make it clear that regulation of membrane trafficking does remain a possibility. If you are able to conduct any additional experiments to support your conclusion, then I would strongly encourage you to do so, but I do accept that you may have taken the analysis as far as you can at this point. Perhaps you can let me know whether you would be able to undertake the biotinylation experiment suggested by the referee, or whether there are other alternative approaches you could take. Referee 1 also has a couple of other minor points that I would ask you to follow, and to modify the manuscript accordingly.

I look forward to hearing from you with regard to this remaining experimental issue, and to receiving the revised version of your manuscript in due course.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I do find that this manuscript was significantly improved by the addition of new results and rewriting.
I do think, however, that it remains formally possible that CTN-1 regulates the trafficking of SLO-1 to the plasma membrane. Overexpression of SLO-1 in HEK293 cells is very different from the physiological conditions in muscle cells. First, is it known whether HEK cells do not endogenously express alpha-catulin? Second, even in the worms, the cellular requirements for SLO 1 expression seem very different between muscle and neuronal cells. The relevance of experiments carried out in transformed human kidney cells is low. Rigorous demonstration would require, for example, similar biotinylation experiments performed on primary muscle cells from WT and ctn-1 mutant. I would suggest to provide the HEK cell results as Supplementary data and make extremely clear in the abstract and in the text that CTN-1 might equally regulate SLO-1 trafficking unless more convincing data are generated.

Minor points:
I would recommend to include figure S1 data in one of the figures of the main text in order to provide a comprehensive set of information to the reader.
Evidence for the stability of SLO 1(1-370)::YFPa provided in response to my previous comments should be briefly mentioned in the text (as data not shown or providing reference to this other study).
If expression of CTN 1 in Xenopus oocytes was tested by western blot, it should be added to Fig S5.

2nd Revision - Authors' Response 19 July 2010

Comment *I do think, however, that it remains formally possible that CTN-1 regulates the trafficking of SLO-1 to the plasma membrane. Overexpression of SLO-1 in HEK293 cells is very different from the physiological conditions in muscle cells. First, is it known whether HEK cells do*

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Response Although our biotinylation assay showed that CTN-1 did not alter SLO-1 total and surface protein levels in transfected HEK293 cells, we agree with the reviewer that more rigorous analyses are needed to confirm that CTN-1 does not play a role in SLO-1 membrane trafficking *in vivo*. Therefore, as suggested by the reviewer, we moved Figure 6E to the supplement (now Figure S3), and acknowledged that a role of CTN-1 in SLO-1 membrane trafficking *in vivo* cannot be excluded based on this observation (page 10).

Comment *I would recommend to include figure S1 data in one of the figures of the main text in order to provide a comprehensive set of information to the reader.*

Response Figure S1 was moved to the main body of the manuscript as suggested (now Figure 3).

Comment *Evidence for the stability of SLO 1(1-370)::YFPa provided in response to my previous comments should be briefly mentioned in the text (as data not shown or providing reference to this other study).*

Response A description about the stability of SLO-1(1-370)::YFPa was added to the legend of Figure 7. (page 34)

Comment *If expression of CTN 1 in Xenopus oocytes was tested by western blot, it should be added to Fig S5.*

Response The western blot for CTN-1 expression in *Xenopus* oocytes was added to Figure S5 (panel C).

