Actin waves transport RanGTP to the neurite tip to regulate non-centrosomal microtubules in neurons

Yung-An Huang, Chih-Hsuan Hsu, Ho-Chieh Chiu, Pei-Yu Hsi, Chris T. Ho, Wei-Lun Lo and Eric Hwang

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MS TITLE: Actin waves transport RanGTP to the neurite tip to regulate non-centrosomal microtubule nucleation in neurons

AUTHORS: Yung-An Huang, Chih-Hsuan Hsu, Ho-Chieh Chiu, Chris Ho, Wei-Lun Lo, and Eric Hwang
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers are positive but still raise a number of substantial criticisms that prevent me from accepting the paper at this stage. Reviewer 1 also feels that the title of your paper is not currently supported by the data. They suggest, however, that a revised version might prove acceptable, if you can address their concerns including providing additional controls and clarifications. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please 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. Please attend to all of the reviewers' comments. 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

Could be a nice story if the authors were more careful with their interpretation of the data

Comments for the author

In this manuscript the authors demonstrate that RanGTP accumulates near axon tips and can influence the number of microtubule plus ends in axons. They also show that actin waves are involved in its distribution. As part of the story they generate a really nice tool (RanTRAP) to locally release Ran from sequestration on mitochondria with light. While the data included is nice, and the approach is very fun, the authors do not present any evidence that the increase in comet number is due to nucleation as claimed in the title and in the text and figures. Comet number is called a nucleation assay, but reports on number of plus ends, which can be regulated in different ways including nucleation, but also including severing. They do not present any data to show that the increase in comet number relies on nucleation.

Additional points
1. In introduction they refer to “inert minus end”
It is becoming increasingly clear that while not as dynamic as the plus end, minus ends are also highly regulated and do exhibit dynamics

2. Although the Anti-RanGTP antibody has been previously used, with the increasing emphasis on reproducibility and rigor it would be helpful to include controls, for example loss of staining when Ran is knocked down.

3. Does the RanGTP Ab recognize the Q69L mutant? Figure S1 Based on the images it looks like there are overall different levels of RanGTP, not just changes at the tip as described in the text and in the graph The description of S1 is somewhat misleading: “Interestingly, expressing RanQ69L or RanT24N specifically alters RanGTP level at the neurite tip” It sounds like the Q69L and RanT24N have similar effects when in fact they are opposite.

4. The Title for figure 2 is “Ran mutants affect microtubule nucleation at the neurite tip.” The data in this figure is EB comet number. While EB comet number can be influenced by nucleation it is equally likely to be influenced by changes in microtubule severing. Thus the data in this figure does not necessarily report on nucleation. It is important to be careful not to confound nucleation and comet number throughout. In figure 3 number of comets is also labeled as nucleation frequency. Could comets be increased by Ran activating a severing protein? Comet number should not be called a nucleation assay anywhere.

5. The rationale for the RanTRAP system is to avoid altering nuclear import and export, which is important as this could lead to cell stress, which could in turn lead to changes in microtubule dynamics. Is the effect on nuclear import ever assessed in neurons?

Reviewer 2

Advance summary and potential significance to field

In this paper, Huang et al., as an extension of their previous work (Chen et al, 2017), show that RanGTP plays an important role in non-centrosomal microtubule nucleation in neurons. In addition, they analyze possible involvement of actin wave in RanGTP transport to neurite tips. As they propose a new interaction between actin cytoskeleton and microtubules in neurons, this paper provide a potentially interesting concept in the field.

Comments for the author

In this paper, Huang et al., as an extension of their previous work (Chen et al, 2017), show that RanGTP plays an important role in non-centrosomal microtubule nucleation in neurons. In addition,

they analyze possible involvement of actin wave in RanGTP transport to neurite tips. As they propose a new interaction between actin cytoskeleton and microtubules in neurons, this paper provide a potentially interesting concept in the field. However, as listed below, I have a number of concerns about their data, discussions and conclusions; I hope my comments are helpful to improve this manuscript.

Major points:
1) Fig. 1B, 4C and S1B: The authors quantify the signal of RanGTP along neurites and conclude that it is enriched in axon and dendrite tips. However it is difficult to conclude from their data that RanGTP is enriched in neurite tips. Distal part of neurites including growth cone is thicker than neurite shaft. So, the apparent increase in the RanGTP signal in the distal neurites may be due to the volume effect. The authors should use a volume marker, for example mRRFP or CMFDA, that localizes diffusely in the cytoplasm, to quantify the thickness of the neurites. The relative concentration, RanGTP/CMFDA, should be calculated. In this context, it is also difficult to conclude that dendrite tips exhibit higher levels of RanGTP compared to axons.

2) Fig. 5A-H: Similarly, without the data of relative concentration, not just RanGTP signal, it is difficult to conclude that RanGTP is concentrated in actin wave and indeed transported with actin wave. Perform “double imaging” with a volume marker and calculate its relative concentration.

3) Fig. 6: The authors use 2.5 μM cytochalasin D to block the propagation of actin waves. Then, based on the reduced microtubule nucleation at the neurite tip, they conclude that ncMT nucleation at the neurite tip is influenced by actin waves. However, 2.5 μM cytochalasin D disrupts not only actin waves but also actin filaments in growth cones (Fig. 4B). In addition, RanGTP appears to co-localize with F-actin in growth cones (Fig. 4A), and the authors discuss a possible interaction of RanGTP with an F-actin binding protein ezrin. Thus, the reduced microtubule nucleation at the neurite tip may be due to a reduced RanGTP level directly caused by the cytochalasin D-induced disruption of F-actin in growth cones. Thus, I feel the authors’ conclusion is too strong. The authors need to discuss their model carefully based on their data. One supportive data for their model would be that actin waves translocate actin and F-actin binding proteins to neurite tips (Flynn, 2009: Katsuno et al, 2015): actin wave contributes to the maintenance of F-actin in growth cones which may be important for the localization of RanGTP in neurite tips.

4) Fig. 6A: The authors conclude that cytochalasin D treatment does not affect MT nucleation within the soma, without quantified data. Please provide the quantitative data to demonstrate it.

5) In Fig. S4A and S4B, it is difficult to see the colocalization of the two signals; enlarged views of the images to demonstrate the colocalization are required.

6) Fig. S6: Provide the quantitative data of the signal.

7) Discussion: As a possible mechanism for RanGTP transport by actin wave authors discuss that ezrin-L1CAM-RanBP9 complex may act as the adaptor for the transport. A previous paper (Katsuno et al, 2015) explains how actin binding proteins, like ezrin, are transported by actin wave.

8) Fig. 5F-H: Indicate the unit of the horizontal values.

Minor points:
1) “Discussion” should be placed before “Materials and Methods”.

2) “Introduction”: as “actin wave” is a key structure analyzed in this paper describe and explain actin wave in “Introduction”.

3) Fig. 4C, 5, 6 and S1B: neurite tip means axon + dendrite?

First revision

Author response to reviewers' comments

Dear Reviewers,

We thank you for critically examining our manuscript and providing us with valuable suggestions. This manuscript has been revised to address all your comments and concerns. The revised text in the manuscript has been highlighted and underlined. We believe all these revisions have greatly improved this manuscript. All revised figures from the new manuscript are shown in this response letter. Here we present the list of your comments and our responses.

Sincerely Yours,

Eric Hwang
Associate Professor
Department of Biological Science and Technology
National Chiao Tung University

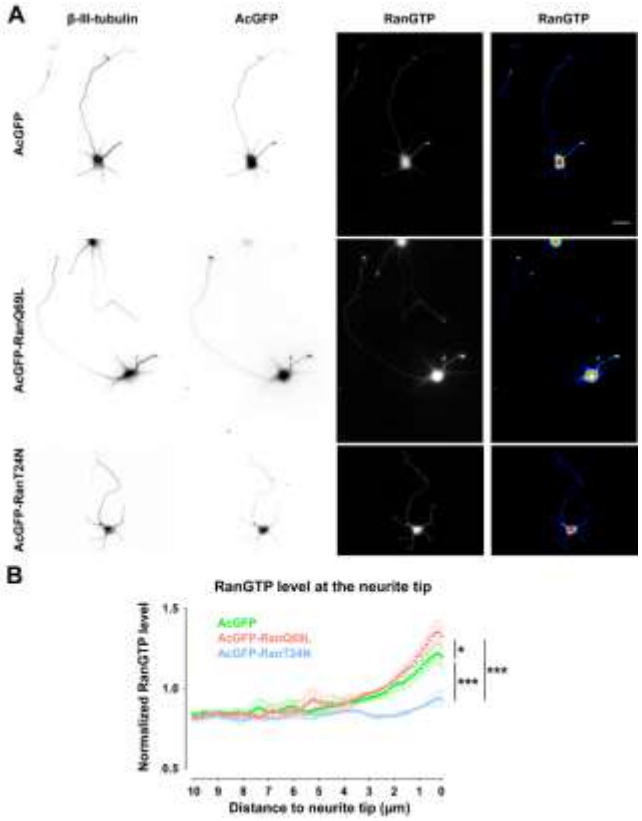
Reviewer 1:

1. In introduction they refer to "inert minus end". It is becoming increasingly clear that while not as dynamic as the plus end, minus ends are also highly regulated and do exhibit dynamics.
Reply: We thank the reviewer for pointing this out and have removed the word "inert" from the sentence. The revised text is shown below:
The ordered assembly of α/ß-tubulin heterodimers gives MTs two distinct ends: a plus-end where rapid polymerization and depolymerization occur, and a minus-end where nucleation event happens.

2. Although the anti-RanGTP antibody has been previously used, with the increasing emphasis on reproducibility and rigor it would be helpful to include controls, for example loss of staining when Ran is knocked down.
Reply: We thank the reviewer for the suggestion to include Ran knockdown as a way to validate the RanGTP (or AR-12) antibody. However, knocking down Ran has been shown to cause drastic changes in neuronal morphology (i.e. excessive neurite branching and neurite blebbing) (Sepp *et al.*, 2008). These morphological changes make reliable quantification of RanGTP at the neurite tip extremely difficult because excessive neurite branching caused neurite tips to intertwine with each other and the neurite blebbing led to the confusion between the neurite shaft and the neurite tip. Instead, we quantified RanGTP at neurite tips when RanGTP-mimic (RanQ69L) or RanGDP-mimic (RanT24N) mutant was expressed in neurons. We believe using RanQ69L or RanT24N to alter the level of RanGTP in neurons is more specific than globally depleting the Ran protein. In addition, expressing these mutants do not have such a drastic effect on neuronal morphology and hence making RanGTP quantification more reliable. As shown in figure S1, expressing RanGTP-mimic (RanQ69L) increases the level of RanGTP at the neurite tips while expressing RanGDP-mimic (RanT24N) decreases it. These data give us the confidence that AR-12 antibody is specific for RanGTP.

3. Does the RanGTP Ab recognize the Q69L mutant? Figure S1
Based on the images it looks like there are overall different levels of RanGTP, not just changes at the tip as described in the text and in the graph.
The description of S1 is somewhat misleading: "Interestingly, expressing RanQ69L or RanT24N specifically alters RanGTP level at the neurite tip" It sounds like the Q69L and RanT24N have similar effects when in fact they are opposite.
Reply: Yes, the RanGTP antibody (AR-12) recognizes the Q69L mutant. This is because AR-12 recognizes a 12-amino acid long peptide (QYEHDLEVAQTT) very close to the C-terminus of the human Ran protein. The C-terminal tail of Ran is only accessible to the antibody in the GTP-bound state (Richards *et al.*, 1995). Based on our quantification, expressing Q69L in neurons led to an increase of AR-12 signal at the neurite tip but not along the neurite

shaft. We can see that the image originally selected in figure S1A gave the impression that expressing Q69L increase RanGTP level along the neurite shaft. To avoid confusing the readers, we selected another image for Q69L in figure S1 to better match our description in the main text. The revised figure S1 is shown below.
As to the description of S1 which the reviewer considered misleading, we have also revised it to be more precise. Here is the revised text: Interestingly, expressing RanQ69L or RanT24N specifically increases or decreases RanGTP level at the neurite tip.



4. The Title for figure 2 is "Ran mutants affect microtubule nucleation at the neurite tip." The data in this figure is EB comet number. While EB comet number can be influenced by nucleation it is equally likely to be influenced by changes in microtubule severing. Thus the data in this figure does not necessarily report on nucleation. It is important to be careful not to confound nucleation and comet number throughout. In figure 3 number of comets is also labeled as nucleation frequency. Could comets be increased by Ran activating a severing protein? Comet number should not be called a nucleation assay anywhere.
Reply: We thank the reviewer for bringing up this important point. Indeed, an increase of EB comet number can be caused by *de novo* microtubule nucleation, polymerization from stable microtubules, or rescue of shrinking microtubules (the latter two situations can be brought about by microtubule severing proteins). The reason we concluded that the increase of EB3 comets is due to microtubule nucleation is because of our previous publication (Chen *et al.*, 2017). In this publication, we found that RanGTP activates TPX2 to produce more growing microtubule plus-ends in neurons. Given that TPX2 is a well-documented microtubule nucleating protein (Petry *et al.*, 2013; Schatz *et al.*, 2003), we concluded that RanGTP promotes microtubule nucleation in neurons. However, we totally understand the reviewer's concern. Therefore, we have revised the title of figure 2, 3, 6 from "microtubule nucleation" to "the formation of growing microtubule plus-ends" so that the title can be directly concluded from our data. Furthermore, we changed "MT nucleation" to "MT plus-end growth" or "growing MT plus-ends" throughout the text. Finally, we changed the title of our manuscript to "Actin waves transport RanGTP to the neurite tip to regulate non-centrosomal microtubules in neurons".

5. The rationale for the RanTRAP system is to avoid altering nuclear import and export, which is important as this could lead to cell stress, which could in turn lead to changes in microtubule dynamics. Is the effect on nuclear import ever assessed in neurons?
Reply: We have not examined the effect of RanTRAP on nuclear import in neurons. This is an interesting question that we can address in the future. One would imagine releasing RanGTP close to the nucleus would disrupt NLS cargo-importin interaction and compromise nuclear import. If releasing RanGTP near the nucleus does impair nuclear import, it will be particularly interesting to determine the minimal distance from the nucleus at which RanGTP release can be tolerated.

Reviewer 2

Major points:

1. Fig. 1B, 4C and S1B: The authors quantify the signal of RanGTP along neurites and conclude that it is enriched in axon and dendrite tips. However, it is difficult to conclude from their data that RanGTP is enriched in neurite tips. Distal part of neurites including growth cone is thicker than neurite shaft. So, the apparent increase in the RanGTP signal in the distal neurites may be due to the volume effect. The authors should use a volume marker, for example mRFP or CMFDA, that localizes diffusely in the cytoplasm, to quantify the thickness of the neurites. The relative concentration, RanGTP/CMFDA, should be calculated. In this context, it is also difficult to conclude that dendrite tips exhibit higher levels of RanGTP compared to axons.
Reply: The reviewer pointed out an important control to help us conclude that RanGTP is enriched at axon or dendrite tips. To address the reviewer's concern, we transfected a plasmid expressing cytosolic EGFP into dissociated hippocampal neurons and quantified the RanGTP signal by normalizing it using the EGFP signal. Consistent with our previous result, the signal of RanGTP is significantly increased at both axon and dendrite tips. Interestingly, we discovered that the volume-corrected RanGTP level is significantly higher at axon tips than dendrite tips. We have revised the Results section, Material and Methods section, and figure legend of our manuscript accordingly. The revised figure 1 and legend are shown below:

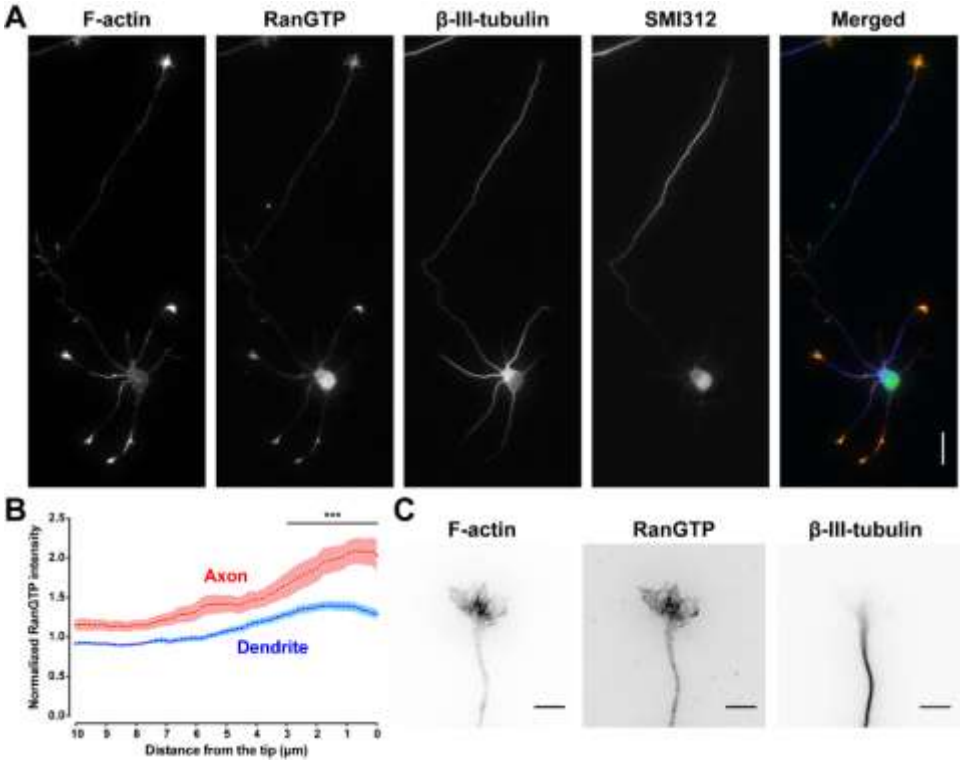


Figure 1. GTP-bound Ran is enriched at both axon and dendrite tips, and colocalized with actin-based structures.
(A) Representative images of a 2DIV hippocampal neuron immunofluorescence stained with RanGTP, ß-III-tubulin, SMI312 antibodies, and phalloidin. The merged image shows phalloidin staining in red, RanGTP in green, and ß-III-tubulin in blue. The scale bar represents 25 µm. (B) Cytoplasmic volume-normalized RanGTP intensity linescan along a 10 µm stretch from axon (red) or dendrite (blue) tips in 2DIV hippocampal neurons. Dots and shaded areas indicate mean and SEM collected from 57 axons and 194 dendrites, *** $p<0.001$, two-way ANOVA followed by Sidak post-hoc analysis. (C) Representative image of the growth cone from a 2DIV hippocampal neuron fixed and stained with RanGTP (middle), ß-III-tubulin (right) antibody, and phalloidin (left). Images were inverted to facilitate visualization. Scale bars represent 10 µm.

2. Fig. 5A-H: Similarly, without the data of relative concentration, not just RanGTP signal, it is difficult to conclude that RanGTP is concentrated in actin wave and indeed transported with actin wave. Perform "double imaging" with a volume marker and calculate its relative concentration.
Reply: We understand the reviewer's concern about the volume effect on RanGTP concentration in the actin wave, and this is the reason why we performed the experiment shown in Fig 5H~J. We reasoned that if the increase of AcGFP-RanQ69L (RanGTP-mimic) signal in actin waves was caused by the increase of the cytoplasmic volume, the signal of cytosolic AcGFP will also show an increase in the actin wave indistinguishable from that of AcGFP-RanQ69L. Our result shows that the increase of AcGFP-RanQ69L signal is significantly higher than that of AcGFP (Fig 5J), arguing against the "volume effect" hypothesis.
We understand the reviewer's suggestion of performing double imaging with a volume marker and calculate RanGTP's relative concentration provides the most accurate assessment, and we did try this approach. However, co-expressing AcGFP-RanQ69L (or AcGFP-RanT24N) and cytosolic mCherry (or tdTomato) in dissociated neurons severely reduces the signal from AcGFP-Ran, making reliable quantification of AcGFP-RanQ69L (or AcGFP-RanT24N) signal infeasible. We hope the reviewer can understand our difficulty and accept our alternative approach to address this cytoplasmic volume issue.

3. Fig. 6: The authors use 2.5 µM cytochalasin D to block the propagation of actin waves. Then, based on the reduced microtubule nucleation at the neurite tip, they conclude that ncMT nucleation at the neurite tip is influenced by actin waves. However, 2.5 µM cytochalasin D disrupts not only actin waves but also actin filaments in growth cones (Fig. 4B). In addition, RanGTP appears to co-localize with F-actin in growth cones (Fig. 4A), and the authors discuss a possible interaction of RanGTP with an F-actin binding protein ezrin. Thus, the reduced microtubule nucleation at the neurite tip may be due to a reduced RanGTP level directly caused by the cytochalasin D-induced disruption of F-actin in growth cones. Thus, I feel the authors' conclusion is too strong. The authors need to discuss their model carefully based on their data. One supportive data for their model would be that actin waves translocate actin and F-actin binding proteins to neurite tips (Flynn, 2009: Katsuno et al, 2015): actin wave contributes to the maintenance of F-actin in growth cones which may be important for the localization of RanGTP in neurite tips.
Reply: The reviewer points to the possibility that "the reduced microtubule nucleation at the neurite tip may be due to a reduced RanGTP level directly caused by the cytochalasin D (CytoD)-induced disruption of F-actin in growth cones". We understand the reviewer's view and revised the Results section to tone down our conclusion. The revised text is shown below:
<u>These findings show that ncMT nucleation at the neurite tip is influenced by the actin cytoskeleton. Given that both actin waves and actin-based structure in the growth cone are disrupted by cytochalasin D treatment, our data suggest that actin cytoskeleton is crucial for either the transportation or anchoring (or both processes) of RanGTP in neurons and provide a new indirect connection between these two cytoskeleton structures.</u>

4. Fig. 6A: The authors conclude that cytochalasin D treatment does not affect MT nucleation within the soma, without quantified data. Please provide the quantitative data to demonstrate it.
Reply: We have revised figure 6 to include the quantification of EB3 dynamics in the soma. The revised figure and legend are shown below. In addition, we included the method used to quantify EB3-mCherry comets in the Materials and Methods section. The revised text is shown here:
For EB3-mCherry comets analysis in the soma, ImageJ plugin TrackMate v5.1.0 was used (Tinevez et al., 2017). Soma region was manually selected, and Differences of Gaussian (DoG) detector model as well as Linear motion LAP tracker method were used to quantify EB3 dynamics. Only EB3-mCherry movement that could be tracked for equal or more than 4 frames (1.5 seconds) were included in the analysis.

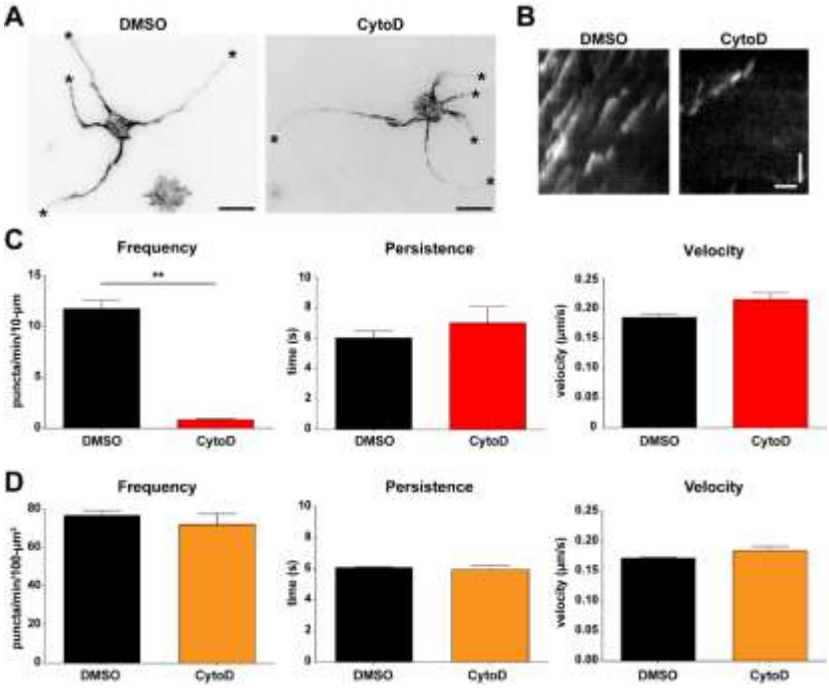


Figure 6. Disrupting actin waves reduces the formation of non-centrosomal microtubule plus-ends at the neurite tip.
(A) Representative maximum projection images (over 2 minute period) of 1DIV EB3-mCherry-expressing hippocampal neurons treated with DMSO (left) or 2.5 µM cytochalasin D (right) for 6 hours. Hippocampal neurons were transfected with plasmids expressing EB3-mCherry and EGFP immediately before plating, incubated for 18 hours, and treated with DMSO or 2.5 µM cytochalasin D for 6 hours before subjected to live cell imaging. Asterisks mark the tips of the neurites, and the scale bars represent 20 µm. (B) Representative kymographs of EB3-mCherry at the neurite tip in DMSO- (left) or 2.5 µM cytochalasin D-treated (right) neurons. The vertical scale bar in the kymograph represents 2 µm and horizontal scale bar represents 10 seconds. (C-D) Quantification of EB3-mCherry dynamics at the neurite tip (C) or in the soma (D) in DMSO- or 2.5 µM cytochalasin D-treated neurons. ** $p<0.01$, two-tailed Student's *t*-test. Error bars represent SEM from 3 independent experiments, with more than 45 neurites or somata analyzed for each condition.

5. In Fig. S4A and S4B, it is difficult to see the colocalization of the two signals; enlarged views of the images to demonstrate the colocalization are required.
Reply: We have revised figure S4 to include enlarged view of the colocalization. The revised figure and legend are shown below.

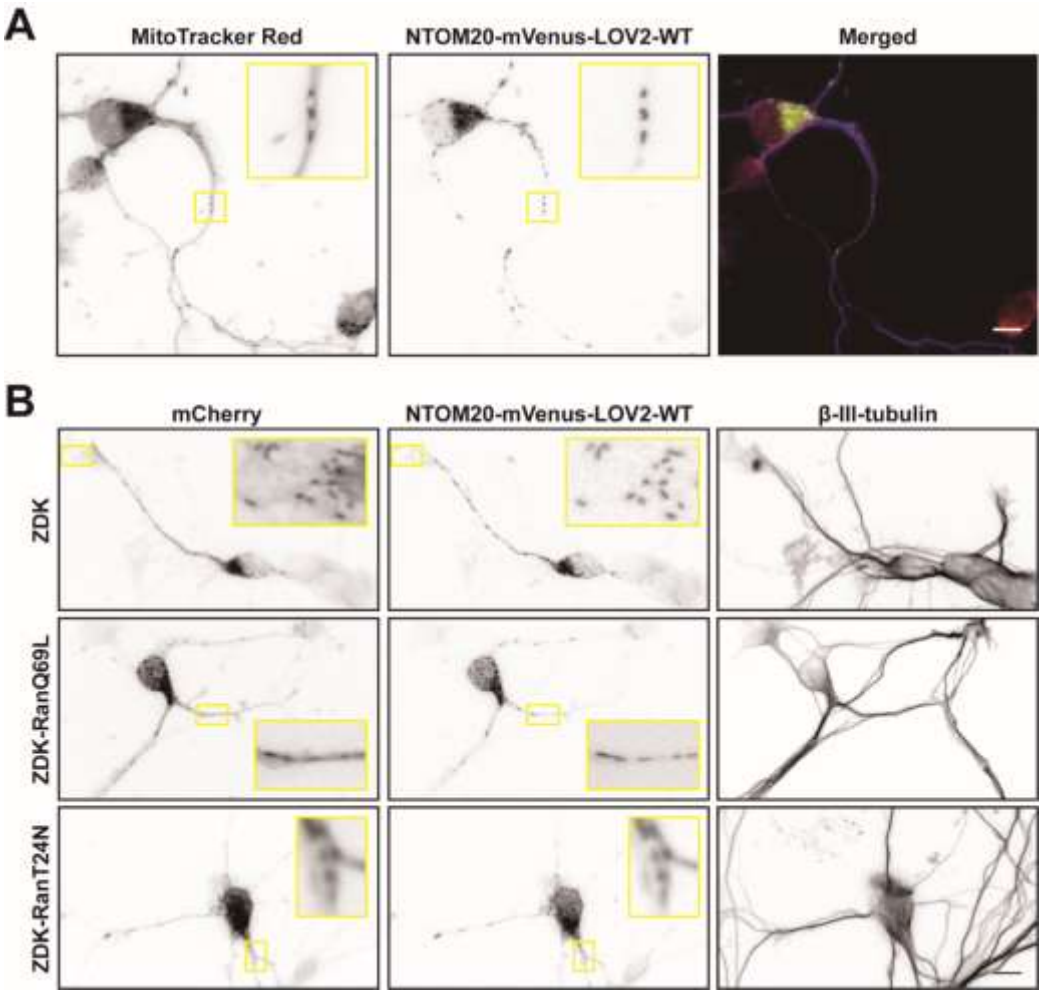


Figure S4. The RanTRAP system targets Ran to mitochondria along the neurite.
(A) Representative images of a mouse cortical neurons transfected with plasmid expressing NTOM20-mVenus-LOV2-WT (green in merged) at 2DIV, fixed and stained with MitoTracker Red (red in merged) and β-III-tubulin antibody (blue in merged) at 4DIV. (B) Representative images of mouse cortical neurons transfected with plasmids expressing NTOM20-mVenus-LOV2-WT and mCherry-ZDK (or mCherry-ZDK-RanQ69L or mCherry-RanT24N) at 2DIV, fixed and stained with the β-III-tubulin antibody at 4DIV. Insets show the magnified view of yellow boxed areas. All scale bars represent 10 μm.

6. Fig. S6: Provide the quantitative data of the signal.
Reply: We have included the quantitative data in figure S6. The revised figure and legend are shown below.

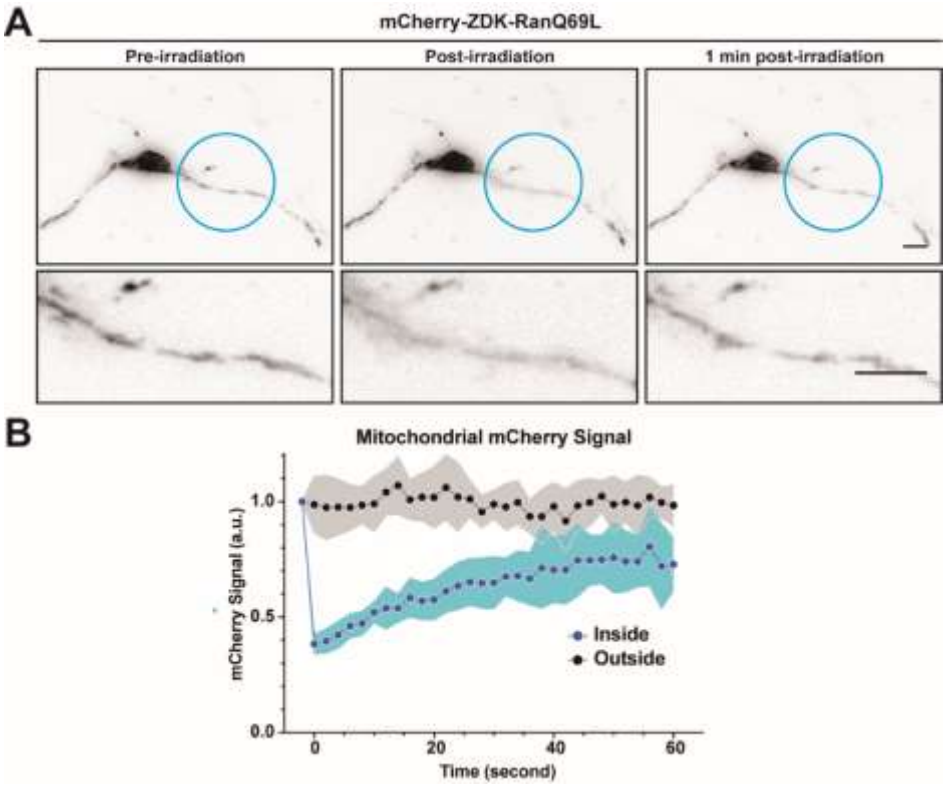


Figure S6. Local release of RanGTP-mimic mutant can be achieved in neurons.
(A) Mouse hippocampal neurons were co-transfected with plasmids expressing NTOM20-mVenus-LOV2-WT and mCherry-ZDK-RanQ69L at 3DIV, incubated for 1 days before subjected to live cell imaging. The blue circle indicated the region of photoactivation. Images on the bottom row show magnified images from the photoactivated region. The mCherry-ZDK-RanQ69L signal before (left), immediately after photoactivation (center), and 1 minute after photoactivation (right) are shown. All scale bars represent 10 μm. (B) Quantification of the mitochondria-localized mCherry-ZDK-RanQ69L signal over time from panel A. 4 selected ROIs on the mitochondria inside (blue line) and outside (black line) the region of photoactivation are analyzed. Dots and shaded areas indicate mean and SD. Note that mCherry-ZDK-RanQ69L only dissociated from the mitochondria inside the photoactivated region.

7. Discussion: As a possible mechanism for RanGTP transport by actin wave, authors discuss that ezrin-L1CAM-RanBP9 complex may act as the adaptor for the transport. A previous paper (Katsuno et al, 2015) explains how actin binding proteins, like ezrin, are transported by actin wave.
Reply: We have included this paper in our revised Introduction section. The added paragraph is shown below:
Neuronal actin waves (or growth cone-like waves) are actin-dependent anterograde movement along the neurite shaft that was originally discovered in cultured hippocampal neurons (Ruthel and Banker, 1998). Actin waves were later observed in organotypic hippocampal or cortical slices (Flynn et al., 2009; Katsuno et al., 2015), demonstrating that they are present both in vitro and in vivo. The neurite undergoes transient retraction when an actin wave approaches its tip, this is followed by a short period of rapid outgrowth as the actin wave reaches the tip (Ruthel and Banker, 1999). It has recently been demonstrated that the anterograde movement of the actin wave is powered by the directional polymerization (oriented toward the tip) and depolymerization (oriented toward the cell body) of the membrane-associated actin filaments (Katsuno et al., 2015). This kind of propagation mechanism allows proteins associated with the actin filaments to be transported within actin waves towards the neurite tip, as a variety of actin-binding proteins, small GTPases, and PIP3 have been documented to co-migrate with or enrich in actin waves (Kakumoto and Nakata, 2013). Although several Ras superfamily GTPases

(Cdc42, Rac1, Rap1) have been demonstrated to concentrate in actin waves (Flynn et al., 2009), whether Ran GTPase can be transported by actin waves remains unknown.

8. Fig. 5F-H: Indicate the unit of the horizontal values.
We did mention the unit of the horizontal scale bars in the figure legend. "(F-H) Time-lapse DIC (top) and AcGFP (middle) images, as well as the AcGFP intensity linescan (bottom) of a single neurite from neurons expressing AcGFP-RanQ69L (F), AcGFP-RanT24N (G), or AcGFP (H). Neurite segments in (F), (G), (H) are derived from the asterisked neurite in (C), (D), (E), respectively. The white arrowheads mark the location of the actin wave. The time stamps (hour:min) indicate the time progressed since the first image. The gray shaded area in the linescan graphs indicates the location of the actin wave. The scale bars represent 10 μm." The scale bars can be found in the rightmost pseudo-colored images.

Minor points:
1. "Discussion" should be placed before "Materials and Methods".
Reply: We thank the reviewers for pointing this out and have rearranged the order accordingly.

2. "Introduction": as "actin wave" is a key structure analyzed in this paper, describe and explain actin wave in "Introduction".
Reply: We have included a paragraph describing the actin wave of neurons in our revised the Introduction section. The added paragraph is shown below:
Neuronal actin waves (or growth cone-like waves) are actin-dependent anterograde movement along the neurite shaft that was originally discovered in cultured hippocampal neurons (Ruthel and Banker, 1998). Actin waves were later observed in organotypic hippocampal or cortical slices (Flynn et al., 2009; Katsuno et al., 2015), demonstrating that they are present both in vitro and in vivo. The neurite undergoes transient retraction when an actin wave approaches its tip, this is followed by a short period of rapid outgrowth as the actin wave reaches the tip (Ruthel and Banker, 1999). It has recently been demonstrated that the anterograde movement of the actin wave is powered by the directional polymerization (oriented toward the tip) and depolymerization (oriented toward the cell body) of the membrane-associated actin filaments (Katsuno et al., 2015). This kind of propagation mechanism allows proteins associated with the actin filaments to be transported within actin waves towards the neurite tip, as a variety of actin-binding proteins, small GTPases, and PIP3 have been documented to co-migrate with or enrich in actin waves (Kakumoto and Nakata, 2013). Although several Ras superfamily GTPases (Cdc42, Rac1, Rap1) have been demonstrated to concentrate in actin waves (Flynn et al., 2009), whether Ran GTPase can be transported by actin waves remains unknown.

3. Fig. 4C, 5, 6 and S1B: neurite tip means axon + dendrite?
Reply: Neurite tip does not mean axon or dendrite tip. The reason we chose neurite tip instead of axon or dendrite tip is because axon specification has not occurred at such an early stage (1~2 days *in vitro*). We felt that it is more accurate to refer to these neuronal protrusions as neurites.

Second decision letter

MS ID#: JOCES/2019/241992

MS TITLE: Actin waves transport RanGTP to the neurite tip to regulate non-centrosomal microtubules in neurons

AUTHORS: Yung-An Huang, Chih-Hsuan Hsu, Ho-Chieh Chiu, Pei-Yu Hsi, Chris Ho, Wei-Lun Lo, and Eric Hwang

ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but still raised a few points that will require amendments to your manuscript. In particular, as both reviewers indicate, I think its essential that all descriptions in the text match the data and assays, in addition to providing the controls requested by reviewer 2. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please 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. Please attend to all of the reviewers' comments. 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

As before this is an interesting study that is improved, but still needs the language corrected in places

Comments for the author

The authors have made substantial improvements to both the quantitation and writing. However, they still conclude that they are looking at nucleation in some places in the manuscript in a way that oversteps the data. For example at the end of the introduction they say that their results inform on nucleation at axon tips. Similarly comet number is still used as an assay for nucleation as on p 4 “To examine whether RanGTP regulates ncMT nucleation, Ran mutants were utilized to alter the level of cytoplasmic RanGTP in neurons.”

At the top of page 5 they describe the EB comet assay as “MT formation.” This is also inaccurate. What they are assaying is formation of a new growing end, but as it could be generated by catastrophe rescue it does not necessarily represent formation of a new microtubule, which is the implication in the current wording. Similarly in the following sentence they say they are using EB3-GFP to monitor “formation of MTs.” It is essential to have the text descriptions match the assays used throughout.

Reviewer 2

Advance summary and potential significance to field

In the revised version, Huang et al., improved the manuscript addressing the comments of the reviewers. However, as listed below, some of their answers are not yet satisfactory, and I still have concerns about their data and text. I hope the authors address my comments thoroughly and improve this paper.

Comments for the author

In the revised version, Huang et al., improved the manuscript addressing the comments of the reviewers. However, as listed below, some of their answers are not yet satisfactory, and I still have

concerns about their data and text. I hope the authors address my comments thoroughly and improve this paper.

Major points:
1) Reply 1 of the authors to my comment: The reviewer pointed out an important control to help us conclude that RanGTP is enriched at axon or dendrite tips. To address the reviewer's concern, we transfected a plasmid expressing cytosolic EGFP into dissociated hippocampal neurons and quantified the RanGTP signal by normalizing it using the EGFP signal. Consistent with our previous result, the signal of RanGTP is significantly increased at both axon and dendrite tips. Interestingly, we discovered that the volume-corrected RanGTP level is significantly higher at axon tips than dendrite tips. We have revised the Results section, Material and Methods section, and figure legend of our manuscript accordingly. The revised figure 1 and legend are shown below:

Major comment 1: Although the authors provided the quantified profile of [RanGTP signal/EGFP signal] (Figure 1B), they do not show the picture of the neurons exhibiting the signals of RanGTP and EGFP. Please show the data of both signals together with the merged image.

2) Reply 2 of the authors to my comment: We understand the reviewer's suggestion of performing double imaging with a volume marker and calculate RanGTP's relative concentration provides the most accurate assessment, and we did try this approach. However, co-expressing AcGFP-RanQ69L (or AcGFP-RanT24N) and cytosolic mCherry (or tdTomato) in dissociated neurons severely reduces the signal from AcGFP-Ran, making reliable quantification of AcGFP-RanQ69L (or AcGFP-RanT24N) signal infeasible. We hope the reviewer can understand our difficulty and accept our alternative approach to address this cytoplasmic volume issue.

Major comment 2: I understood that "double live imaging" of AcGFP-RanQ69L and mCherry is not easy. Then, please perform double staining of fixed neurons with RanGTP antibody and EGFP to calculate relative RanGTP concentration. Show the data of both signals in actin waves together with the merged image. Provide the profile of relative RanGTP concentration along axon including actin wave, as in the case of Figure 1B. These data would demonstrate that RanGTP is indeed concentrated in actin waves.

3) Reply 6 of the authors to my comment: We have included the quantitative data in figure S6. The revised figure and legend are shown below.

Major comment 3: It is difficult to identify where are the mitochondria in Figure S6A (enlarged lower panels). Indicate where, in the enlarged panels, the authors quantified the signals of mitochondria inside and outside.

Minor points:
Reply 8 of the authors to my comment: We did mention the unit of the horizontal scale bars in the figure legend. "(F-H) Time-lapse DIC (top) and AcGFP (middle) images, as well as the AcGFP intensity linescan (bottom) of a single neurite from neurons expressing AcGFP-RanQ69L (F), AcGFP-RanT24N (G), or AcGFP (H). Neurite segments in (F), (G), (H) are derived from the asterisked neurite in (C), (D), (E), respectively. The white arrowheads mark the location of the actin wave. The time stamps (hour:min) indicate the time progressed since the first image. The gray shaded area in the linescan graphs indicates the location of the actin wave. The scale bars represent 10 μm." The scale bars can be found in the rightmost pseudo-colored images.

Minor comment 1: I asked to indicate the unit of the horizontal values of the graphs in Figure 5F-H, not the length of the scale bars. What do "0", "10", "20", "5" and "15' of the graphs mean?

Minor comment 2: Correct "□"s in the legends for Figures 4 and 5.

Second revision

Author response to reviewers' comments

Reviewer 1:

The authors have made substantial improvements to both the quantitation and writing. However, they still conclude that they are looking at nucleation in some places in the manuscript in a way that oversteps the data. For example at the end of the introduction they say that their results inform on nucleation at axon tips. Similarly comet number is still used as an assay for nucleation as on p 4 “To examine whether RanGTP regulates ncMT nucleation, Ran mutants were utilized to alter the level of cytoplasmic RanGTP in neurons.”
At the top of page 5 they describe the EB comet assay as “MT formation.” This is also inaccurate. What they are assaying is formation of a new growing end, but as it could be generated by catastrophe rescue it does not necessarily represent formation of a new microtubule, which is the implication in the current wording. Similarly in the following sentence they say they are using EB3-GFP to monitor “formation of MTs.” It is essential to have the text descriptions match the assays used throughout.
Reply:
1) We revised the end of the introduction to the following statement. These observations confirm the role of Ran GTPase in regulating ncMTs and identify a novel mechanism of moving the active RanGTP molecules towards the neurite tip.
2) We revised page 4 to the following statement. To examine whether RanGTP regulates ncMTs, Ran mutants were utilized to alter the level of cytoplasmic RanGTP in neurons.
3) We revised page 5 regarding the description of the EB comet assay to the following statement. We next examined the formation of growing MT plus-ends at the neurite tip in neurons expressing these Ran mutants. The CNS-enriched MT plus-end tracking protein EB3 was used to assess the amount of growing MT plus-ends (Nakagawa et al., 2000).

Reviewer 2:

Major comment 1: Although the authors provided the quantified profile of [RanGTP signal/EGFP signal] (Figure 1B), they do not show the picture of the neurons exhibiting the signals of RanGTP and EGFP. Please show the data of both signals together with the merged image.
Reply: We added the image the reviewer requested to Figure 1 (panel B) and revised the figure legend as well as the main text accordingly. The revised figure and legend are shown below:

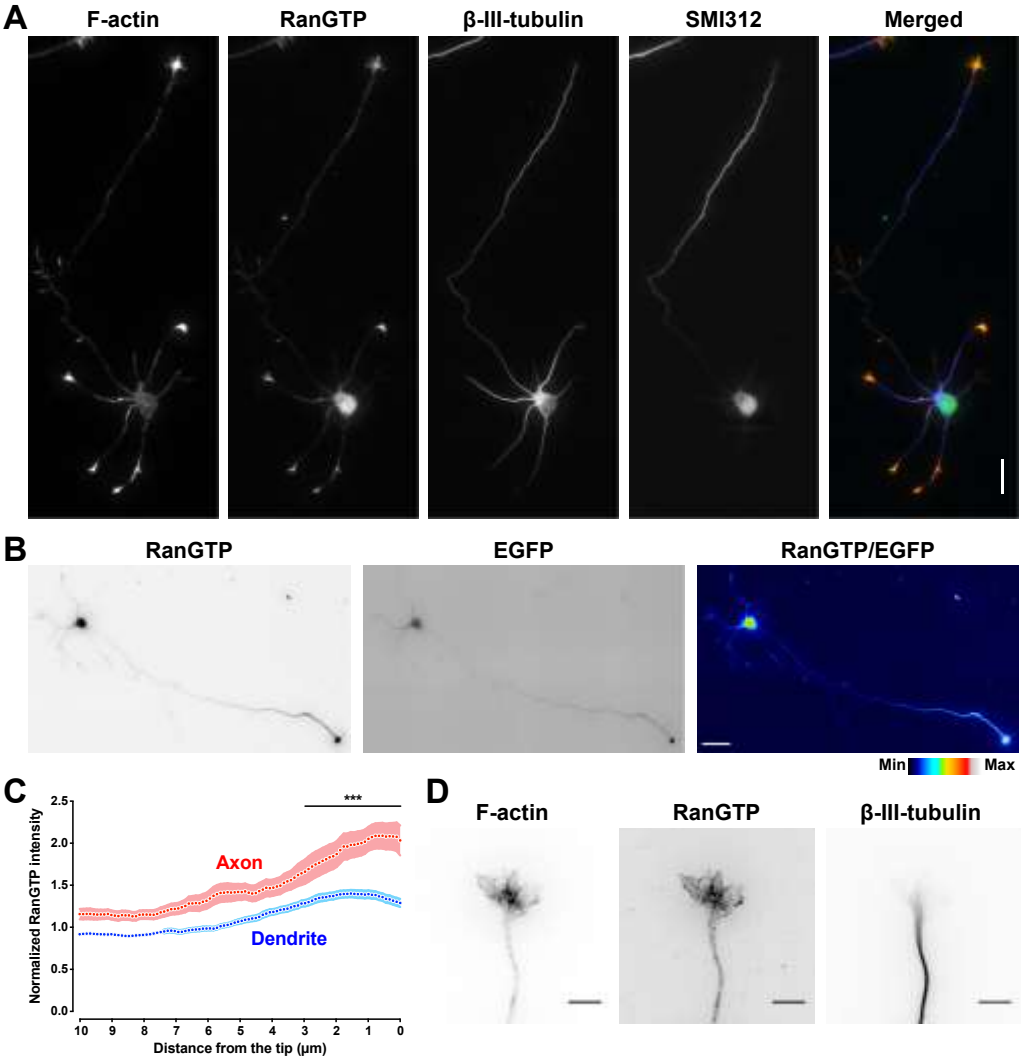


Figure 1. GTP-bound Ran is enriched at both axon and dendrite tips, and colocalized with actin-based structures.
(A) Representative images of a 2DIV hippocampal neuron immunofluorescence stained with RanGTP, ß-III-tubulin, SMI312 antibodies, and phalloidin. The merged image shows phalloidin staining in red, RanGTP in green, and ß-III-tubulin in blue. The scale bar represents 25 μm. (B) Representative images of a 2DIV hippocampal neuron expressing cytosolic EGFP immunofluorescence stained with RanGTP antibody. The ratio image is pseudo-colored and the scale bar represents 30 μm. (C) Cytoplasmic volume-normalized RanGTP intensity linescan along a 10 μm stretch from axon (red) or dendrite (blue) tips in 2DIV hippocampal neurons. Dots and shaded areas indicate mean and SEM collected from 57 axons and 194 dendrites, *** p<0.001, two-way ANOVA followed by Sidak post-hoc analysis. (D) Representative image of the growth cone from a 2DIV hippocampal neuron fixed and stained with RanGTP (middle), ß-III-tubulin (right) antibody, and phalloidin (left). Images were inverted to facilitate visualization. Scale bars represent 10 μm.

Major comment 2: I understood that “double live imaging” of AcGFP-RanQ69L and mCherry is not easy. Then, please perform double staining of fixed neurons with RanGTP antibody and EGFP to calculate relative RanGTP concentration. Show the data of both signals in actin waves together with the merged image. Provide the profile of relative RanGTP concentration along axon including actin wave, as in the case of Figure 1B. These data would demonstrate that RanGTP is indeed concentrated in actin waves.
Reply: We added the quantification of RanGTP/EGFP ratio around the actin wave from fixed neurons in a new supplementary figure (Figure S6). Consistent with our previous data, RanGTP/EGFP ratio exhibits significant increase at the wave compared to the neurite region in front or behind the wave. In addition, comparing to neurite regions without an actin wave,

those that contain an actin wave also exhibit a significant increase of RanGTP/EGFP. We added a new supplementary figure (Figure S6) and revised the main text and Methods section accordingly. Figure S6 and legend are shown below:

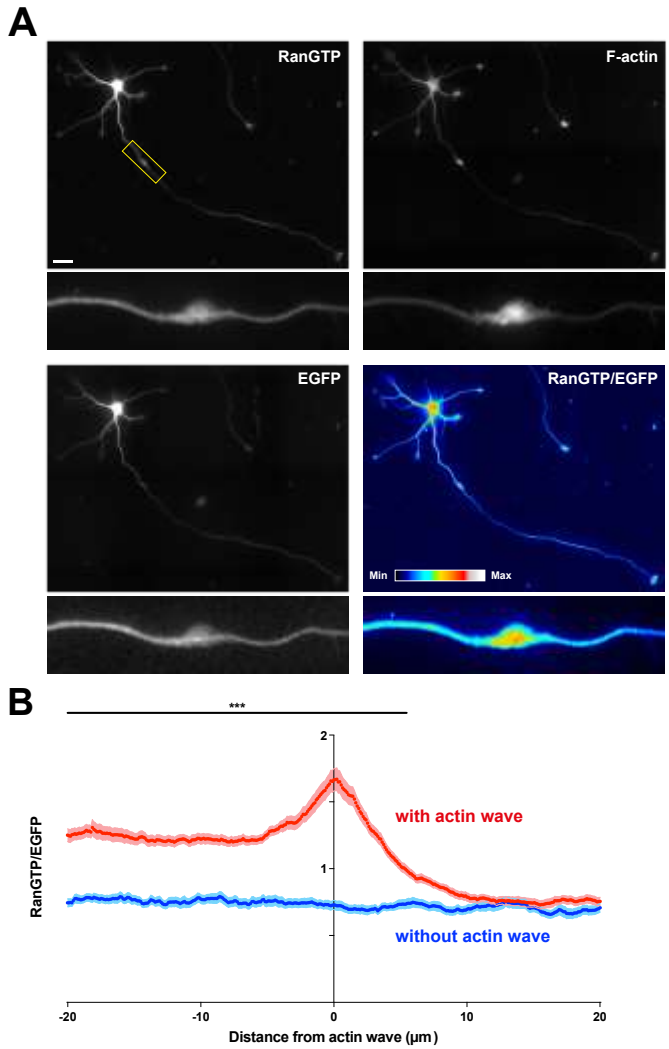


Figure S6. RanGTP level is elevated in the actin wave in neurons.
(A) Representative image of a 2DIV mouse hippocampal neurons expressing EGFP (bottom left) and immunofluorescence stained with antibody against RanGTP (top left) and phalloidin (top right). The ratio image of RanGTP/EGFP was pseudo-colored (bottom right). The boxed area was used to generated the magnified images. All images have the same scale and the scale bar represents 20 µm. (B) Cytoplasmic volume-normalized RanGTP intensity linescan along a 40 µm stretch centered at the actin wave (red) or a random location without the actin wave (blue) in 2DIV hippocampal neurons. The origin of the X-axis is selected using the neurite location with the highest phalloidin staining, negative or positive value of the X-axis indicate the neurite region towards or away from the soma. Dots and shaded areas indicate mean and SEM collected from 47 neurites, *** $p<0.001$, two-way ANOVA followed by Sidak post-hoc analysis between the red and the blue curves.

Major comment 3: It is difficult to identify where the mitochondria in Figure S6A are (enlarged lower panels). Indicate where, in the enlarged panels, the authors quantified the signals of mitochondria inside and outside.
Reply: We included the 4 ROIs from which the mitochondrial mCherry signal was quantified. The revised figure and legend are shown below. We want to bring to your attention that because J Cell Science does not allow more supplementary figures than main-text figures, we merged Figure S1 and S2 together. This causes the original Figure S6 to become Figure S5.

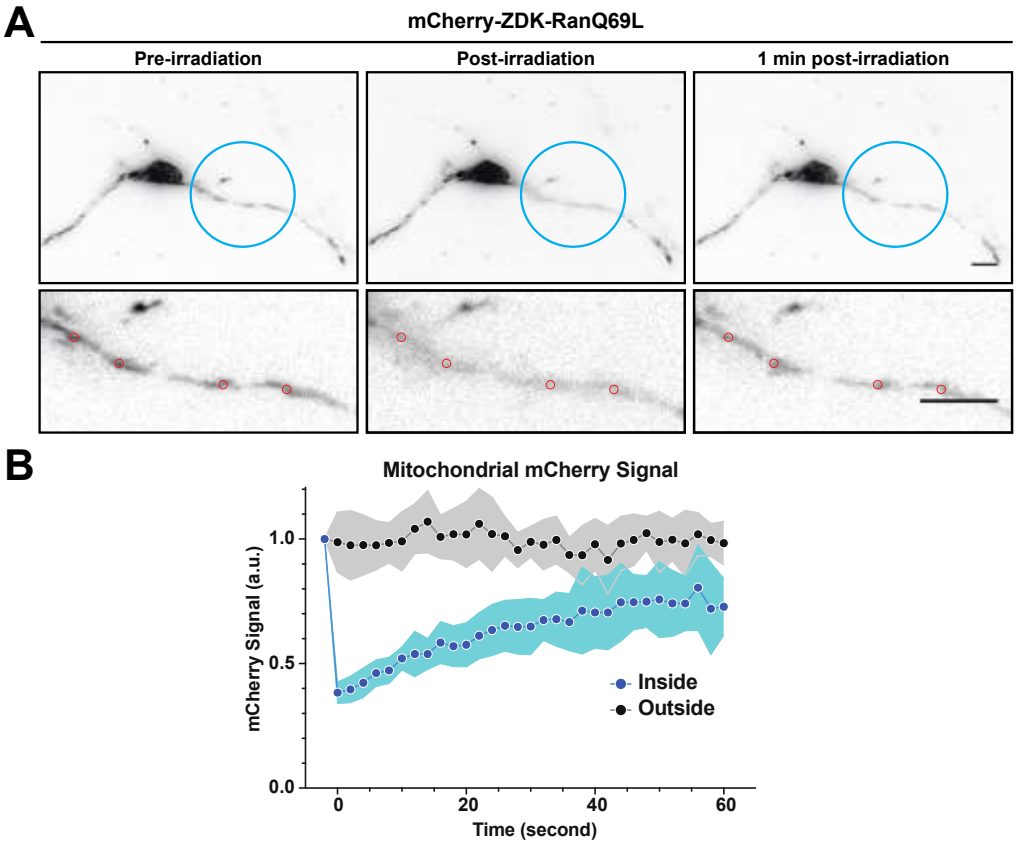


Figure S5. Local release of RanGTP-mimic mutant can be achieved in neurons.
(A) Mouse hippocampal neurons were co-transfected with plasmids expressing NTOM20- mVenus-LOV2-WT and mCherry-ZDK-RanQ69L at 3DIV, incubated for 1 days before subjected to live cell imaging. The blue circle indicated the region of photoactivation. Images on the bottom row show magnified images from the photoactivated region. The mCherry-ZDK-RanQ69L signal before (left), immediately after photoactivation (center), and 1 minute after photoactivation (right) are shown. All scale bars represent 10 μm. (B) Quantification of the mitochondria-localized mCherry-ZDK-RanQ69L signal over time from panel A. 4 selected ROIs (red circles in panel A) on the mitochondria inside (blue line) and outside (black line) the region of photoactivation are analyzed. Dots and shaded areas indicate mean and SD. Note that mCherry-ZDK-RanQ69L only dissociated from the mitochondria inside the photoactivated region.

Minor points:
Minor comment 1: I asked to indicate the unit of the horizontal values of the graphs in Figure 5F-H, not the length of the scale bars. What do “0”, “10”, “20”, “5” and “15’ of the graphs mean?
Reply: We thank the reviewer for clarify her/his initial question. We added the information in the figure legend. The X-axis of the linescan graphs indicates the distance (in μm) from the base of the neurite.

Minor comment 2: Correct “□”s in the legends for Figures 4 and 5.
Reply: We thank the reviewer for carefully reading through our manuscript and discovering these odd boxes “□”. However, we do not see them in the legends of Figure 4 or 5 in any of our files (.docx or .pdf). We will communicate with the editorial office to make sure they are removed during the proofing process.

Third decision letter

MS ID#: JOCES/2019/241992

MS TITLE: Actin waves transport RanGTP to the neurite tip to regulate non-centrosomal microtubules in neurons

AUTHORS: Yung-An Huang, Chih-Hsuan Hsu, Ho-Chieh Chiu, Pei-Yu Hsi, Chris Ho, Wei-Lun Lo, and Eric Hwang
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.