WAVE1 and WAVE2 have distinct and overlapping roles in controlling actin assembly at the leading edge

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RE: E19-12-0705
TITLE: WAVE1 and WAVE2 have distinct roles in regulating actin assembly at the leading edge

Dear Bruce,

Two experts in the field reviewed your manuscript. You will see that both reviewers found your study, on the role of Wave 1 and Wave 2 in regulating actin assembly at the leading edge, interesting. However, you will see that both reviewers found the effect of WAVE 1 on WAVE 2 KO cells somehow surprising. They suggested some very interesting experiments in particular over-expression of WAVE1 that should strengthen the main conclusion of your manuscript.

Do not hesitate to contact me via the editorial office (MBoC@ascb.org) if you want to discuss these experiments.

All the best,
Laurent

Laurent Blanchoin
Monitoring Editor

Dear Prof. Goode:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Tang et al describes the characterisation of B16F1 melanoma cells lines deficient in WAVE1 or WAVE2 and double knockout for WAVE1 and WAVE2.
The authors report that WAVE1 and WAVE2 are redundant for lamellipodia formation and they conclude from their data that they have unique mechanistic roles in controlling the rate of actin network growth at the leading edge, with WAVE2 promoting assembly and WAVE1 restricting it.
Previously, individually, WAVE 1 or WAVE 2 MEF knockout cell lines have been reported and characterised but these have been not thoroughly characterised to draw in depth mechanistic conclusions. In addition, WAVE1 and WAVE 2 have not been knocked out simultaneously in the same cell line. Therefore, the current work is important and we have no clear understanding of the differences of the molecular functions of the WAVE family members. In addition, the authors had previously reported in a biochemical study that the VCA domain of WAVE1, but not WAVE2, slows the rate of elongation at actin filament barbed ends, even in the absence of Arp2/3 complex depending on the WH2 domain of WAVE1 (Sweeney et al., 2015).
Overall the current study was performed with great care and partly appropriately controlled. Most importantly, a rescue of the phenotypes by re-expression of the knocked out cDNA is missing to control for off target effects of the Cas9 strategy which can be substantial. Furthermore, two experiments have been only repeated two times.
In addition, I am concerned that the main claim from the study that WAVE2 promoting assembly and WAVE1 restricting it is currently not sufficiently backed by the data provided and the current data would also allow the opposite conclusion:
How can you conclude that WAVE1 restricts actin network assembly at the leading edge if its expression level of 10% (compared to WAVE2) can compensate for a loss of 90% of WAVE2? Of course, it cannot fully compensate and shows slightly reduced F-actin intensity in the lamellipodium and actin incorporation rates, and consequently also lamellipodia protrusion speeds. The increase in F-actin density in the WAVE1 KO cells is also very small and only reaches significance due to pooling of large number of data points and not using biological repeats as individual N's.
My interpretation of this data is that WAVE1 could be more active than WAVE2. This is supported by unchanged Arp2/3 incorporation into lamellipodia suggesting higher WAVE1 activity to compensate for WAVE2 loss.
Fair enough, an alternative interpretation as pointed out in this manuscript could be that only a small fraction of WAVE2 is recruited to the leading edge and in this scenario WAVE1 could have a lower activity - but with your current data you can't distinguish this.
In addition, the increase in retrograde flow in the WAVE1 KO cells could be due to effects of WAVE1 on focal complex formation/adhesion rather than on Arp2/3 activation which is supported by the unchanged incorporation of Arp2/3.
To distinguish these possibilities, it would be essential to test over-expression of WAVE1 to levels

similar as WAVE2 in wild-type or in WAVE2 KO cells to test for this supposedly negative role of WAVE1 on actin nucleation at the leading edge.
The results by Bieling et al 2017 also concur with an interpretation that WAVE1 accelerated barbed end actin filament elongation.
In general, I am supportive of publication of this manuscript in the MBoC but only after more experiments (especially overexpression and rescue experiments) have been performed to allow definitive conclusions to be made.

Detailed comments:

Which commercial anti-W2 antibody was used?

Fig. 1 show "was confirmed by genomic sequencing at the targeted loci"
New W1 and commercial W2 specific antibodies: show whole blots to show how specific they are and use on lysates of DKO cells re-expressing W1-EGFP or W2-EGFP to show specificity.
Is there compensation in the W1, W2 or W1/w DKO cells through increased expression levels of WAVE3? Show blot or Q-PCR.
Fig 2B: Data are from {greater than or equal to} 90 cells per cell line, pooled from at only two independent experiments. This needs to be repeated at least 3 times as it is common.
It would be better to show means of means of the three biological repeats.

Fig 3 Actin assembly rates:
Data were combined from two independent experiments (analyzing a total of at least 49 cells per condition).
This needs to be repeated at least 3 times as it is common.
Fig. 3F,G: Please be consistent and use scatter plots and SD as in the other figures.

Fig. S2: It would be useful to also report Mean Square Displacement.
The frame rate of this random migration experiment was quite high 2 min very frame and this may cause an aberrantly high tracking error from tracking noise without real displacement of the cells. Of course, at the high frame rate "pausing" of the cells may just point to a too high frame rate. For both reasons it would be important to understand the algorithm behind the AI automated tracking.

Reviewer #2 (Remarks to the Author):

This manuscript uses mouse B16-F1 cells to examine the roles of two Arp2/3 complex activators (NPFs), WAVE1 and WAVE2, which are closely related and for whom distinct functions have not been ascribed. The authors measured Abi1 intensity at the leading edge in order to show that WAVE1 KO (which according to them makes up ~10% of total WAVE in B16 cells) only slightly decreases the levels of WRC (Supplementary figure 1A). Following from that result, the KO of WAVE2 (which presumably comprises ~90% of total WAVE) had a more dramatic decrease of Abi1 intensity at lamellipodia tips. The authors also demonstrated that WAVE2 KOs display less efficient protrusion and leading edge actin assembly compared to wildtype. Interestingly, WAVE2 KOs have higher leading edge actin assembly compared to WT. These results suggest some non-overlapping functions for WAVE1 and WAVE2.

Overall, the manuscript is well done, and provides some interesting new findings. There are a couple of things that would be good to address.

1) Faster protruding lamellipodia often correlate with increased accumulation of actin assembly factors, such as VASP (Rottner et al. Nat Cell Biol. 1999) and WAVE complex. This in turn means that reduced lamellipodia protrusion efficiency in WAVE2 KOs could account for decreased amounts of Abi1 levels at the tip membrane, thus it does not necessarily reflect the expression of WRC in theses cells. If the authors want to make a statement about overall WRC levels, they should rather test for the expression of other WRC subunits, such as Sra1 or Nap1 by western blotting. It is well established that loss of one subunit affects the expression of the remaining components (Schaks et al. Curr Biol. 2018, Litschko et al. Eur J Cell Biol. 2017). Is the expression of other WRC components altered upon loss of WAVE1 or -2? If so, is it affected similarly in WAVE1 and WAVE2 KOs? Is the small residual amount of WAVE1 (~10%) enough to 'stabilize' the expression of the other WRC subunits?

2) It is surprising that WAVE2 KOs don't have apparent effects on lamellipodia formation given that presumably 90% of total WAVE protein is missing. This suggests that a small amount of WAVE1 is sufficient to activate the Arp2/3 complex comparably well to high levels of WAVE2. Is it possible that WAVE1 has a higher turnover at the leading edge membrane, thus being able to activate Arp2/3 complex more efficiently? Two possible experiments would provide information on this point.
a) Test and compare the dynamics of WAVE1 and WAVE2 using FRAP. I suggest expressing WAVE1 or WAVE2 in a W1/W2 KO clone in order to avoid disturbing effects from residual WAVE proteins.
b) Overexpress WAVE1 in WAVE2 KO cells to increase the amount of WAVE1 to more than ~10%. Would an increased amount of WAVE1 reinforce the observed effects on actin density, polymerization and/or retrograde flow?

Other Comments:
Figure 1E: Changing the color of shown F-actin staining to black and white would improve visibility.

We wish to thank both reviewers for carefully reading our manuscript and bringing to our attention many valuable points, which we have attempted to address both in the revised manuscript and in our point-by-point responses below.

Reviewer #1 (Remarks to the Author):

Tang et al describes the characterisation of B16F1 melanoma cells lines deficient in WAVE1 or WAVE2 and double knockout for WAVE1 and WAVE2.

The authors report that WAVE1 and WAVE2 are redundant for lamellipodia formation and they conclude from their data that they have unique mechanistic roles in controlling the rate of actin network growth at the leading edge, with WAVE2 promoting assembly and WAVE1 restricting it.

Previously, individually, WAVE 1 or WAVE 2 MEF knockout cell lines have been reported and characterised but these have been not thoroughly characterised to draw in depth mechanistic conclusions. In addition, WAVE1 and WAVE 2 have not been knocked out simultaneously in the same cell line. Therefore, the current work is important and we have no clear understanding of the differences of the molecular functions of the WAVE family members. In addition, the authors had previously reported in a biochemical study that the VCA domain of WAVE1, but not WAVE2, slows the rate of elongation at actin filament barbed ends, even in the absence of Arp2/3 complex depending on the WH2 domain of WAVE1 (Sweeney et al., 2015).

Overall the current study was performed with great care and partly appropriately controlled. Most importantly, a rescue of the phenotypes by re-expression of the knocked out cDNA is missing to control for off target effects of the Cas9 strategy which can be substantial.

To control for off-target effects of the Cas9 genome editing approach, we selected two independent clones for each cell line and showed that the effects were consistent between the two clones, which helps ensure the specificity of the effects resulting from loss of one or both WAVE isoforms in the cells. We also demonstrated that WAVE expression from a plasmid could rescue the gross phenotypes resulting from the double knockout, demonstrating that the phenotypes arise specifically from the loss of WAVE. We did not individually test the more subtle rescue of WAVE1 KO and WAVE2 KO with separate WAVE1 and WAVE2 plasmids, because the expression of these WAVEs from plasmids is highly variable, cell to cell, which would make such rescue attempts very difficult to interpret.

In the revised manuscript, we now show that plasmid-based re-expression of EGFP-WAVE1 and EGFP-WAVE2 in WAVE1/2 double KO cells rescues the defects in lamellipodia formation (Supplemental Figure 3A) and the defects in velocity of cell motility (Supplemental Figure 3E). This demonstrates that the phenotypes we are reporting are due to the loss of the WAVE proteins. Note, while the rescue was impressive, it was not complete (Figure 3E), which we believe is the result of cell-to-cell variability in expression levels using plasmid-based rescue.

Furthermore, two experiments have been only repeated two times.

Before the COVID shutdown, we managed to complete a third independent experiment for one of these (the Arp2/3 staining at the leading edge), but not the other (FRAP analysis of GFP-actin dynamics at the leading edge). We hope that this is sufficient given the circumstances.

In addition, I am concerned that the main claim from the study that WAVE2 promoting assembly and WAVE1 restricting it is currently not sufficiently backed by the data provided and the current data would also allow the opposite conclusion:

How can you conclude that WAVE1 restricts actin network assembly at the leading edge if its expression level of 10% (compared to WAVE2) can compensate for a loss of 90% of WAVE2?

Although the total cellular level of WAVE1 is only 10% that of WAVE2, this does not necessarily mean that WAVE2 is 10 times more abundant specifically at the leading edge. It is possible that only a fraction of the total cellular pool of WAVE is found at the leading edge, or needed there to achieve proper levels of Arp2/3-mediated actin nucleation. We are unaware of any published findings that contradict this possibility. We gave serious consideration to trying to quantify endogenous WAVE1 vs. WAVE2 levels at the leading edge, but the only tools available are two different antibodies (to WAVE1 and WAVE2) for cell staining, which makes this virtually impossible, and is likely why no studies have yet succeeded in answering this question.

In addition, we have now shown that increasing the levels of WAVE1 in W2-KO cells (by ectopic expression) restricts actin assembly at the leading edge even further, with lamellipodial F-actin density as the readout (Supplemental Figure 3E and F). This is highly consistent with our conclusions (see also below).

Of course, it cannot fully compensate and shows slightly reduced F-actin intensity in the lamellipodium and actin incorporation rates, and consequently also lamellipodia protrusion speeds. The increase in F-actin density in the WAVE1 KO cells is also very small and only reaches significance due to pooling of large number of data points and not using biological repeats as individual N's.

The differences we measure between WT and WAVE1 KO cells may be modest, but they are statistically significant. For this reason, we believe it is appropriate to consider them as meaningful. We believe that having a large ‘n’ (number of data points) shows good sampling and makes our analysis more accurate and robust. We handled these data and performed the statistical analysis based on well-established methods in the field, e.g., which were used in these studies: Fort et al., 2018, *Nature Cell Biol*; Gateva et al., 2017, *Curr Biol*; Kage et al., 2017, *Nature Commun*,; Litschko et al., 2017, *Eur J Cell Biol*; Rotty et al., 2017, *Dev Cell*; Young et al, 2018, *J Cell Sci*.

My interpretation of this data is that WAVE1 could be more active than WAVE2. This is supported by unchanged Arp2/3 incorporation into lamellipodia suggesting higher WAVE1 activity to compensate for WAVE2 loss.

This is certainly possible, yet it is only one interpretation, and it assumes that there is less WAVE1 than WAVE2 at the leading edge, which (see above) is difficult to validate. Therefore, the field still does not know the relative abundance of WAVE1 and WAVE2 at the leading edge. We have provided the first quantitative western analysis of endogenous WAVE1 and WAVE2

levels in any cell type, revealing a 9:1 ratio of WAVE2:WAVE1 protein in B16-F1 cells. However, this difference is in the TOTAL cellular levels, and does not necessarily reflect differences in levels at specific locations in the cell, such as at the leading edge. Thus, one cannot interpret the unchanged Arp2/3 levels (in WAVE2 KO cells) as suggesting that WAVE1 is more active than WAVE2. We have previously shown that in vitro WAVE1 and WAVE2 have similar NPF activities on Arp2/3 complex (Sweeney et al., 2015; *MBC*).

Fair enough, an alternative interpretation as pointed out in this manuscript could be that only a small fraction of WAVE2 is recruited to the leading edge and in this scenario WAVE1 could have a lower activity - but with your current data you can't distinguish this.

As mentioned above, we do not think that WAVE1 has lower (NPF) activity compared to WAVE2. Importantly, WAVE1 has two separate effects on actin filament assembly dynamics. First, it robustly promotes actin nucleation by Arp2/3 (similar to WAVE2), i.e., it has strong NPF activity. Second, WAVE1 slows elongation at filament barbed ends (by about 3-fold in vitro), and this effect is seen both in the presence and absence of Arp2/3 complex. In contrast, WAVE2 has no detectable effect on filament elongation. Therefore, we interpret the ability of WAVE1 to recruit/activate Arp2/3 complex at the leading edge and support lamellipodia formation (in WAVE2 KO cells) as indicating that WAVE1 and WAVE2 are redundant as NPFs. On the other hand, our observation that the lamellipodial networks extend/grow faster in WAVE1 KO cells, and conversely extend slower in WAVE2 KO cells, correlates with the differences between WAVE1 and WAVE2 on filament elongation.

Importantly, the inhibitory effects of WAVE1 on filament elongation are not in conflict with WAVE1 being a robust NPF. The fact that WAVE2 KO cells form lamellipodia (and that WAVE1 levels did not increase in response to WAVE2 KO) also suggests that the cells require only a relatively small amount of active WAVE protein at the leading edge to robustly stimulate nucleation by Arp2/3, and thereby form a branched actin network. These points are clarified in the revised manuscript (page 2 paragraph 2, page 6 paragraph 2).

In addition, the increase in retrograde flow in the WAVE1 KO cells could be due to effects of WAVE1 on focal complex formation/adhesion rather than on Arp2/3 activation which is supported by the unchanged incorporation of Arp2/3.

We acknowledge this point, as there is a relationship between focal adhesions and retrograde flow (Gardel et al., 2008; *J Cell Biol*), and disrupting WAVE or WRC can alter focal adhesions (Silva et al., 2009, *Cell*; Tang et al., 2013, *Curr Biol*; Yamazaki et al, 2005, *Genes to Cell*). We now mention this near the end of the Results/Discussion, i.e., that our data do not exclude the possibility of WAVE1 KO effects on focal adhesions contributing to the changes we see in actin network dynamics. However, we also make the point that for this to account for the changes we see in retrograde actin flow, we would also expect to see a change in motility rate in the WAVE1 KO cells (since motility depends strongly on adhesion), but we do not. Instead, the changes we see in WAVE1 KO cells are primarily at the leading edge, e.g., an increase in F-actin density and an increase in the rate of actin incorporation at the leading edge. These observations are most readily explained by loss of WAVE1 function at the leading edge.

Notably, we do not think that the increase in retrograde flow in WAVE1 KO cells is due to a change in “Arp2/3 activation”, but rather a loss of the Arp2/3-independent WAVE1 interactions with the barbed ends of filaments. Our data suggest that WAVE1 helps couple the barbed ends

of actin networks to the membrane, an idea supported by earlier studies showing that VCA domains can have this effect (Co et al, 2007, Cell).

To distinguish these possibilities, it would be essential to test over-expression of WAVE1 to levels similar as WAVE2 in wild-type or in WAVE2 KO cells to test for this supposedly negative role of WAVE1 on actin nucleation at the leading edge.

The reviewer asks that we overexpress WAVE1 (in WAVE2 KO cells) by 9-fold, so that it matches endogenous WAVE2 levels. Unfortunately, plasmid-based over-expression of WAVE proteins is very difficult to tune, typically leading to higher expression levels compared to the endogenous proteins, and always producing great cell-to-cell variability in expression level. In addition, when WAVEs are over-expressed to levels higher than the endogenous WAVE regulatory complex (WRC) components, the 'free' unregulated WAVE can sequester actin from the lamellipodia network by activating Arp2/3 in the cytosol, making this type of experiment very difficult to interpret.

With these caveats in mind, we performed an experiment requested by reviewer 2 (see below), in which we overexpressed EGFP-WAVE1 in WAVE2 KO cells (this was also mentioned above). We observed that EGFP-WAVE1 expression led to reduced F-actin density at the lamellipodia compared to control WAVE2 KO cells (Supplemental Figure 3 E and F). Again, this result supports our model that WAVE1 restricts actin assembly at the leading edge.

The results by Bieling et al 2017 also concur with an interpretation that WAVE1 accelerated barbed end actin filament elongation.

The in vitro results in Bieling et al 2017 (EMBO J) are opposite to our previous in vitro observations for WAVE1 (Sweeney et al., 2015, *Mol Biol Cell*), and reach an opposite conclusion to our new in vivo observations. The Bieling study showed that WAVE1 (if concentrated on a 2D surface, and specifically in the presence of profilin) accelerated rather than slowed actin filament elongation. There is currently no in vivo evidence to support a model in which WAVE1 accelerates filament elongation in cells. To the contrary, our in vivo evidence is consistent with WAVE1 slowing elongation.

In general, I am supportive of publication of this manuscript in the MBoC but only after more experiments (especially overexpression and rescue experiments) have been performed to allow definitive conclusions to be made.

Detailed comments:

Which commercial anti-W2 antibody was used?

WAVE-2 (D2C8) XP Rabbit mAb (Cell Signaling, Catalog# 3659). This information is now added to the Methods.

Fig. 1 show "was confirmed by genomic sequencing at the targeted loci"
We have included the targeted loci sequences showing the sequence changes leading to early stop codon (Supplemental Figure 1A).

New W1 and commercial W2 specific antibodies: show whole blots to show how specific they are and use on lysates of DKO cells re-expressing W1-EGFP or W2-EGFP to show specificity.

As requested, we have included the whole blots corresponding to Figure 1A (see Supplemental Figure 1C). The blots were cut, so they only include MW range 50-150 kDa.

To further demonstrate WAVE1 and WAVE2 antibody specificity, we also included new blots (Supplemental Figure 1B) showing that our WAVE1 antibody reacts with a band at the appropriate MW for endogenous WAVE1, and more robustly with a higher MW band in cells overexpressing EGFP-WAVE1, but not cells over-expressing EGFP-WAVE2. Further, we show the converse for our WAVE2 antibody. Thus, there is no cross-reactivity between the antibodies.

Is there compensation in the W1, W2 or W1/w DKO cells through increased expression levels of WAVE3? Show blot or Q-PCR.

To address this, we have added a new blot using a WAVE3 antibody (Supplemental Figure 1D) showing that WAVE3 is undetectable in B16-F1 WT and WAVE1/2 KO cells, but detectable in MDA-MB-231 cells, as reported (Spence et al, 2012; *Biochem J*). Moreover, we show that when EGFP-WAVE3 is ectopically expressed in our WAVE1/2 KO cells, lamellipodia formation is restored (Supplemental Figure 3A). This demonstrates that WAVE3, when expressed ectopically, will compensate as an NPF for the combined loss of WAVE1 and WAVE2, but that B16-F1 cells normally have undetectable levels of WAVE3. This is now mentioned in the text (on page 4 and page 5). These results are also consistent with microarray studies showing that WAVE3 transcripts are undetectable in B16-F1 cells, and that WAVE1 and WAVE2 are the major isoforms in these cells (Block et al, 2008; *Journal of Microscopy*).

Fig 2B: Data are from {greater than or equal to} 90 cells per cell line, pooled from at only two independent experiments. This needs to be repeated at least 3 times as it is common.
It would be better to show means of means of the three biological repeats.

As requested, we performed a third repeat of Arp2/3 staining on WT vs W1KO (#1) and W2KO (#11), and these data are now included in the relevant panel (Figure 2B). The other experiment in Figure 2B, i.e., Arp2/3 staining in WT vs W1KO (#6) and W2KO (#16), already had three repeats. We compared the mean of means and there is no significant difference among the cell lines.

Fig 3 Actin assembly rates:
Data were combined from two independent experiments (analyzing a total of at least 49 cells per condition).
This needs to be repeated at least 3 times as it is common.

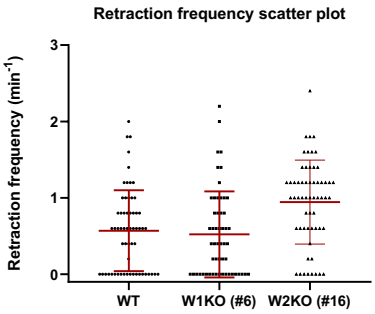
The FRAP analysis for this study has all been performed in the lab of our co-author Klemens Rottner, and we do not have the appropriate microscope with FRAP capabilities for mammalian cell work at our institute. The Rottner lab was planning to do the third FRAP trial, but did not

manage to get this done before the COVID shutdown. We hope that this is acceptable given the circumstances.

Fig. 3F,G: Please be consistent and use scatter plots and SD as in the other figures.

We now show SD (below the plots) for Figure 3F and 3G.

For Figure 3F, scatter plots were not appropriate, as the frequencies of retraction events are low. In the 5 min observation window, the leading edge on average retracts only ~3-5 times. Graphing these frequencies in a scatter plot (example shown here) makes the data difficult to interpret. We also considered using histograms, but they make it more difficult to compare conditions. Other studies (e.g., Haynes et al, 2015; *J Cell Biol*) have displayed retraction frequency data using Box and Whisker plots, which we now have done for Figure 3F.



For Figure 3G, we had a large number of data points (141-314 per condition), which led to the data points being obscured in scatter plots. In addition, there are a few outlier points that compromise the display of the data as a scatter plot (shown here). Therefore, we displayed these data in Box and Whisker (10-90 percentile) plots in Figure 3G.

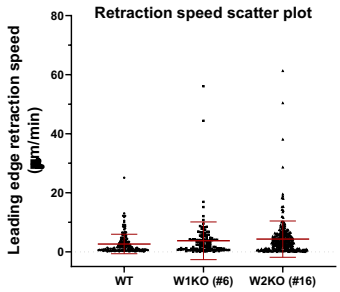


Fig. S2: It would be useful to also report Mean Square Displacement.
The frame rate of this random migration experiment was quite high 2 min very frame and this may cause an aberrantly high tracking error from tracking noise without real displacement of the cells. Of course, at the high frame rate "pausing" of the cells may just point to a too high frame rate. For both reasons it would be important to understand the algorithm behind the AI automated tracking.

As requested, we have added MSD plots (Supplemental Figure 2F), which show that WT and single WAVE KO cells are similar in their migration directedness. B16-F1 cells migrate relatively fast (~1.5 μm/min or even higher), and therefore 2 min image capture intervals were appropriate. In our initial trials, we tried the analysis using 5-min frame intervals, but we ran into problems with the automated tracking AI picking up other cells crossing the path of the cell being analyzed. The tracking algorithm is proprietary from Metavi labs, but it recognizes and tracks the center of the nucleus based on image contrast. We acknowledge that the pausing frequency of the cells might be slightly over-estimated due to the higher frame rate, but the tracking algorithm was applied to all conditions equally, which means that our comparison of our WT, WAVE1 KO, WAVE2 KO, and WAVE1/2 KO cell lines is internally controlled.

Reviewer #2 (Remarks to the Author):

This manuscript uses mouse B16-F1 cells to examine the roles of two Arp2/3 complex activators (NPFs), WAVE1 and WAVE2, which are closely related and for whom distinct functions have not been ascribed. The authors measured Abi1 intensity at the leading edge in order to show that WAVE1 KO (which according to them makes up ~10% of total WAVE in B16 cells) only slightly decreases the levels of WRC (Supplementary figure 1A). Following from that result, the KO of WAVE2 (which presumably comprises ~90% of total WAVE) had a more dramatic decrease of Abi1 intensity at lamellipodia tips. The authors also demonstrated that WAVE2 KOs display less efficient protrusion and leading edge actin assembly compared to wildtype. Interestingly, WAVE2 KOs have higher leading edge actin assembly compared to WT. These results suggest some non-overlapping functions for WAVE1 and WAVE2.

Overall, the manuscript is well done, and provides some interesting new findings. There are a couple of things that would be good to address.

1) Faster protruding lamellipodia often correlate with increased accumulation of actin assembly factors, such as VASP (Rottner et al. Nat Cell Biol. 1999) and WAVE complex. This in turn means that reduced lamellipodia protrusion efficiency in WAVE2 KOs could account for decreased amounts of Abi1 levels at the tip membrane, thus it does not necessarily reflect the expression of WRC in these cells. If the authors want to make a statement about overall WRC levels, they should rather test for the expression of other WRC subunits, such as Sra1 or Nap1 by western blotting. It is well established that loss of one subunit affects the expression of the remaining components (Schaks et al., 2018; *Curr Biol*, Litschko et al., Eur J Cell Biol. 2017). Is the expression of other WRC components altered upon loss of WAVE1 or -2? If so, is it affected similarly in WAVE1 and WAVE2 KOs? Is the small residual amount of WAVE1 (~10%) enough to 'stabilize' the expression of the other WRC subunits?

This is a valid point, that other subunits of WRC complex may be at least partially lost upon removal of SCAR/WAVE.

As requested, we now include western blots of Nap-1 and Sra-1 protein levels in our cell lines (Supplemental Figure 1G). Nap-1 levels are modestly reduced in our KO lines, compared to WT, and Sra-1 is more drastically reduced according to the loss of WAVE, i.e., more severely for WAVE2 (which is more abundant) and less severely for WAVE1 (which is less abundant).

2) It is surprising that WAVE2 KOs don't have apparent effects on lamellipodia formation given that presumably 90% of total WAVE protein is missing. This suggests that a small amount of WAVE1 is sufficient to activate the Arp2/3 complex comparably well to high levels of WAVE2. Is it possible that WAVE1 has a higher turnover at the leading edge membrane, thus being able to activate Arp2/3 complex more efficiently? Two possible experiments would provide information on this point.
a) Test and compare the dynamics of WAVE1 and WAVE2 using FRAP. I suggest expressing WAVE1 or WAVE2 in a W1/W2 KO clone in order to avoid disturbing effects from residual WAVE proteins.

This is an interesting suggestioni, although a technically challenging experiment given the comparably weak signals of GFP-WAVE fusions that we would have to monitor - to avoid cytoplasmic Arp2/3 sequestration and thus lamellipodia inhibition observed upon WAVE over-expression previously (e.g., see Machesky and Insall, 1998, *Curr Biol*). More importantly though, it is not clear how determining the turnover dynamics for WAVE proteins at the leading edge would inform our model. The timescale of WAVE2 turnover at the leading edge is slow compared to actin network assembly and Arp2/3 complex incorporation (Lai et al, 2008, *EMBO J*), making it likely that an individual WAVE molecule will repeatedly stimulate many Arp2/3-driven branching events. Moreover, WCA/PWCA-coated beads or ActA positioned at the surface of *Listeria* don't require any turnover to the best of our knowledge, in spite of mediating continuous, Arp2/3-dependent motility. Considering all this, it would be difficult to imagine how WAVE turnover could affect the efficiency of Arp2/3 activation, and indeed, data from the Vaccinia system suggests the opposite to be the case, i.e. active Arp2/3-dependent actin assembly drives NPF turnover, in this case N-WASP (Weisswange et al., 2009, *Nature*). All together, there is no evidence at present to suggest if (or how) potential differences in WAVE isoform turnover, should they exist, would translate into changes of their respective activities. Thus, any changes in turnover dynamics of WAVEs would be difficult to interpret at least concerning their activities referred to here.

b) Overexpress WAVE1 in WAVE2 KO cells to increase the amount of WAVE1 to more than ~10%. Would an increased amount of WAVE1 reinforce the observed effects on actin density, polymerization and/or retrograde flow?

To address this point, as mentioned earlier (see response to reviewer 1) we overexpressed EGFP-WAVE1 in WAVE2 KO cells and observed a decrease in F-actin density at the leading edge compared to control WAVE2 KO cells.

Other Comments:
Figure 1E: Changing the color of shown F-actin staining to black and white would improve visibility.

We have changed the images to black and white.

RE: Manuscript #E19-12-0705R
TITLE: "WAVE1 and WAVE2 have distinct and overlapping roles in controlling actin assembly at the leading edge"

Dear Bruce,

You should be able to address the reviewer#1 comments #2 (first part) and 4 in a revised version of your manuscript.
If you could also address the other comments in your rebuttal, I should be able to take rapidly the final decision on your manuscript.

Sincerely,

Laurent Blanchoin
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Goode,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable

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Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The revised manuscript by Tang et al has improved and the authors have addressed several of the points raised.
I am very sympathetic to the argument regarding current reduced access to labs due to the covid situation. However, I am very sorry, but I cannot support a publication in which experiments have been repeated less then 3 times as this will reduce the validity of this publication and will cause readers to question its reproducibility. This manuscript should never have been submitted last December reporting preliminary results which had not been repeated at least 3 times.

Therefore, I unfortunately have to ask for another revision in which the following points need to be addressed before I can recommend publication in the MBoC.

1. The following experiments need to be repeated at least 3 times: Fig. 1B; Fig. S1E,F; Fig. 3A-C, The new data in Fig. S3F
2. The new data in Fig. S3A: The loss and rescue of lamellipodia in W1/2 KO cells by W1, W2 and W3 needs to be quantified. This should be a fairly easy quantification on percentage of cells that form lamellipodia from existing images from the (hopefully) three repeats.
3. It should be indicated for all experiments including western blots in the figure legends that the experiments or blots have been repeated at least 3 times.
4. The authors did not give the exact sample size in the figure legends: The MBoC requires that the exact sample size (n) for each experimental group/condition, given as a number, not a range.
5. In the abstract it should be stated that the requirements for W1 and W2 in B16-F1 differ from the results obtained in MEFs earlier (Suetsugu et al., 2003)
Not necessary (since I did not ask for this in the first round) but helpful to make the story more complete would be:

Quantification of filament density/organisation in EM.
Quantification of lamellipodia width from Phalloidin stained images in Fig. 2C.
Rescue of W1/2 KO cells with W1 delta PP, or WH2 domain swapping (W1 vs W2) or W1 with K207-mut of WH2 to further explore the discrepancy with Biehling et al 2017.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments very well, and I think this manuscript is ready for publication.

The revised manuscript by Tang et al has improved and the authors have addressed several of the points raised. I am very sympathetic to the argument regarding current reduced access to labs due to the covid situation. However, I am very sorry, but I cannot support a publication in which experiments have been repeated less then 3 times as this will reduce the validity of this publication and will cause readers to question its reproducibility. This manuscript should never have been submitted last December reporting preliminary results which had not been repeated at least 3 times.
As discussed with the Editor, it is not feasible for us to perform a third repeat of these experiments. Further, there is no official rule in the field, or in the MBoC checklist and guidelines for authors, saying that an experiment repeated 3 times is valid, while an experiment repeated 2 times is invalid. How many times an experiment needs be repeated in order to yield a meaningful result depends on many things, including sample size and the precise nature of the experiment. In cases where we performed two rather than three trials, we analyzed a very large number of cells and obtained very similar results. We stated in the legends how many times we did each experiment and provided the sample size (n). Our graphs are transparent in showing our many data points, along with means, error bars, and statistical tests to verify any claimed differences between conditions.

Therefore, I unfortunately have to ask for another revision in which the following points need to be addressed before I can recommend publication in the MBoC.

1. The following experiments need to be repeated at least 3 times: Fig. 1B; Fig. S1E,F; Fig. 3A-C, The new data in Fig. S3F.
No longer relevant. See above.

2. The new data in Fig. S3A: The loss and rescue of lamellipodia in W1/2 KO cells by W1, W2 and W3 needs to be quantified. This should be a fairly easy quantification on percentage of cells that form lamellipodia from existing images from the (hopefully) three repeats.
We have quantified this, and report the values in the main text.

3. It should be indicated for all experiments including western blots in the figure legends that the experiments or blots have been repeated at least 3 times.
No longer relevant. See above.

4. The authors did not give the exact sample size in the figure legends: The MBoC requires that the exact sample size (n) for each experimental group/condition, given as a number, not a range.
We have fixed this in all of the legends.

5. In the abstract it should be stated that the requirements for W1 and W2 in B16-F1 differ from the results obtained in MEFs earlier (Suetsugu et al., 2003)
As discussed with the Editor, referencing this in our abstract is not appropriate in this case, as our story is not directly related to the 2003 paper (different techniques, different cell types, different starting questions and rationale, etc). Further, we cite the 2003 paper in our manuscript and openly discuss the relationship between our findings and theirs.

Not necessary (since I did not ask for this in the first round) but helpful to make the story more complete would be:
Quantification of filament density/organisation in EM.
Quantification of lamellipodia width from Phalloidin stained images in Fig. 2C.
Rescue of W1/2 KO cells with W1 delta PP, or WH2 domain swapping (W1 vs W2) or W1 with K207-mut of WH2 to further explore the discrepancy with Biehling et al 2017.
As discussed with the Editor, these suggested revisions are not required.

RE: Manuscript #E19-12-0705RR
TITLE: "WAVE1 and WAVE2 have distinct and overlapping roles in controlling actin assembly at the leading edge"

Dear Bruce:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,

Laurent Blanchoin
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Goode:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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We are pleased that you chose to publish your work in MBoC.

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Eric Baker
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