N-WASP-Arp2/3 signaling controls multiple steps of dendrite maturation in Purkinje cells *in vivo*

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AUTHORS: Koichi Hasegawa, Takeshi K Matsui, Junpei Kondo, and Ken-ichiro Kuwako

I have now received the reports of two referees on your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, referee 1 requests that the data obtained with dominant negative constructs are confirmed using a knockdown approach, and referee 2 asks for additional controls for the specificity of the antibodies you use in immunocytochemistry experiments. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referees' comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Hasegawa et al. Investigated the involvement of N-WASP and Arp2/3 in cerebellar Purkinje cells (PCs) early dendrite development.

They first show by immunohistofluorescence that N-WASP, phospho-N-WASP, Arp2 Arp3 and the upstream small GTPase Cdc42 are present in the soma of PCs at postnatal day 6 (P6) and are also present in dendrites at P14 (Figure 1).

Time lapse videomicroscopy with cultured PCs revealed that Arp3-mCherry accumulated at branching sites prior to the onset of de novo dendritic protrusion (Figure 2A). Using PCs cultured in the presence of small chemical inhibitors, they showed that 21 days treatments with inhibitors of NWASP, Arp2/3 or Cdc42 reduced dendritic length and branching (Figure 2B).

(Figure 3) In order to study the implication of Arp2/3 in vivo, the authors electroporated animals in utero at embryonic day 12 (E12) with two dominant negative forms of NWASP that sequester Arp2/3 in the cytoplasm (named VCA and PRDVCA). This is therefore an inhibition of Arp2/3. Observation at P21 showed PCs with shorter and less branched dendrites. They also used another dominant negative form of NWASP (NWASP deltaVCA) that interacts with PIP2, Cdc42 and many of its other interactors but fails to activate Arp2/3. This is how the authors inhibited NWASP. The observed phenotype is similar to the two other dominant negative forms.

Finally, the authors showed a rescue of the defect induced by VCA when co-overexpressed with a wildtype NWASP but not with NWASP(H208D), that cannot interact with Cdc42, suggesting the importance of the Cdc42 upstream activation in PC dendritic development (Figure 4).

Hasegawa et al. then decided to investigate this pathway by inhibiting it at a later point using AAV directed expression which is only detected at P10 (Figure S5). Observation at P21 showed an attenuated dendritic growth (Figure 5).

In order to show that the overactivation of the pathway is also deleterious, the authors overexpressed the mutant memNWASP(Y253E) that is freed from its autoinhibition and also forced to be membrane anchored. Observation at P21 showed a decreased dendritic development and appearance of multiple primary dendrites (Figure 6).

While this manuscript brings interesting results, I would request the following experiments in order to strengthen the data and support further the authors conclusion:

Comments for the author

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Major requests:

1) Figure 1: After separating the RGB colors from the figures, it is strange to see that the calb+ cells do not have a nucleus. At least this is what I saw when separating the colors. The figures are supposed to be in RGB. Please provide true RGB with no leakage of colors in the other channels. It makes it otherwise difficult to assess the localization of each signal and difficult to make comments on them.

2) Supplemental movie: The authors should provide the same movie but with the colors separated. It is difficult to see the Arp2-mCherry with such a bright GFP.

3) Figure 2D-G: Please show that wiskostatin is inhibiting NWASP at that 1µM concentration and for 21 days in these culture conditions. Please show that it did not affect the health of the cells. Conclusions need to be made with caution, knowing that wiskostatin was shown as not very

selective (Guerriero et al., 2007; Bompard et al., 2008). The same caution should be taken for the other inhibitors.

4) Please give us some references that characterized the dominant negative mutants. The references provided by the authors are not helpful: Kurisu and Takenawa, 2009 do not mention any of these mutants; Miki et al., 1996 only used the deltaVCA and do not really characterize its mechanism of action.

5) You mention that the mutant form called here VCA sequester Arp2/3 in the cytoplasm. Does it therefore activate high uncontrolled actin polymerization in the cytoplasm?
This might be responsible for some of the observed phenotype. Would you get similar results with shRNAs?

6) While the approach used by the authors using dominant negative forms is a powerful tool to dissect the mechanism, dominant negative forms can have non-specific effects. The authors should support their data with the knockdown of Cdc42, NWASP, Arp2/3.

7) The authors conclude that their inhibition data in vivo (in utero electroporation at E12 and observations at P21) suggest a function of Arp2/3 in the early phase of PC dendrite maturation, particularly in the establishment of an apical stem dendrite. But is it arrested or delayed? To answer this question they could assess the phenotype at a much later stage.

8) Figure S3: The authors mention and show what they called an abnormal accumulation of the Golgi apparatus. Please provide a control PC picture with a normal Golgi apparatus for comparison.

9) In figure 4, the authors showed that the phenotype due to expression of VCA is rescued by the co-overexpression of wildtype NWASP. Could the authors explain how NWASP can rescue the defects when NWASPVCA sequester Arp2/3 in the cytoplasm which is therefore not available???

10) In figure 4, the authors showed that the NWASP(H208D) is not able to rescue the VCA-induced phenotype and conclude that Cdc42 must be involved. A conclusion for the involvement of Cdc42 should be reinforced by an inhibition of Cdc42 that should also give a defect in PC dendritic growth.

11) In figure 5, the authors expressed the mutants NWASP using AAV (injected at P7 and observations at P21). They show that the expression is only visible by immunohistofluorescence at P10. While it is not visible by IHF, it might still be expressed and have an effect earlier. It would be nice to have an earlier analysis such as at P10. Indeed, comparison of a control P10 (Fig S2) and a PRDVCA-expressing P10 (Fig S5) show a visible decrease in dendritic growth. This is suggesting that, even when using this approach, it already has an effect earlier than you thought. It seems to me that, with this in mind, you cannot yet really separate the effect on early and late PC dendritic development. Maybe a solution would be to repeat this experiment and inject later, maybe at P10.

12) Figure 6: The figure legend explains that abnormal orientation is when there are several primary dendrites going in several directions (they refer us to the drawing in fig 3H). To me then, the PCs showing more than one primary dendrite should all be abnormally oriented as by the authors definition. But there is only 23.4% abnormally oriented and 31.7% of PC with more than one primary dendrite (Figure 6B). Maybe you should show a PC with multiple primary dendrites but that is still well oriented and another one abnormally oriented so the reader can understand the difference?

Minor comment:

1) In the introduction: “...probably through the abnormalities in ciliogenesis of ependymal cells or migration of radial glial cells...” Radial glia cells do not migrate. Please correct this sentence.

Reviewer 2

Advance summary and potential significance to field

This is an interesting analysis of the role of N-Wasp and Arp2/3 complex on different aspects of Purkinje cell development. The study shows that adequate levels of active N-Wasp are needed for proper dendritic development in Purkinje cells. The study includes both in vitro and in vivo approaches. Quantifications are adequate.

Conclusions are important in the field and appropriate.

Comments for the author

My only concern is on Figure 1. The immunolocalization gives diffuse staining for some of the endogenous proteins, which is kind of expected. On the other hand important controls for the specificity of the signals are missing and should be shown including secondary Abs alone, to determine the background. Also, to be convinced of the specificity of the stainings, especially when pAbs are used, controls of specificities like asbence/decrease of signals in KO sections or at least after Kd of endogenous proteins in cells would be important.

First revision

Author response to reviewers' comments

Responses to Reviewers

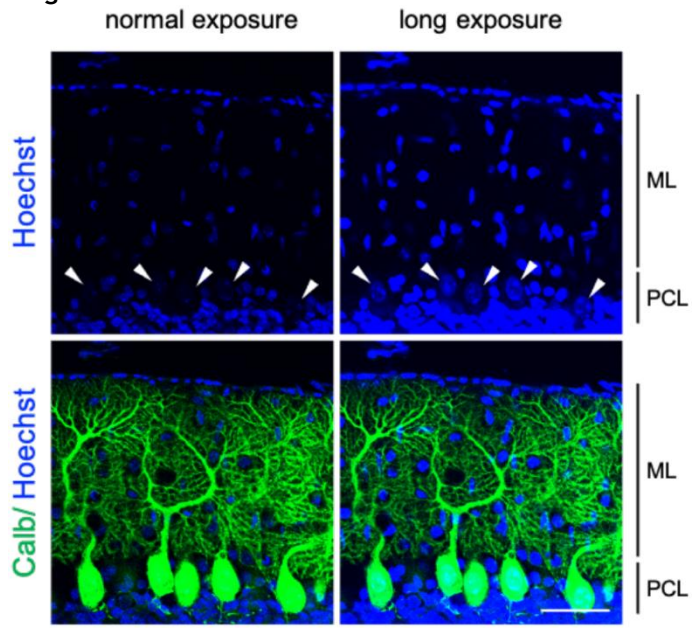
Responses to reviewer 1

1) Figure 1: After separating the RGB colors from the figures, it is strange to see that the calb+ cells do not have a nucleus. At least this is what I saw when separating the colors. The figures are supposed to be in RGB. Please provide true RGB with no leakage of colors in the other channels. It makes it otherwise difficult to assess the localization of each signal and difficult to make comments on them.

   Response

   We verified that all images were RGB. We also performed color-separation of all images in Fig. 1 to ensure that there is no leakage. In addition, it is a well- known fact that the intensity of Hoechst staining in Purkinje cells (PCs) is significantly lower than in surrounding cells (Reviewer's figure 1, left). If we increase the exposure time significantly, the PC nuclei become visible (Reviewer's figure 1, right).

Reviewer's figure 1



Section of P14 cerebellum was stained with anti-Calbindin (Calb) antibody and Hoechst33342. Note that PC nuclei were barely detectable at normal exposure time (left), but became visible at longer exposure time (right). Arrowheads indicate nuclei of PCs. ML: molecular layer, PCL: Purkinje cell layer. Scale bar represents 50 μm.

2) Supplemental movie: The authors should provide the same movie but with the colors separated. It is difficult to see the Arp2-mCherry with such a bright GFP.

Response

According to the reviewer's suggestion, we added the single-color movies. Supplemental movies 2 and 3 are GFP and Arp3-mCherry alone, respectively. Supplemental movie 1, which includes both GFP and Arp3-mCherry, remains unchanged.

3) Figure 2D-G: Please show that wiskostatin is inhibiting NWASP at that 1μM concentration and for 21 days in these culture conditions. Please show that it did not affect the health of the cells. Conclusions need to be made with caution, knowing that wiskostatin was shown as not very selective (Guerriero et al., 2007; Bompard et al., 2008). The same caution should be taken for the other inhibitors.

Response

While we understand the reviewer's concern, unfortunately, it is impossible to prove the effect of wiskostatin in PCs by common biochemical methods because PC is co-cultured with a large number of granule cells. In general, immunocytochemical approaches can also evaluate effect of wiskostatin by labeling F-actin with a chemical labeling reagent, such as Lifeact-GFP, and measuring the total amount of its signal. However, since previous paper has shown that the majority of Lifeact signals in cultured PCs are located in the dendrites but not in the cell body (Fukumitsu et al., 2015), it would be difficult to accurately evaluate the effect of wiskostatin by comparing wiskostatin- treated PCs, which have significantly stunted dendrites, with control PCs that have highly-developed dendrites. Instead, previous studies have shown that wiskostatin is effective in inhibiting N-WASP-mediated actin dynamics even at 1 μM in a variety of cells (Ganeshan et al., 2007; King et al., 2011; Serrano- Pertierra et al., 2012). We also confirmed that 1 uM wiskostatin, as well as 50 μM CK-666 and 10 μM ML-141, had no effect on the health of PCs (new Fig. 2H).

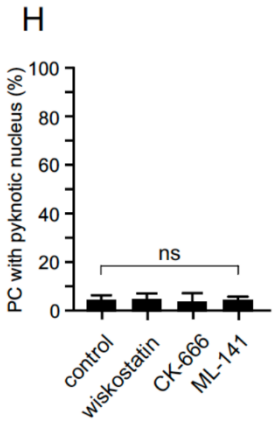
The two papers cited by reviewer 1 claim that wiskostatin is nonspecific because of the following reasons; (1) wiskostatin treatment affects ATP metabolism in which N-

WASP has not been reported to be involved. (2) wiskostatin inhibits cytokinesis, whereas N-WASP knockdown has no effect on it. However, a possibility that N-WASP is indirectly involved in ATP metabolism cannot be excluded because cytoskeletal regulation has a strong influence on diverse cellular phenomena including gene expression. It is also possible that the remaining N-WASP may be sufficient to cause cytokinesis since siRNA generally does not completely deplete endogenous proteins. Moreover, these papers did not identify alternative targets for wiskostatin. Therefore, we consider that this fact does not negate the specificity of wiskostatin. Of course, nonspecific effects of wiskostatin cannot be completely ruled out, but this inhibitor is currently considered the best N-WASP inhibitor and has been used in a great number of studies (Orange et al., 2011, Baranov et al., 2014, Soykan et al., 2017, Yoshihara et al., 2020, Keb et al., 2021). As with wiskostatin, we scrutinized previous papers on Arp2/3 and Cdc42 inhibitors (Hong et al., 2013, Maldonado and Dharmawardhane, 2018, Fokin et al., 2022) and selected the highly-specific inhibitors; CK- 666 and ML-141.

< References for the reviewer >

Ganeshan et al., *Biochim. Biophys Acta* 1773, 192-200 (2007).
King et al., *EMBO J.* 30, 1705-1718 (2011).
Orange et al., *J. Clin. Invest.* 121, 1535-1548 (2011).
Serrano-Pertierra et al., *Eur. J. Immunol.* 42, 2142-2151 (2012).
Hong et al., *J. Biol. Chem.* 288, 8531-8543 (2013).
Baranov et al., *J. Cell Sci.* 127, 1052-1064 (2014).
Fukumitsu et al., J neurosci. 35, 5707-5723 (2015).
Soykan et al., *Neuron* 93, 854-866 (2017).
Yoshihara et al., *Exp. Cell Res.* 392, 112011 (2020).
Keb et al., *mBio* 12, e02861-20 (2021).
Maldonado and Dharmawardhane., *Cancer Res.* 78, 3101-3111 (2021).
Fokin et al., *Front. Pharmacol.* 13, 896994 (2022).

Fig. 2H



(E-H) Quantification of total dendrite length (E), branch number (F), dendrite area (G), and percentage of PC with pyknotic nucleus (H) at 21 DIV in the experiment shown in (D). The data represent the means ± SEM. n=74 cells (control), n=77 cells (wiskostatin), n=74 cells (CK-666), and n=74 cells (ML-141) in (E-G). n=3 experiments (40- 55 cells were quantified in each experiment) for all conditions in (H). ****p<0.0001; ns: not significant (non-repeated one-way ANOVA with a post hoc Bonferroni correction).

4) Please give us some references that characterized the dominant negative mutants. The references provided by the authors are not helpful: Kurisu and Takenawa, 2009 do not mention any of these mutants; Miki et al., 1996 only used the deltaVCA and do not really characterize its mechanism of action.

Response

In the original manuscript, we cited the two papers (Kurisu and Takenawa 2009, Miki et al., 1996) pointed by reviewer 1 to mention the domain function of N-WASP and its binding proteins, but not to describe the dominant-negative mutants. The references on the N-WASP dominant-negative mutants used in this study are cited in the revised manuscript. The list of references is as follows.

N-WASP VCA
Machesky and Insall, 1998 (page 9 line 3), Strasser et al., 2004 (page 9 line 4), Wegner et al., 2008 (page 9 line 4)
N-WASP PRDVCA
Pinyol et al., 2007 (page 9 line 18)
N-WASP ΔVCA
Pinyol et al., 2007 (page 10 line 24), Wegner et al., 2008 (page 10 line 24)
N-WASP H208D
Miki et al., 1998, (page 11 line 17), Rohatgi et al., 1999, (page 11 line 18)
N-WASP Y253E
Suetsugu et al., 2002 (page 13 line 6)

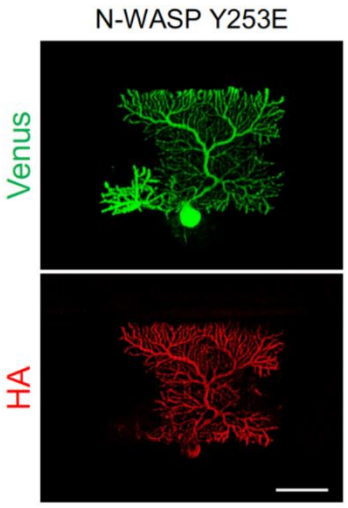
5) You mention that the mutant form called here VCA sequester Arp2/3 in the cytoplasm. Does it therefore activate high uncontrolled actin polymerization in the cytoplasm? This might be responsible for some of the observed phenotype. Would you get similar results with shRNAs?

Response

Unfortunately, we have no evidence whether the Arp2/3 sequestered in the cytoplasm causes aberrant actin polymerization in N-WASP VCA-expressing PCs. As shown in Fig. 6, the forced expression of membrane-anchored constitutively-active N-WASP Y253E (mem N-WASP Y253E) causes abnormal dendrite development in PCs. In contrast, we found that N-WASP Y253E without membrane-anchored signal did not cause apparent defect in PC dendrites (Reviewer's figure 2). This fact suggests that hyperactivation of Arp2/3 outside the juxtamembrane region may not impede dendrite development in PCs. Therefore, we consider that the abnormal dendrite formation in N-WASP VCA-expressing PCs is not due to uncontrolled activation of Arp2/3 in the cytoplasm, but rather to the inability of Arp2/3 to function near the plasma membrane.

Although we have not performed a knockdown approach, since both N- WASP VCA and N-WASP RNAi increase axon length in the same way (Pinyol et al., 2007), we consider that knockdown of N-WASP in PCs would yield the same result as N-WASP VCA.

Reviewer's figure2



Z section of N-WASP Y253E-expressing PCs at P21. HA-tagged N-WASP Y253E was specifically expressed in PCs through *in utero* electroporation. Venus was coexpressed in PCs to visualize dendrite morphology. Sections were immunostained with antibodies against GFP (for Venus) and HA. Scale bar represents 50 μm.

6) While the approach used by the authors using dominant negative forms is a powerful tool to dissect the mechanism, dominant negative forms can have non- specific effects. The authors should support their data with the knockdown of Cdc42, NWASP, Arp2/3.

Response

The dominant-negative mutants based on structural and functional evidence of N-WASP have been well established as powerful tools for inhibition of N- WASP-Arp2/3 signaling and used in many studies. In addition, previous studies have reported the same effect of N-WASP dominant-negative mutants as its shRNAs (Legg et al, 2007, Pinyol et al, 2007, Pommereit and Wouters, 2007). While we agree that the knockdown approach is an excellent tool, it always comes with concerns about efficiency and off-target effects. Therefore, we consider that our analyses with the dominant-negative mutants is sufficient to draw the conclusion of this study.

< References for the reviewer >

Legg et al., *Mol. Biol. Cell.* 18, 678-687 (2007).
Pinyol et al., *PLoS ONE* 2, e400 (2007).
Pommereit and Wouters, *J. Cell Sci.* 120, 2694-2705 (2007).

7) The authors conclude that their inhibition data in vivo (in utero electroporation at E12 and observations at P21) suggest a function of Arp2/3 in the early phase of PC dendrite maturation, particularly in the establishment of an apical stem dendrite. But is it arrested or delayed? To answer this question, they could assess the phenotype at a much later stage.

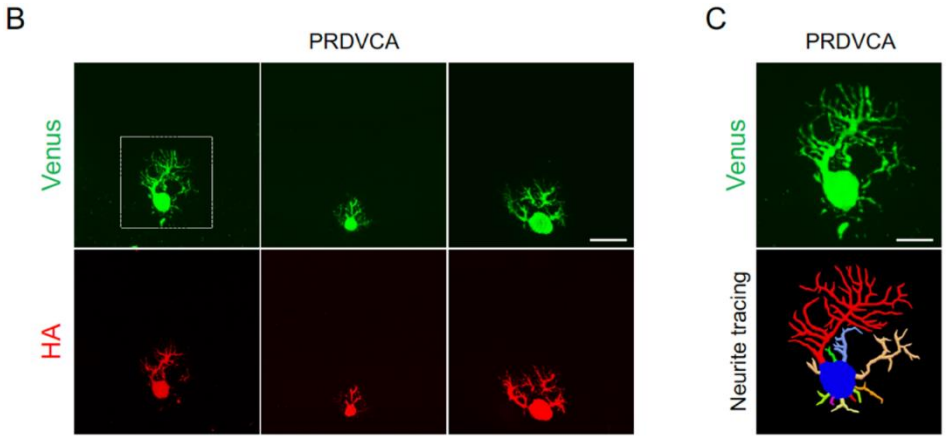
Response

According to the reviewer's suggestion, we analyzed dendrites of N-WASP PRDVCA or mem ΔVCA-expressing PCs at P35 and confirmed that stem dendrites were still not formed in those PCs. These results strongly suggest that the inhibition of N-WASP-Arp2/3 signaling causes arrest of stem dendrite formation in PCs. We added the images of N-WASP PRDVCA or mem ΔVCA- expressing PCs at P35 in the revised manuscript (Fig. S3B,C and Fig. S5B). We also mentioned this fact in the revised manuscript as follows.

< page 11, line 7-10 >

N-WASP PRDVCA or mem ΔVCA-expressing PCs at P35 showed similar dendritic abnormalities as those at P21 (Fig. S3B, Fig. S5B), suggesting that inhibition of N-WASP-Arp2/3 signaling causes arrest, but not delay, of dendritic maturation after stellate cell stage.

Fig. S3B,C



(B) Z sections of N-WASP PRDVCA-expressing PCs at P35. HA-tagged N-WASP PRDVCA was specifically expressed in PCs through *in utero* electroporation. Venus was coexpressed in PCs to visualize dendrite morphology. Sections were immunostained with antibodies against GFP (for Venus) and HA. Dotted box indicates the position of the high-magnification image shown in (C). (C) High-magnification image of PC in (B). Bottom panel represents neurite reconstruction of a PC using Neurolucida. Scale bars represent 20 μm (A) and (C), or 50 μm (B).

Fig. S5B

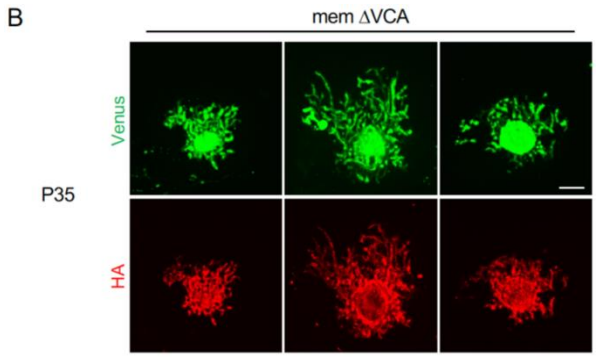


Fig. S5. Inhibition of N-WASP severely impairs maturation of PC dendrites.
Examples of N-WASP mem ΔVCA- expressing PCs. Sections of N-WASP mem ΔVCA and Venus-expressing PCs at P21 (A) and P35 (B). HA-tagged N-WASP mem ΔVCA and Venus were coexpressed in PCs by *in utero* electroporation. Sections were immunostained with antibodies against GFP (for Venus) and HA. Three examples of N-WASP mem ΔVCA- expressing PCs are shown. Scale bars represent 20 μm.

8) Figure S3: The authors mention and show what they called an abnormal accumulation of the Golgi apparatus. Please provide a control PC picture with a normal Golgi apparatus for comparison.

Response

According to the reviewer's comment, we added the data of control PC in new Fig. S4, as below. Note that, in control PC, the Golgi apparatus was localized to the cell body and no abnormal accumulation in the dendrites was observed as in N-WASP PRDVCA-expressing PCs.

Fig. S4

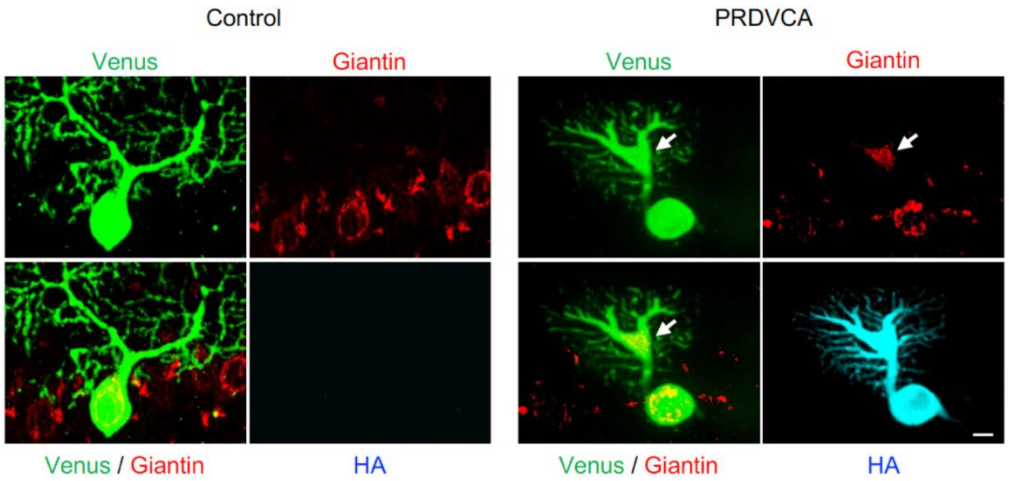


Fig. S4. Inhibition of Arp2/3 causes aberrant hypertrophic structures in the stem-like dendrites in PCs.
Z sections of Venus-expressing PCs (control) and N-WASP PRDVCA and Venus-coexpressing PCs (PRDVCA) at P21. HA-tagged N-WASP PRDVCA and Venus were expressed in PCs using *in utero* electroporation. Sections were immunostained with antibodies against GFP (for Venus), HA, and giantin (a marker for the Golgi apparatus). Representative images of PC expressing N-WASP PRDVCA with stem-like dendrite are shown. Arrowheads indicate the hypertrophic structure in the stem-like dendrite. Note that the giantin-labeled golgi apparatuses are highly accumulated in the hypertrophic structure. Scale bar represents 10 μm.

9) In figure 4, the authors showed that the phenotype due to expression of VCA is rescued by the co-overexpression of wildtype NWASP. Could the authors explain how NWASP can rescue the defects when NWASPVCA sequester Arp2/3 in the cytoplasm which is therefore not available???

Response

In this experiment, wild-type N-WASP was expressed simultaneously with N- WASP VCA, and both are thought to bind competitively to endogenous Arp2/3. Thus, Arp2/3 bound to wild-type N-WASP would function normally, thereby partially rescuing the abnormal phenotype.

10) In figure 4, the authors showed that the NWASP(H208D) is not able to rescue the VCA-induced phenotype and conclude that Cdc42 must be involved. A conclusion for the involvement of Cdc42 should be reinforced by an inhibition of Cdc42 that should also give a defect in PC dendritic growth.

Response

Since Cdc42 has many downstream targets, inhibition of Cdc42 itself will have a greater impact on pathways other than the N-WASP-Arp2/3 signaling. Therefore, phenotypic analysis of Cdc42 inhibition is expected to be very difficult to interpret. In contrast, because N-WASP H208D is unable to bind Cdc42 by a point mutation (Miki et al., 1998; Rohatgi et al., 1999), we considered N-WASP H208D to be the best tool to clarify the relationship between Cdc42 and N-WASP. And we believe that our clear data using N-WASP H208D (Fig. 4) is sufficient to conclude the relationship between Cdc42 and N-WASP in PCs.

11) In figure 5, the authors expressed the mutants NWASP using AAV (injected at P7 and observations at P21). They show that the expression is only visible by immunohistofluorescence at P10. While it is not visible by IHF, it might still be expressed and have an effect earlier. It would be nice to have an earlier analysis such

as at P10. Indeed, comparison of a control P10 (Fig S2) and a PRDVCA-expressing P10 (Fig S5) show a visible decrease in dendritic growth. This is suggesting that, even when using this approach, it already has an effect earlier than you thought. It seems to me that, with this in mind, you cannot yet really separate the effect on early and late PC dendritic development. Maybe a solution would be to repeat this experiment and inject later, maybe at P10.

Response

While we understand the reviewer's concerns, we consider that our experiment with AAV administration at P7 is appropriate due to the multiple rationales listed below.

First, in the experiment in which HA-tagged N-WASP PRDVCA was expressed by electroporation (Fig. 3), no abnormal phenotype was observed in PCs with a detectable but very weak HA signal. This fact strongly suggests that HA-N-WASP PRDVCA below the detection limit by IHC has no inhibitory effect. Second, when AAV was administered at P7, none of the PCs expressing HA-N-WASP PRDVCA showed abnormal stem dendrite formation. This suggests that the effect, if any, of HA-tagged N-WASP PRDVCA was very minimal before P10. Third, delaying the administration of AAV prevents us from fully detecting the effect of functional inhibition by N-WASP-PRDVCA, since the development of PC dendrites proceeds before sufficient expression of N-WASP PRDVCA is achieved (it takes at least a week for AAV to reach sufficient expression, but even this is still not yet the maximal expression level.). In addition, we had performed preliminary experiments comparing AAV administration at P7, P8, and P9 and had identified P7 as the optimal time of AAV administration at which sufficient dominant-negative effects could be observed and did not affect stem cell dendrite formation. Finally, it would be impossible to infer effects on dendrites from a single image in Fig. S3A and Fig. S6B, since the size of PC dendrites depends on the lobe in which they reside and their location within the lobe. Indeed, we observed a number of PCs at P10 and found no obvious differences between control PCs and HA-N-WASP PRDVCA- expressing PCs (AAV was administrated at P7).

12) Figure 6: The figure legend explains that abnormal orientation is when there are several primary dendrites going in several directions (they refer us to the drawing in fig 3H). To me then, the PCs showing more than one primary dendrite should all be abnormally oriented as by the authors definition. But there is only 23.4% abnormally oriented and 31.7% of PC with more than one primary dendrite (Figure 6B). Maybe you should show a PC with multiple primary dendrites but that is still well oriented and another one abnormally oriented so the reader can understand the difference?

Response

As the Reviewer points out, some PCs with multiple dendrites have normal and abnormal orientation, which makes the difference between 23.4 % and 31.7 %. According to the reviewer's suggestion, we added the images of PCs with normally or abnormally oriented multiple dendrites in Fig. S7, as below.

Fig. S7

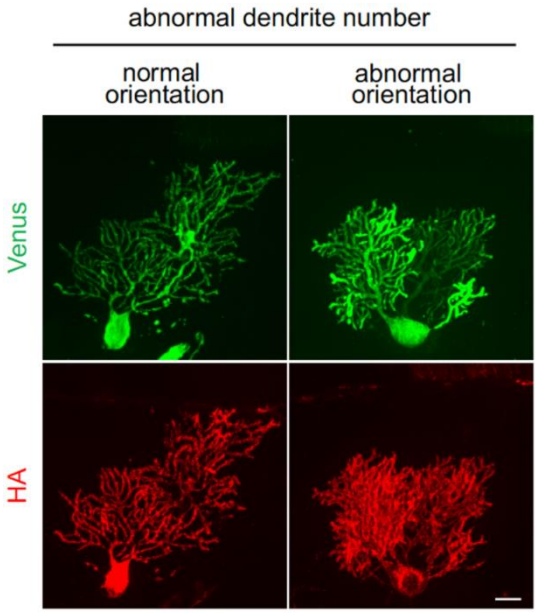


Fig. S7. Abnormalities of mem N-WASP Y253E-expressing PCs.
Z sections of membrane-anchored N-WASP Y253E (mem Y253E)-expressing PCs at P21. HA-tagged N-WASP mem Y253E was specifically expressed in PCs by *in utero* electroporation. Venus was expressed in PCs to visualize dendrite morphology. Sections were coimmunostained with antibodies against GFP (for Venus) and HA. Two PCs with multiple dendrites expressing N-WASP mem Y253E are shown. Note that the PC in the left panels has normal dendritic orientation (i.e., apical), while the PC in the right panels has abnormal orientation (i.e., apical and lateral). Scale bar represents 20 μm.

Minor comment 1)

In the introduction: "...probably through the abnormalities in ciliogenesis of ependymal cells or migration of radial glial cells..." Radial glia cells do not migrate. Please correct this sentence.

Response

We appreciate the reviewer's point. We have made the following correction in the page 5 line 18.

Before : "...probably through the abnormalities in ciliogenesis of ependymal cells or migration of radial glial cells..."
After : "...probably through the abnormalities in ciliogenesis of ependymal cells or migration of neural precursor cells..."

Responses to reviewer 2

My only concern is on Figure 1. The immunolocalization gives diffuse staining for some of the endogenous proteins, which is kind of expected. On the other hand important controls for the specificity of the signals are missing and should be shown, including secondary Abs alone, to determine the background. Also, to be convinced of the specificity of the stainings, especially when pAbs are used, controls of specificities like asbence/decrease of signals in KO sections or at least after Kd of endogenous proteins in cells would be important.

Response

According to the reviewer's suggestion, we performed immunohistochemistry without primary antibodies against N-WASP pathway molecules to confirm the signal specificity. As shown below, non-specific signals were not detected in any conditions (Fig. S1A,B).

Anti-N-WASP antibody (ab126626 Abcam) is knockout validated by the manufacturer (URL below). The specificity of the anti-phospho-N-WASP antibody (PA5-105307 Invitrogen) is

guaranteed by an analysis with a phospho-blocking peptide. (URL below). The specificity of anti-Arp2 (sc-166103 Santa Cruz Biotechnology), anti-Arp3 (A5979 Sigma-Aldrich), and anti-Cdc42 (ACD03 Cytoskeleton) antibodies has been confirmed using shRNAs in previous papers (Kailiang et al., 2020, Almeida-Souza et al., 2018, Singh et al., 2019).

Based on the above evidence, we consider that the antibodies used in this study are specific to their targets.

Fig. S1

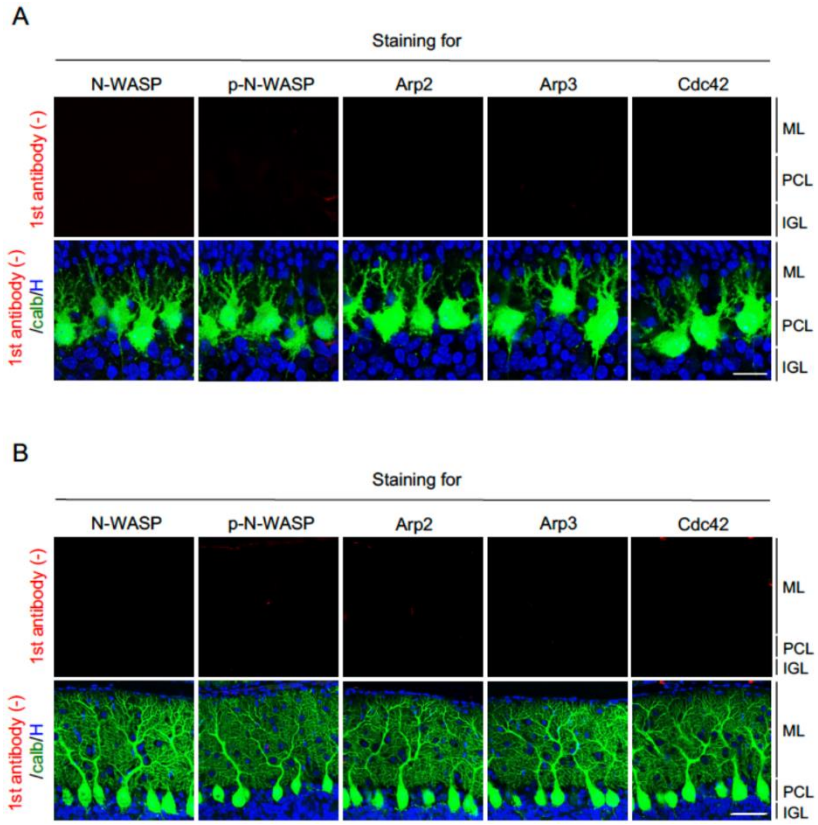


Fig. S1. Validation of the specificity of antibodies used in Fig. 1.
For validation of immunosignals for N-WASP, phospho-N-WASP, Arp2, Arp3, and Cdc42 in Fig.1, sections of P6 (A) and P14(B) cerebella were immunostained without primary antibodies except for anti-calbindin (calb, a PC marker). Note that no signal was detected by Alexa594-conjugated fluorescent secondary antibody alone (upper panels). H: Hoechst, ML: molecular layer, PCL: Purkinje cell layer, IGL: internal granule cell layer. Scale bars represent 20 μm (A) or 50 μm (B).

< References for the reviewer >

Anti-N-WASP antibody (ab126626 Abcam)
https://www.abcam.com/n-wasp-antibody-epr6959-ab126626.html
Anti-phospho-N-WASP antibody (PA5-105307 Invitrogen)
https://www.thermofisher.com/antibody/product/Phospho-N-WASP-Tyr256-Antibody- Polyclonal/PA5-105307
Anti-Arp2 antibody (sc-166103 Santa Cruz Biotechnology)
Kailiang et al., *EMBO Rep*. 21, e49269 (2020).
Anti-Arp3 antibody (A5979 Sigma-Aldrich)
Almeida-Souza et al., *Cell* 174, 325-337 (2018).
Anti-Cdc42 antibody (ACD03 Cytoskeleton)
Singh et al., *Arterioscler Thromb. Vasc. Biol*. 39, 137-149 (2019).

Second decision letter

MS ID#: DEVELOP/2022/201214

MS TITLE: N-WASP-Arp2/3 signaling controls multiple steps of dendrite maturation in Purkinje cells in vivo

AUTHORS: Koichi Hasegawa, Takeshi K Matsui, Junpei Kondo, and Ken-ichiro Kuwako

I have now received the reports of the two referees who reviewed the earlier version of your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The reviewers' evaluation is overall positive and we would like to publish a revised manuscript in Development. However you will see that referee 1 still has some concerns about the lack of evidence establishing the specificity of some of your reagents in your particular system. I am not asking you at this stage to do new experiments but to respond to the referees' comments and acknowledge in the manuscript the potential caveats with these reagents.

Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The authors answered satisfactorily to most of my concerns. It is however unfortunate that the authors did not show the effect of the 3 small molecule compounds on their specific targets specifically in their experimental setup. It is unfortunate that the authors did not perform controls for the specificity of their dominant negative form approach for instance using shRNA. I agree that shRNA approach is not perfect, which is why it is always used with its set of controls to confirm the efficiency and specificity of the effect. The use of dominant negative approach is not perfect either and should also come with its set of controls. Several controls exist and one of them is by obtaining similar results with different inhibitory molecules such as for instance shRNAs. The fact that, in previous studies, other authors controlled the specificity in their study system (using Dom neg and shRNA as I requested here) does not mean that it will be the case in your hands in your study system. For instance, a higher expression of the dominant negative protein would have a higher chance to trigger non-specific effects, and this will depend on the cell type too, and many other factors. So, you cannot compare their studies with yours. However, I would say that, in the case of NWASP, the rescue with the wild type NWASP supports the specificity of the approach. I might be overly cautious with all the controls here and I do not want to further delay the publication of this manuscript.

Comments for the author

The authors answered satisfactorily to most of my concerns. It is however unfortunate that the authors did not show the effect of the 3 small molecule compounds on their specific targets specifically in their experimental setup. It is unfortunate that the authors did not perform controls for the specificity of their dominant negative form approach for instance using shRNA. I agree that shRNA approach is not perfect, which is why it is always used with its set of controls to confirm the efficiency and specificity of the effect. The use of dominant negative approach is not perfect either and should also come with its set of controls. Several controls exist and one of them is by obtaining similar results with different inhibitory molecules such as for instance shRNAs. The fact that, in previous studies, other authors controlled the specificity in their study system (using Dom neg and shRNA as I requested here) does not mean that it will be the case in your hands in your study

system. For instance, a higher expression of the dominant negative protein would have a higher chance to trigger non-specific effects, and this will depend on the cell type too, and many other factors. So, you cannot compare their studies with yours. However, I would say that, in the case of NWASP, the rescue with the wild type NWASP supports the specificity of the approach. I might be overly cautious with all the controls here and I do not want to further delay the publication of this manuscript.

Reviewer 2

Advance summary and potential significance to field

Authors have partially answered to my concerns on Figure 1.

Comments for the author

Authors have partially answered to my concerns on Figure 1.

Second revision

Author response to reviewers' comments

Responses to Reviewers

Responses to reviewer 1

The authors answered satisfactorily to most of my concerns. It is however unfortunate that the authors did not show the effect of the 3 small molecule compounds on their specific targets specifically in their experimental setup. It is unfortunate that the authors did not perform controls for the specificity of their dominant-negative form approach for instance using shRNA. I agree that shRNA approach is not perfect, which is why it is always used with its set of controls to confirm the efficiency and specificity of the effect. The use of dominant-negative approach is not perfect either and should also come with its set of controls. Several controls exist and one of them is by obtaining similar results with different inhibitory molecules such as for instance shRNAs. The fact that, in previous studies, other authors controlled the specificity in their study system (using Dom neg and shRNA as I requested here) does not mean that it will be the case in your hands in your study system. For instance, a higher expression of the dominant-negative protein would have a higher chance to trigger non-specific effects, and this will depend on the cell type too, and many other factors. So, you cannot compare their studies with yours. However, I would say that, in the case of NWASP, the rescue with the wild type NWASP supports the specificity of the approach. I might be overly cautious with all the controls here and I do not want to further delay the publication of this manuscript.

Response

Although it is difficult to experimentally prove the specificity of the inhibitors in our PC culture system because of the reasons that we noted in the previous "Response to Reviewers", we deeply understand the concerns of reviewer 1.

In addition, we fully agree with reviewer 1's comments on our approach using dominant-negative mutants. As reviewer 1 pointed, adding shRNA data would be helpful in eliminating concerns about dominant-negative mutants. We consider that the fact that overexpression of wild-type N-WASP or N-WASP Y253E without membrane-anchored signal did not hamper PC dendrite development, partially indicates that expression of N-WASP mutants does not result in nonspecific effects. We newly described about this point and general concern about dominant-negative approach in page 14, line 5-14.

Third decision letter

MS ID#: DEVELOP/2022/201214

MS TITLE: N-WASP-Arp2/3 signaling controls multiple steps of dendrite maturation in Purkinje cells in vivo

AUTHORS: Koichi Hasegawa, Takeshi K Matsui, Junpei Kondo, and Ken-ichiro Kuwako
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.