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MTCL1 plays an essential role in maintaining Purkinje neuron axon initial segment

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1st Editorial Decision 26 September 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see, the referees find the analysis reporting a role for MTCL1 in MT organisation and AIS regulation interesting. They raise a number of concerns that are clearly outlined below. I will not repeat them all here but would like to highlight an issue that both referees raise namely that we need more experimental data to address if MTCL1 is important for AIS initiation or maintenance.

Given the referees' comments, I would like to invite you to submit a revised version addressing the comments raised in full. I should add that it is EMBO Journal policy to allow a single major round of revision only and that it is therefore important to resolve the raised issues at this stage.

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, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of 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.

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

REFEREE REPORTS

Referee #1:

In this manuscript, Satake et al. are using a large range of techniques and models to study the role of a microtubule (MT)-associated protein, MTCL1, in the organization of the AIS and proximal axon of Purkinje Cells (PCs) of the cerebellum. MTCL1 KO and conditional KO (cKO) mice are examined, as well as PCs where MTCL1 has been suppressed by shRNA after in utero electroporation (IUE). The authors first find a specific location of MTCL1 along stabilized MTs in the axon hillock and proximal axon at P7, that disappears in older animals. In KO and cKO mice, absence of MTCL1 causes defects of the AIS scaffold at P21 and after (shorter ankG concentration further from the soma), as well as a change in microtubule organization at the ultrastructural level. Using shRNA agains MTCL1 introduced by IUE, they observe a perturbation of the AIS morphology, with the apparition of spinules reminiscent of the structures observed in ankG KO PCs by Sobotzik et al. (PNAS 2009). They interpret this as resulting from a defect in proper maintenance of polarity by the AIS. The IUE technique allows them to perform rescue experiments, with a partial phenotype rescue by the WT MTCL1, and varying degrees of rescue by mutants lacking the MT-binding or -regulating domains.
Interestingly, deficits at the AIS are correlated to abnormal motor coordination. Finally, they identify a mutation in spinocerebellar ataxia patients that has a defective MT-stabilizing activity, and is slightly less capable of rescuing shRNA defects in MTCL1-knockdown PCs.

I found the manuscript very interesting. This subject of what organizes MTs into the axon, and the relation between this organization and the AIS scaffold has been a hot topic lately, with new MT partners identified (Nudel, CAMSAP2, TRIM46 from the Hoogenraad lab) and new models put forward (such as the pre-axonal exclusion zone, Farias et al. Cell Rep 2015). The role of MTCL1 would be a novel and meaningful addition to this subject, even if generalizing its role to all neurons will require additional scrutiny (see MP3 below). the range of different models used here (KO, cKO, IUE, EM...) is staggering, and make the detailed understanding of the work a significant task. Although there are differences between the results obtained with each models, overall the point of a role of MTCL1 in MT organization and AIS regulation is convincing. I have an issue with the main interpretation, i.e. the role of MTCL1 on the initial formation of the AIS upstream the recruitment of ankG (see MP2), and think this can be modified to better represent the data, or strengthened with additional data in younger animals.

Major point 1: temporal details of MTCL1 localization and effect
The KO and cKO mice demonstrate that MTCL1 is necessary for the maturation of the AIS, with an effect on AIS length and width (as measured by ankG labeling) occurring at P21 and after for the KO (Fig. 3E-F) and the cKO (Fig. 7C-D). However, MTCL1 presence along the stabilized MTs in the hillock and proximal AIS is only detected at P7 (Fig. 3A-C and Fig. 7A), with the ankG labeling being normal at that point (Fig. 3D-F, not shown for the cKO). The question is then, how come MTCL1 absence in the KO and cKO mice has no effect at the time when it is present at the hillock and AIS (P7), but has one when it is present in the somatodendritic compartment (>P21)?

Major point 2: proposed role of MTCL1 in AIS formation.
The main message of the manuscript is that MTCL1 has a MT-related function upstream of ankG accumulation for the formation of the AIS (stated numerous times in the text). AIS presence (ankG concentration) is reported as early as P2 in PCs (Jenkins et al. JCB 2001). However, there is no data in the present manuscript on the localization of ankG and MTCL1 before P7, a time when the AIS is already formed. Data at earlier times (before and after the reported P2 point where ankG is present at the AIS according to Jenkins et al.) would be useful here.
Regarding perturbation experiments, the data on KO and cKO mice show an effect long after AIS

formation (>P21, see above), which is more consistent with an effect of MTCL1 on AIS maintenance. There is no effect on ankG localization (Fig. 3E-F) and no effect on MT bundles (Fig. S4) in the KO mouse at P7. In the IUE shRNA experiments, the AIS is strongly affected or absent at the first time point shown, which is P21. However, an effect at P21 can also be related to maintenance and not formation (as is the case for the KO and cKO models). The effect of MTCL1 knockdown before P21, ideally as soon as P2, would be necessary to support a bona fide effect on AIS formation.
The proposed model (Fig. 7E) where MTCL1 has a role (yellow, center) between the initial organization of MTs by TRIM46 (pink, left) and the recruitment of ankG at the AIS (purple, right) is thus not supported by the available data. This model should either be modified to reflect the effect on AIS maturation maintenance, or data showing an effect on ankG initial concentration at the AIS (i.e. between P2 and P7) should be provided.

Major point 3: generalization of MTCL1 role
In the brain, MTCL1 is mainly expressed in the cerebellum (Fig. 1D), with a preferential expression in PCs (Fig. 3A). As MT bundling and AIS formation are common to virtually all neuronal types, how general is MTCL1 role for the proximal axon organization? Is there a related protein (like SOGA, cited in the Discussion) that could play the role of MTCL1 in brain regions where MTCL1 has a much lower expression? It would be interesting to have data on the AIS and morphology of the KO mouse in other regions (hippocampus or cortex), to directly assess if MTCL1 role is dependent on the higher expression in PCs.

Minor points

Introduction
The introduction does not cite several recent papers that have brought decisive advances on microtubule organization in the axon hillock and the AIS: Yau et al. Neuron 2014 on CAMSAP2, van Beuningen et al. Neuron 2015 on TRIM46, Kujipers et al. Neuron 2016 on Nudel. The introduction and results (for example the introduction for Fig. 5 on page 14) should take these into account, as well as recent work about the pre-axonal exclusion zone (PAEZ, Farias Cell Rep 2016) that is cited later on in the Discussion.

p.4 l.2: "Axonal identity is established by a special domain..." is ambiguous. Axon specification occurs before AIS assembly, and the AIS has more a role in maintaining axonal identity than establishing it.

p.5 l.12: "Nonetheless, the factors and mechanisms responsible for directing AnkG localization are largely unknown". Here it would be useful to cite Galiano et al. Cell 2012.

p.6 l.4: "Thus, formation of uniformly oriented and stabilized MT bundles is supposed to be a critical event for AIS formation". The arguments for a causal relationship, with MT bundling necessary for AIS formation are quite elusive: only a temporal sequence in Jones et al. JCB 2014 (but in an extracted preparation that collapses the whole structure on the cytoskeleton). By contrast, a reverse causal relationship, with ankG concentration at the AIS necessary for MT bundling is clearly demonstrated in Sobotzik et al. PNAS 2009). The causality should be more balanced here.

Results
p.11 l.1: It would be interesting to have the MTCL1 immunolabeling in parallel with the ankG and calbindin in FIg. 3D, to understand better the difference between MTCL presence at the hillock/proximal AIS (P7) and the timing of effect on AIS morphology (P21+).

p.14, l.1: The conclusion that MTCL1 has a role in ankG localization during AIS formation should be modified given that the effects are only reported at P21 (see MP2 above).

p.15, l.1: It would be interesting for the localization of MTCL1 and mutants (Fig. 5B) to be quantitatively analyzed (length, position) similar to what is done for the ankG labeling in Fig. 5C-D). How come the WT MTCL1 localizes at the hillock and AIS in P21 neurons (Fig. 5B), whereas endogenous MTCL1 is not localized in this way (Fig. 3A)? This should be discussed (an generally the issue with MTCL1 variation of localization with time and timing of effects, see MP1).

p.17 l.1: It is not really clear what is the nature of the MT perturbation that is seen at the EM level (Fig. 6D & Fig. S4). From what I understand, the MT bundles are still present in the KO mouse ("MTs extended from the axon hillock, gathered into bundles, and were then funneled into the AIS in both WT and GT cells"). The difference seem to be in the distal AIS, where MTs do not follow the plasma membrane in a longitudinal way as in the WT mouse. Given the shorter AIS in the GT mouse, this distal AIS region could be where ankG is downregulated/absent, and this disorganization of MTs close to the membrane could be due to ankG perturbation, rather than a direct effect of MTCL1 absence (given the interaction of ankG via EBs). Is it possible to closely examine the submembrane density (thought to be the ankyrin/spectrin scaffold) on the EM images and see if there is a disruption of it at the places of MT perturbation in the GT mouse?

Discussion
p.23 l.5: An example of statement about the role of MTCL1 in ankG localization and AIS formation (see MP2): "in the later stages, MTCL1 works in the stabilization of MT bundles to initiate AnkG localization at the proximal axon (Fig 7E)".

p.24 l.6: An interesting reference about the presence of MTs spanning from the Golgi to the axon entrance is Matsumura & Kohno Anat Embryol 1991, where such a microtubule population is described in goldfish PCs.

Referee #2:

The manuscript by Satake et al. reports that MTCL1 is found at axon initial segments (or at least near the AIS) during development where it participates in the AIS assembly. The findings are quite interesting and novel. They extend our understanding of the role of the microtubule cytoskeleton in AIS maintenance (note the dramatic loss of neuronal polarity and the establishment of VGLUT1-labeled spines in the AIS of mice lacking MTCL1 - Fig. 4D). Overall, the experiments are well done and convincing. There is mechanistic insight into the domains of MTCL1 that may be important for maintenance of the AIS. The potential relevance to human disease is also emphasized. I think the conceptual advance is high and this paper is appropriate for EMBO Journal. However, I have a few points that need to be addressed as they reduce my enthusiasm for this story as presented.

1. Throughout the paper the authors claim that MTCL1 'directs AIS formation.' For example, in the abstract it says, 'MTCL1-mediated formation of the stable MT bundles is crucial for AnkG localization.' I don't agree with this interpretation. As shown in Fig. 3D, AnkG is properly localized to the nascent AIS at 1 week of age. All of the phenotypes reported are consistent with a role for MTCL1 in maintaining the AIS, not in its initial assembly. I think the entire paper needs to be rewritten with this different interpretation. It is absolutely clear that AnkG can cluster at the AIS without MTCL1. However, over time its localization becomes disrupted and this is most consistent with a defect in maintenance of the AIS. The title, abstract, etc. should be revised to reflect a focus on maintenance rather than initial clustering and assembly of the AIS.
2. The authors have only examined the role of MTCL1 in Purkinje neurons. This paper would be of even broader interest if the authors would also examine the distribution and role of MTCL1 in other cell types... like excitatory pyramidal neurons in the cortex. If there is no effect on the structure of the AIS in those cells that is quite interesting and important. It would imply this is a specific role for MTCL1 in Purkinje neurons. They should look in the cortex and use cultured hippocampal neurons to examine the distribution and roles of MTCL1. Otherwise, they need to emphasize that this unique to Purkinje neurons.
3. Although the EM pictures are lovely (Fig 6), they do not adequately demonstrate the microtubule cross-linking that occurs in the AIS. Cross sections of the AIS are needed for this.

1st Revision - authors' response 21 December 2016

Responses to Reviewer #1:

1) *Major point 1: temporal details of MTCL1 localization and effect*
The KO and cKO mice demonstrate that MTCL1 is necessary for the maturation of the AIS, with an effect on AIS length and width (as measured by ankG labeling) occurring at P21 and after for the

KO (Fig. 3E-F) and the cKO (Fig. 7C-D). However, MTCL1 presence along the stabilized MTs in the hillock and proximal AIS is only detected at P7 (Fig. 3A-C and Fig. 7A), with the ankG labeling being normal at that point (Fig. 3D-F, not shown for the cKO). The question is then, how come MTCL1 absence in the KO and cKO mice has no effect at the time when it is present at the hillock and AIS (P7), but has one when it is present in the somatodendritic compartment (>P21)?

First of all, please see revised Fig. 5B, in which V5-MTCL1 ectopically expressed in Purkinje cells under suppression of endogenous MTCL1 was simultaneously stained with anti-V5-tag antibody and anti-MTCL1 antibody. As was shown in the previous Fig. 5B, V5-MTCL1 strongly accumulates in the hillock and proximal AIS of P21 Purkinje cells. Significantly, similar accumulation of V5-MTCL1 was not detected using anti-MTCL1 antibody (Fig. 5B), suggesting that the epitope for this antibody (central region of MTCL1), but not the N-terminal V5-tag, is masked in these regions at this stage. This suggests that MTCL1 continuously accumulates in the hillock and proximal AIS from P7 (and even at P21 or P28), but its detection is hampered at these stages. If this is the case, MTCL1 may function continuously after P7, and we can assume that AIS defects in KO mice are gradually induced after P7 due to lack of normal MTCL1.

The possibility that endogenous MTCL1 continuously localizes to the hillock and proximal AIS, ever after P7, was mentioned in the context of Fig. 3A, by referring to Fig. 5B (page 10, lines 8–14). In addition, we have included an enlarged view of P21 Purkinje cells (in Fig. 3C) to demonstrate, albeit weakly, that substantial localization of MTCL1 can be detected even using anti-MTCL1 antibody.

We have also mentioned in the revised manuscript that expression of the N-terminal MTCL1 fragment containing N-MTBD may partially compensate for lack of normal MTCL1 expression at P7 (see page 12, lines 11–13), as the fragment accumulates to the proximal AIS at P7, but not P21 (see Appendix Figure S1D and E).

With regards the cKO, retardation of AIS defects can be attributed to activation of the *Pcp2* promoter (used for Purkinje cell-specific knockout of MTCL1) around P7 (see page 18, line 13–18). This was shown in the previous manuscript by inclusion of Fig. 7A, which demonstrates that cKO mice retain MTCL1 expression at P7 but gradually lose it afterwards.

2) *Major point 2: proposed role of MTCL1 in AIS formation.*
The main message of the manuscript is that MTCL1 has a MT-related function upstream of ankG accumulation for the formation of the AIS (stated numerous times in the text). AIS presence (ankG concentration) is reported as early as P2 in PCs (Jenkins et al. JCB 2001). However, there is no data in the present manuscript on the localization of ankG and MTCL1 before P7, a time when the AIS is already formed. Data at earlier times (before and after the reported P2 point where ankG is present at the AIS according to Jenkins et al.) would be useful here.
Regarding perturbation experiments, the data on KO and cKO mice show an effect long after AIS formation (>P21, see above), which is more consistent with an effect of MTCL1 on AIS maintenance. There is no effect on ankG localization (Fig. 3E-F) and no effect on MT bundles (Fig. S4) in the KO mouse at P7. In the IUE shRNA experiments, the AIS is strongly affected or absent at the first time point shown, which is P21. However, an effect at P21 can also be related to maintenance and not formation (as is the case for the KO and cKO models). The effect of MTCL1 knockdown before P21, ideally as soon as P2, would be necessary to support a bona fide effect on AIS formation.
The proposed model (Fig. 7E) where MTCL1 has a role (yellow, center) between the initial organization of MTs by TRIM46 (pink, left) and the recruitment of ankG at the AIS (purple, right) is thus not supported by the available data. This model should either be modified to reflect the effect on AIS maturation maintenance, or data showing an effect on ankG initial concentration at the AIS (i.e. between P2 and P7) should be provided.

Thank you for highlighting this critical issue. We completely agree that our data do not necessarily indicate a role for MTCL1 in the initial stage of AIS development. To address this, we attempted to examine the AIS in MTCL1 knockdown Purkinje cells at P2, but unfortunately did not obtain sufficient data within the short period for revision. Therefore, we have chosen not to discuss this issue in the revised manuscript, and instead argue that MTCL1 plays an essential role in AIS maintenance. Accordingly, we have modified any sentences (including the title and running title)

suggesting that MTCL1 works in initial AIS formation upstream of AnkG recruitment (modified sentences are colored in red in the revised text). We have also added a sentence stating that our data only indicate a role for MTCL1 in maintaining the AIS, and its involvement in the initial stage should be clarified in future studies (see Discussion, page 22, line 14–18).

We have also altered the schematic model in Fig. 7E to correctly illustrate the role of MTCL1 in AIS maintenance.

3) *Major point 3: generalization of MTCL1 role*
In the brain, MTCL1 is mainly expressed in the cerebellum (Fig. 1D), with a preferential expression in PCs (Fig. 3A). As MT bundling and AIS formation are common to virtually all neuronal types, how general is MTCL1 role for the proximal axon organization? Is there a related protein (like SOGA, cited in the Discussion) that could play the role of MTCL1 in brain regions where MTCL1 has a much lower expression? It would be interesting to have data on the AIS and morphology of the KO mouse in other regions (hippocampus or cortex), to directly assess if MTCL1 role is dependent on the higher expression in PCs.

In accordance with the reviewer's suggestion, we investigated MTCL1 expression and AIS morphology in cortical and hippocampal neurons by comparing MTCL1 KO mice and their WT siblings at P21. In the cortex, MTCL1 was expressed in neurons in all layers (I–VI). Subsequently, we focused on neurons in layer II/III. Similar to Purkinje cells, MTCL1 was mainly distributed in cell bodies, and localized to the hillock and proximal AIS. We did not detect any obvious abnormalities in AIS morphology. Similarly, AIS morphology in hippocampal pyramidal neurons of KO mice was little affected, yet they also expressed MTCL1. These results suggest that MTCL1 mainly plays a role in AIS morphology in Purkinje cells. We have included these findings in Appendix Figure S5, and discuss them in the Discussion (page 24, line 7–15) as follows:

"MTCL1 is expressed not only in Purkinje cells, but also in other neuronal cells (Appendix Figure S5). Thus, it will be intriguing to determine whether MTCL1 plays a general role in AIS development in other neurons. From our examination, abnormalities of AnkG localization and AIS morphology were not detected in cortical and hippocampal neurons in GT mice at 3 weeks of age (Appendix Figure S5). This suggests that MTCL1 mainly plays a role in AIS morphology in Purkinje cells. The MTCL1 paralog (registered as SOGA) may compensate for MTCL1 dysfunction in other neuronal types. Alternatively, characteristic features of Purkinje cells (e.g., their size) may necessitate stronger support from stabilized MTs to stably maintain the AIS."

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4) *Minor points*
> Introduction
The introduction does not cite several recent papers that have brought decisive advances on microtubule organization in the axon hillock and the AIS: Yau et al. Neuron 2014 on CAMSAP2, van Beuningen et al. Neuron 2015 on TRIM46, Kujipers et al. Neuron 2016 on Nudel. The introduction and results (for example the introduction for Fig. 5 on page 14) should take these into account, as well as recent work about the pre-axonal exclusion zone (PAEZ, Farias Cell Rep 2016) that is cited later on in the Discussion.

We have cited all the suggested papers in the Introduction (page 6, lines 9–10) and Results (page 14, line 18).

> p.4 l.2: "Axonal identity is established by a special domain..." is ambiguous. Axon specification occurs before AIS assembly, and the AIS has more a role in maintaining axonal identity than establishing it.

In accordance with the reviewer's suggestion, we have changed the verb in the sentence from "established" to "maintained" (page 4, lines 4–5).

> p.5 l.12: "Nonetheless, the factors and mechanisms responsible for directing AnkG localization are largely unknown". Here it would be useful to cite Galiano et al. Cell 2012.

We have cited the suggested reference. (page 5, line 13)

> p.6 l.4: "Thus, formation of uniformly oriented and stabilized MT bundles is supposed to be a critical event for AIS formation". The arguments for a causal relationship, with MT bundling necessary for AIS formation are quite elusive: only a temporal sequence in Jones et al. JCB 2014 (but in an extracted preparation that collapses the whole structure on the cytoskeleton). By contrast, a reverse causal relationship, with ankG concentration at the AIS necessary for MT bundling is clearly demonstrated in Sobotzik et al. PNAS 2009). The causality should be more balanced here.

Based on our understanding, we do not feel that the paper by Sobotzik *et al*. (PNAS 2009) demonstrates that AnkG concentration at the AIS is necessary for MT bundling. Instead, it reports on the absence of AIS MT bundles in Purkinje cells of adult mice lacking AnkG. However, because we agree with Reviewer #1's notion that the causality should be more balanced, we have altered our description on causality by changing "is supposed to" to "might be" in the relevant sentence. (page 6, lines 5–6)

> Results
p.11 l.1: It would be interesting to have the MTCL1 immunolabeling in parallel with the ankG and calbindin in FIg. 3D, to understand better the difference between MTCL presence at the hillock/proximal AIS (P7) and the timing of effect on AIS morphology (P21+).

As mentioned above, we have included MTCL1 immunostaining data in parallel with AnkG and calbindin for P21 Purkinje cells only (see Fig. 3C), as immunostaining of endogenous MTCL1 at the hillock and proximal AIS is complicated.

> p.14, l.1: The conclusion that MTCL1 has a role in ankG localization during AIS formation should be modified given that the effects are only reported at P21 (see MP2 above).

In accordance with the suggestion, we have changed the sentence as follows (page 14, lines 10–12):

"**MTCL1 plays an essential role in maintaining AIS structure and function in developing Purkinje cells by regulating AnkG localization to the AIS region.**"

> p.15, l.1: It would be interesting for the localization of MTCL1 and mutants (Fig. 5B) to be quantitatively analyzed (length, position) similar to what is done for the ankG labeling in Fig. 5C-D). How come the WT MTCL1 localizes at the hillock and AIS in P21 neurons (Fig. 5B), whereas endogenous MTCL1 is not localized in this way (Fig. 3A)? This should be discussed (an generally the issue with MTCL1 variation of localization with time and timing of effects, see MP1).

For discrepant localization of endogenous and exogenous MTCL1, see the response to MP1. We have discussed this issue again in the context of Fig. 5B as follows (page 16, lines 1–7):

"As already mentioned, localization of V5-MTCL1-WT to the hillock and proximal part of the AIS region was clearly detected when using anti-V5 antibody, but not anti-MTCL1 antibody, whose epitope is located at the central region of MTCL1 (Fig. 5B; Satake and Suzuki, our unpublished observations). This may imply that not only both MTBDs, but also the central region (including the Par-1b binding region) (Fig. 1B), is involved in MTCL1 localization to the hillock and proximal AIS."

As for the suggested experiments, it would certainly be interesting to quantitatively analyze localization of ectopically-expressed MTCL1 WT and mutants. However, unfortunately, we did not have sufficient time to perform such experiments, and instead will endeavor to address this in our future studies.

> p.17 l.1: It is not really clear what is the nature of the MT perturbation that is seen at the EM level (Fig. 6D & Fig. S4). From what I understand, the MT bundles are still present in the KO mouse ("MTs extended from the axon hillock, gathered into bundles, and were then funneled into the AIS in both WT and GT cells"). The difference seem to be in the distal AIS, where MTs do not follow the plasma membrane in a longitudinal way as in the WT mouse. Given the shorter AIS in the GT mouse, this distal AIS region could be where ankG is downregulated/absent, and this

disorganization of MTs close to the membrane could be due to ankG perturbation, rather than a direct effect of MTCL1 absence (given the interaction of ankG via EBs). Is it possible to closely examine the submembrane density (thought to be the ankyrin/spectrin scaffold) on the EM images and see if there is a disruption of it at the places of MT perturbation in the GT mouse?

We included EM data in the present paper to support our immunofluorescence data at the ultrastructural level, and not to extract anything highly significant on its own. Although we think that the suggested experiments are interesting, they are not sufficient to identify causality between AnkG perturbation and MT perturbation (as discussed above in the paper by Sobotzik *et al*.). Given the above, we sought to address Reviewer #1's comment by modifying the text to clearly describe the aim of our EM analysis (see page 17, lines 15–18).

> *Discussion*
p.23 l.5: An example of statement about the role of MTCL1 in ankG localization and AIS formation (see MP2): "in the later stages, MTCL1 works in the stabilization of MT bundles to initiate AnkG localization at the proximal axon (Fig 7E)".

We have changed the sentence as follows (page 24, lines 3–6):

"TRIM46 works first in formation of uniformly orientated MT arrays for axon specification **and subsequent initiation of AIS formation**, while in the later stages, MTCL1 works in stabilization of MT bundles to **maintain** AnkG localization at the proximal axon (Fig. 7E)".

> *p.24 l.6: An interesting reference about the presence of MTs spanning from the Golgi to the axon entrance is Matsumura & Kohno Anat Embryol 1991, where such a microtubule population is described in goldfish PCs.*

We have added a sentence and cited the paper as follows (page 25, lines 15–17):

"Interestingly, it has been shown that the cross-linked MT bundles are extended from Golgi area of cell body to the AIS in goldfish Purkinje cells (Matsumura & Kohno, 1991)."

Responses to Reviewer #2:

1) *Throughout the paper the authors claim that MTCL1 'directs AIS formation.' For example, in the abstract it says, 'MTCL1-mediated formation of the stable MT bundles is crucial for AnkG localization.' I don't agree with this interpretation. As shown in Fig. 3D, AnkG is properly localized to the nascent AIS at 1 week of age. All of the phenotypes reported are consistent with a role for MTCL1 in maintaining the AIS, not in its initial assembly. I think the entire paper needs to be rewritten with this different interpretation. It is absolutely clear that AnkG can cluster at the AIS without MTCL1. However, over time its localization becomes disrupted and this is most consistent with a defect in maintenance of the AIS. The title, abstract, etc. should be revised to reflect a focus on maintenance rather than initial clustering and assembly of the AIS.*

Thank you for highlighting this critical issue. We completely agree with the comment that our data do not necessarily indicate a role for MTCL1 in the initial stage of AIS development. To directly interpret our present data, we have modified any sentences in the text (including the paper title) suggesting that MTCL1 works in initial AIS formation upstream of AnkG recruitment (modified sentences are colored red in the revised manuscript). We have also included sentences in the Discussion stating that our data only indicate a role for MTCL1 in maintaining the AIS as follows (page 22, lines 14–18):

"In mice, AnkG localization to the proximal axon is observed in the majority of Purkinje cells (82.7 %) two days after birth (Satake and Suzuki, our unpublished observations). As we did not examine the effect of MTCL1 depletion at this stage, our present results do not show an indispensable role for MTCL1 in initial targeting of AnkG to the proximal axon. The role of MTCL1 at this stage should be clarified in future studies (Fig 7E)."

In addition, we have changed the schematic model in Fig. 7 to correctly illustrate the role of MTCL1 in AIS maintenance.

2) *The authors have only examined the role of MTCL1 in Purkinje neurons. This paper would be of even broader interest if the authors would also examine the distribution and role of MTCL1 in other cell types... like excitatory pyramidal neurons in the cortex. If there is no effect on the structure of the AIS in those cells that is quite interesting and important. It would imply this is a specific role for MTCL1 in Purkinje neurons. They should look in the cortex and use cultured hippocampal neurons to examine the distribution and roles of MTCL1. Otherwise, they need to emphasize that this unique to Purkinje neurons.*

In accordance with the reviewer's suggestion, we investigated MTCL1 expression and AIS morphology in cortical and hippocampal neurons by comparing MTCL1 KO mice and their WT siblings at P21. In the cortex, MTCL1 was expressed in neurons in all layers (I–VI). Subsequently, we focused on neurons in layer II/III. Similar to Purkinje cells, MTCL1 was mainly distributed in cell bodies, and localized to the hillock and proximal AIS. We did not detect any obvious abnormalities in AIS morphology. Similarly, AIS morphology in hippocampal pyramidal neurons of KO mice was little affected, yet they also expressed MTCL1. These results suggest that MTCL1 mainly plays a role in AIS morphology in Purkinje cells. We have included these findings in Appendix Figure S5, and discuss them in the Discussion as follows (page 24, line 7–15):

"MTCL1 is expressed not only in Purkinje cells, but also in other neuronal cells (Appendix Figure S5). Thus, it will be intriguing to determine whether MTCL1 plays a general role in AIS development in other neurons. From our examination, abnormalities of AnkG localization and AIS morphology were not detected in cortical and hippocampal neurons in GT mice at 3 weeks of age (Appendix Figure S5). This suggests that MTCL1 mainly plays a role in AIS morphology in Purkinje cells. The MTCL1 paralog (registered as SOGA) may compensate for MTCL1 dysfunction in other neuronal types. Alternatively, characteristic features of Purkinje cells (e.g., their size) may necessitate stronger support from stabilized MTs to stably maintain the AIS."

3) *Although the EM pictures are lovely (Fig 6), they do not adequately demonstrate the microtubule cross-linking that occurs in the AIS. Cross sections of the AIS are needed for this.*

We believe that the recommended analysis is outside the scope of our present study, as we do not argue that MTCL1 is a MT crosslinking protein for AIS MT bundles. Rather, we propose that MTCL1 critically affects AIS MT bundles via MT stabilizing activity attributable to its C-terminal MT-binding domain. This is based on the fact that MT stabilization is frequently coupled with formation of tight MT bundles without any MT cross-linking activities (see Chapin *et al*., 1991; MacRae, 1992). We have attempted to more clearly convey this idea by modifying the appropriate part of the Discussion (page 21, lines 13–18, and page 22, lines 1–4).

2nd Editorial Decision 30 January 2017

Thank you for submitting your revised manuscript to The EMBO journal. Your study has now been seen by the two referees and their comments are provided below.

As you can see, both referees appreciate that the analysis has been strengthened and are supportive of publication here. There are just a few issues that need to be sorted out. Referee #1 has some relative minor suggestions while referee #2 has some more significant text ones. I agree with referee #2 that some of the statements concerning the function of MTCL in AIS development and ankG localization need to be toned down. Please take a look at the specific comments and make sure that you have a balanced presentation of the findings.

When you submit the revised version would you also upload

- a synopsis of the paper (for examples please see our website http://emboj.embopress.org/). The manuscript should have a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels). If it becomes too much work I can also choose an image from the paper.

REFEREE REPORTS

Referee #1:

In this revised manuscript, Satake et al. have addressed the major and minor points of my referee report. With the refocused message on AIS maintenance, I think the data is compellingly supporting the conclusions. The additional data on neurons from the hippocampus and cortex is interesting, as it show a clear AIS concentration of MTCL1, but no obvious effect of MTCL1 in the GT mouse. As suggested by the authors, this emphasizes a potentially specific molecular organization of the AIS in PCs, which is an intriguing prospect for future studies. I'd have been very interested in more data on younger animals (P2-P7), but I agree that this will better be addressed in follow-up studies, given the significant amount of work already present in this manuscript.

Thus, I think this manuscript is suitable for publication in the EMBO Journal. I only have two small suggestions at this point:
- It is not very clear how the ankG and MTCL1 staining overlap in the lower-magnification images of Appendix Figure 5 (labeling in the cortex and hippocampus). A color overlay of ankG and MTCL1 would be very helpful here.
- In several instances the authors refer to microtubules as "MT filaments" ie "microtubule filaments". I think "microtubules" are the filaments themselves, ie "microtubules" are "tubulin filaments" in the same way "microfilaments" (as they used to be called) are "actin filaments". So I think "MT filaments" should be replaced simply by "MTs".

Referee #2:

I have carefully read the authors responses and revised manuscript. While several of my concerns have been addressed, the revision to address my major concern has not. The major concern is in the interpretation of what MTCL1 is doing. The manuscript still implies MANY places that MTCL1 is involved in the developing AIS and participates in the localization of ankG. There is no evidence to support these statements. The authors must revise the whole manuscript to carefully remove these statements. For example:

Abstract: "These results indicate that MTCL1-mediated formation of stable MT bundles is crucial for AnkG localization." This should be revised to state "crucial for maintenance of ankG localization."

Intro: "Nonetheless, the factors and mechanisms responsible for directing AnkG localization are largely unknown (Galiano et al, 2012)." The authors simply add the reference without any explanation - their response should be more nuanced. In fact Galiano shows that AnkG localization depends on the sub membranous spectrin cytoskeleton. So the authors need to be more thorough and nuanced in their introduction.

"Subsequent analysis revealed that MTCL1 is essential for postnatal development of AIS in Purkinje cells." No, maintenance.

"By performing in utero electroporation knockdown and rescue experiments, we also show that MTCL1 plays an indispensable role in AnkG localization by mediating AIS MT bundle formation." No evidence it plays a role in ankG localization. In fact the data presented in Fig. 3E shows the opposite. AnkG is localized just fine without MTCL1.

Results: "...developing Purkinje cells by regulating AnkG localization to the AIS region." No evidence MTCL1 regulates AnkG localization. Only evidence is that it maintains it.

Everywhere it says that MTCL1 affects postnatal AIS development. This is not shown. What is shown is that MTCL1 affects maintenance of the AIS. There is no causal relation between MTCL1 and AnkG targeting or regulating AIS development.

Discussion: "how does the MTCL1-mediated formation of stabilized MT bundles facilitate AnkG localization to the proximal axon?" No evidence that it does. It plays a role in maintenance.

The authors need to revise their manuscript accordingly.

Finally, the title of the paper should be revised to state that MTCL1's function at the AIS is limited to Purkinje neurons: "MTCL1 plays an essential role in maintaining Purkinje neuron axon initial segments."

2nd Revision - authors' response 07 February 2017

Responses to Referee #1:
1) *It is not very clear how the ankG and MTCL1 staining overlap in the lower-magnification images of Appendix Figure 5 (labeling in the cortex and hippocampus). A color overlay of ankG and MTCL1 would be very helpful here.*

We have added overlay images of AnkG and MTCL1 staining in Appendix Figure 5.

2) *In several instances the authors refer to microtubules as "MT filaments" ie "microtubule filaments". I think "microtubules" are the filaments themselves, ie "microtubules" are "tubulin filaments" in the same way "microfilaments" (as they used to be called) are "actin filaments". So I think "MT filaments" should be replaced simply by "MTs".*

Thank you for your correction. We have changed “MT filaments” into “MTs”.

Responses to Referee #2:
1) *I have carefully read the authors responses and revised manuscript. While several of my concerns have been addressed, the revision to address my major concern has not. The major concern is in the interpretation of what MTCL1 is doing. The manuscript still implies MANY places that MTCL1 is involved in the developing AIS and participates in the localization of ankG. There is no evidence to support these statements. The authors must revise the whole manuscript to carefully remove these statements.*

As shown in Figure 3, the length and width of AnkG-positive region progressively became smaller, reaching a plateau around 3 weeks in WT, indicating that the AIS structure undergoes a developmental change after initial targeting of AnkG. Since this developmental change of the AIS was disturbed in MTCL1 GT mice, we believe that we can argue that “MTCL1 is involved in the developing AIS”. On the other hand, Fig.4 revealed that acute depletion of MTCL1 by in utero electroporation knockdown resulted in the dissociation of AnkG from proximal axon. This clearly indicates that “MTCL1 participates in the localization of AnkG” even if this participation occurs not in the initial targeting of AnkG but in the maintenance and developmental phase of its localization. Therefore, we did not agree with the referee’s comment to remove these statements.

2) *Abstract: "These results indicate that MTCL1-mediated formation of stable MT bundles is crucial for AnkG localization." This should be revised to state "crucial for maintenance of ankG localization."*

In accordance with the referee’s suggestion, we have changed the sentence as follows.

“These results indicate that MTCL1-mediated formation of stable MT bundles is crucial for maintenance of AnkG localization during AIS development.”

3) *Intro: "Nonetheless, the factors and mechanisms responsible for directing AnkG localization are largely unknown (Galiano et al, 2012)." The authors simply add the reference without any explanation - their response should be more nuanced. In fact Galiano shows that AnkG localization depends on the sub membranous spectrin cytoskeleton. So the authors need to be more thorough and nuanced in their introduction.*

In accordance with the referee's suggestion, we added the explanation of the reference by changing the sentence as follows.

"Galiano et al. demonstrated that AnkG localization at the proximal axon is restricted by the distal axonal cytoskeleton comprising AnkB, aII- and bII-Spectrin (Galiano et al, 2012). Nonetheless, the factors and mechanisms responsible for directing and maintaining AnkG localization are largely unknown."

4) *Intro: "Subsequent analysis revealed that MTCL1 is essential for postnatal development of AIS in Purkinje cells." No, maintenance.*

Because of the reason stated above, we left the sentence unchanged.

5) *Intro: "By performing in utero electroporation knockdown and rescue experiments, we also show that MTCL1 plays an indispensable role in AnkG localization by mediating AIS MT bundle formation." No evidence it plays a role in ankG localization. In fact the data presented in Fig. 3E shows the opposite. AnkG is localized just fine without MTCL1.*

We changed the above sentence as follows to response the referee's comment.

"By performing in utero electroporation knockdown and rescue experiments, we also show that MTCL1 plays an indispensable role in maintenance of AnkG localization by mediating AIS MT bundle formation."

6) *Results: "...developing Purkinje cells by regulating AnkG localization to the AIS region." No evidence MTCL1 regulates AnkG localization. Only evidence is that it maintains it.*

As shown in Fig.4B, MTCL1 knockdown frequently disrupts AnkG localization at the AIS region. We believe that we can conclude a role of MTCL1 in regulating AnkG localization from this result, even if MTCL1 exerts this function after initial targeting of AnkG. Therefore, we left the sentence unchanged.

7) *Everywhere it says that MTCL1 affects postnatal AIS development. This is not shown. What is shown is that MTCL1 affects maintenance of the AIS. There is no causal relation between MTCL1 and AnkG targeting or regulating AIS development.*

Because of the reason stated above, we do not agree with this comment of the referee #2.

8) *Discussion: "how does the MTCL1-mediated formation of stabilized MT bundles facilitate AnkG localization to the proximal axon?" No evidence that it does. It plays a role in maintenance.*

In accordance with the referee's suggestion, we changed the sentence as follows.

"how does the MTCL1-mediated formation of stabilized MT bundles maintain AnkG localization to the proximal axon?"

9) *Finally, the title of the paper should be revised to state that MTCL1's function at the AIS is limited to Purkinje neurons: "MTCL1 plays an essential role in maintaining Purkinje neuron axon initial segments."*

We changed the title as the referee suggested.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Atsushi Suzuki
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-95630

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to determine sample size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was chosen based on the minimum number of animals needed to obtain statistical power.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded. Mice were choosen according to their genotype only.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Litters coming from different parents were used in all experiments.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was applied.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Mice were monitored for ataxia by two or three persons.
5. For every figure, are statistical tests justified as appropriate?	Yes. See every figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Normal distribution was assessed by histograms and normal plots.
Is there an estimate of variation within each group of data?	Yes. We tested variance within each group of data by F test.
Is the variance similar between the groups that are being statistically compared?	Yes. In case variance was similar between the groups, student t-test was used.

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers and/or clone numbers of all antibodies used in this study are listed in the Materials and Methods section (Page 29) and Appendix Supplementary Methods section (Page 14)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lline used in this study is the HeLa-K cells (See Appendix Supplementary Methods section, Page 21)

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Details are stated in the materials and methods section (page 28) and Appendix Supplementary Methods (page 10-11)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All mice used in this study were maintained and handled in accordance with the Institutional Animal Care and Use Committees at Yokohama City University, Medical Life Science and RIKEN Kobe Branch (see page 28).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirmed the compliance to ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Experimental protocols were approved by the Institutional review board of Yokohama City University School of Medicine (see page 31).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Written informed consent was obtained from all the patients or their parents (see page 31). Experiments were conformed to the principles set out in the WMA Declaration of Helsinki and the Dept. of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA