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PAR-1 Promotes Microtubule Breakdown During Dendrite Pruning in *Drosophila*

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(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 November 2016

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

As you can see from the comments the referees find the analysis interesting. However, they also find that further work is needed to support the key conclusions. In addition they also find that the manuscript contains too many parallel stories that are not always well integrated or sufficiently conclusive to stand on their own. Referee #2 suggest to remove the last part of the manuscript "Microtubule disruption affects endocytosis and dendrite thinning" and to focus the revisions on strengthening the other parts. I think this is a very suggestion - this will also help to streamline the manuscript.

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

Let me know if we need to discuss any specifics further

Please note that when preparing your letter of response to the referees' comments 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

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

revision.

REFEREE REPORTS

Referee #1:

In this manuscript, Herzmann and colleagues focus on an important and not well understood question in biology which is how microtubule dynamics affects neuronal remodeling. It is well established that MT disassembly is the first observable step in remodeling of axons and dendrites in both vertebrates and invertebrates. However, whether this disassembly is required for pruning and how it is regulated is not well understood. In this study, Herzmann et al first identify the Par-1 kinase as required for the normal progress of dendrite pruning in the Drosophila da sensory neurons. They propose that Par-1 functions, at least in part, by regulating the stability of the MT stabilization protein Tau. Next they continue to probe MT related proteins such as kinesins and to this end find two motors (khc and klp64d) that are required for normal dendrite pruning. Then they link between the kinesins to the uniform organization of MT, which is plus end facing the soma in dendrites. They make the observation that in the khc mutant the orientation of the MT is less uniform and further propose that this mixed orientation might be the cause for slower MT disassembly and pruning. Next (although not really presented in this order), the authors link between Par-1 mediated dendrite thinning and cytoskeletal stability as well as Ca transients. Finally, the authors also test another hypothesis as for the role of khc during pruning, which is the trafficking of golgi outposts.
All in all, I find that each piece of data is interesting and largely supports the statements provided in the text (although not always). My strongest reservation is that this paper seems like a combination of at least three (interesting but incomplete) projects. The most developed project, which could relatively quickly be upgraded to a complete story, is the Par-1-Tau story (see specific comments for suggestions on how). The second and third stories are the roles of kinesins - from keeping the dendrite orientation uniform to trafficking of golgi-outposts. While such a descriptive study (encompassing all three stories) is of interest, I strongly urge the authors to focus on one, make it complete with deep mechanistic insights, and publish as a smaller (but stronger) story.

Specific comments:

1) The most important point is, as stated above, the fact that this study includes many descriptive findings, not totally relevant to each other and mechanistic insights are largely missing. Here are a few suggestions to deepen the Par-1/Tau story:

a) If indeed the major role for Par-1 is to phosphorylate Tau and by this cause its dissociation from the MTs, then overexpression of Tau should inhibit pruning by itself. The data in Fig 3G, which is consistent with this hypothesis, shows that overexpressing Tau exacerbates the Par1 RNAi phenotype. While this is nice, it would be very interesting to do a much more serious attempt to find the Par-1 phosphorylation site (in vitro and in vivo) and show that over expressing Tau that cannot be phosphorylated by Par-1 inhibits pruning even more. Likewise - in the experiments where overexpression of Tau did NOT inhibit pruning by itself - what is the state of MTs?

b)Along the same lines, and as discussed by the authors themselves, it would make sense to try and dissect the relationship between Tau/Par1 and Kat60L.

c) Finally, a new study from the Misgeld lab has shown that Spastin is required for the MT severing and disassembly in NMJ branch pruning. One important aspect of their paper is the observation of interesting MT modifications enrichment in the loosing branch - but it remains completely open as how and whether these modifications regulate Spastin recruitment, activity and MT disassembly. While I think that including Spastin in this study might be beyond its scope - testing a few antibodies of different MT modifications and see if this changed in Par1 mutants should provide complimentary data in a relatively short time.

2) There are several cases in which I feel statements, especially those attempting to indicate casualty, are over interpreting. A few examples:

a) "Conversely, removal of one copy of tau with the small deficiency tauMR22 (Doerflinger et al.,

2003) led to a significant suppression of the par- 1 RNAi phenotype (Figure 3 G), indicating that Tau is a target for PAR-1 during c4da neuron dendrite pruning" - on one hand, the authors showed that Par1 CAN phosphorylate Tau (using purified proteins, which is even less biologically relevant than in vitro and obviously in vivo). But to say that the fact that reducing the Tau levels by half, which led to a suppression of the par1 RNAi phenotype means that this INDICATES that Tau is a target for par... - this is over statement. Doing some experiments with Tau mutants should clear this up, hopefully.

b)"However, overexpression of tauHA S184A did not cause dendrite pruning defects and ...indicating that dTau S184 phosphorylation is not required for dendrite pruning" - how can a negative result using a transgene indicate that a specific phosphorylation on Tau is not required for dendrite pruning? I would say that expressing TauS184 is not sufficient to inhibit pruning.

c) "Expression of par-1 RNAi completely abrogated Ca2+ transients (Figure 8 F), indicating that PAR-1 is required for dendrite thinning formation" - the authors are using the transients as a proxy for thinning - I think that while this is likely, I don't think its a certainty.

d) "Thus, dendrite branch points might have less stable microtubules than dendrite shafts. PAR-1 and Kat60-L1 are mostly localized to the soma (Figure EV7)." - this is misleading as these are transgenes, not endogenous proteins so what does it really mean

e) There are a lot of instances where mechanistic interpretations are superimposed on vague "genetic interactions" - what does genetic interaction even mean? I am not sure one can deduce something mechanistic from these experiments.

3) Quantifications: First, it is unclear based on the figures themselves what you are quantifying - % of unpruned dendrites or % of brains that contain unpruned dendrites - I know it is the latter but this is confusing. One caveat with this quantification is that it does not really measure the SEVERITY of the phenotype but rather the PENETRANCE or EXPRESSIVITY - is there any way one could directly measure the severity? By, for example, actually counting the number of remaining branches in combination with the total length of unpruned neurites?

4) In some cases - khc mutant, for example - there are defects in the dendritic arbor even at L3 - does that affect the pruning? How can one exclude that?

Minor comments
1) when discussing the different da neurons, it would be advisable to keep a consistent nomenclature between the text (where its "the class 1 da ") and the figure (ddaD/ddaE).
2) EV6E - lacks a Y axis
3) Order of EV figures is confusing.

To summarize - I think this is an interesting paper with solid data and a lot of potential. I would focus on one story, beef it up with a few mechanistic experiments and modify the text to carefully reflect the data but no more than that.

Referee #2:

In the manuscript "PAR-1 and Kinesins Promote Directional Microtubule Breakdown During Dendrite Pruning in Drosophila" Herzmann et al. generate a satisfying model for how microtubule polarity and microtubule destabilization can together promote proximal dendrite clipping from the cell body. They also identify par-1, tau and two kinesins as new regulators of pruning. Overall the story is an important contribution to understanding controlled disassembly of dendrites. There are, however, some key points that need to be resolved. The most important of these is the last set of conclusions in the section "Microtubule disruption affects endocytosis and dendrite thinning." The connection of microtubules to Ca transients and endocytosis is based solely on manipulation of par-1. While par-1 is important for microtubule regulation, it is a kinase with many substrates and so it is not clear whether a microtubule regulator is the key substrate that alters Ca transients or endocytosis during pruning. To really demonstrate that it is the effect of par-1 on microtubules that

is important here would require a lot more experiments. I therefore recommend removing this section and shoring up some of the data in the core part of the story.

Specific points that need to be addressed:

1. The effect of Par-1 loss on microtubules in uninjured neurons is not described. Because the authors later demonstrate that changes in MT polarity reduce pruning it is important to determine whether Par-1 reduction results in changes in microtubule polarity. If it does this could contribute to the pruning defect.

2. For RNAi control experiments is a control RNAi included? This transgene number matching is particularly important for double RNAi experiments like those in Figure 5 as inclusion of additional Gal4 driven transgenes can dilute the effect of each, and so phenotypes can be reduced simply by inclusion of another RNA hairpin rather than any specific effect of target knockdown. It is therefore essential to have identical Gal4-driven transgene numbers in these experiments, so for example par-1 RNAi cannot be compared to par-1 RNAi + ens RNAi. Instead the comparison should be par-1 RNAi + control RNAi compared to par-1 RNAi + ens RNAi. The same is true for RNAi + other transgene experiments like those in Figure 3. An alternate explanation for the effect of tau expression here is that it simply reduces expression of the par-1 RNAi by competing for Gal4.

3. The data that microtubule markers leave the proximal dendrite before other markers or disassembly is not very convincing. For example, In Figure 2 GFP-td-tomato and GFP-a-tubulin are both dramatically reduced at the proximal dendrite region and there is no quantitation of ratios of fluorescence intensity in proximal vs distal dendrites to back this up. The genetic data is much more convincing than any images where it is suggested MTs disappear before the dendrite is completely broken down. It might be helpful to include more detailed images of microtubule vs other marker loss.

4. They have a lot of genetic evidence of the interaction between Par-1 and Tau. And they know in Drosophila Par-1 can phosphorylate Tau. However the identification of the phosphorylation site is not possible, and the authors description of this is a little hedged. Do they think there is a different phosphorylation site? I think it would be helpful to just be a bit more direct in the wording here.

5. % Attached dendrites 18hpf is shown for par-1 RNAin in Figures 1 and 5F, but the numbers are quite different. Can the reason for this be made clear in the figures and text? Is an additional control RNAi included in Fig 5?

6. Strong pruning defects result from Khc RNAi. But they didn't show the polarity data from this RNAi. Instead they show the polarity data from khc mutant, it would be good to include both. The sample sizes are very small for the polarity data in Figure 6. They also try to figure out why kinesin-1 has a much stronger pruning phenotype that kinesin-2. An important part of this argument is looking at MT polarity in kinesin-2 knockdown in class IV neurons. Is the polarity phenotype just much weaker with reduction of kinesin-2?

7. Overexpressed man-II is used a Golgi marker in Figure 7, but it is clearly visible in the axon where no Golgi is thought to be present. If the authors want to argue that this marker specifically labels the Golgi then they need to address this. Another interpretation is that it labels the Golgi + other membranes when overexpressed. This could be addressed with careful wording or use of additional markers or staining of endogenous Golgi.

8. The title does not reflect the essential points of the model. It sounds like kinesins are directly involved in pruning, while really the authors suggest correct MT polarity is the key. Could the title be reworked to reflect this.

Referee #3:

In this paper, the authors found that kinase PAR-1 is required for pruning in Drosophila. sensory neurons. By excluding that PAR-1 regulates pruning via its activity in the known PAR-1 polarity pathway or ecdysone signaling, they hypothesize that PAR-1 might regulate MT disassembly during

dendrite pruning. The authors show that in PAR-1 RNAi c4da neurons which fail to prune GFP-tubulin stays, while it disappears in proximal dendrites in the control neurons. The authors show that PAR1 phosphorylates tau and provide evidence for a genetic interaction between PAR1 and tau. Based on these results the authors claim that that tau is a target of PAR1 and PAR-1 regulates pruning by inactivation of tau.

In addition to PAR-1, they identify the uniform plus-end in MT orientation in dendrites to be crucial for pruning. In a search specific for MT regulating factors in pruning they found kinesin-1 and kinesin-2 to be required for dendrite pruning. Interestingly they can show that not only kinesin-2, but also kinesin-1 is essential for uniform MT orientation and suggest that this uniform orientation may facilitate the proximal-to-distal MT disassembly as initiation of dendrite pruning. By showing genetic interactions of PAR-1 and kinesin1 and PAR-1 and endocytosis factors, a sequential model of the cellular mechanisms in pruning is proposed.

In sum, the authors develop a plausible model of dendrite pruning during Drosophila metamorphosis which is based on the identification of PAR-1 and uniform MT orientations, likely regulated by kinesin-1 and kinesin-2 as required factors for pruning. The authors provide profound work which - after rewriting and some additional experiments - is probably suitable for the EMBO journal. Particular some of the proposed cellular and molecular mechanisms need a more direct proof to uphold the (too) strong mechanistic claims the authors make.

Major points

1) Some claims are too strong. Especially the abstract/title claims are too strong: e.g. PAR-1 regulates MT disassembly or PAR-1 inactivates Tau. However, in the text (and more based on shown results), it is said that PAR-1 might regulate MT disassembly. This all needs to be tuned down. Also make more clear in the title and abstract the main points of the paper. The authors screened for mechanisms in pruning and found two involved mechanisms with no direct link. Otherwise, it seems like two (or more) stories combined into one. They might consider taking the transport part out or re-writing it.

2) There is no direct evidence that MT disassembly is regulated by PAR-1 (and not by other mechanisms which initialize dendritic pruning perhaps tau-independent...)

3) There is no direct evidence that PAR-1 does not only phosphorylate but indeed inactivates Tau in sensory neurons. The causal link between PAR-1 and Tau is not proven. Since these genetics were done in a partial loss of PAR-1 background, it cannot be used to prove there is a direct link. It should be done in a full (strong loss of function). The strong enhancement of the PAR-1 phenotype by mild overexpression of Tau (not seen in wildtype PAR-1 animals) actually suggest parallel pathways. Same applies to tau, why not use the homozygous mutant next to the heterozygous animal? Additionally, although the authors find that drosophila Tau can be phosphorylated by PAR-1 the most obvious phosphorylation site tau mutant acted the same as the wildtype. Is this mutated Tau still phosphorylated by PAR-1?

4) At the end of the paper the authors show there is a clear genetic interaction between endocytosis and PAR-1. However, since PAR-1 cannot directly be linked to microtubules one cannot say: "This strong genetic interaction indicates a close coupling between microtubule breakdown and local endocytosis during dendrite pruning." To address this one should look more directly at microtubules, either with mutant (e.g. for Tau) or with microtubule drugs.

5) Kinesin-1 transport of Golgi outposts is confusing. The phenotype of the kinesin-1 mutant might be due to its effect on microtubule organization and/or cargo transport. Therefore the authors studied static localization of Golgi outpost to address transport defects. Although they found differences, this might just as well be an indirect effect of the microtubule organization defect. Therefore this paragraph is confusing. Furthermore the choice of using Golgi outposts seems unfortunate, since these have also been implicated in organizing dendritic microtubules (Ori-McKenney at al).

6) To study the role of kinesin-1 transport in dendritic pruning more specifically one could think of studying the KLC loss of function mutant, which in C. elegans seem not essential for dendritic microtubule organization (although this should also be checked for Drosophila). Or alternatively the

Gelfand lab recently reported an endogenous kinesin-1 tail mutant in Drosophila which specifically affects microtubule sliding but not transport. Although there the effect on the dendritic microtubule organization has not been addressed.

7) In the discussion the authors propose a model in which the MT plus ends depolymerize during pruning explaining the proximal to distal pruning. However, the first sings of pruning/microtubule loss is in the distal dendrites where microtubules might be more mixed (Stone et al). Did the authors check the microtubule polarity in these dendritic side-branches?

Detailed major points

8) Drosophila PAR-1 is required for sensory neuron dendrite pruning. Figure 1. Error bars missing/undefined?

9) Defects in Microtubule breakdown upon par-1 downregulation. They show that GFP-tubulin is gone in the proximal parts, however this is no direct proof that PAR-1 is regulating microtubule disassembly. GFP-tubulin overexpression is not an optimal tool, as this can have a number of other effects on the MT cytoskeleton.

I) Could the authors express a constitutively active PAR-1 kinase mutant under control of temporal/spatial promoters: Early driving promoter to induce PAR1ßregulated MT disassembly earlier in APF as in wt situation? A promoter to have specific expression in the distal parts of c4da dendrites to induce MT disassembly ectopically at the distal end (even if it might be slower there due to MT orientation)?
II) Show actual MT dynamics/disassembly by live cell imaging in PAR1 RNAi and kinase active mutant background? e.g. with: a) EB1-GFP to visualize MT polymerization/depolymerization and orientation (perhaps rather changes in MT dynamics/ orientation regulated by kinesins cause the deattachment of tau in the dendrites as PAR1 driven inactivation of tau?). b) Jupiter-RFP to visualize (the loss of) stable MTs. c) EB1int-GFP (Weiner et al., 2016) to show MTs live. d) Optimally combined with lifeAct to show that specifically MT stability changes, but not actin and the entire dendritic structure (yet).e) Or use MT modifying drugs like taxol /nocodazole at 3rd larval stadium before AFP where they should still penetrate (I asked Martin about this and he told me in worms it is somehow possible...) and try to counteract with Par1 kinase ON (active) or DEAD mutants? f) Perhaps they could also perform Kinesin_betaGal imaging here to dissect if Par1 depends on uniform MT orientation or is also involved in the regulation of uniform MT orientation and to get a more complete picture of the two mechanisms they discovered and describe in their study.
10) PAR-1 inhibits Tau during dendrite pruning Kinesin family members are required for dendrite pruning and microtubule disassembly. The authors can show that PAR1 phosphorylates tau and provide a genetic interaction between PAR1 and tau and suggest that tau is a target of PAR1. However, their genetic interaction does not show a causal link and shows neither that tau is the target, nor that it is inactivated by PAR1. a) check for other P-sites in Tau and express these S to A or to D mutants like they did for S184 and confirm biochemically the newly generates Par1 SxxxA/D mutants . b) suppress PAR1 in a tau deficient background to show that the genetic interaction is indeed a causal link. (Which we doubt a bit, since the strong synergistic effect they found suggests parallel pathways. c) Fig3: also the shown Par1delta16 dendrite looks like it would prune; very dotty and discontinuous Tau signal. Perhaps they would like to show a different example picture
11) Uniform dendritic microtubule orientation is required for dendrite pruning. Fig6D table not clear. How many neurons/dendrites/experiments?

12) Transport defects upon kinesin inhibition. We suggest to re-write this section in a way that it rather shows that the kinesin1/2 mediated regulation of dendrite pruning is based on their function in controlling MT orientation and not primarily in transport (that kinesin inhibition also causes transport defects is not too surprising but does not clarify the mechanisms of pruning....). We doubt if Golgi outposts are the optimal model cargo, esp. since there appears to be also a Golgi (morphology) phenotype! Moreover they also claim that kinesin1 is more important for the cargo transport than kinesin2, however that might be caused by a higher cargo-motor-affinity of kinesin1 and Golgi outposts. Therefore a more general model for transport should be used. To dissect if transport defects after kap3 RNAi are only secondary effects of MT orientation: downregulation of

kinesin1 (changes MT orientation) and image kinesin2 specific cargo. Compare to transport of same cargo after kinesin2 downregulation or just take this part out

13) Microtubule disruption affects endocytosis and dendrite thinning. Could they depolymerize MTs in a PAR1 deficient background to trigger membrane breakdown?

Minor points:
1) What is physiological relevance of Par1 regulated pruning in sensory neurons for fly development? (Does the Par1 RNAi driving in sensory neurons inhibit development of adult flies?)
2) Just curious: do APC RNAi flies have pruning defects, since APC is involved in uniform MT orientation?
3) Would be interesting to know what/how candidate pruning factors were chosen
4) The introduction of the MARCM assay is not clear (to non-fly people)
5) Arrows in figure 3A, seems to point to a different dendrite. It might help to have the arrows also in the merge
6) The primary/secondary branching quantification is confusing. How was this done exactly? It is important to understand the difference between the kinesins
7) What is the evidence for calling Ens/Map7 a subunit of kinesin-1?
8) C. elegans has mainly uniform minus end MTs in dendrites (Maniar et al 2011)
9) Asterisk is missing in figure 2D

1st Revision - authors' response 06 March 2017

Point-by-point response to reviewers:

We would like to thank all three reviewers for their critical and constructive comments. We have addressed them as thoroughly as possible and we now present an improved manuscript. We are confident our experiments will satisfy the reviewers. Briefly, all three reviewers asked to back up the evidence that PAR-1 acts on microtubules, to investigate the Tau phosphorylation sites more, and to shorten the paper. To address these questions, we added measured MT stability and dynamics by analysis of MT modifications and photoconversion experiments, showed that the dTau serines analogous to hTau phosphorylation sites are dispensable for in vitro Tau phosphorylation, and moved the analysis of Golgi oupost trafficking to the Appendix. We are confident our experiments will satisfy the reviewers.

Referee #1:

In this manuscript, Herzmann and colleagues focus on an important and not well understood question in biology which is how microtubule dynamics affects neuronal remodeling. It is well established that MT disassembly is the first observable step in remodeling of axons and dendrites in both vertebrates and invertebrates. However, whether this disassembly is required for pruning and how it is regulated is not well understood. In this study, Herzmann et al first identify the Par-1 kinase as required for the normal progress of dendrite pruning in the Drosophila da sensory neurons. They propose that Par-1 functions, at least in part, by regulating the stability of the MT stabilization protein Tau. Next they continue to probe MT related proteins such as kinesins and to this end find two motors (khc and klp64d) that are required for normal dendrite pruning. Then they link between the kinesins to the uniform organization of MT, which is plus end facing the soma in dendrites. They make the observation that in the khc mutant the orientation of the MT is less uniform and further propose that this mixed orientation might be the cause for slower MT disassembly and pruning. Next (although not really presented in this order), the authors link between Par-1 mediated dendrite thinning and cytoskeletal stability as well as Ca transients. Finally, the authors also test another hypothesis as for the role of khc during pruning, which is the trafficking of golgi outposts.
All in all, I find that each piece of data is interesting and largely supports the statements provided in the text (although not always). My strongest reservation is that this paper seems like a combination of at least three (interesting but incomplete) projects. The most developed project, which could relatively quickly be upgraded to a complete story, is the Par-1-Tau story (see specific comments for suggestions on how). The second and third stories are the roles of kinesins - from keeping the dendrite orientation uniform to trafficking of golgi-outposts. While such a descriptive study

(encompassing all three stories) is of interest, I strongly urge the authors to focus on one, make it complete with deep mechanistic insights, and publish as a smaller (but stronger) story.

Response 1: We agree that our manuscript is long. We think of the Par-1/Tau and the kinesin/orientation parts as belonging to the same story, i. e., „Mechanisms that contribute to microtubule breakdown during pruning".
The Golgi outpost analysis (formerly Figure 7) was thought as a control for potential additional kinesin functions. We moved this part to the supplementary figures to adjust to the reviewers comments.
The analysis of membrane thinning defects (Figure 8) is in a way an outlook from the microtubule and pruning theme. We kept this Figure but tried to shorten the text to better integrate it.

Specific comments:

1) The most important point is, as stated above, the fact that this study includes many descriptive findings, not totally relevant to each other and mechanistic insights are largely missing. Here are a few suggestions to deepen the Par-1/Tau story:

Q: If indeed the major role for Par-1 is to phosphorylate Tau and by this cause its dissociation from the MTs, then overexpression of Tau should inhibit pruning by itself. The data in Fig 3G, which is consistent with this hypothesis, shows that overexpressing Tau exacerbates the Par1 RNAi phenotype. While this is nice, it would be very interesting to do a much more serious attempt to find the Par-1 phosphorylation site (in vitro and in vivo) and show that over expressing Tau that cannot be phosphorylated by Par-1 inhibits pruning even more. Likewise - in the experiments where overexpression of Tau did NOT inhibit pruning by itself - what is the state of MTs?

Response 2: We do show a dose-dependent effect of tau. When we overexpressed a very strong UAS-tau::GFP transgene, this caused significant pruning defects (Figure 6 F). However, our hypothesis also poses that PAR-1 inactivates tau at least to some degree, and inactive (i. e., likely phosphorylated) tau just might not inhibit pruning. The best evidence for epistasis is our finding that par-1 RNAi can be suppressed by tau heterozygosity. In order to demonstrate better the specificity of the genetic interactions between *par-1* and *tau*, we also included data with another MAP, futsch/MAP1B, which, while expressed and localized in a similar pattern to dTau, does not interact strongly with Par-1 (Figure 4 G). Of particular note, loss of *futsch* does not suppress *par-1*, as is the case with *tau*. We also reference Figure EV1, where we show that dtau heterozygosity also suppresses a *par-1* mutation in c1da neurons.
To address the tau phosphorylation sites, we asked whether the tau S184A mutant could still be phosphorylated by PAR-1. In addition, we tested a mutant lacking S184 and S305, the serine corresponding to htau S356, a second known Par-1 target. Both of these dTau mutants are still efficiently phosphorylated by PAR-1 (Figure EV2), strongly indicating that dTau still has other phosphorylation sites, and that dTau might be different from hTau in this respect. We considered an unbiased mass-spec approach, but this was not doable do to time constraints and for lack of established mass-spec connections here in Münster.

Q: b)Along the same lines, and as discussed by the authors themselves, it would make sense to try and dissect the relationship between Tau/Par1 and Kat60L.

Response 3: We addressed this comment by showing that *kat-60L1* acts as an enhancer of *par-1* during dendrite pruning (Figure 4). This observation fits again with the above genetic interactions (katanins are sensitive to Tau levels but not MAP1B levels, Qiang et al., 2006) and hence strengthens our point that tau is the likely PAR-1 target.

Q: c) Finally, a new study from the Misgeld lab has shown that Spastin is required for the MT severing and disassembly in NMJ branch pruning. One important aspect of their paper is the observation of interesting MT modifications enrichment in the loosing branch - but it remains completely open as how and whether these modifications regulate Spastin recruitment, activity and MT disassembly. While I think that including Spastin in this study might be beyond its scope - testing a few antibodies of different MT modifications and see if this changed in Par1 mutants should provide complimentary data in a relatively short time.

Response 4: this question touches on the role of microtubule dynamics (of which microtubule modifications are often a reflection) during the early pupal phase. After reading the comments, we felt ourselves that we hadn't addressed this point enough. We therefore stained dendrites with antibodies against acetylated tubulin (Figure 2 E – H) and polyglutamylated tubulin (Appendix Figure S3). These analyses showed that loss of *par-1* leads to persisting stable microtubules at 5 h APF that are positive for both acetylated and polyglutamylated tubulin. Other antibodies (e. g., tyrosinated tubulin) did not work in our samples. In order to further address microtubule dynamics, we used tdEOS::αtubulin for photoconversion experiments. These experiments showed that microtubules become more dynamic at the onset of the pupal phase, and that this effect depends on PAR-1 (new Figure 3). We think that these experiments indeed strengthen our claims about Par-1 and microtubules – thanks!

2) There are several cases in which I feel statements, especially those attempting to indicate casualty, are over interpreting. A few examples:

Q: a) "Conversely, removal of one copy of tau with the small deficiency tauMR22 (Doerflinger et al., 2003) led to a significant suppression of the par- 1 RNAi phenotype (Figure 3 G), indicating that Tau is a target for PAR-1 during c4da neuron dendrite pruning" - on one hand, the authors showed that Par1 CAN phosphorylate Tau (using purified proteins, which is even less biologically relevant than in vitro and obviously in vivo). But to say that the fact that reducing the Tau levels by half, which led to a suppression of the par1 RNAi phenotype means that this INDICATES that Tau is a target for par... - this is over statement. Doing some experiments with Tau mutants should clear this up, hopefully.

Response 5: we toned down this part and state instead: „...the strong and specific genetic interactions between PAR-1 and Tau, and especially the fact that a reduction of *tau* levels can suppress *par-1* phenotypes, suggest that Tau is a target for PAR-1 during dendrite pruning.“ (p. 8) This is more careful and still reflects the multitude of genetic interactions that we have for par and tau. We also addressed the genetic interactions between Par-1 and katanin (see responses 2 and 3).

Q: b)"However, overexpression of tauHA S184A did not cause dendrite pruning defects and ...indicating that dTau S184 phosphorylation is not required for dendrite pruning" - how can a negative result using a transgene indicate that a specific phosphorylation on Tau is not required for dendrite pruning? I would say that expressing TauS184 is not sufficient to inhibit pruning.

Response 6: we rephrased accordingly:“ ... indicating that inhibition of S184 phosphorylation is not sufficient to inhibit pruning“. (p. 8)

Q: c) "Expression of par-1 RNAi completely abrogated Ca2+ transients (Figure 8 F), indicating that PAR-1 is required for dendrite thinning formation" - the authors are using the transients as a proxy for thinning - I think that while this is likely, I don't think its a certainty.

Response 7: we rephrased: „Consistent with the idea that microtubule disruption is required for thinning formation, expression of *par-1* RNAi abrogated Ca^{2+} transients (Figure 8 F).“ (p. 14)

Q: d) "Thus, dendrite branch points might have less stable microtubules than dendrite shafts. PAR-1 and Kat60-L1 are mostly localized to the soma (Figure EV7)." - this is misleading as these are transgenes, not endogenous proteins so what does it really mean

Response 8: We agree that this Figure does not add much to the understanding. We therefore removed it. There is still a PAR-1 staining in Appendix Figure S6.

Q: e) There are a lot of instances where mechanistic interpretations are superimposed on vague "genetic interactions" - what does genetic interaction even mean? I am not sure one can deduce something mechanistic from these experiments.

Response 9: We would like to disagree respectfully – strong enhancing genetic interactions of the kind presented in Figures 4 and 6 – 8 are very strong indicators that the genes involved act in common or parallel pathways, while suppression is often an indication of epistasis, i. e., the two factors being in the same pathway. In cases where both apply – like the interaction between PAR-1

and tau, this is a very strong basis for a mechanistic interpretation. A number of examples are included where there is no interaction with Par-1: futsch/MAP1B (Figure 4, despite similar function and distribution as tau), ecdysone receptor EcR, proteasome subunit Mov34 (despite both being required for dendrite pruning, Appendix Figure S6).

Q: 3) Quantifications: First, it is unclear based on the figures themselves what you are quantifying - % of unpruned dendrites or % of brains that contain unpruned dendrites - I know it is the latter but this is confusing. One caveat with this quantification is that it does not really measure the SEVERITY of the phenotype but rather the PENETRANCE or EXPRESSIVITY - is there any way one could directly measure the severity? By, for example, actually counting the number of remaining branches in combination with the total length of unpruned neurites?

Response 10: the description of the axes was indeed confusing. We changed it to „Neurons with attached dendrites at 18 h APF [%]. We hope this helps!
In most Par-1 genetic interaction experiments, the main difference between genotypes was indeed in the penetrance, not in the severity, (possibly indicating that a certain threshold had to be reached). We therefore included only those data. In other cases, e. g., when we compare the par-1 mutant with the RNAi, or when we compare different kinesin mutants, the number of unpruned primary and secondary branches is a very good readout of severity. However, we included dendrite length for Figures 1 (par-1 phenotype) and 5 (kinesin phenotypes).

Q: 4) In some cases - khc mutant, for example - there are defects in the dendritic arbor even at L3 - does that affect the pruning? How can one exclude that?

Response 11: For Par-1 RNAi, we showed that microtubule stability is not significantly different from controls at the larval stage, but only at the pupal stage. (new Figure 3). For the khc mutants, we are actually proposing that a pre-existing defect (in dendritic microtubule orientation) underlies the pruning defects. It is conceivable that this defect could also cause defects in the dendritic arbor. We can in this system sometimes address acute functions with inducible expression systems. In our experience, this works well with dominant-negative effectors, but not RNAi, because the latter needs time to exert effects.

Minor comments
Q: 1) when discussing the different da neurons, it would be advisable to keep a consistent nomenclature between the text (where its "the class 1 da ") and the figure (ddaD/ddaE).

Response 12: we removed the ddaD/E captions from the figures and explain once in the text/legend.

Q: 2) EV6E - lacks a Y axis

Response 13: we added a Y axis.

Q: 3) Order of EV figures is confusing.

Response 14: We adapted the order of EV figures and moved some to the Supplementary material.

To summarize - I think this is an interesting paper with solid data and a lot of potential. I would focus on one story, beef it up with a few mechanistic experiments and modify the text to carefully reflect the data but no more than that.

Referee #2:

In the manuscript "PAR-1 and Kinesins Promote Directional Microtubule Breakdown During Dendrite Pruning in Drosophila" Herzmann et al. generate a satisfying model for how microtubule polarity and microtubule destabilization can together promote proximal dendrite clipping from the cell body. They also identify par-1, tau and two kinesins as new regulators of pruning. Overall the story is an important contribution to understanding controlled disassembly of dendrites. There are, however, some key points that need to be resolved. The most important of these is the last set of conclusions in the section "Microtubule disruption affects endocytosis and dendrite thinning." The

connection of microtubules to Ca transients and endocytosis is based solely on manipulation of par-1. While par-1 is important for microtubule regulation, it is a kinase with many substrates and so it is not clear whether a microtubule regulator is the key substrate that alters Ca transients or endocytosis during pruning. To really demonstrate that it is the effect of par-1 on microtubules that i s important here would require a lot more experiments. I therefore recommend removing this section and shoring up some of the data in the core part of the story.

Response 1: We have added a number of data pieces that strengthen our finding that Par-1 acts on microtubules during dendrite pruning. For example, we show that stable acetylated MTs accumulate upon loss of Par-1, and we use photoconversion to show that Par-1 RNAi increases MT dynamics specifically during the pupal phase. But our wording was very strong and we obviously cannot uphold the idea that MT breakdown triggers membrane thinning. Therefore, and because we technically cannot exclude that Par-1 does have other targets during pruning, we have toned down our wording in this part. We now ask whether „...microtubule disruption is required (i. e., permissive or even triggering) for thinning formation..." (p. 14) and conclude: „Thus, microtubule breakdown is required for, and might be very tightly linked to, local membrane destabilization during dendrite pruning." (p. 14). We hope that this addresses the reviewer's concerns.
Specific points that need to be addressed:

Q: 1. The effect of Par-1 loss on microtubules in uninjured neurons is not described. Because the authors later demonstrate that changes in MT polarity reduce pruning it is important to determine whether Par-1 reduction results in changes in microtubule polarity. If it does this could contribute to the pruning defect.

Response 2: This is a great point. We have now addressed the effects of Par-1 on various aspects of microtubule dynamics. Most importantly, we measured the effect of loss-of-Par-1 on microtubule stability in larval and pupal neurons. Using photoconvertible tdEOS-tubulin, we found that Par-1 does not significantly affect the stability of larval dendritic microtubules. In contrast, microtubules were more stable upon loss of Par-1 at the onset of the pupal phase (new Figure 3). Consistently, pupal neurons lacking Par-1 had higher levels of microtubules with posttranslational modifications (acetyl-, polyglutamyl-tubulin, Figure 2 E – H, Supplementary Figure 3). We also used EB1::GFP tracking and found no effect of Par-1 on microtubule orientation in larval dendrites (new Figure 7). These analyses demonstrate a high degree in specificity of the Par-1 phenotype.

Q: 2. For RNAi control experiments is a control RNAi included? This transgene number matching is particularly important for double RNAi experiments like those in Figure 5 as inclusion of additional Gal4 driven transgenes can dilute the effect of each, and so phenotypes can be reduced simply by inclusion of another RNA hairpin rather than any specific effect of target knockdown. It is therefore essential to have identical Gal4-driven transgene numbers in these experiments, so for example par-1 RNAi cannot be compared to par-1 RNAi + ens RNAi. Instead the comparison should be par-1 RNAi + control RNAi compared to par-1 RNAi + ens RNAi. The same is true for RNAi + other transgene experiments like those in Figure 3. An alternate explanation for the effect of tau expression here is that it simply reduces expression of the par-1 RNAi by competing for Gal4.

Response 3: We did include a control RNAi in Figure 6 (former Figure 5) and also in a new double-RNAi experiment in Figure 4 (former Figure 3). The enhancement by UAS-tau in Figure 4 (former Figure 3) seems unlikely to be caused by a reduction of the effect of Par-1 RNAi.

Q: 3. The data that microtubule markers leave the proximal dendrite before other markers or disassembly is not very convincing. For example, In Figure 2 GFP-td-tomato and GFP-a-tubulin are both dramatically reduced at the proximal dendrite region and there is no quantitation of ratios of fluorescence intensity in proximal vs distal dendrites to back this up. The genetic data is much more convincing than any images where it is suggested MTs disappear before the dendrite is completely broken down. It might be helpful to include more detailed images of microtubule vs other marker loss.

Response 4: We added the stainings against posttranslationally modified tubulin (Figure 2 E – H, Appendix Figure S3). Examples of dendrites without microtubules can also be seen in Figure 7 A – C.

Q: 4. They have a lot of genetic evidence of the interaction between Par-1 and Tau. And they know in Drosophila Par-1 can phosphorylate Tau. However the identification of the phosphorylation site is not possible, and the authors description of this is a little hedged. Do they think there is a different phosphorylation site? I think it would be helpful to just be a bit more direct in the wording here.

Response 5: We did attempt to identify the phosphorylation site in kinase assays with recombinant proteins. However, dTau lacking the two main candidate Par-1 target serines was still efficiently phosphorylated by Par-1 (Figure EV2). We did not take a mass-spec approach to identify the site, because we do not currently have a good mass-spec connection, and wouldn't have been able to perform this task and the ensuing genetics within the time for the revision.

Q: 5. % Attached dendrites 18hpf is shown for par-1 RNAin in Figures 1 and 5F, but the numbers are quite different. Can the reason for this be made clear in the figures and text? Is an additional control RNAi included in Fig 5?

Response 6: The difference is mostly due to the fact that we used different *ppk-GAL4* driver insertions on the second and third chromosomes, respectively. We indicate this now in the legends where applicable. We also included a control RNAi in Figure 5 (now Figure 6).

Q: 6. Strong pruning defects result from Khc RNAi. But they didn't show the polarity data from this RNAi. Instead they show the polarity data from khc mutant, it would be good to include both. The sample sizes are very small for the polarity data in Figure 6. They also try to figure out why kinesin-1 has a much stronger pruning phenotype that kinesin-2. An important part of this argument is looking at MT polarity in kinesin-2 knockdown in class IV neurons. Is the polarity phenotype just much weaker with reduction of kinesin-2?

Response 7: We added additional genotypes and increased the N for the control in the EB1::GFP analysis (new Figure 7). kap3 RNAi causes an intermediate phenotype between control and khc mutant. Surprisingly, it turns out that khc RNAi does not cause a orientation defect, while still causing a strong pruning defect. We do not believe that this is due to a dominant effect of the mutant which has been described as a null. Rather we think this reflects two distinct functions of Khc in dendrite pruning as discussed in the section about Golgi outpost distribution. We are keeping the Golgi outpost analysis in the Supplementary to underscore this idea. We don't think this casts doubt on the role of MT orientation during MT disassembly pruning, as there still are the genetic interactions between the MT disassembly factor Par-1 (this conclusion has gotten stronger) and khc as well as Par-1 and EB1.

Q: 7. Overexpressed man-II is used a Golgi marker in Figure 7, but it is clearly visible in the axon where no Golgi is thought to be present. If the authors want to argue that this marker specifically labels the Golgi then they need to address this. Another interpretation is that it labels the Golgi + other membranes when overexpressed. This could be addressed with careful wording or use of additional markers or staining of endogenous Golgi.

Response 8: This is an interesting point – we used to have a debate in the Jan lab as to whether there wer no outposts at all in the axon, or just very few. We have now chosen a more careful wording by saying that manII::cherry labels „...Golgi outposts and similar vesicles...", (p. 13) thus acknowledging a potential lack of specificity.

8. The title does not reflect the essential points of the model. It sounds like kinesins are directly involved in pruning, while really the authors suggest correct MT polarity is the key. Could the title be reworked to reflect this.

Response 9: we changed the title to „Par-1 and Uniform Microtubule Orientation Promote Microtubule Breakdown During Dendrite Pruning in *Drosophila"*

Referee #3:

In this paper, the authors found that kinase PAR-1 is required for pruning in Drosophila sensory neurons. By excluding that PAR-1 regulates pruning via its activity in the known PAR-1 polarity

pathway or ecdysone signaling, they hypothesize that PAR-1 might regulate MT disassembly during dendrite pruning. The authors show that in PAR-1 RNAi c4da neurons which fail to prune GFP-tubulin stays, while it disappears in proximal dendrites in the control neurons. The authors show that PAR1 phosphorylates tau and provide evidence for a genetic interaction between PAR1 and tau. Based on these results the authors claim that that tau is a target of PAR1 and PAR-1 regulates pruning by inactivation of tau.

In addition to PAR-1, they identify the uniform plus-end in MT orientation in dendrites to be crucial for pruning. In a search specific for MT regulating factors in pruning they found kinesin-1 and kinesin-2 to be required for dendrite pruning. Interestingly they can show that not only kinesin-2, but also kinesin-1 is essential for uniform MT orientation and suggest that this uniform orientation may facilitate the proximal-to-distal MT disassembly as initiation of dendrite pruning. By showing genetic interactions of PAR-1 and kinesin1 and PAR-1 and endocytosis factors, a sequential model of the cellular mechanisms in pruning is proposed.

In sum, the authors develop a plausible model of dendrite pruning during Drosophila metamorphosis which is based on the identification of PAR-1 and uniform MT orientations, likely regulated by kinesin-1 and kinesin-2 as required factors for pruning. The authors provide profound work which - after rewriting and some additional experiments - is probably suitable for the EMBO journal. Particular some of the proposed cellular and molecular mechanisms need a more direct proof to uphold the (too) strong mechanistic claims the authors make.

Major points

Q: 1) Some claims are too strong. Especially the abstract/title claims are too strong: e.g. PAR-1 regulates MT disassembly or PAR-1 inactivates Tau. However, in the text (and more based on shown results), it is said that PAR-1 might regulate MT disassembly. This all needs to be tuned down. Also make more clear in the title and abstract the main points of the paper. The authors screened for mechanisms in pruning and found two involved mechanisms with no direct link. Otherwise, it seems like two (or more) stories combined into one. They might consider taking the transport part out or re-writing it.

Response 1: In order to strengthen our point that Par-1 acts on microtubules, we added two sets of experiments: (1) stainings of microtubule posttranslational modifications which demonstrate that stable microtubules persist upon loss of Par-1 (new Figures 2 E – H), and (2) EOS-tubulin photoconversion experiments to address the effect of Par-1 on MT dynamics (new Figure 3). These experiments show that Par-1 is required for MT destabilization at the onset of the pupal phase.
We also changed the title to more directly reflect the findings of the paper. We rephrased the abstract to state more carefully„...Here, we show that the kinase PAR-1 is required for pruning and dendritic microtubule breakdown. Our data suggest that PAR-1 increases microtubule dynamics at the onset of metamorphosis, likely via a negative effect on *Drosophila* Tau...“ and „...PAR-1 interacts genetically with endocytic factors required for dendritic thinning, suggesting that microtubule breakdown is required for ensuing plasma membrane alterations...“
We also moved the transport part to the Appendix to make the paper more concise.

Q: 2) There is no direct evidence that MT disassembly is regulated by PAR-1 (and not by other mechanisms which initialize dendritic pruning perhaps tau-independent...)

Response 2: This is clearly a good point. While we think the circumstantial evidence was very clear, we did not provide too much direct evidence (even though we feel you are playing the advocate of the devil a bit here). But to be on the safe side, we added a lot of experiments to address this. Our new photoconversion analysis shows that loss of Par-1 affects MT dynamics (new Figure 3), and the stainings for tubulin modifications demonstrate the persistence of stable microtubules in the absence of PAR-1 (Figure 2 E – H). The genetic interactions with tau are strong – the enhancement is the strongest we have seen, and tau is the only suppressor, both of par-1 RNAi and the par-1 mutant. As discussed in response 9 to reviewer 1, enhancement usually reflects functions in the same or parallel pathways, whereas suppression often indicates epistasis. Each data point by itself could be interpreted in various ways, but since tau is both a enhancer and a suppressor of par-1, this is very strong evidence of epistasis, and of Par-1 being upstream of tau. Now we think the evidence is compelling.

3) There is no direct evidence that PAR-1 does not only phosphorylate but indeed inactivates Tau in sensory neurons. The causal link between PAR-1 and Tau is not proven. Since these genetics were done in a partial loss of PAR-1 background, it cannot be used to prove there is a direct link. It should be done in a full (strong loss of function). The strong enhancement of the PAR-1 phenotype by mild overexpression of Tau (not seen in wildtype PAR-1 animals) actually suggest parallel pathways. Same applies to tau, why not use the homozygous mutant next to the heterozygous animal? Additionally, although the authors find that drosophila Tau can be phosphorylated by PAR-1 the most obvious phosphorylation site tau mutant acted the same as the wildtype. Is this mutated Tau still phosphorylated by PAR-1?

Response 3: We also did the experiments in a full loss of par-1 background – in the MARCM analysis of c1da neuron dendrite pruning in Figure EV1. Also here, tau heterozygosity suppresses the par-1 mutant to a degree comparable with a UAS-Par-1 rescue construct. Unfortunately, we couldn't use a full loss of tau background as the tau locus is on the third chromosome where a number of our other transgenes for these experiments are also located, and could not be recombined with the other markers used. Plus, the used tau mutant is lethal, and we would have had to use a double MARCM to address such interactions. However,we think that the fact that suppression even occurs with heterozygous tau actually shows how specific this interaction is.

4) At the end of the paper the authors show there is a clear genetic interaction between endocytosis and PAR-1. However, since PAR-1 cannot directly be linked to microtubules one cannot say: "This strong genetic interaction indicates a close coupling between microtubule breakdown and local endocytosis during dendrite pruning." To address this one should look more directly at microtubules, either with mutant (e.g. for Tau) or with microtubule drugs.

Response 4: We hope that this is answered by the new experiments in Figure 2 E – H (acetyl-tub stainings) and in the new Figure 3 (loss of Par-1 stabilizes MTs as shown by EOS-tub photoconversion) that clearly demonstrate that Par-1 acts on MTs. The fact that tau can specifically suppress the loss of PAR-1 also argues that tau is the target. Enhancement of the type in Figure 8 can result from parallel pathways, but suppression and enhancement as with tau is usually reflective of epistasis.

Q: 5) Kinesin-1 transport of Golgi outposts is confusing. The phenotype of the kinesin-1 mutant might be due to its effect on microtubule organization and/or cargo transport. Therefore the authors studied static localization of Golgi outpost to address transport defects. Although they found differences, this might just as well be an indirect effect of the microtubule organization defect. Therefore this paragraph is confusing. Furthermore the choice of using Golgi outposts seems unfortunate, since these have also been implicated in organizing dendritic microtubules (Ori-McKenney at al).

Response 5: Since our data now show more clearly that there must be multiple functions for kinesin-1, we would like to keep this part. But in order to present the data in a more concise way, we moved this part to the Supplementary Material. We also added the Ori-McKenney reference.

Q: 6) To study the role of kinesin-1 transport in dendritic pruning more specifically one could think of studying the KLC loss of function mutant, which in C. elegans seem not essential for dendritic microtubule organization (although this should also be checked for Drosophila). Or alternatively the Gelfand lab recently reported an endogenous kinesin-1 tail mutant in Drosophila which specifically affects microtubule sliding but not transport. Although there the effect on the dendritic microtubule organization has not been addressed.

Response 6: We tested the kinesin-1 tail mutant from Gelfand's lab. It shows a small increase in the plus-end-out dendritic microtubules, and a very mild pruning defect (Appendix Figure S7).

7) In the discussion the authors propose a model in which the MT plus ends depolymerize during pruning explaining the proximal to distal pruning. However, the first sings of pruning/microtubule loss is in the distal dendrites where microtubules might be more mixed (Stone et al). Did the authors check the microtubule polarity in these dendritic side-branches?

Response 7: We have looked in a relatively systematic way, and the first signs of microtubule loss occur at the first or second branchpoints, counting from the soma (e. g., Figure 7 A – C). By the definition of Ori-McKenney (2012) most of these branches still qualify as primary branches where the microtubule orientation is almost uniformly plus-end-in. These are also the regions where we assessed EB1::GFP comets in Figure 7 D. These regions are quite proximal. But we toned down the interpretation in the Discussion, saying „...Preferred shrinkage from plus ends might also explain why microtubules become preferentially depleted from proximal dendrite regions and thus represent a spatial cue for dendrite breakpoint selection.“ (p. 16)

Detailed major points

Q: 8) Drosophila PAR-1 is required for sensory neuron dendrite pruning. Figure 1. Error bars missing/undefined?

Response 8: In the graph in Figure 1 E, we assessed the percentage of neurons with attached dendrites. This is a yes/no question where error bars cannot be given. The statistic test chosen (Fisher's exact test) reflects this type of question.

Q: 9) Defects in Microtubule breakdown upon par-1 downregulation. They show that GFP-tubulin is gone in the proximal parts, however this is no direct proof that PAR-1 is regulating microtubule disassembly. GFP-tubulin overexpression is not an optimal tool, as this can have a number of other effects on the MT cytoskeleton.

Response 9: This is a valid point. We have now also tested antibodies against acetylated (Figure 2 E – H) and polyglutamylated (Supplementary Figure 3) tubulin to show the existence of stable microtubules, and we have assessed microtubule dynamics using photoconvertible EOS-tubulin. All data support our idea that Par-1 regulates microtubule disassembly.

I) **Q:** Could the authors express a constitutively active PAR-1 kinase mutant under control of temporal/spatial promoters: Early driving promoter to induce PAR1ßregulated MT disassembly earlier in APF as in wt situation? A promoter to have specific expression in the distal parts of c4da dendrites to induce MT disassembly ectopically at the distal end (even if it might be slower there due to MT orientation)?

Response 10: Unfortunately, constitutively active Par-1 cannot be made. This was shown in a 2007 J. Neurosci paper by Bingwei Lu's lab (Wang et al.). When they changed the activatory threonine to aspartate, mimicking phosphorylation, the protein became inactive.

Q: II) Show actual MT dynamics/disassembly by live cell imaging in PAR1 RNAi and kinase active mutant background? e.g. with: a) EB1-GFP to visualize MT polymerization/depolymerization and orientation (perhaps rather changes in MT dynamics/ orientation regulated by kinesins cause the deattachment of tau in the dendrites as PAR1 driven inactivation of tau?). b) Jupiter-RFP to visualize (the loss of) stable MTs. c) EB1int-GFP (Weiner et al., 2016) to show MTs live. d) Optimally combined with lifeAct to show that specifically MT stability changes, but not actin and the entire dendritic structure (yet).e) Or use MT modifying drugs like taxol /nocodazole at 3rd larval stadium before AFP where they should still penetrate (I asked Martin about this and he told me in worms it is somehow possible...) and try to counteract with Par1 kinase ON (active) or DEAD mutants? f) Perhaps they could also perform Kinesin_betaGal imaging here to dissect if Par1 depends on uniform MT orientation or is
also involved in the regulation of uniform MT orientation and to get a more complete picture of the two mechanisms they discovered and describe in their study.

Response 11: we used EOS-tub to assess microtubule stability (new Figure 3), and we also assessed the effect of Par-1 RNAi on microtubule orientation using EB1::GFP (Figure 7 D). The results show that Par-1 does not affect orientation but stability of microtubules.

Q: 10) PAR-1 inhibits Tau during dendrite pruning Kinesin family members are required for dendrite pruning and microtubule disassembly. The authors can show that PAR1 phosphorylates tau

and provide a genetic interaction between PAR1 and tau and suggest that tau is a target of PAR1. However, their genetic interaction does not show a causal link and shows neither that tau is the target, nor that it is inactivated by PAR1. a) check for other P-sites in Tau and express these S to A or to D mutants like they did for S184 and confirm biochemically the newly generates Par1 SxxxA/D mutants . b) suppress PAR1 in a tau deficient background to show that the genetic interaction is indeed a causal link. (Which we doubt a bit, since the strong synergistic effect they found suggests parallel pathways. c) Fig3: also the shown Par1delta16 dendrite looks like it would prune; very dotty and discontinuous Tau signal. Perhaps they would like to show a different example picture

Response 12: the time given for a revision is not sufficient for a mass-spec approach and genetic follow-up. We tested a dTau mutant lacking the homologous sites to human S262 and S356, and found that this mutant was still efficiently phosphorylated.
The argument that the genetic interactions do not imply epistasis, or a causal link. While we agree that this would be the case for any single genetic interaction, the combination of enhancing (Figure 4 G) and suppressing (Figure 4 G, Figure EV1) interactions strongly suggests epistasis, and a negative effect of Par-1 on Tau.
We could not assess the effects of a complete loss of tau function on the loss-of-Par-1 phenotype because we had too many transgenes and mutations on the third chromosome in the corresponding analyses (Figure 4 G, EV1). However, we think the fact that tau heterozygosity is sufficient to cause suppression argues for specificity, and hence for epistasis.
We did tone down the initial sentence of the corresponding paragraph. Instead of saying, „...in order to establish a causal link...“, we now say „...We next employed a genetic test to see whether PAR-1 and these MAPs might act in a common pathway during dendrite pruning....“ (p. 7).
11) Uniform dendritic microtubule orientation is required for dendrite pruning. Fig6D table not clear. How many neurons/dendrites/experiments?

Response 13: We added more genotypes, and increased N, and stated how many animals.

Q: 12) Transport defects upon kinesin inhibition. We suggest to re-write this section in a way that it rather shows that the kinesin1/2 mediated regulation of dendrite pruning is based on their function in controlling MT orientation and not primarily in transport (that kinesin inhibition also causes transport defects is not too surprising but does not clarify the mechanisms of pruning....). We doubt if Golgi outposts are the optimal model cargo, esp. since there appears to be also a Golgi (morphology) phenotype! Moreover they also claim that kinesin1 is more important for the cargo transport than kinesin2, however that might be caused by a higher cargo-motor-affinity of kinesin1 and Golgi outposts. Therefore a more general model for transport should be used. To dissect if transport defects after kap3 RNAi are only secondary effects of MT orientation: downregulation of kinesin1 (changes MT orientation) and image kinesin2 specific cargo. Compare to transport of same cargo after ki
nesin2 downregulation or just take this part out

Response 14: The comparison of EB1::GFP tracking data and the dendrite pruning defects (Figures 5 - 7) still suggest that there is a second function for kinesin during dendrite pruning. To account for this, we kept the figure (which does point to the same direction). However, in order to avoid confusion and enhance readability, we have now transfered these data to the Supplementary Material.

13) Microtubule disruption affects endocytosis and dendrite thinning. Could they depolymerize MTs in a PAR1 deficient background to trigger membrane breakdown?

Response 15: We agree with this and the other reviewers that we are potentially overinterpreting the data when we say that microtubule breakdown triggers membrane thinning (response 7 to reviewer 1, response 1 to reviewer 2). We therefore tone this part down to say that MT break down is „...upstream of...“ and „... required for...“ membrane remodeling. These are interpretations that can be drawn, also based on the stable unthinned dendrites seen essentially in all loss-of-Par-1 pictures from 5 h APF. The proposed triggering experiment is certainly a very good idea and we will do it in the future.

Minor points:

1) What is physiological relevance of Par1 regulated pruning in sensory neurons for fly development? (Does the Par1 RNAi driving in sensory neurons inhibit development of adult flies?)

Response 16: while complete null alleles of Par-1 are embryonic lethal, even hypomorphs are viable. RNAi would be too weak to draw conclusions.

2) Just curious: do APC RNAi flies have pruning defects, since APC is involved in uniform MT orientation?

Response 17: we tested APC by RNAi and it does not have an effect on c4da neuron dendrite pruning (not shown). But APC RNAi has only a very weak effect on MT orientation in c4da, and only slightly stronger in c1da (Mattie et al., 2010).

3) Would be interesting to know what/how candidate pruning factors were chosen

Response 18: no major pre-choice, basically all RNAi lines available at the institute. Later, MT-related factors.

4) The introduction of the MARCM assay is not clear (to non-fly people)

Response 19: We explain now: „...Because strong *par-1* loss-of-function alleles like *par-1*^{16} (Cox et al., 2001) are embryonic lethal, we used MARCM (Mosaic Analysis with a Repressible Cell Marker) (Lee and Luo, 1999) a mitotic recombination technique, to generate fluorescently labeled homozygous *par-1*^{16} mutant clones in otherwise heterozygous animals.“ (p. 4)

5) Arrows in figure 3A, seems to point to a different dendrite. It might help to have the arrows also in the merge

Response 20: we added the arrows in the merge

6) The primary/secondary branching quantification is confusing. How was this done exactly? It is important to understand the difference between the kinesins

Response 21: All primary and secondary dendrites still attached to the soma were counted and divided by the total number of neurons in the sample. This is a good measure of severity as it reflects how many dendrites are still attached per soma, and whether the dendrites attached are still branched. In our hands, this analysis matches the results of dendritic length measurements. For the kinesins, the results are saying, about 50% of kinesin-1 mutant neurons still have dendrites attached, most of them several branches. In the case of kinesin-2, a similar percentage have dendrites attached, but it is usually only one last branch. Explanation is now added in the Experimental Procedures.

7) What is the evidence for calling Ens/Map7 a subunit of kinesin-1?

Response 22: Gelfand’s lab calls it an essential cofactor – we now call it a „kinesin-1 recruitment factor“, after the results in Sung et al., 2008. (p.10)

8) C. elegans has mainly uniform minus end MTs in dendrites (Maniar et al 2011)

Response 23: Thanks for pointing this out. We added the reference and changed the text.

9) Asterisk is missing in figure 2D

Response 24: we added the asterisk (Figure 2 A’).

2nd Editorial Decision 10 April 2017

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

As you can see, the referees appreciate the introduced revisions and are supportive of publication. They still raise issues with the Kinesin part. They don't have any issues with the data but more with the interpretations and they suggest to remove that part. I have looked at everything and I think their suggestion is a good one. The manuscript has a lot of data in it anyways and removing that part would also make it for a more compact paper. This would also free up the discussion a bit and allow you to expand it.

I have provided a link below for you to upload the final revised version. Let me know if we need to discuss anything further.

REFEREE REPORTS

Referee #1:

In this revised manuscript Herzmann et al addressed a significant part of my concerns. However, some issues remain and there are a few ways to address them.

The first part of this paper, focusing on PAR-1 and Tau is the most well developed and the text now nicely reflects the results (even the genetic interactions, which I am not a big fan of...). I think that Figure 1-4 could be wrapped up to a small and interesting paper without any additional experiments (that said, it would have been nice to see if overexpression of Tau that is doubly mutant for both potential phosphorylation sites did inhibit pruning - without this experiment, one cannot REALLY say that PAR1 likely phosphorylates OTHER residues in the context of pruning). This would be my recommendation to the authors - wrap this up and publish as is.

Option 2 would be to maintain also the kinesin part but here I have many more issues arising:
The fact that several kinesins exhibit a pruning defect (figure 5) is in itself interesting but not necessarily linked to the first part. I am not sure what to take from Figure 6 - the fact that unpruned dendrites also contain MT is circumstantial and the genetic interactions not too informative (I agree with reviewer 3, by the way, that for proper genetic interactions one has to use complete nulls - otherwise the interpretation of the results might be confounded - in fact the same experiment with a hypomorph or null might result in opposite results --> hence my preference against these sort of experiments - and especially from making strong mechanistic arguments from them).
Figure 7 actually argues against a direct link between MT orientation in dendrites and pruning. The fact that both Par-1 and khc-RNAi don't exhibit MT orientation defects is inconsistent with the story. The twist that trafficking might be an ADDITIONAL role of khc in pruning is interesting but unsatisfying. To substantiate this potential link one could do one of the following: the authors themselves claim that khc^mutA (whose nature is unsatisfactorily described) does not result in a pruning defect - how about MT orientation? Furthermore, APC is also know to affect MT orientation - but in the response to reviewers' letter, the authors mentioned it does not exhibit a pruning defect. Thus - at this point the link seems circumstantial.
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Taken together - a shortened version of the paper focusing on Par1/Tau, which should be accepted as is, could provide a nice and important progress in our understanding of how dendrites are pruned. However, trying to expand beyond that would need varying levels of additional experiments to substantiate claims.

Finally, I would strongly encourage the authors to more carefully read their response to the reviewer's letter before submitting it (which should actually be treated as a response, not rebuttal). I hope that reviewer 3 was able to receive with humor, as I did, the following statement: "While we think the circumstantial evidence was very clear, we did not provide too much direct evidence (even though we feel you are playing the advocate of the devil a bit here). But to be on the safe side, we added a lot of experiments to address this." We, as reviewers, spend a substantial chunk of our valuable time reviewing papers - and while we might disagree on specific points and even might make mistakes, I think that being very careful in these letters would help in the future.

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They show that futsch disappears with similar timing to tau and is also dependent on par-1 (Figure EV3). If both depend on par-1 in a similar way, then it is tough to conclude that only tau is the relevant target. The tau suppression and enhancement data indicates that it likely has an important role in the pathway, but it is important to not dismiss futsch as its removal also depends on par-1. This really is just a wording issue to check.

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1. Chen L, Stone MC, Tao J, Rolls MM. Axon injury and stress trigger a microtubule-based neuroprotective pathway. Proceedings of the National Academy of Sciences of the United States of America. 2012. Epub 2012/06/27. doi: 10.1073/pnas.1121180109. PubMed PMID: 22733771.
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The authors addressed most points that were raised in the initial review. Especially their additional experiments addressing the microtubule disassembly by PAR-1 are a strong addition. They also toned down most of their 'overstated' conclusions. The title, abstract and text flow much better now and reflects the presented data. The data suggest a strong genetic interaction between PAR-1 and Tau, however their argumentation in the rebuttal letter is still not convincing. The enhancement/suppression is not the final proof. Finally, I would still advice to remove the kinesis part. Without out this data it will make a stronger, more compact story.

2nd Revision - authors' response 21 April 2017

Referee #1:

In this revised manuscript Herzmann et al addressed a significant part of my concerns. However, some issues remain and there are a few ways to address them.

The first part of this paper, focusing on PAR-1 and Tau is the most well developed and the text now nicely reflects the results (even the genetic interactions, which I am not a big fan of...). I think that Figure 1-4 could be wrapped up to a small and interesting paper without any additional experiments (that said, it would have been nice to see if overexpression of Tau that is doubly mutant for both potential phosphorylation sites did inhibit pruning - without this experiment, one cannot REALLY say that PAR1 likely phosphorylates OTHER residues in the context of pruning). This would be my recommendation to the authors - wrap this up and publish as is.

Response: Since both reviewer 1 and 3 felt we should remove the second part of our manuscript, we are following this suggestion. The reviewers are absolutely right that it makes for a more compact „story“ that is easier to follow. We are changing the title accordingly. We are leaving the data on the effects of PAR-1 on Calcium transients and the endocytosis interactions. We feel that the experimental effects here are large and a strong basis for an informed speculation on the epistasis between microtubule and membrane events during dendrite pruning. This is an obvious and pressing question in the field, especially after the spectacular description of the membrane alterations leading to calcium transients (Kanamori et al., 2015). The reviewers cautioned that our observations might reflect again other functions of PAR-1 during dendrite pruning. We reflect this criticism by stating more specifically that PAR-1, not microtubule breakdown, is required for these events (p. 9). We feel that the idea that microtubule breakdown is epistatic over endocytosis is the most obvious interpretation for our data. We are therefore presenting it now as the most likely option in the text but caution that we cannot exclude other targets. Specifically, we are now writing in the corresponding experimental section (p. 9, last paragraph): „Thus, PAR-1 is required for both microtubule breakdown and local membrane destabilization during dendrite pruning. Since our genetic data have indicated that Tau is the most likely PAR-1 target during dendrite pruning, these data suggest an epistatic relationship between microtubule breakdown and membrane thinning.“

Option 2 would be to maintain also the kinesin part but here I have many more issues arising:
The fact that several kinesins exhibit a pruning defect (figure 5) is in itself interesting but not necessarily linked to the first part. I am not sure what to take from Figure 6 - the fact that unpruned dendrites also contain MT is circumstantial and the genetic interactions not too informative (I agree with reviewer 3, by the way, that for proper genetic interactions one has to use complete nulls - otherwise the interpretation of the results might be confounded - in fact the same experiment with a hypomorph or null might result in opposite results --> hence my preference against these sort of experiments - and especially from making strong mechanistic arguments from them).
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Response: We now removed most of the corresponding Figures. In contrast to Ank2XL, a cytosplasmic marker would still be visible in this region (see, e. g., Figure 2 B').

Taken together - a shortened version of the paper focusing on Par1/Tau, which should be accepted as is, could provide a nice and important progress in our understanding of how dendrites are pruned. However, trying to expand beyond that would need varying levels of additional experiments to substantiate claims.

Finally, I would strongly encourage the authors to more carefully read their response to the reviewer's letter before submitting it (which should actually be treated as a response, not rebuttal). I hope that reviewer 3 was able to receive with humor, as I did, the following statement: "While we think the circumstantial evidence was very clear, we did not provide too much direct evidence (even though we feel you are playing the advocate of the devil a bit here). But to be on the safe side, we added a lot of experiments to address this." We, as reviewers, spend a substantial chunk of our valuable time reviewing papers - and while we might disagree on specific points and even might make mistakes, I think that being very careful in these letters would help in the future.

Response: We rather meant to strongly defend our conclusions than to personally attack a reviewer. We certainly are aware that reviewing is a service to the community, and grateful for it.

Referee #2:

The new data on microtubules before pruning (both stability and orientation) and during pruning (stability) significantly strengthen the manuscript. The writing and presentation of a more unified story is also a nice improvement. There are still some issues with making statements that over-reach the results- see several examples below. The interpretation of kinesin-1 data is also difficult- I think the authors do a good job acknowledging this and also fleshing out the main points with the EB1 RNA and dominant negative as well as kap3 RNAi. I agree that the most likely explanation is that the kinesin-1 loss is doing something other than controlling microtubule organization. I have one alternative explanation to consider for this (see below).

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mutants cause a similar response, indeed the kymograph in Figure 7 shows many more comets per length in the khc mutants than in the control, so this is something that should be considered. Kinesin-2 has been shown to work directly on microtubules to control polarity rather than indirectly through JNK, so this result is much cleaner.

Response: This part is now removed - this is a nice idea and we will consider this when we rewrite this part – thanks!

Additional comments:
They show that futsch disappears with similar timing to tau and is also dependent on par-1 (Figure EV3). If both depend on par-1 in a similar way, then it is tough to conclude that only tau is the relevant target. The tau suppression and enhancement data indicates that it likely has an important role in the pathway, but it is important to not dismiss futsch as its removal also depends on par-1. This really is just a wording issue to check.

Response: It has been well-documented in the literature that futsch/22C10 distribution closely follows microtubules in the fly PNS. It is therefore not surprising if futsch leaves MT-free regions. This was also used as a MT proxy in Williams and Truman, 2005 and Kanamori et al., 2015. It is actually a better MT marker than the Tau markers we have used. These show some characteristics of a microtubule marker (such as loss from thinning dendrites), but also characteristics of cytoplasmic markers, e. g., high concentration in the cell body (in fact the on/off rates of the tau/MT interaction have been shown to be extremely high). For these reasons, we relied more on the genetic data in our conclusions.

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Some of the genetic interaction data is still over-interpreted. One example is: "Neither overexpression of Ensconsin nor Khc ameliorated the pruning defects
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Response: We took out the corresponding figures in the manuscript.

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Response: We agree that this is a interpretation rather than the experiment. We therefore tried to separate our experimental design and interpretation more clearly. We rephrased the corresponding results section to ask if PAR-1 affects, Ank2XL, transients etc. We then state at the end of the section that the most likely explanantion for the results is an indirect effect via microtubules (Pages 8-9). We hope this clarifies the issue.

1. Chen L, Stone MC, Tao J, Rolls MM. Axon injury and stress trigger a microtubule-based neuroprotective pathway. Proceedings of the National Academy of Sciences of the United States of America. 2012. Epub 2012/06/27. doi: 10.1073/pnas.1121180109. PubMed PMID: 22733771.
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Response: We removed the kinesin part. We also took another effort to rephrase the parts on the PAR-1/Tau interaction. While we agree that the genetics alone is not the final proof, we think it is a strong basis for our interpretation. So we tried to separate experimental results and interpretation more carefully. Specifically, we renamed the corresponding paragraph: ”PAR-1 is linked to Drosophila tau during dendrite pruning” (p. 5), and we state at the end of the section: “Taken together, our genetic data are consistent with a model where PAR-1 alters microtubule dynamics during dendrite pruning via inhibition of Tau, thus enhancing microtubule accessibility to katanin.” (p. 7). In the introduction, we rephrased: “…we found that PAR-1 interacts genetically with *Drosophila* Tau in a manner consistent with Tau being a PAR-1 target during dendrite pruning.” (p. 2). Also, we are upgrading former Figure EV1 to new Figure 5. This Figure on the effects of PAR-1 and Tau during c1da neuron pruning shows genetic interactions also between a par-1 mutant and tau and therefore confirms, and also extends, our c4da data on PAR-1 and Tau. (p. 8)
We hope this meets your criticism.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sebastian Rumpf
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-95890R

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?	Based on previous experience. Similar to other studies in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	No
For animal studies, include a statement about randomization even if no randomization was used.	NA
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
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	For statistical analysis of categorial data (dendrites attached vs. not attached) we used the Fisher's exact test. For statistical analysis of continuous data (e.g. legth of unpruned dendrites) we used non-parametric tests (Wilcoxon-Mann Whitney test) since the data is not normally distributed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	As test for normality the D'Agostino-Pearson omnibus test was used.
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	anti-acetylated Tubulin antibody : Tao et al. 2016 JCS ; anti-PAR-1 antibody: McDonald et al. 2008 Current Biology; anti-ANK2XL: Koch et al 2002 Neruon; anti-22C10: Hummel et al. 2000 ;anti-Sox14: Ritter and Beckstead DevDyn 2010
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
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