EMBO reports

Protein phosphatase PP2A regulates microtubule orientation and dendrite pruning in *Drosophila*

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

1st Editorial Decision 4 September 2019

Thank you for the submission of your manuscript to EMBO reports. I am sorry for the delay in getting back to you which is due to seasonal traveling. We have now received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge that the findings are potentially interesting. However, they also all raise several technical concerns and point out that the 2 back to back studies should be better aligned. I think that all concerns make sense and should therefore be addressed. Please let me know if you disagree and we can discuss the revisions further.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 7 main figures, I suggest that you layout the manuscript as a full article.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images. And please note that the EMBO reports reference style is numbered (it is in EndNote).

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Ng and colleagues demonstrate here that PP2A is required for dendrite pruning of class 4 multidendritic neurons in Drosophila. They confirm this by using multiple RNAis targeting multiple subunits of the complex as well as analyze mutant clones for key subunits. They found that the expression of the EcR targets Sox14 and MICAL is reduced in PP2A mutants, and interestingly in KD of one regulatory unit (wdb) but not another (tws). Overexpression of MICAL partly suppresses the pruning defect which leads the authors to conclude that PP2A regulates pruning at least in part by controlling MICAL levels via an unknown mechanism. Finally, they also investigate the role of PP2A in cytoskeletal organization and find that MT polarity is disturbed in PP2A KD and mutants. Interestingly, expression of klp2a, a MT severing enzyme whose elevated expression somehow inhibits pruning (as previously shown by this group), is increased in PP2A mutants. KD Klp2a suppresses the pruning defects of PP2A.

While the quality of the experiments is high, the link between the two parts is currently weak and should be at the very least strengthened in the text. There are major discrepancies between this manuscript and an accompanying paper from the Rumpf group and trying to resolve at least some of these is important.

* there is a lack of statistic analysis here - should be added to all figures
* There are a few cases in which pruning is considered to be severe. However, in most of the instances, they refer to the penetrance of the phenotype and not to its severity. The figures actually include both aspects of the pruning defects and therefore I think it would be important to be more accurate.
* "We also observed that the number of dendritic termini was significantly reduced in mtsxe-2258 mutant neurons from the wandering 3rd instar larvae, compared to that in the wild-type ones" - why use a different mutant than in other analyses? What does this result mean? Its not informative as its presented.
* how do the authors explain the unchanged EcR levels in Wdb mutants while reduced levels of Sox14 and MICAL in these mutant clones?
* over expression of MICAL results in a partial suppression of the pruning defect. This is indeed consistent with the hypothesis that PP2A regulates pruning via MICAL levels or activity but this definitely does not prove this and therefore the word "indicate", as written by the authors is a major overstatement.
* I find the terminology Kin-β-gal and Nod-β-gal somewhat confusing and the explanations definitely incomplete. In flybase, these are called Khc::LacZ and Khc::Nod::LacZ.
* the description of the live imaging (figure 6) is super confusing... the 1% completely confused me until I read the rest of the text for about three times...
* does KD of Klp10a also suppress the pruning defects? This would be highly expected in light of their own recent eLife paper.
* The discussion is highly repetitive and the interesting parts are very limited. I would shorten this tremendously and keep to the interesting stuff. Also - this would be a great place to discuss the similarities but also the differences between this and the Rumpf paper.
* talking about the Rumpf paper - in their manuscript, they do not see a change in Sox14 or MICAL expression in KD of PP2A. This could, in principle, be a result of using KD vs MARCM clone but it would be important to test. Have the authors also tested other MT components? It is surprising that Rumpf group did not find a link to MT but rather to actin dynamics.

Typos etc:
* "However, it remains elusive about the function of protein phosphatases in pruning" - in the abstract - really elusive sentence... grammatically and logically, in the context of the previous text.
* what does the word "notable" mean? - relates to the stats required, as mentioned.
* "Our results demonstrate that the PP2A core enzyme, via the regulatory subunit Wdb, facilitates the activation of ecdysone signaling to promote dendrite pruning, whereas it, via a second regulatory subunit, Tws, also regulates the minus-end-out orientation of dendritic MTs which is required for dendrite pruning" - rewrite this sentence... What does "it" refer to? long and convoluted.

Referee #2:

The manuscript by Ng and colleagues addresses the role of protein phosphatase PP2A in the orientation of dendritic microtubules and dendritic pruning in Drosophila ddaC sensory neurons. The authors dissect the role of catalytic, regulatory and scaffolding subunits in pruning of dendrite, demonstrate that PP2A regulates the activation of ecdysone signaling in ddaC neurons prior to dendrite's pruning and show that regulatory subunit Tws is important for establishment of 'minus-end outside' polarity of dendritic microtubules. This manuscript is focused on the microtubule cytoskeleton as the major target of PP2A in the pruning process.
This is interesting and important work but there are several issues and gaps which need to be addressed before it becomes suitable for publication in EMBO Reports.

Major comments:
1. In the Figures 1-3 RNAi-expressing neurons are compared to wild type neurons. It would be more appropriate to include RNAi control. The authors state they compared several RNAi constructs for the knock-down, however they only show one of them. The analysis / comparison of the other constructs should be shown in the supplement.

2. In the Figure 3, the authors say they performed a knock-down of all four PP2A regulatory subunits, however they only show the results for two of them (wdb and tws). They should include the other (negative) ones in the supplement regardless.

3. The microscopy images shown in the Figure 4 are not of very high quality, and the methods for this figure are completely missing. Which microscope was used? How were images acquired? To compare fluorescence values, it would be important to take images from Z-stack to cover the entire volume of the cell. It is not clear whether this was done. Some of the images look overexposed, hence it is questionable how accurate intensity data can be extracted from them. It is also not explained how fluorescence intensities were measured (which software was used / what values were compared?), and how the data analysis was performed. How / what was normalized to what? The same applied for Figure 5 and Figure 7. Furthermore, it is not clear how the fluorescence within the outlined cell bodies was measured especially when there are big red blobs covering up the region of interest. Or was fluorescence only measured in the nucleus, in the case of EcR-B1 and Sox14?
Finally, the authors decided to put the results from tws knockdown from the same experiment into the supplement (Figure S4). However, it would be beneficial for the flow of reading and understanding to combine all data from one dataset into the main figure, instead of placing pieces of it in the supplement, as these are important findings that pin-point the observed effect to the wdb regulatory subunit.

4. Figure S5: The figure shows that overexpression of Mical in mts- and 29B-knockout background rescues the pruning phenotype to a large extent. To complete the data, it would be nice to show this effect also in a wdb-knockout background. In general, it is not clear why this data was put into the supplement. EcR-B1 and Sox14 have many downstream targets, and this figure pinpoints the effect (largely) to one protein, Mical, which is an actin severing enzyme. This is a highly interesting finding. However, this line of evidence for involvement of the actin cytoskeletal system, or the regulation of actin dynamics, in dendrite pruning is completely disregarded by the authors. Obviously, the focus of this paper is more on the microtubule cytoskeleton. However, the authors do not even comment on the possibility of actin involvement or discuss potential ramifications. Instead, from this they jump abruptly to the topic of microtubule orientation, abandoning the EcR-B1-Sox14-Mical line completely. The text flow should be improved, and the author's reasoning to move away from the EcR-B1-Sox14-Mical line and to microtubules should be better explained. Alternatively, it would be highly interesting to include an actin staining (phalloidin staining of fixed cells, or if that gets too crowded expression of LifeAct), to get a first idea whether the actin cytoskeleton could be compromised in PP2A knockout background, and set the focus of the paper on both the actin and microtubule cytoskeleton.

5. Figure 5 shows that knock-down of mts, 29B and knock-out of tws affect the localization of the microtubule-minus-end marker Nod-β-gal (Fig. 5A-I), while knock-down of wdb did not have any effect. Fig. 5 J-M shows the effect on the microtubule-plus-end marker Kin-β-gal, although it is

unclear why the authors switched from mts RNAi to the dominant negative mts over-expression system, or why they did not test tws knock-out in this experiment. This should be explained in the text. Once again, part of the results were included into the supplement Figure S6 (wdb knock-down). As for Figure 4, it would be better to combine the data in the main figure for a better overview. While the measured fluorescence intensity did not statistically differ from the control (Fig. S6D), the shown image looks as if the distribution of the signal is different from the control, it is focused more towards proximal dendrites and not as evenly spread, i.e. wdb knock-down might also have an effect on the MT cytoskeleton. Moreover, the authors showed in Figure 3 that MARCM knock-down of wdb had a much more dramatic effect as knock-down via RNAi. It might be that the low RNAi knock-down efficiency of mts it not sufficient to bring out a phenotype in this experiment. It would therefore be interesting to look at the Nod-β-gal and Kin-β-gal distribution in the wdb MACRM background (as it was already done for tws MARCM).

6. Figure 6: EB1-GFP imaging / kymographs: The authors show that under Mts, 29B and tws knock-down conditions, the growth direction microtubules in dendrites, which usually move retrogradely, is switched around and now a subset of microtubules move away from the soma. These very interesting results go in line with Fig. 5 and suggest a switched orientation of MT polarity, with Mts and 29B knock-down having a much stronger effect that tws knock-down alone. This hints at the involvement of another regulatory subunit. Wdb RNAi knock-down yielded non-significant results. However, as mentioned in the above comment, wdb involvement in MT polarity should be tested more stringently, using the MARCM knock-out additionally to the RNAi knock-down. If possible, it would be interesting to combine tws and wdb knock-down and see if that exacerbates the phenotype. As none of the other tested MT polymerization parameters were affected, several interesting possibilities open up including the mis-localization of microtubule nucleation factors in a PP2A deficient background, a change in MT cathastrophy or post-translational modifications of MTs. It would be good to discuss this in relation to known regulators of MT orientation and nucleation in Drosophila sensory neurons, and especially how those might be downstream of PP2A activity.

7. The authors state they "analysed several MT regulators", however they only show results for Klp10. They should at least name the other factors tested, and even better show the (negative) results in the supplement.

8. The MT depolymerizing kinesin Klp10 was previously shown to be involved in MT polarity regulation by the same authors. Klp10 overexpression impaired MT orientation in dendrites, which is detrimental for dendrite pruning, and knock-down of the Klp10 antagonist patronin/CAMSAP had the same effect. However, knock-down of Klp10 by itself did not affect MT orientation (Wang, Y., Rui, M., Tang, Q., Bu, S., and Yu, F. (2019). Elife 8.) Here, the authors found that in mts and 29B knock-down backgrounds, the intensity of Klp10 antibody staining was increased compared to the control. Additionally, knock-down of Klp10 suppressed the observed MT phenotype in a mts, 29B or tws knockdown background. Accordingly, the authors suggest PP2A might somehow inhibit the function or levels of Klp10A, thereby affecting MT orientation and, by extension, dendrite pruning. However, since data from a different publication shows Klp10 is negatively regulated by phosphorylation, the authors speculate PP2A-mediated dephosphorylation could activate Klp10, making it "more stable and potent", which contradicts their earlier statement and does not make too much sense considering the evidence presented in this and earlier publications. Although, if PP2A truly removes an inhibitory phosphorylation on Klp10, the observed increase in Klp10 intensity upon PP2A inhibition could be due to a compensatory over-expression of Klp10. This issue should be commented in Discussion.

9. The authors do not really discuss their finding that PP2A affects levels of EcR-B1, Sox14, and Mical in depth. The fact that EcR-B1 levels are affected by mts and 29B knock-out hints at PP2A functioning UPSTREAM of EcR-B1 signaling, or maybe affecting a positive feedback loop. However, knock-out of the regulatory subunit wdb only affected Sox14 and Mical levels, hence probably acting downstream of EcR-B1. Alternatively, this could hint at the involvement of several regulatory subunits. The results from the first part of the study should be discussed more in detail, especially, like mentioned above, the involvement of actin regulation (via Mical), which was completely disregarded by the authors, and possible ways in which PP2A might interfere in the EcR-B1-Sox14-Mical pathway. Including more adequate model into the manuscript will also help the readers to follow complexity of the pathways involved in dendritic pruning.

Minor Points:
1. The abbreviation "WP stage" is not spelled out. It would be nice to explain the Drosophila model system used in one or two sentences for readers that are not familiar with this field.

2. Fig. 1A +S1A is not referred to in the text.

3. Fig. 2A is not referred to in the text.
4. Fig. 3A could be shown / mentioned earlier in the text to help explain PP2A structure, best in Fig. 1.

5. Fig. 3B is not referred to in the text.

6. Fig. 4: the green channel should also be shown for control (4A-C); in Fig. S4, cells are additionally stained with HRP to visualize cell outline, this can be extended to Fig. 4.

7. In the discussion, the authors state "We found that the dendrite-pruning phenotypes in various pp2a mutants are reminiscent of those of EcR-B1, sox14 or mical mutants" this is neither shown in this paper nor referenced. Some literature references should be added here.

8. In the discussion, the authors mistakenly write "Par-1 kinesin". This should be corrected to "Par-1 kinase".

9. Supplementary figures are missing figure captions.

10. Reference list needs to be properly formatted.

Referee #3:

The work by Ng and colleagues on the role of PP2A in dendrite pruning is convincing in its fundamental aspects but becomes a bit blurred with regard to the regulatory aspects. The authors propose a model in Fig. 7I that has the major deficit of Wdb not having an effect on EcR expression levels but on Sox14 and Mical. Either the ecdyson link should be proper investigated and understood or left out. Sox14 and Mical should be mentioned instead. A further weakness is that the model would suggest that Wdb and Tws act in parallel and should therefore enhance each other's LOF phenotypes - this has not been tested; the fact that phenotypes of wdb or tws are as strong as those of mts or pp2a-29B are puzzling in this context. A further weakness is that Mical's potential role in the regulation of Klp10 and MT orientation has not been tested - prediction would be that it does not play a role but that it acts through a very different route.

Overall the manuscript could be written much more concisely by grouping together statements with different genetic approaches which come to the same result. Figure choices as to whether data are shown in the main part or as Suppl. Mat. are not always clear to me, and at least most data could be shown in the bar graphs of the main part, whereas Figures become redundant in their appearance and could be shown in Suppl. Mat. I appreciate that pupal dissections are cumbersome but sample numbers below 10 become questionable. Also, the readouts and means of generating the bar graphs need to be far better explained. Data for white pupal images are mostly missing, and in certain figures statistical analyses are left out. The introduction is not well written and lacks a clear rationale for this work. Authors should use the fact that they found PP2A in a screen as a clear motivation for this work.

In sum, the manuscript has clear potential, but it is not acceptable in its current form. I recommend to give authors the possibility to resubmit a more thorough and concise manuscript. Leave out confusing aspects (ecdyson signalling) in favour of a more thorough analyses of the regulatory subunits and actual downstream players, such as Mical and Klp10.

Detailed comments:

Abstract: protein phosphatases as suspected angle does not sell very well. Change 3rd sentence into:

However, the understanding of the underlying molecular mechanisms remains incomplete.

Abstract: simplify 5th sentence

p.4: transition to PP2A does not make sense. There must be a rationale for thinking about PP2A in this context, be it a screen, be it expected function due to certain observations in other contexts.

p5: Par1 and Ptrn are suddenly mentioned, then leading over to PP2A in a non-convincing way, detached from other pruning regulators on page 4. I would like to see a clear argumentative structure and clear rationale for this study.

p.5: last para of intro: I would prefer if authors did not repeat their abstract but rather stated in 2-3 sentences what the key outcome of this study is and why it is important for the context explained above.

All figures: throughout the text, images of whit pre-pupa show very different degrees of morphological aberration, yet they seem not to have been quantified at all. Quantification would help to clarify whether the mutant conditions cause complexity changes during development, too.

Fig.1: "The number of samples (n) in each group is shown on the bars"; it needs to be explained in methods and figure legend what a sample is: one ROI in a dendritic tree? Overall sample numbers are very low. Statistical evaluation for section 1 is not indicated (only in Fig.S2). It is not clear to me what the "percentage of severing defects" is - not explained in methods either. How does it differ from the bar graph F?

p.6: integrate data for RNAi #2 into the graph of Fig.1

p6,1st+2nd para: since phenotypes are consistent, this section could be written far more efficiently.

p.6: what is the mts[xe-2258] allele? Explain. Add the measured data to the bars in Fig.1. Images could be shown together with RNAi #2 in Fig.S1

p.6: When talking about DN experiments, at the idea of: "to test whether PP2A is required at the time of pruning, and phenotypes are not caused by ..."

p.7: Please, briefly remind of the three subunits (together with fly names) but do not repeat info from introduction.

p.7: add data for pp2a/29b RNAi #2 and 3 (shown in S1) in bar graphs of Fig.2

p.8: data for Wrd and PR72 should be shown in a supplementary figure; all data for tws and wdb should be show in bar graphs of Fig. 3

p.9: at least comment on what it might mean that wdb does not affect EcR-B1 expression. This also needs to be confirmed with other LOF conditions for wdb. Later you conclude that PP2A via Wdb regulated Ecdyson signalling? Something here seems contradictory?

p.9: The role of Mical as an actin regulator needs to be explained, and findings need to be woven conceptually into the explanations for PP2A roles.

p.9: Fig.S5 should be integrated into the main part since it is an important experiment for this work; comparisons to controls are missing

p.8: Nod-beta-Gal is not a minus end marker, but it indicates presence of minus-end out MTs; Ptrn is a marker for minus ends and should be considered to be used here. Similar for Kin-beta-Gal.

p.10: With the Nod-beta-Gal readout, things start becoming a bit messy, because it addresses a very different level of regulation., without involving the regulators in a consequent way. If Tws but not Wdb influence Nod-beta-Gal, would this mean that Mical overexpression would not rescue this phenotype?

p.11: "delocalisation" of Kin-beta-Gal; the conclusion should be that the puncta potentially indicate that there are plus-end-out MTs in the mutant dendrites

p.12: "PP2A appears to be crucial for the maintenance of Klp10A level" - this sounds wrong and should rather be "negatively regulates"

p.13: What about klp10A in wdb mutant neurons? How do you explain that Klp10A has an effect even though it is not increased upon loss of Tws? Authors need to provide thoughts here.

1st Revision - authors' response 3 January 2020

Reviewer #1:

Ng and colleagues demonstrate here that PP2A is required for dendrite pruning of class 4 multidendritic neurons in Drosophila. They confirm this by using multiple RNAis targeting multiple subunits of the complex as well as analyze mutant clones for key subunits. They found that the expression of the EcR targets Sox14 and MICAL is reduced in PP2A mutants, and interestingly in KD of one regulatory unit (wdb) but not another (tws). Overexpression of MICAL partly suppresses the pruning defect which leads the authors to conclude that PP2A regulates pruning at least in part by controlling MICAL levels via an unknown mechanism. Finally, they also investigate the role of PP2A in cytoskeletal organization and find that MT polarity is disturbed in PP2A KD and mutants. Interestingly, expression of klp10a, a MT severing enzyme whose elevated expression somehow inhibits pruning (as previously shown by this group), is increased in PP2A mutants. KD Klp10a suppresses the pruning defects of PP2A.

While the quality of the experiments is high, the link between the two parts is currently weak and should be at the very least strengthened in the text. There are major discrepancies between this manuscript and an accompanying paper from the Rumpf group and trying to resolve at least some of these is important.

We thank the reviewer for positive endorsement and helpful suggestions.

* There is a lack of statistic analysis here - should be added to all figures

We have now included the statistical analyses for all the figures.

* There are a few cases in which pruning is considered to be severe. However, in most of the instances, they refer to the penetrance of the phenotype and not to its severity. The figures actually include both aspects of the pruning defects and therefore I think it would be important to be more accurate.

We have deleted “severe” in most of phenotypical analyses except for the case of Mts-dn induction (the strongest pruning defect in this study). We have included the percentage of dendrite pruning phenotypes (penetrance) to made the phenotypic descriptions more accurate (p5 and p7).

* "We also observed that the number of dendritic termini was significantly reduced in mtsxe-2258 mutant neurons from the wandering 3rd instar larvae, compared to that in the wild-type ones" - why use a different mutant than in other analyses? What does this result mean? It’s not informative as its presented.

mts^{xe2258}, a *mts* null allele, harbors a 16-bp deletion (bases -7 to 9) that spans the translation start site (Wassarman DA, et al., *Genes Dev* 1996). We have also provided the phenotypic analysis of mts^{xe2258} allele in terms of dendrite pruning. Similar to those in mts^{299} mutant neurons, all mts^{xe2258} mutant clones exhibited dendrite pruning defects, which were fully rescued by the overexpression of full-length Mts. I have now included the data in the revised text (p5-6) and Figure EV1A.

We have also conducted the sholl analysis for the dendrite morphology of *mts*^{xe2258} ddaC neurons (Figure EV1C), indicating that both dendrite branch points and termini in *mts*^{xe2258} neurons are strongly reduced in number, compared to those in the control neurons.

* How do the authors explain the unchanged EcR levels in Wdb mutants while reduced levels of Sox14 and MICAL in these mutant clones?

We have confirmed the expressions of EcR, Sox14 and Mical using the other *wdb* allele *wdb*^{14}. In *wdb*^{14} ddaC neurons, EcR levels remained unchanged but Sox14 and Mical levels were significantly reduced (Appendix Fig S3A), similar to those in *wdb*^{dw} mutant. We have now included the following explanations in the revised Discussion part (p15) and the *wdb*^{14} data in Appendix Fig S3A.— "Different from the catalytic (Mts) and scaffolding subunits (PP2A-29B), the regulatory subunit Wdb is important for Sox14 and Mical expression, but is not important for EcR-B1 expression. Wdb might regulate Sox14 and Mical expression in parallel to or downstream of EcR-B1. Alternatively, multiple regulatory subunits of PP2A might act redundantly with Wdb to facilitate EcR-B1 expression.".

* Over expression of MICAL results in a partial suppression of the pruning defect. This is indeed consistent with the hypothesis that PP2A regulates pruning via MICAL levels or activity but this definitely does not prove this and therefore the word "indicate", as written by the authors is a major overstatement.

We have now changed "indicate" to "suggest" in the revised manuscript (p9).

* I find the terminology Kin-β-gal and Nod-β-gal somewhat confusing and the explanations definitely incomplete. In flybase, these are called Khc::LacZ and Khc::Nod::LacZ.

The terminology Kin-β-gal and Nod-β-gal has been also used in the previous studies (Reuter, JE, et al., 2003; Yang et al., 2008 and Wang et al., 2019). These terms are interchangeable with Khc::LacZ and Khc::Nod::LacZ, respectively. We have included the following description "also known as Khc::LacZ and Khc::Nod::LacZ, respectively" (p10). We have also provided the residues of Nod and Khc to explain the details of the Kin-β-gal and Nod-β-gal constructs in the revised text (p10-11).

* the description of the live imaging (figure 6) is super confusing... the 1% completely confused me until I read the rest of the text for about three times...

We have included more detailed description (p12) and rephased the sentence to "In the control ddaC neurons, almost all of dendritic EB1-GFP comets migrated towards the soma (retrograde), whereas approximately 1% of the dendritic comets moved away from the soma (anterograde), indicating a uniform minus-end-out MT orientation in the major dendrites".

* does KD of Klp10a also suppress the pruning defects? This would be highly expected in light of their own recent eLife paper.

We have now examined the suppression effect of Klp10A knockdown in *mts* RNAi or *pp2a-29b* RNAi neurons. Klp10A knockdown slightly suppressed the dendrite pruning defects in *mts* or *pp2a-29b* RNAi neurons (Fig EV5A), however, did not restore Mical expression (Fig EV5B). The partial suppression suggests that PP2A regulates dendrite pruning partly via Klp10-mediated MT orientation. We have now included the new data in the revised text (p14) and Figure EV5A-B.

* The discussion is highly repetitive and the interesting parts are very limited. I would shorten this tremendously and keep to the interesting stuff. Also - this would be a great place to discuss the similarities but also the differences between this and the Rumpf paper.

We have now rewritten the Discussion section with the focus on some interesting points. Moreover, we have also discussed the actin cytoskeletons during dendrite pruning and briefly compared our MT study and the Rumpf study in the revised Discussion section (p17)

* talking about the Rumpf paper - in their manuscript, they do not see a change in Sox14 or MICAL expression in KD of PP2A. This could, in principle, be a result of using KD vs MARCM clone but it would be important to test.

We agree with the reviewer. In our study, we depleted most of *PP2A* gene functions, as we either generated mutant clones using the null/strong hypomorphic alleles (mts^{xe2258}, $pp2a\text{-}29b^{rs}$ and wdb^{dw}) or used two copies of *ppk-Gal4* driver. Moreover, in the revised manuscript, we have also examined the levels of Sox14 and Mical using Mts-DN (dominant negative) (with 2 copies of *ppk-Gal4*) and the other *wdb* allele wdb^{14} (Appendix Figure S3A). Consistently, our new results further support the conclusion that loss of *mts*, *PP2A-29B* or *wdb* function leads to significant decreases in Sox14 and Mical expression. Moreover, we also utilized a *mical-β*-gal reporter that drives the LacZ expression under the control of a *mical* enhancer to examine the *mical* transcription. The LacZ expressions were absent or strongly reduced in *mts* or *pp2a-29b* RNAi neurons (Appendix Figure S3B), suggesting that Mts and PP2A-29B likely regulate the *mical* transcription in ddaC neurons. Taken together, our data indicate that PP2A is required for the expression of Mical in ddaC neurons during early metamorphosis. We have included these new data in the revised text (p8-9) and Appendix Figure S3A-B.

Have the authors also tested other MT components? It is surprising that Rumpf group did not find a link to MT but rather to actin dynamics.

In addition to Klp10A, we also tested other MT regulators including Patronin, TACC and Mini spindles (Msps). We did not detect any alteration in the expression level and distribution pattern in *mts* RNAi or *pp2a-29b* RNAi ddaC neurons, suggesting that PP2A selectively regulates the Klp10 level in sensory neurons. We have now included these data in the revised text (p13) and Appendix Figure S4A-B.

Typos etc:
* "However, it remains elusive about the function of protein phosphatases in pruning" - in the abstract - really elusive sentence... grammatically and logically, in the context of the previous text.

We have rephrased the sentences to “However, the understanding of the molecular mechanisms underlying dendrite pruning remains incomplete.”.

* What does the word "notable" mean? - relates to the stats required, as mentioned.

We have deleted "notable" and also added the statistical analyses for all the figures.

* "Our results demonstrate that the PP2A core enzyme, via the regulatory subunit Wdb, facilitates the activation of ecdysone signaling to promote dendrite pruning, whereas it, via a second regulatory subunit, Tws, also regulates the minus-end-out orientation of dendritic MTs which is required for dendrite pruning" - rewrite this sentence... What does "it" refer to? long and convoluted.

We have rephrased the sentences to “Our data suggest that PP2A, via Wdb, facilitates the expression of Sox14 and Mical, two downstream targets of ecdysone signalling, whereas PP2A, via Tws, regulates dendritic minus-end-out MT orientation which is required for dendrite pruning” in p14.

Reviewer #2:

The manuscript by Ng and colleagues addresses the role of protein phosphatase PP2A in the orientation of dendritic microtubules and dendritic pruning in Drosophila ddaC sensory neurons. The authors dissect the role of catalytic, regulatory and scaffolding subunits in pruning of dendrite, demonstrate that PP2A regulates the activation of ecdysone signaling in ddaC neurons prior to dendrite's pruning and show that regulatory subunit Tws is important for establishment of 'minus-end outside' polarity of dendritic microtubules. This manuscript is focused on the microtubule cytoskeleton as the major target of PP2A in the pruning process.
This is interesting and important work but there are several issues and gaps which need to be addressed before it becomes suitable for publication in EMBO Reports.

We are greatly grateful to the reviewer for recognizing our work interesting and important.

Major comments:
1. In the Figures 1-3 RNAi-expressing neurons are compared to wild type neurons. It would be more appropriate to include RNAi control. The authors state they compared several RNAi constructs for the knock-down, however they only show one of them. The analysis / comparison of the other constructs should be shown in the supplement.

We have now included proper control neurons for both RNAi or MARCM experiments in Figure 1-3. To make our main figures concise, we had previously included other RNAi lines, including *mts* RNAi #2 and *pp2a-29b* RNAi (#2 and #3) in the original Figure S1 (currently in Appendix Figure S1). For the cases of *wdb* and *tws*, we have now included the images for *wdb* RNAi #2 and *tws* RNAi #2 in Figure EV2A.

2. In the Figure 3, the authors say they performed a knock-down of all four PP2A regulatory subunits, however they only show the results for two of them (wdb and tws). They should include the other (negative) ones in the supplement regardless.

We have now included the knockdown images of two other regulatory subunits, namely Wrd and Pr72. We examined three RNAi lines for *wrd* and two independent RNAi lines for *PR72*. Knockdown of *wrd* or *PR72* did not affect normal dendrite pruning. We have included these negative data in Figure EV2A.

3. The microscopy images shown in the Figure 4 are not of very high quality, and the methods for this figure are completely missing. Which microscope was used? How were images acquired? To compare fluorescence values, it would be important to take images from Z-stack to cover the entire volume of the cell. It is not clear whether this was done.

We have now included the details of the methods for Figures 4, 5 and 7 in the revised Materials and Methods part (p19-20). Briefly, we imaged the immunostained fillets with proper exposure in sensory neurons using the confocal microscopy Leica TSC SP2. The images were acquired from projected Z-stacks (at 1.5 μm intervals) to cover the entire volume of the cells.

Some of the images look overexposed, hence it is questionable how accurate intensity data can be extracted from them. It is also not explained how fluorescence intensities were measured (which software was used / what values were compared?), and how the data analysis was performed. How / what was normalized to what? The same applied for Figure 5 and Figure 7.

In Figure 4, we imaged ddaC and ddaE sensory neurons to acquire their fluorescent signals with proper exposure. With the same confocal setting, the epidermal signals could be overexposed. However, the epidermal overexposure did not affect our results in neurons, as we only quantified the fluorescence signals in ddaC/ddaE neurons in Figure 4. The fluorescence intensity was measured with Image J and the normalized intensity means the ratio of ddaC to ddaE mean intensity as shown in the graphs. Likewise, the images in Figure 6 and 7 were taken with proper exposure in sensory neurons.

Furthermore, it is not clear how the fluorescence within the outlined cell bodies was measured especially when there are big red blobs covering up the region of interest. Or was fluorescence only measured in the nucleus, in the case of EcR-B1 and Sox14?

The EcR-B1/Sox14 signals were only measured in the nuclei of ddaC neurons, therefore the red blobs did not affect their signals in the nucleus. For Mical, Klp10A and other cytoplasmic proteins, their fluorescence signals were measured in the entire soma. We have now included the information in the revised Materials and Methods part (p19-20).

Finally, the authors decided to put the results from tws knockdown from the same experiment into the supplement (Figure S4). However, it would be beneficial for the flow of reading and understanding to combine all data from one dataset into the main figure, instead of placing pieces of it in the supplement, as these are important findings that pin-point the observed effect to the wdb regulatory subunit.

We have now included the data on EcR-B1, Sox-14 and Mical levels in *tws*^{60} MARCM clones and combined these data into the revised Figure 4M-O.

4. Figure S5: The figure shows that overexpression of Mical in mts- and 29B-knockout background rescues the pruning phenotype to a large extent. To complete the data, it would be nice to show this effect also in a wdb-knockout background. In general, it is not clear why this data was put into the supplement.

We have examined the effect of Mical overexpression in *wdb* RNAi ddaC neurons. Overexpression of Mical significantly suppressed the dendrite pruning defects in *wdb* RNAi neurons. We have now integrated these new rescue data into the revised manuscript (p9 and Figure 5G-I) and moved all rescue data to the main Figure 5.

EcR-B1 and Sox14 have many downstream targets, and this figure pinpoints the effect (largely) to one protein, Mical, which is an actin severing enzyme. This is a highly interesting finding. However, this line of evidence for involvement of the actin cytoskeletal system, or the regulation of actin dynamics, in dendrite pruning is completely disregarded by the authors. Obviously, the focus of this paper is more on the microtubule cytoskeleton. However, the authors do not even comment on the possibility of actin involvement or discuss potential ramifications. Instead, from this they jump abruptly to the topic of microtubule orientation, abandoning the EcR-B1-Sox14-Mical line completely. The text flow should be improved, and the author's reasoning to move away from the EcR-B1-Sox14-Mical line and to microtubules should be better explained. Alternatively, it would be highly interesting to include an actin staining (phalloidin staining of fixed cells, or if that gets too crowded expression of LifeAct), to get a first idea whether the actin cytoskeleton could be compromised in PP2A knockout background, and set the focus of the paper on both the actin and microtubule cytoskeleton.

We previously examined the distribution of F-actin in *mts* RNAi or *pp2a-29b* RNAi neurons by using phalloidin staining in fixed larval tissues. At that time, we focused on the phalloidin staining at the proximal dendrites/somas and did not observe any obvious difference between wild-type and *mts*/*pp2a-29b* RNAi ddaC neurons from the 3rd instar larvae (Fig EV3A-B). Therefore, we focused our study on the role of PP2A in regulating the MT cytoskeleton in our manuscript.

We have now revised our text flow (p10)--"It has been shown that Mical functions as an actin disassembly factor to disassemble F-actin *in vitro* and in bristles [43]. However, we did not observe any obvious defect in the phalloidin staining in the proximal dendrites and soma of *mts* and *pp2a-29b* RNAi ddaC neurons from the 3rd instar larvae (Fig EV3A-B). Recent studies have also documented that MT dynamics, stability, and orientation play important roles….".

5. Figure 5 shows that knock-down of mts, 29B and knock-out of tws affect the localization of the microtubule-minus-end marker Nod-β-gal (Fig. 5A-I), while knock-down of wdb did not have any effect. Fig. 5 J-M shows the effect on the microtubule-plus-end marker Kin-β-gal, although it is unclear why the authors switched from mts RNAi to the dominant negative mts over-expression system, or why they did not test tws knock-out in this experiment. This should be explained in the text.

We have now examined the localization of Kin-β-gal in *mts* RNAi and *tws* RNAi ddaC neurons. Kin-β-gal was also mislocalized as punctate structures in the dendrites upon *mts* RNAi knockdown, consistent with that in Mts^{DN}-overexpressing neurons. Likewise, knockdown of *tws* also resulted in dendritic localization of Kin-β-gal in ddaC neurons. These new data consistently support the conclusion that Mts and Tws are required for proper distribution of the axonal MT marker Kin-β-gal. We have included these data in the revised text (p11) and Figure 6G, 6I and replaced the Mts^{DN} data with the *mts* RNAi data.

Once again, part of the results were included into the supplement Figure S6 (wdb knock-down). As for Figure 4, it would be better to combine the data in the main figure for a better overview. While the measured fluorescence intensity did not statistically differ from the control (Fig. S6D), the shown image looks as if the distribution of the signal is different from the control, it is focused more

towards proximal dendrites and not as evenly spread, i.e. wdb knock-down might also have an effect on the MT cytoskeleton.

We checked our original images and confirmed that the Nod-β-gal distribution in *wdb* knock-down neurons is similar to that in the control neurons. Nevertheless, we have now replaced the *wdb* knockdown result with that of wdb^{dw} mutant (see the next point).

Moreover, the authors showed in Figure 3 that MARCM knock-down of wdb had a much more dramatic effect as knock-down via RNAi. It might be that the low RNAi knock-down efficiency of mts it not sufficient to bring out a phenotype in this experiment. It would therefore be interesting to look at the Nod-β-gal and Kin-β-gal distribution in the wdb MACRM background (as it was already done for tws MARCM).

We have generated wdb^{dw} mutant ddaC clones for detecting the Nod-β-gal distribution and wdb^{dw}/wdb^{14} transheterozygous mutant neurons for detecting the Kin-β-gal distribution. Both Nod-β-gal and Kin-β-gal were properly localized in dendrites and axons of *wdb* mutant neurons. Since the results from *wdb* mutants are more conclusive than those from *wdb* RNAi knockdown, we have now replaced the *wdb* RNAi results with those of wdb^{dw} mutants. Thus, these new data strongly support the conclusion that Wdb is dispensable for the distribution of dendritic and axonal microtubule markers (Nod-β-gal and Kin-β-gal) in ddaC neurons. We have now included these data in the revised text (p11) and Figure 6E, 6J.

6. Figure 6: EB1-GFP imaging / kymographs: The authors show that under Mts, 29B and tws knock-down conditions, the growth direction microtubules in dendrites, which usually move retrogradely, is switched around and now a subset of microtubules move away from the soma. These very interesting results go in line with Fig. 5 and suggest a switched orientation of MT polarity, with Mts and 29B knock-down having a much stronger effect that tws knock-down alone. This hints at the involvement of another regulatory subunit. Wdb RNAi knock-down yielded non-significant results. However, as mentioned in the above comment, wdb involvement in MT polarity should be tested more stringently, using the MARCM knock-out additionally to the RNAi knock-down.

We have now examined EB1-GFP comet behaviour in wdb^{dw}/wdb^{14} mutant ddaC neurons. Consistently, loss of *wdb* function did not influence the retrograde movement of EB1-GFP comets in the mutant dendrites, supporting that Wdb is dispensable for the minus-end-out MT orientation in ddaC dendrites. The new data are shown in the main Figure 7I-J and revised text (p12).

If possible, it would be interesting to combine tws and wdb knock-down and see if that exacerbates the phenotype.

We have now examined the orientation of dendritic EB-GFP comets in double knockdown of *tws* and *wdb* (*tws* RNAi + *wdb* RNAi), and compared it with *tws* RNAi + control RNAi. knockdown of *wdb* in the *tws* RNAi background did not significantly enhance the dendritic MT misorientation phenotype, suggesting that Tws and Wdb unlikely act redundantly to regulate dendritic MT orientation. We have now included the new data in the revised text (p12) and Appendix Figure S6B.

As none of the other tested MT polymerization parameters were affected, several interesting possibilities open up including the mis-localization of microtubule nucleation factors in a PP2A deficient background, a change in MT cathastrophy or post-translational modifications of MTs. It would be good to discuss this in relation to known regulators of MT orientation and nucleation in Drosophila sensory neurons, and especially how those might be downstream of PP2A activity.

We have now discussed the potential roles of PP2A in distributing the MT nucleator γ-tubulin and regulating MT orientation in dendrites. We have now included the detailed discussion in the revised text (p16).

7. The authors state they "analysed several MT regulators", however they only show results for Klp10. They should at least name the other factors tested, and even better show the (negative) results in the supplement.

In addition to Klp10A, we had also tested other MT regulators including Patronin, TACC and Msps in our study. We observed no detectable alteration in the expression levels of Patronin, TACC and Msps in *mts* RNAi and *pp2a-29b* RNAi ddaC neurons, suggesting that PP2A selectively regulates the Klp10 level in sensory neurons. We have now included the results in p13 and Appendix Figure S4A-B.

8. The MT depolymerizing kinesin Klp10 was previously shown to be involved in MT polarity regulation by the same authors. Klp10 overexpression impaired MT orientation in dendrites, which is detrimental for dendrite pruning, and knock-down of the Klp10 antagonist patronin/CAMSAP had the same effect. However, knock-down of Klp10 by itself did not affect MT orientation (Wang, Y., Rui, M., Tang, Q., Bu, S., and Yu, F. (2019). Elife 8.) Here, the authors found that in mts and 29B knock-down backgrounds, the intensity of Klp10 antibody staining was increased compared to the control. Additionally, knock-down of Klp10 suppressed the observed MT phenotype in a mts, 29B or tws knockdown background. Accordingly, the authors suggest PP2A might somehow inhibit the function or levels of Klp10A, thereby affecting MT orientation and, by extension, dendrite pruning. However, since data from a different publication shows Klp10 is negatively regulated by phosphorylation, the authors speculate PP2A-mediated dephosphorylation could activate Klp10, making it "more stable and potent", which contradicts their earlier statement and does not make too much sense considering the evidence presented in this and earlier publications. Although, if PP2A truly removes an inhibitory phosphorylation on Klp10, the observed increase in Klp10 intensity upon PP2A inhibition could be due to a compensatory over-expression of Klp10. This issue should be commented in Discussion.

We have now deleted the speculation and included the following discussion in the revised text (p16-17)— “Therefore, loss of PP2A function phenocopies Klp10A overexpression in terms of dendrite pruning and dendritic MT orientation [23]. It is conceivable that elevated Klp10A might be able to attack the MT ends and depolymerize long MTs to short filaments. Short MTs was proposed to re-orient randomly and serve as seeds for polymerization [63], potentially resulting in a mixed MT polarity in dendrites.”.

9. The authors do not really discuss their finding that PP2A affects levels of EcR-B1, Sox14, and Mical in depth. The fact that EcR-B1 levels are affected by mts and 29B knock-out hints at PP2A functioning UPSTREAM of EcR-B1 signaling, or maybe affecting a positive feedback loop. However, knock-out of the regulatory subunit wdb only affected Sox14 and Mical levels, hence probably acting downstream of EcR-B1. Alternatively, this could hint at the involvement of several regulatory subunits. The results from the first part of the study should be discussed more in detail, especially, like mentioned above, the involvement of actin regulation (via Mical), which was completely disregarded by the authors, and possible ways in which PP2A might interfere in the EcR-B1-Sox14-Mical pathway. Including more adequate model into the manuscript will also help the readers to follow complexity of the pathways involved in dendritic pruning.

We have now included the following discussion in the revised Discussion part (p15)-- “Different from the catalytic (Mts) and scaffolding subunits (PP2A-29B), the regulatory subunit Wdb is important for Sox14 and Mical expression, but is not important for EcR-B1 expression. Wdb might regulate Sox14 and Mical expression in parallel to or downstream of EcR-B1. Alternatively, multiple regulatory subunits of PP2A might act redundantly with Wdb to facilitate EcR-B1 expression.”.

We have also discussed a possible role of actin dynamics and Mical in dendrite pruning in the revised text (p17)—" In an accompanying manuscript by the Rumpf laboratory, the role of PP2A in dendrite pruning is also reported with the focus on its function in regulating the actin dynamics in the dendrites. F-actin undergoes disassembly at the proximal dendrites of ddaC neurons at the onset of dendrite pruning [17]. Since Mical can directly disassemble F-actin via its N-terminal flavoprotein monooxygenase (FM) domain *in vitro* and in bristles [43], it remains an excellent candidate for promoting F-actin disassembly during dendrite pruning.”.

Minor Points:

1. The abbreviation "WP stage" is not spelled out. It would be nice to explain the Drosophila model system used in one or two sentences for readers that are not familiar with this field.

We have now included “at the white prepupal (WP)” in the revised text (p6) and also briefly explained the dendrite pruning system in the Introduction section (p3-4).

2. Fig. 1A +S1A is not referred to in the text.

We have now referred to them in the revised text (p4).

3. Fig. 2A is not referred to in the text.

We have now referred to it in the revised text (p6).

4. Fig. 3A could be shown / mentioned earlier in the text to help explain PP2A structure, best in Fig. 1.

We have now moved the schematic representation of PP2A structure in the revised Appendix Figure S1A.

5. Fig. 3B is not referred to in the text.

We have now included it in the revised text (p7).

6. Fig. 4: the green channel should also be shown for control (4A-C); in Fig. S4, cells are additionally stained with HRP to visualize cell outline, this can be extended to Fig. 4.

In Figure 4, we conducted the MARCM analyses to generate GFP-positive mutant ddaC clones in an otherwise heterozygous background. We normally used their neighboring GFP-negative heterozygous neurons as the controls. These internal controls are better and more reliable, as GFP-negative and positive neurons in the same fillets were processed under the same experimental conditions and also visualized with the same confocal settings. I hope that the reviewer agrees with us on this point.

Since it is quite easy for us to identify ddaC neurons based on their position and shape, we conducted HRP staining for some of our experiments. To make the figures consistent and concise, we have removed the HRP images from all the immunostaining figures.

7. In the discussion, the authors state "We found that the dendrite-pruning phenotypes in various pp2a mutants are reminiscent of those of EcR-B1, sox14 or mical mutants" this is neither shown in this paper nor referenced. Some literature references should be added here.

We have now added the references in the revised text (p15).

8. In the discussion, the authors mistakenly write "Par-1 kinesin". This should be corrected to "Par-1 kinase".

We have now changed “Par-1 kinesin” to “Par-1 kinase”.

9. Supplementary figures are missing figure captions.

We had included all the supplementary figure legends after the main figure legends.

10. Reference list needs to be properly formatted.

We have now formatted the references according to the journal’s style.

Reviewer #3:

The work by Ng and colleagues on the role of PP2A in dendrite pruning is convincing in its fundamental aspects but becomes a bit blurred with regard to the regulatory aspects. The authors propose a model in Fig. 7I that has the major deficit of Wdb not having an effect on EcR expression

levels but on Sox14 and Mical. Either the ecdyson link should be proper investigated and understood or left out. Sox14 and Mical should be mentioned instead.

In the revised manuscript, we have now modified the model by highlighting Sox14 and Mical, instead of ecdysone signalling (Figure 8I).

A further weakness is that the model would suggest that Wdb and Tws act in parallel and should therefore enhance each other's LOF phenotypes - this has not been tested; the fact that phenotypes of wdb or tws are as strong as those of mts or pp2a-29B are puzzling in this context.

The dendrite pruning phenotypes in *wdb* mutant neurons are indeed as strong as those in *mts* or *pp2a-29b* mutants. These data suggest that Wdb-mediated Mical pathway contributes more significantly to normal dendrite pruning, compared to Tws-mediated MT pathway. Alternatively, the severity of the mutant phenotypes from the MARCM clonal analyses is also dependent on the protein perdurance of each gene (Lee T, *Neuron* 1999), which may complicate the comparisons between the pruning phenotypes of *mts*, *pp2a-29b* or *wdb* mutants.

Moreover, we have now examined the effects of double knockdown of *tws* and *wdb* (*tws* RNAi + *wdb* RNAi), and compared it with either *tws* RNAi or *wdb* RNAi knockdown. Our results indicate that double knockdown of *wdb* and *tws* exacerbated the dendrite pruning phenotype compared to either of single knockdown. This result is consistent with the model that Wdb and Tws act in parallel to regulate dendrite pruning. We have now included in the revised text (p7) and Figure EV2B.

A further weakness is that Mical's potential role in the regulation of Klp10 and MT orientation has not been tested - prediction would be that it does not play a role but that it acts through a very different route.

We have now examined Klp10A level and dendritic MT orientation in *mical* RNAi ddaC neurons. Indeed, knockdown of Mical did not affect Klp10A level, moreover, neither dendritic distribution of Nod-β-gal nor EB1-GFP comet directionality was disturbed. Thus, Mical is dispensable for the regulation of Klp10A and dendritic MT orientation. These new data are shown in the revised text (p13), Figure EV4G-H and Appendix Figure S7A-B.

Overall the manuscript could be written much more concisely by grouping together statements with different genetic approaches which come to the same result. Figure choices as to whether data are shown in the main part or as Suppl. Mat. are not always clear to me, and at least most data could be shown in the bar graphs of the main part, whereas Figures become redundant in their appearance and could be shown in Suppl. Mat. I appreciate that pupal dissections are cumbersome but sample numbers below 10 become questionable. Also, the readouts and means of generating the bar graphs need to be far better explained. Data for white pupal images are mostly missing, and in certain figures statistical analyses are left out. The introduction is not well written and lacks a clear rationale for this work. Authors should use the fact that they found PP2A in a screen as a clear motivation for this work.

In sum, the manuscript has clear potential, but it is not acceptable in its current form. I recommend to give authors the possibility to resubmit a more thorough and concise manuscript. Leave out confusing aspects (ecdyson signalling) in favour of a more thorough analyses of the regulatory subunits and actual downstream players, such as Mical and Klp10.

We are grateful to the reviewer for positive endorsement and constructive suggestions. We have provided a significant amount of new data to addressed all the concerns in the following point-by-point response letter. We have also re-written the manuscript in a more thorough and concise way.

Detailed comments:

Abstract: protein phosphatases as suspected angle does not sell very well. Change 3rd sentence into: However, the understanding of the underlying molecular mechanisms remains incomplete.

We have changed the sentence to "However, the understanding of the molecular mechanisms underlying dendrite pruning remains incomplete.".

Abstract: simplify 5th sentence

We have rephrased the 5th sentence to "We show that the catalytic (Microtubule star, Mts), scaffolding (PP2A-29B), and two regulatory subunits (Widerborst/Wdb and Twins/Tws) play important roles in dendrite pruning."

p.4: transition to PP2A does not make sense. There must be a rationale for thinking about PP2A in this context, be it a screen, be it expected function due to certain observations in other contexts.

We have now indicated the identification of PP2A in our RNAi screen for a transition to PP2A—"In a genome-wide RNA interference (RNAi) screen, we isolated Microtubule star (Mts), a catalytic subunit of Protein phosphatase 2A (PP2A), which is required for dendrite pruning of ddaC neurons." in the revised text (p4).

p5: Par1 and Ptrn are suddenly mentioned, then leading over to PP2A in a non-convincing way, detached from other pruning regulators on page 4. I would like to see a clear argumentative structure and clear rationale for this study.

We have moved the Par1/Ptrn sentence into the preceding paragraph (p4) as follows --"It has been recently reported that PAR-1 and Patronin play important roles in regulating MT stability and orientation during dendrite pruning.". This makes the text flow clear.

p.5: last para of intro: I would prefer if authors did not repeat their abstract but rather stated in 2-3 sentences what the key outcome of this study is and why it is important for the context explained above.

We have significantly shortened the last paragraph of the Introduction part (p5).

All figures: throughout the text, images of white pre-pupa show very different degrees of morphological aberration, yet they seem not to have been quantified at all. Quantification would help to clarify whether the mutant conditions cause complexity changes during development, too.

Loss or knockdown of *mts*, *pp2a-29b* or *wdb* led to different degrees of simplified dendrite arbors at white prepupal (WP) stage. However, the rescue experiments or Gene-switch experiments show normal dendrite arbors at WP stage. For direct comparisons, we have now quantified the number of primary and secondary dendrites at WP stage from Figure 1-3 and their respective supplemental figures. The quantification results have been shown in Appendix Figure S1C.

Fig.1: "The number of samples (n) in each group is shown on the bars"; it needs to be explained in methods and figure legend what a sample is: one ROI in a dendritic tree? Overall sample numbers are very low. Statistical evaluation for section 1 is not indicated (only in Fig.S2). It is not clear to me what the "percentage of severing defects" is - not explained in methods either. How does it differ from the bar graph F?

We have now included "The number of neurons (n) examined in each group is shown on the bars." in the Materials and Methods part (p19) and all the figure legends. For MARCM clonal analyses, the clone-making efficiency was very low (<0.5 clones per animal), which required our tremendous efforts. Therefore, the number of the MARCM clones was much smaller than those of RNAi and dominant negative analyses. Importantly, our conclusions are consistent and reliable, as they are supported by multiple genetic approaches. Nevertheless, we have repeated the clonal experiments and increased the sample number over 10. The new data are consistent with the previous results and thus further strengthen the conclusions.

We had previously included some of the definition and now made them clearer (p19)—"The severing defect was defined by the presence of dendrites that remain attached to the soma at 16 h APF. The severing defect in wild-type or mutant ddaC neurons were quantified in a 275 μm x 275 μm region of the dorsal dendritic field, originating from the abdominal segments 2-5. The

percentage of severing defects is the percentage of ddaC neurons with larval dendrites attached to the soma at 16 h APF". We have also included the above definition in the revised Materials and Methods section (p19).

"The percentage of severing defects" indicates the penetrance of the defects, whereas "the number of primary and secondary dendrites attached to soma" (in the original bar graph 1F) indicates the severity of the defects.

p.6: integrate data for RNAi #2 into the graph of Fig.1

We have integrated the data for *mts* RNAi #2 into the graph of Figure 1I, 1J. Similarly, we have included all the statistical data in the graphs of Figure 2-3.

p6,1st+2nd para: since phenotypes are consistent, this section could be written far more efficiently.

We have now combined the first and second paragraphs (p5-6). We have also deleted some repetitive sentences and written the paragraph more efficiently (p5-6).

p.6: what is the mts[xe-2258] allele? Explain. Add the measured data to the bars in Fig.1. Images could be shown together with RNAi #2 in Fig.S1

mts^{xe2258}, a *mts* null allele, harbors a 16-bp deletion (bases -7 to 9) that spans the translation start site. We have cited the original paper for the allele information (Wassarman DA, *Genes Dev* 1996). We have also provided the phenotypic analysis of mts^{xe2258} allele in terms of dendrite pruning (Figure EV1A). Similar to mts^{299} allele, mts^{xe2258} exhibited dendrite pruning defects with full penetrance, which were fully rescued by the expression of full-length Mts protein. I have now included these new data in p5-6 and Figure EV1A.

p.6: When talking about DN experiments, at the idea of: "to test whether PP2A is required at the time of pruning, and phenotypes are not caused by ..."

We have now rephrased the sentence to " To examine whether PP2A is required at the timing of pruning, and the dendrite pruning defect in *mts* mutant neurons is not caused by the initial morphology defect…" in p6.

p.7: Please, briefly remind of the three subunits (together with fly names) but do not repeat info from introduction.

We have now included the information only in the Introduction part (p4).

p.7: add data for pp2a/29b RNAi #2 and 3 (shown in S1) in bar graphs of Fig.2

We have now included the quantification data for *pp2a-29b* RNAi #2 and 3 in bar graphs of Figure 2F-G.

p.8: data for Wrd and PR72 should be shown in a supplementary figure; all data for tws and wdb should be show in bar graphs of Fig. 3

We have now included the knockdown images of two other regulatory subunits, namely Wrd and Pr72. We had examined three RNAi lines for *wrd* and two independent RNAi lines for *PR72*. Knockdown of *wrd* or *PR72* did not affect normal dendrite pruning. We have included these negative data in Figure EV2A.

We have also included all the images and quantification data for *tws* and *wdb* RNAi phenotypes in Figure 3I-J graphs and Figure EV2A.

p.9: at least comment on what it might mean that wdb does not affect EcR-B1 expression. This also needs to be confirmed with other LOF conditions for wdb. Later you conclude that PP2A via Wdb regulated Ecdyson signalling? Something here seems contradictory?

We have confirmed the expressions of EcR, Sox14 and Mical using the other *wdb* allele wdb^{14}. In wdb^{14} ddaC neurons, EcR levels remained unchanged but Sox14 and Mical levels were significantly reduced (Appendix Fig S3A), similar to those in wdb^{dw} mutant. We have now included the following comment in the revised Discussion part (p15)—" Different from the catalytic (Mts) and scaffolding subunits (PP2A-29B), the regulatory subunit Wdb is important for Sox14 and Mical expression, but is not important for EcR-B1 expression. Wdb might regulate Sox14 and Mical expression in parallel to or downstream of EcR-B1. Alternatively, multiple regulatory subunits of PP2A might act redundantly with Wdb to facilitate EcR-B1 expression.".

We have also revised the model (Figure 8I) by showing that PP2A, via Wdb, regulates the expression of Sox14 and Mical, two downstream targets of ecdysone signalling.

p.9: The role of Mical as an actin regulator needs to be explained, and findings need to be woven conceptually into the explanations for PP2A roles.

We have now included the role of Mical as an actin regulator in the revised text (p10)-- "It has been shown that Mical functions as an actin disassembly factor to disassemble F-actin *in vitro* and in bristles [43]. However, we did not observe any obvious defect in the phalloidin staining in the proximal dendrites and soma of *mts* and *pp2a-29b* RNAi ddaC neurons from the 3rd instar larvae (Fig EV3A-B). Recent studies have also documented that MT dynamics, stability, and orientation play important roles….".

p.9: Fig.S5 should be integrated into the main part since it is an important experiment for this work; comparisons to controls are missing

We have now included the MARCM controls and moved the rescue data into the main Figure 5, as the reviewers suggested.

p.8: Nod-beta-Gal is not a minus end marker, but it indicates presence of minus-end out MTs; Ptrn is a marker for minus ends and should be considered to be used here. Similar for Kin-beta-Gal.

We have now changed to "Nod-β-gal was … used as a marker for potentially detecting the presence of minus-end-out MTs in dendrites" (p10) and "suggesting the presence of plus-end-out MTs in the mutant dendrites" in the revised text (p11).

We have also investigated the distribution of Patronin in wild-type, *mts/pp2a-29b* RNAi neurons using the anti-Patronin antibody, however, we did not observe any punctate distribution on individual minus ends in dendrites, axons and soma. Since neuronal MT bundles are compactly arranged into the compartments of neurons, we were not able to detect individual minus ends with the standard resolution from a normal confocal microscopy. We have now included these Patronin images in Appendix Figure S4A-B.

p.10: With the Nod-beta-Gal readout, things start becoming a bit messy, because it addresses a very different level of regulation., without involving the regulators in a consequent way. If Tws but not Wdb influence Nod-beta-Gal, would this mean that Mical overexpression would not rescue this phenotype?

We have used three markers (Nod-β-gal4, Kin-β-gal4 and EB1-GFP) to detect dendritic MT orientation in *pp2a* RNAi or mutant neurons. Our data from these three markers consistently support the conclusion that Mts, PP2A-29B and Tws are required to regulate the minus-end-out MT orientation in dendrites, whereas Wdb is dispensable for the dendritic MT orientation.

We have also tested the possibility of whether Mical overexpression can rescue Nod-β-Gal defect in *tws* RNAi neurons. As expected, overexpression of Mical was not able to rescue the Nod-β-Gal defect observed in *tws* RNAi neurons (Appendix Figure S5B). In addition, we have also examined the Nod-β-Gal distribution and EB1-GFP comet orientation in *mical* RNAi neurons. Consistently, our results indicate that Mical is dispensable for dendritic MT orientation and Klp10A level in ddaC neurons (Appendix Figure S7A-B, Figure EV4G-H). We have now included the data in the revised text (p11 and p13).

p.11: "delocalisation" of Kin-beta-Gal; the conclusion should be that the puncta potentially indicate that there are plus-end-out MTs in the mutant dendrites

We have now modified the sentence to “suggesting the presence of plus-end-out MTs in the mutant dendrites” in p11.

p.12: "PP2A appears to be crucial for the maintenance of Klp10A level" - this sounds wrong and should rather be "negatively regulates"

We have now rephrased the sentences to “PP2A appears to negatively regulate Klp10A level” in the revised text (p13).

p.13: What about klp10A in wdb mutant neurons? How do you explain that Klp10A has an effect even though it is not increased upon loss of Tws? Authors need to provide thoughts here.

We have now examined the Klp10A level in wdb^{dw} mutant neurons and did not observe any significant alteration in Klp10A level in those mutant neurons. Thus, Wdb appears to be dispensable for proper Klp10A level in ddaC neurons. We have included the data in p13 and Figure EV4E-F.

Our explanation is that subtle alterations in the Klp10A level in *tws* RNAi neurons might be below the detection limit in the immunostaining assays with the anti-Klp10A antibody. We have now included this potential explanation in the revised manuscript (p14).

2nd Editorial Decision 12 February 2020

Thank you for the submission of your revised manuscript. We have now received the enclosed comments from all referees, and I am happy to say that all support its publication now. Please address all remaining referee concerns in a final manuscript file, and please co-submit a point-by-point response with your final submission.

A few other changes are also required:

Shufeng Bu is missing from the author contributions. Please add.

On page 14 you mention "data not shown", which we do not allow. Please either show the data or rephrase.

The figure panel callouts need to appear in sequential order. Please correct.

Please upload all source data as 1 file per figure.

Please discuss and cite the accompanying paper from Rumpf's lab also in the reference list.

I attach to this email a manuscript word file with comments by our data editors. Please address all comments in the final manuscript file.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I would like to suggest a few minor changes to the abstract that needs to be written in present tense:

Pruning that selectively eliminates inappropriate projections is crucial for sculpting neural circuits during development. During Drosophila metamorphosis, ddaC sensory neurons undergo dendrite-specific pruning in response to the steroid hormone ecdysone. However, the understanding of the molecular mechanisms underlying dendrite pruning remains incomplete. Here, we show that protein

phosphatase 2A (PP2A) is required for dendrite pruning. The catalytic (Microtubule star/Mts), scaffolding (PP2A-29B), and two regulatory subunits (Widerborst/Wdb and Twins/Tws) play important roles in dendrite pruning. Functional analyses indicate that PP2A, via Wdb, facilitates the expression of Sox14 and Mical prior to dendrite pruning. Furthermore, PP2A, via Tws, governs the minus-end-out orientation of microtubules (MTs) in the dendrites. Moreover, the levels of Klp10A, a MT depolymerase, increase when PP2A is compromised. Attenuation of Klp10A fully rescues the MT orientation defects in mts or pp2a-29b RNAi ddaC neurons, suggesting that PP2A governs dendritic MT orientation by suppressing Klp10A levels and/or function. Taken together, this study sheds light on a novel function of PP2A in regulating dendrite pruning and dendritic MT polarity in sensory neurons.

Please let me know if you agree with these changes.

I look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have any questions or comments.

REFEREE REPORTS

Referee #1:

In this revised manuscript, Yu and colleagues have properly addressed, or at least tempted to address, my concerns as well as all other reviewers. I support publication and back to back publication with the Rumpf story should be preferred.
One point that I still feel could be improved is the link between the two parts of the manuscript - still very much reads like two unlinked stories. I am unsure of how to do this and therefore leave it completely up to the authors (and editor) if they can think of a way to make the main message more unifying.

Referee #2:

In the revised version, the authors have adequately addressed most of the initial concerns. However, one big problem that remains and that was mentioned already in the first round of revision is the fact that some of the data is inconclusive or even contradictory, and the two halves of the paper are still not logically connected. The biggest problems is in the first half (the "actin part"), while the second half (microtubules) is well presented and conclusive in itself.

1. 1) From the data that the authors present, it is evident that the involvement of PP2A in the Ecdysone-Sox14-Mical pathway is crucial for correct dendrite pruning. While overexpression of Mical does not fully rescue the pruning defect in a PP2A-deficient background, the phenotype is significantly milder.
Mical is best known for its role in actin disassembly, which suggests that the observed pruning defect ultimately lies in some misregulation of the actin cytoskeleton. However, the authors present a phalloidin-staining of PP2A-deficient neurons (Fig. EV3) that does not show any differences in F-actin content compared to control neurons. Hence, the mechanism behind PP2A (and ultimately Mical) involvement in dendrite pruning remains completely elusive and also not commented on by the authors.
Another unresolved issue is the question where in the Ecdysone-Sox14-Mical pathway PP2A acts, and which of its (regulatory) subunits is primarily required. In Figure 4 (as already mentioned in the first revision), the knock-down of both the PP2A catalytic and scaffolding unit leads to a disappearance of the Ecdysone-Receptor signal (and consequently also Sox14 and Mical), suggesting that PP2A acts upstream of EcR, or is somehow directly involved in controlling EcR levels.
It gets quite unclear when it comes to the question which of PP2As regulatory subunits is required in this mechanism.
The two regulatory subunits of PP2A that are initially found responsible the pruning defect are tws and wdb, whereas wdb knock-down consistently produces a stronger phenotype (more residual dendrites and higher penetrance) than tws knock-down. Double-knock-out of wdb+tws seems to exacerbate the phenotype (Figure EV2B), hinting at a redundancy, or parallel function, of wdb and

tws. However, the data shown for the effect of wdb knock-down (RNAi) are inconsistent between Figure 3 and Figure EV2. Figure 3 shows that wdb RNAi neurons have on average ~2 dendrites attached, with 85 % penetrance, and figure EV2 shows that wdb RNAi neurons have on average 0.5 neurons attached, with ~ 30 % penetrance. It seems that the wdb RNAi phenotype is quite inconsistent and variable. The "exacerbation" of the phenotype in the double-knock-down condition shown in Figure EV2 (wdb+tws; on average 1.5 dendrites attached with 70% penetrance) "only" lifts up the phenotype to a similar level already seen in Fig. 3 with wdb knock-down alone. It is unclear how these data should be interpreted.
Coming back to EcR-Sox14-Mical, in contrast to the PP2A scaffolding or catalytic subunit, knock-down of wdb only reduces Sox14 (and consequently Mical) levels, while it seems to have no effect on the EcR. At the same time, tws knock-down has no effect on either EcR, Sox14 or Mical levels. As the authors mention in the discussion, in line with the (possibly) "exacerbated" dendrite pruning defect seen in a double knock-down, it is possible that wdb and tws act redundantly in this pathway, and that a double knock-down might have an effect on the EcR levels. Unfortunately, the authors did not test this possibility, and it is hard to draw a conclusion from the presented data.

2) In the second half of the paper, the authors show that knock-down of PP2A leads to a defect in microtubule polarity. They show very conclusively that tws, rather than wdb, is the responsible regulatory subunit for this effect. Further, they show that PP2A/tws seems to be required to down-regulate the MT-depolymerizing kinesin Klp10A, as its levels are enhanced in a PP2A-knock-down background. Additional knock-down of Klp10A in a PP2A-deficient background rescue the MT polarity defect. These data are nicely presented and conclusive. In the revised version the authors now show that the MT polarity defect seems to be inconsequential for dendrite pruning, which is an interesting finding in itself, but is completely unconnected to the first half of the paper. Klp10 knock-down in a PP2A-deficient background, although it restores MT polarity, does not rescue the highly penetrant pruning defect, and neither has any effects on Mical expression levels (Figure EV5). Nonetheless, in the discussion the authors state "Further knockdown of klp10a suppressed the defects in both dendritic MT orientation and dendrite pruning in these RNAi backgrounds", referring to figure EV5A. While the effect is statistically significant, it is still very minor, the pruning defect is still very strong, and saying "PP2A regulates dendritic MT orientation and dendrite pruning via suppressing the levels or functions of Klp10A" could be an overstatement.

Referee #3:

Overall, I feel that the manuscript is much improved and reads well. There are a number of issues that still need some consideration at the discretion of the authors, but which I feel would further improve the manuscript:

Firstly, I may have missed it, but I could not find tws wdb double knock-down to see whether both pathways are truly separate and phenotypes are enhanced. I still think that this would be an important experiment to do, but leave this to authors/editors to decide.

Secondly, the data around Ecd-R are shaky (see comments below about low sample numbers, data representation and statistical comparisons) and authors should tone it down further. In the end, it is not relevant for this paper whether Wdb and the others work through different mechanisms in Sox14/Mical upregulation, whereas the reduction in expression of those two proteins is very convincing.

Third, the discussion is quite repetitive to the results part, and it would be helpful to see a more focussed line of discussion, aiming at the fact that PP2A activity splits into two (separate?) pathways during dendrite pruning, followed by thoughts about how Klp10 and Mical may act in this context. As said before, a double-mutant experiment would help to refine these thoughts.

Finally, the authors mention the parallel paper by the Rumpf group and seem to be aware. Please, could they discuss how the different sensory neurons used (ddaC versus c4da) compare to each other, and whether data match? This would be helpful for future readers which want to make sense by integrating results from both stories.

In conclusion, I would recommend publication, but ideally ask authors to improve their manuscript

further to give it a better standing in the field.

Detailed comments

p.9: I think the data need to be viewed with a bit less differentiation. It is clear that tws is not involved in Sox14/Mical regulation, but a differential statement about mts, pp2a-29b and wdb is over-interpreted when considering the rather low number of neurons samples (without a clear statement about how many animals were used in each case). If there were three runs for each experiment then, in one case (n=6), there were only two neurons analysed in each run.

p.9, last para: if tws does not influence Mical, would Mical overexpression also not rescue tws mutant phenotypes?

p.15 middle: I do not buy into the statement that Wdb regulates Sox14/Mical not via EcR-B1, because data are just too shaky (see my comments on Fig.4) and more investigations would be necessary to make this clear. Authors should tone down this statement and express it as a possibility that will require further investigation.

p.16, middle: If Patronin and Klp10A both promote minus end out MTs, how can they still antagonise each other? Please, rewrite this passage towards more clarity, since it is essential to your model.

Fig.4 Provide statistical comparisons also between groups, in particular between mts, pp2a-29b and wdb in P. It would be more informative to see data plotted in addition to showing mean and SED, given that sample numbers are quite low.

Fig.8: comments on bar graph in B similar to Fig.4P

2nd Revision - authors' response 20 February 2020

Referee #1:

In this revised manuscript, Yu and colleagues have properly addressed, or at least tempted to address, my concerns as well as all other reviewers. I support publication and back to back publication with the Rumpf story should be preferred.

One point that I still feel could be improved is the link between the two parts of the manuscript - still very much reads like two unlinked stories. I am unsure of how to do this and therefore leave it completely up to the authors (and editor) if they can think of a way to make the main message more unifying.

We are grateful to the reviewer for strong support and helpful suggestion. In the current manuscript, we show that PP2A plays dual roles in regulating dendrite pruning via two distinct regulatory subunits, Wdb and Tws. PP2A, via Wdb, regulates the expression of Sox14 and Mical, two important downstream targets of ecdysone signalling, which are required for dendrite pruning. Moreover, PP2A, via the other regulatory subunit Tws, regulates dendritic microtubule orientation and thereby dendrite pruning. In our opinion, this study provides a relatively complete understanding of PP2A functions in dendrite pruning. We will continue to explore the roles of microtubule orientation and Mical-mediated actin disassembly in dendrite pruning. Future investigation will help to determine the crosstalk between these two pathways.

Referee #2:

In the revised version, the authors have adequately addressed most of the initial concerns. However, one big problem that remains and that was mentioned already in the first round of revision is the fact that some of the data is inconclusive or even contradictory, and the two halves of the paper are still

not logically connected. The biggest problems is in the first half (the "actin part"), while the second half (microtubules) is well presented and conclusive in itself.

We thank the reviewer for positive endorsement and helpful suggestion. In the current manuscript, we show that PP2A plays dual roles in regulating dendrite pruning via two distinct regulatory subunits, Wdb and Tws. PP2A, via Wdb, regulates the expression of Sox14 and Mical, two important downstream targets of ecdysone signalling, which are required for dendrite pruning. Moreover, PP2A, via the other regulatory subunit Tws, regulates dendritic microtubule orientation and thereby dendrite pruning. The focus of our manuscript is mainly on the role of PP2A in regulating dendritic microtubule orientation, whereas the accompanying Rumpf manuscript highlights a second role of PP2A in regulating actin dynamics. Thus, these two manuscripts are complementary and provide a complete understanding of the PP2A functions in regulating both microtubule orientation and actin dynamics.

1. 1) From the data that the authors present, it is evident that the involvement of PP2A in the Ecdysone-Sox14-Mical pathway is crucial for correct dendrite pruning. While overexpression of Mical does not fully rescue the pruning defect in a PP2A-deficient background, the phenotype is significantly milder. Mical is best known for its role in actin disassembly, which suggests that the observed pruning defect ultimately lies in some misregulation of the actin cytoskeleton. However, the authors present a phalloidin-staining of PP2A-deficient neurons (Fig. EV3) that does not show any differences in F-actin content compared to control neurons. Hence, the mechanism behind PP2A (and ultimately Mical) involvement in dendrite pruning remains completely elusive and also not commented on by the authors.

We have now commented on the phalloidin result in the revised text (p10) as follows--"suggesting that PP2A does not regulate overall F-actin level. However, PP2A is shown to modulate F-actin dynamics in a separate study". In the Rumpf paper, they report that PP2A is required for the distribution the GFP-Cofilin marker, suggesting that PP2A regulates F-actin dynamics in ddaC neurons.

Overall, the focus of our manuscript is mainly on the role of PP2A in regulating dendritic microtubule orientation during dendrite pruning, whereas the accompanying Rumpf manuscript highlights a second role of PP2A in regulating actin dynamics. Thus, these two manuscripts are complementary and provide a complete understanding of the PP2A functions in regulating both microtubule orientation and actin dynamics.

Another unresolved issue is the question where in the Ecdysone-Sox14-Mical pathway PP2A acts, and which of its (regulatory) subunits is primarily required. In Figure 4 (as already mentioned in the first revision), the knock-down of both the PP2A catalytic and scaffolding unit leads to a disappearance of the Ecdysone-Receptor signal (and consequently also Sox14 and Mical), suggesting that PP2A acts upstream of EcR, or is somehow directly involved in controlling EcR levels.
It gets quite unclear when it comes to the question which of PP2As regulatory subunits is required in this mechanism. The two regulatory subunits of PP2A that are initially found responsible the pruning defect are tws and wdb, whereas wdb knock-down consistently produces a stronger phenotype (more residual dendrites and higher penetrance) than tws knock-down. Double-knock-out of wdb+tws seems to exacerbate the phenotype (Figure EV2B), hinting at a redundancy, or parallel function, of wdb and tws. However, the data shown for the effect of wdb knock-down (RNAi) are inconsistent between Figure 3 and Figure EV2. Figure 3 shows that wdb RNAi neurons have on average ~2 dendrites attached, with 85 % penetrance, and figure EV2 shows that wdb RNAi neurons have on average 0.5 neurons attached, with ~ 30 % penetrance. It seems that the wdb RNAi phenotype is quite inconsistent and variable. The "exacerbation" of the phenotype in the double-knock-down condition shown in Figure EV2 (wdb+tws; on average 1.5 dendrites attached with 70% penetrance) "only" lifts up the phenotype to a similar level already seen in Fig. 3 with wdb knock-down alone. It is unclear how these data should be interpreted.

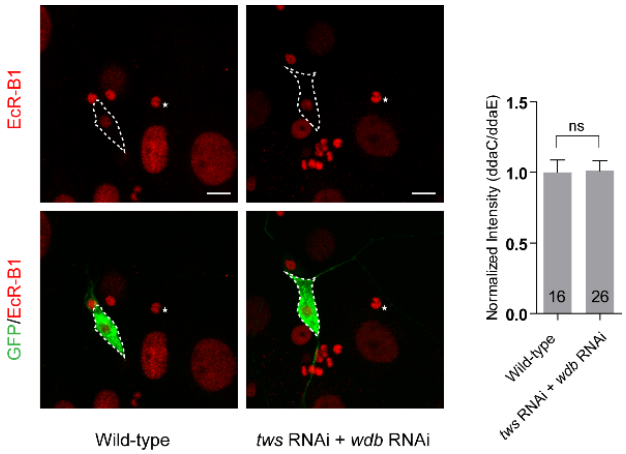
In Figures 3 and EV2B, the different penetrance and severity of the *wdb* RNAi phenotypes are caused by different copies of the *wdb* RNAi transgene. Two copies of *wdb* RNAi transgenes were expressed under the control of two copies of *ppk-Gal4* driver in Figure 3, whereas only a copy of *wdb* RNAi transgene, together with the control RNAi transgene, was expressed under the same

copies of *ppk-Gal4* driver in Figure EV2B. Given more copies of *wdb* RNAi transgenes and less diluted *ppk-Gal4* driver in Figure 3, its knockdown efficiency is higher than that in Figure EV2B. Therefore, the pruning defects in Figure 3 are more severe than those in Figure EV2B. Because of the different genetic manipulations, we cannot simply compare the severity of the phenotypes between Figure 3 and Figure EV2.

Coming back to EcR-Sox14-Mical, in contrast to the PP2A scaffolding or catalytic subunit, knock-down of wdb only reduces Sox14 (and consequently Mical) levels, while it seems to have no effect on the EcR. At the same time, tws knock-down has no effect on either EcR, Sox14 or Mical levels. As the authors mention in the discussion, in line with the (possibly) "exacerbated" dendrite pruning defect seen in a double knock-down, it is possible that wdb and tws act redundantly in this pathway, and that a double knock-down might have an effect on the EcR levels. Unfortunately, the authors did not test this possibility, and it is hard to draw a conclusion from the presented data.

We have tested this possibility. However, our result did not show a reduction in EcR level when both *wdb* and *tws* were knocked down. This result implies the existence of other unknown regulatory subunits that regulates EcR expression. We have now included this negative data below.

We have convincingly demonstrated that PP2A is required to regulate the expression of Sox14 and Mical, two important downstream targets of ecdysone signalling. The potential mechanisms whereby PP2A regulates the EcR expression are not the major focus of our manuscript.



2) In the second half of the paper, the authors show that knock-down of PP2A leads to a defect in microtubule polarity. They show very conclusively that tws, rather than wdb, is the responsible regulatory subunit for this effect. Further, they show that PP2A/tws seems to be required to down-regulate the MT-depolymerizing kinesin Klp10A, as its levels are enhanced in a PP2A-knock-down background. Additional knock-down of Klp10A in a PP2A-deficient background rescue the MT polarity defect. These data are nicely presented and conclusive. In the revised version the authors now show that the MT polarity defect seems to be inconsequential for dendrite pruning, which is an interesting finding in itself, but is completely unconnected to the first half of the paper. Klp10 knock-down in a PP2A-deficient background, although it restores MT polarity, does not rescue the highly penetrant pruning defect, and neither has any effects on Mical expression levels (Figure EV5). Nonetheless, in the discussion the authors state "Further knockdown of klp10a suppressed the defects in both dendritic MT orientation and dendrite pruning in these RNAi backgrounds", referring to figure EV5A. While the effect is statistically significant, it is still very minor, the pruning defect is still very strong, and saying "PP2A regulates dendritic MT orientation and dendrite pruning via suppressing the levels or functions of Klp10A" could be an overstatement.

We thank the reviewer for the positive endorsement. We have now rephrased the statement as "PP2A regulates dendritic MT orientation and dendrite pruning at least partially via suppressing the levels or functions of Klp10A" in p16.

Referee #3:

Overall, I feel that the manuscript is much improved and reads well. There are a number of issues that still need some consideration at the discretion of the authors, but which I feel would further improve the manuscript:

Firstly, I may have missed it, but I could not find tws wdb double knock-down to see whether both pathways are truly separate and phenotypes are enhanced. I still think that this would be an important experiment to do, but leave this to authors/editors to decide.

The *tws*, *wdb* double knock-down experiments had been shown in the text (p7) and Figure EV2B. We observed that the double knockdown of *tws* and *wdb* significantly exacerbated the dendrite pruning phenotypes, compared to either *wdb* or *tws* knockdown (Fig EV2B), suggesting two separate pathways.

Secondly, the data around Ecd-R are shaky (see comments below about low sample numbers, data representation and statistical comparisons) and authors should tone it down further. In the end, it is not relevant for this paper whether Wdb and the others work through different mechanisms in Sox14/Mical upregulation, whereas the reduction in expression of those two proteins is very convincing.

We thank the reviewer for the helpful suggestion. We have highlighted the requirement of PP2A in regulating the expression of Sox14 and Mical, two downstream effectors of EcR-B1. Our manuscript demonstrates a critical role of PP2A in regulating the expression of Sox14 and Mical and thereby the activation of ecdysone signalling. The potential mechanisms whereby PP2A regulates the EcR expression are not relevant for our manuscript.

Third, the discussion is quite repetitive to the results part, and it would be helpful to see a more focussed line of discussion, aiming at the fact that PP2A activity splits into two (separate?) pathways during dendrite pruning, followed by thoughts about how Klp10 and Mical may act in this context. As said before, a double-mutant experiment would help to refine these thoughts.

We have now made the Discussion part more concise. We had included the *wdb, tws* double knockdown experiment in the text (p7) and Figure EV2B, supporting the notion that PP2A plays dual roles in regulating dendrite pruning via Wdb-dependent and Tws-dependent pathways.

Finally, the authors mention the parallel paper by the Rumpf group and seem to be aware. Please, could they discuss how the different sensory neurons used (ddaC versus c4da) compare to each other, and whether data match? This would be helpful for future readers which want to make sense by integrating results from both stories.

ddaC and C4da neurons are the same neurons. We have now included (also known as C4da neurons) in the revised manuscript (p3). Moreover, we also expanded the discussion on the major points of these two manuscripts in the Discussion part (p16-17).

In conclusion, I would recommend publication, but ideally ask authors to improve their manuscript further to give it a better standing in the field.

We are greatly grateful to the reviewer for the strong support and constructive suggestions.

Detailed comments

p.9: I think the data need to be viewed with a bit less differentiation. It is clear that tws is not involved in Sox14/Mical regulation, but a differential statement about mts, pp2a-29b and wdb is over-interpreted when considering the rather low number of neurons samples (without a clear statement about how many animals were used in each case).

We have removed “drastically” or “strongly” in the revised text (p8-9), as the reviewer suggested.

If there were three runs for each experiment then, in one case (n=6), there were only two neurons analysed in each run.

Clones were randomly generated with the relatively low efficiency. On average, one clone every two animals. We usually set up three separate crosses for generating mutant clones. In this case (n=6), only few clones were recovered from our MARCM analysis. Given that the reduction in Sox14 level is very consistent among six clones, we are very confident of the results shown in Figure 4 as well as other figures. Moreover, all our data were repeated by at least two authors independently.

p.9, last para: if tws does not influence Mical, would Mical overexpression also not rescue tws mutant phenotypes?

We speculate that Mical overexpression unlikely rescues the *tws* mutant phenotypes. But we have not conducted this experiment to confirm it.

p.15 middle: I do not buy into the statement that Wdb regulates Sox14/Mical not via EcR-B1, because data are just too shaky (see my comments on Fig.4) and more investigations would be necessary to make this clear. Authors should tone down this statement and express it as a possibility that will require further investigation.

We have now modified the sentence to "but appears dispensable for EcR-B1 expression" in p15.

p.16, middle: If Patronin and Klp10A both promote minus end out MTs, how can they still antagonise each other? Please, rewrite this passage towards more clarity, since it is essential to your model.

We have rephrased the sentence to "The kinesin-13 MT depolymerase Klp10A antagonizes the function of Patronin and thereby negatively regulates dendritic MT orientation during dendrite pruning" in p16.

Fig.4 Provide statistical comparisons also between groups, in particular between mts, pp2a-29b and wdb in P. It would be more informative to see data plotted in addition to showing mean and SED, given that sample numbers are quite low.

We have now provided statistical comparisons between *mts*, *pp2a-29b* and *wdb* in the revised Figure 4P.

Fig.8: comments on bar graph in B similar to Fig.4P

We have also provided statistical comparisons between the rest of groups in the revised Figure 8B.

Accepted 4 March 2020

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Corresponding Author Name: Fengwei Yu
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-48843V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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/

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?	For all our experimental analyses, we normally need to collect around 10-30 ddaC neurons for quantifications.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For MARCM clonal analysis, we normally need to more than 10 larvae/pupae to collect around 10 ddaC neurons, as the clonal effciiecncy is low (0.5 clone each animal). For RNAi or overexpression analysis, the sample size is about 4-6 larvae/pupae to acquire 15-30 neurons
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude any samples during the analysis.
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.	We stained and processed all the samples with the relevant controls in the same eppendorf tube under the same condition. We also acquired our confocal image data with the same confocal setting.
For animal studies, include a statement about randomization even if no randomization was used.	All our larval/pupal sampes from the same developmental stage were randomly chosen for the immunostaining and imaging.
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.	The results were conducted and repeated by two co-first authors independently. Our data were from at least three independent experiments.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We did not conduct blind assays in most of our experiments. Multiple authors conducted some of the key expeirments independently.
5. For every figure, are statistical tests justified as appropriate?	Yes. They are appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. For pairwise comparison, two-tailed Student's T-test was applied to determine statistical significance. One-way ANOVA with Bonferroni test was applied to determine significance when multiple groups were present. Error bars in all graphs represent standard error of the mean (SEM). Statistical significance was defined as ***p<0.001, **p<0.01, *p<0.05, n.s., not significant

Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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).	Yes.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N.A.

* 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.	Drosophila melanoganster; Larvae and adult; Maintaine on standard food according to Bloomington Stock Centre at 25°C; RNAi flies from Bloomington Stock Centre and Vienna Drosophila RNAi Centre, transgenic and mutant flies are generated in F.W.Yu lab or from other labs (indicated in the Materials and Methods in the revised manuscript).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N.A. We worked on invertebrates.
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.	N.A.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N.A. We worked on invertebrates.
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.	N.A.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N.A.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N.A.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N.A.
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.	N.A.
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.	N.A.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	We will upload the source data and add "source data are avilable online at the end of the each figure legend"
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).	Source data are avilable for each figure
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).	N.A.
21. 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.	N.A.

G- Dual use research of concern

22. 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.	No