Actomyosin dynamics modulate microtubule deformation and growth during T cell activation

Ivan Rey-Suarez, Nate Rogers, Sarah Kerr, Hari Shroff, and Arpita Upadhyaya

Corresponding author(s): Arpita Upadhyaya, University of Maryland

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

RE: Manuscript #E20-10-0685
TITLE: Actomyosin dynamics modulate microtubule deformation and growth during T cell activation

Dear Prof. Upadhyaya:

Your manuscript, entitled " Actomyosin dynamics modulate microtubule deformation and growth during T cell activation" has been seen by two referees whose verbatim comments are enclosed. Both referees felt that your findings, in principle, would be of interest to our MBC readership. However, both reviewers raised some important points that need to be addressed. Most of these can be addressed by changes in the text. In particular, more detail is needed in the description of the data analysis and other section need revision for clarity. We look forward to receiving your revised manuscript and a letter indicating your response to the referees in the near future.

Sincerely,

Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Upadhyaya,

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, as described in the Monitoring Editor's decision letter above and the reviewer comments 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).

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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):

The manuscript "Actomyosin dynamics modulate microtubule deformation and growth during T cell activation" by Rey-Suarez and co-authors explores the crosstalk between microtubules and actin networks during T cell activation. They use high-resolution fluorescence microscopy and quantitative image analysis to study the spatial distribution of actin and the growth and dynamics of microtubules (MTs) in different regions of a reconstituted inmune synapse (IS).

The authors have found that the different actin architectures present in the IS modulate microtubule growth rates differently: the growing of MTs in the distal region is slower and less radially directed than MTs in the central or peripheral region. By analyzing the lateral fluctuations and deformations of individual MTs, they have found that MTs dynamics is affected by the crosstalk with the different actin structures, with peripheral actin associated with more bended and fluctuating MTs.

By selectively perturbing the actin network the authors have explored the contribution of Arp2/3 and formin to MTs behavior, showing that the disruption of actin architecture affects MTs dynamics. Furthermore, inhibition of myosin activity led to significantly less deformed and less dynamic MTs.

From these results the authors conclude the central role of the actomyosin cytoskeleton in consortium with the distinct actin regions in the modulation of MTs dynamics in the IS.

Overall, this is an interesting study which provides additional information on the interactions between cytoskeleton networks during T cell activation.

However there are some points related to the methodological aspects and the results presentation that need to be clarified or improved:

1. Explain the procedure followed to calculate the mean intensities represented in Figures 1E-G. Are these mean intensities an average of the intensities within each of the three regions of the profiles shown in Fig. 1 B? Do the data points represented in each boxplot come from the 90 cells referenced in Fig. 1B?

2. Please, add error bars to represent the dispersion of the averaged intensity profiles shown in Figure 1B. Is this profile the average of 50 lines from 90 cells? Was the variability in the intensity values between different cells and images taken into account?

3. Boxplots: include the data points in the figure (swarm boxplot) and/or add the information about the number of data points in the figure captions.

4. Statistical analysis: the Kruskal-Wallis test is preferred to the KS test to determine the statistical significance of 3 or more samples. When comparing 2-samples at a time, the authors should mention if they have considered the corrections necessary to compensate for the increase in the likelihood of incorrectly rejecting a null hypothesis when performing multiple comparisons (e.g. Bonferroni correction).

5. In Page 7: "Interestingly, the presence of VCAM-1 on the activating substrate induced a significant increase in the amount of F-actin over the entire contact zone (Figure 3F) "
The authors should explain how F-actin levels were quantified and give an estimate of their variability between different cells or images.

6. In Page 9: "...taken 30 seconds apart (to ensure that MT shapes were uncorrelated) ".
Add reference or explain why 30 seconds is enough time to ensure that MT shapes were uncorrelated.

7. Revise and improve the writing of the Data Analysis section, specially the description of EB3 tracking. Include a definition of Temporal Autocorrelation (presented in Page 8) and the formula used to compute it.

Mistakes/typos:

- Page 6. "Figure 3H" should be "Figure 2H"

- Page 8: Please check this sentence: "Notably, while the correlation decay time at the periphery was reduced for CK666 and SMIFH2 treated cells, in both cases the correlation decay time was still larger than that at the center, indicating that MT filaments are more dynamic at the cell periphery even with inhibition of either actin nucleator "
I think that "time" should be replaced by "rate", or alternatively "reduced" by "increased".

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"and" is repeated.

- Figures 2E and 2H: angle units are missing.

- Figures 4A, 5E and 5H: check colorbar display. The color-codes do not agree with the colors of the filaments shown in the figures.

Further comments:

- Figure 1A: Tracing the cell border, the centroid and one or two representative lines across the cell could help to improve the understanding of the procedures used to obtain the intensity profiles.

- Page 6: "The lower EB3 radial speeds observed in the distal region may be attributed to the opposing force produced by the centripetal actin retrograde flow, which is highest in this region (Babich et al., 2012). "
Retrograde flow could also explain the observed differences in the EB3 directionality.

Supplementary information:
- Add N data in Supplementary Tables captions

Reviewer #2 (Remarks to the Author):

In this study Rey-Suarez et al. describe the interactions between the actin and microtubule cytoskeletons during immune synapse formation in T cells. Briefly, the authors demonstrate that microtubule growth dynamics differ in the subdomains of the immune synapse, with microtubules displaying slower growth speeds and increased deformation in the dSMAC region. Through the use of inhibitors, the properties of the growing microtubules are shown to be impacted in different ways by the distinct actin-dependent systems that characterize the pSMAC and the dSMAC. Formin inhibtion moderately slows growth and reduces microtubule straightness, while Arp2/3 inhibition increases growth and increases microtubule straightness. Overall the work is nicely done and convincingly demonstrates the interconnectedness of these cytosksletal systems. The area is of interest as the interactions of these two cytoskeletal systems are not well characterized in the context of the immune synapse. In additional, the study develops broadly useful tools for the quantitative analysis of microtubule architecture. Although the work lacks functional data, the characterization of these cytoskeletal interactions lays the groundwork for such studies in the future.

Figure 3A-B: Why is the narrow but dense lamellipodium at the boundary of the immune synapse in 3A not evident as an increase in F-actin intensity at R=1 in 3B? This is clear in 1A-B.

Supplementary Movie 4: The images of F-actin rather startlingly reveal a slowly contracting ring structure in the SMIFH2-treated cells on CD3+VCAM. In contrast, the actin structures in the equivalent DMSO-treated cell are more uniform and static. Ring structures of this sort are generally assumed to be formin-dependent and are not expected to assemble following SMIFH2 treatment. Is it possible that these are pre-existing actomyosin-arcs that are decoupled from the substrate by formin inhibition? In either case, it suggests that contractile rings have not been disabled by SMIFH2. Pre-treatment time may be the issue. This should be discussed.

Figure 4, text, page 8 (1): "The parameter provides the correlation decay rate parameters and corresponds to the correlation amplitude and decay rate at later times" should probably be "The parameter provides the correlation decay rate AND parameters and correspond to the correlation amplitude and decay rate at later times"

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Figure 5E & H: The color scales at the right of each image are presumably meant to convey a smooth color gradient corresponding to 'log curvature', but are instead displaying as a series of randomly colored horizontal bars on black bankground. Presumably blue indicates a lower 'log curvature' and teal-yellow-orange increasing 'log curvatures'. Please clarify.

Sup Fig 5: The rationales behind the curvature cosine analyses should be explained in greater detail. The Bicek paper is not open access and is not available via my institution. I don't doubt the analyses are correct. But please translate the underlying equations into plain english. For example, it would be helpful to state that the cosine metric should be 1 for a straight line and will drop towards -1 when the segment tangents are separated by 180 degrees. Diagrams would be hepful. Similarly, it would be

helpful tranlate statements such as "reduced the distance-dependent decay in correlations, particularly for filaments at the periphery" to "filaments were straighter in the periphery"?

- For filament curvature, if the segment spacing is uniform, then wouldn't the segment lengths be constant, rendering curvature simply a measure of the angle between adjacent segments? And if the segment contour lengths differ from the inter-segment distances, then wouldn't the increased lengths be suggestive of more curvature, even though these deviations would lower the curvature metric?

- For the cosine analysis, define the derivation of the angle theta beyond the "angle between segments". Is theta vectorial?

Overall, the effects of the myosin inhibitor Y27632 exceed those of the formin inhibitor SMIFH2. Y27632 reduces peripheral microtubule curvature more effectively than SMIFH2 (5F & G). Similarly, Y27632 increases peripheral microtubule decay time, stabilizing MTs, to a greater extent than SMIFH2 (4G & H). And as noted for Supplementary Movie 4, SMIFH2 is not completely suppressing the contraction of actin rings. Is it possible that the length of SMIFH2 pretreatment in inadequate?

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Your manuscript, entitled " Actomyosin dynamics modulate microtubule deformation and growth during T cell activation" has been seen by two referees whose verbatim comments are enclosed. Both referees felt that your findings, in principle, would be of interest to our MBC readership. However, both reviewers raised some important points that need to be addressed. Most of these can be addressed by changes in the text. In particular, more detail is needed in the description of the data analysis and other section need revision for clarity. We look forward to receiving your revised manuscript and a letter indicating your response to the referees in the near future.

We thank you for the opportunity to revise the manuscript. We have clarified the details of the data analysis as requested by the reviewers and updated the methods section accordingly. We have modified the text to address the questions raised by the reviewers. A detailed response to the comments is provided below.

Reviewer #1 (Remarks to the Author):

The manuscript "Actomyosin dynamics modulate microtubule deformation and growth during T cell activation" by Rey-Suarez and co-authors explores the crosstalk between microtubules and actin networks during T cell activation….
Overall, this is an interesting study, which provides additional information on the interactions between cytoskeleton networks during T cell activation.

We thank the reviewer for the kind comments and the constructive feedback.

However there are some points related to the methodological aspects and the results presentation that need to be clarified or improved:

We have addressed these points below.

1. Explain the procedure followed to calculate the mean intensities represented in Figures 1E-G. Are these mean intensities an average of the intensities within each of the three regions of the profiles shown in Fig. 1 B? Do the data points represented in each boxplot come from the 90 cells referenced in Fig. 1B?

Yes, the intensities presented in Figures 1E-G correspond to an average intensity within each region - the summed intensity of all pixels within a region divided by the total number of pixels in that region. We thus obtain a single value of mean intensity per region per cell. This analysis is applied to the same 90 cells used to generate Figure 1B and so the data points in each boxplot come from the same 90 cells as in Figure 1B. We have now indicated this in the caption. This procedure has now been explained in more detail in the Methods section and with a diagram in Supplementary Figure 7A.

2. Please, add error bars to represent the dispersion of the averaged intensity profiles shown in Figure

1B. Is this profile the average of 50 lines from 90 cells? Was the variability in the intensity values between different cells and images taken into account?

We thank the reviewer for this comment. We have now included shaded error bars for the averaged intensity profiles in Figure 1B. We have indicated how the profiles were calculated from the population of cells in the Methods section. We verified that the variability between cells was low – the mean pixel-wise fluorescence intensities for all cells were well within one standard deviation of each other.

3. Boxplots: include the data points in the figure (swarm boxplot) and/or add the information about the number of data points in the figure captions.

Swarm boxplots have been included in all plots where the number of data points is less than one hundred. We have included the number of data points for all figure captions and tables.

4. Statistical analysis: the Kruskal-Wallis test is preferred to the KS test to determine the statistical significance of 3 or more samples. When comparing 2-samples at a time, the authors should mention if they have considered the corrections necessary to compensate for the increase in the likelihood of incorrectly rejecting a null hypothesis when performing multiple comparisons (e.g. Bonferroni correction).

We agree with the reviewer and have performed all the statistical tests now using the Kruskal-Wallis test to compare multiple samples. Bonferroni correction was applied for multiple pairwise comparisons. We have now stated this in the Methods section 'Statistical analysis'.

5. In Page 7: "Interestingly, the presence of VCAM-1 on the activating substrate induced a significant increase in the amount of F-actin over the entire contact zone (Figure 3F) "
The authors should explain how F-actin levels were quantified and give an estimate of their variability between different cells or images.

To quantify the F-actin levels, cells were stained using Rhodamine-Phalloidin and the fluorescence intensity of the pixels within the cell contour was summed and divided by the total number of pixels in the cell to obtain a *mean intensity,* as shown in Figure 3F. We have now explained this in the Methods section (page 14). The variability in F-actin intensities across cells can now be seen in the bee-swarm plots (Figure 3F). Immunostaining was performed on the two samples simultaneously with identical imaging conditions. We found that cells in CD3+VCAM display overall higher actin intensities and had a greater variability in intensities.

6. In Page 9: "...taken 30 seconds apart (to ensure that MT shapes were uncorrelated) ".
Add reference or explain why 30 seconds is enough time to ensure that MT shapes were uncorrelated.

Brangwynne et al. (PNAS, 2007) have shown that the shapes of microtubules displaying short wavelength fluctuations ($\lambda \sim 6$ μm) and become uncorrelated within 10 seconds. Additionally, we found that the correlation of the microtubule intensity pattern within a region drops to zero between 10-30 seconds (see Figure 4F). We have now explained this in the Methods section and added this reference (page 14).

7. Revise and improve the writing of the Data Analysis section, specially the description of EB3 tracking. Include a definition of Temporal Autocorrelation (presented in Page 8) and the formula used to compute it.

We have now re-written the methods section and included diagrams in the Supplementary information section to better describe the analysis workflow. The MATLAB function used to calculate the temporal autocorrelation (*corrcoeff*) has been indicated in the caption of Supplementary Figure 4.

Mistakes/typos:

- Page 6. "Figure 3H" should be "Figure 2H"

- Page 8: Please check this sentence: "Notably, while the correlation decay time at the periphery was reduced for CK666 and SMIFH2 treated cells, in both cases the correlation decay time was still larger than that at the center, indicating that MT filaments are more dynamic at the cell periphery even with inhibition of either actin nucleator "
I think that "time" should be replaced by "rate", or alternatively "reduced" by "increased".

- Page 10: "... led to significantly less deformed and and a reduction in their fluctuations ..."
"and" is repeated.

We thank the reviewer for noting the above errors. We have now corrected these in the revised manuscript.

- Figures 2E and 2H: angle units are missing.

We have now fixed this.

- Figures 4A, 5E and 5H: check colorbar display. The color-codes do not agree with the colors of the filaments shown in the figures.

There seems to have been a problem with the color display of two of the figures during the process of uploading the paper into the submissions website. We will confirm that the conversion is correct in the revision.

Further comments:

- Figure 1A: Tracing the cell border, the centroid and one or two representative lines across the cell could help to improve the understanding of the procedures used to obtain the intensity profiles.

We have now explained this in the Methods section with a new Supplementary Figure 7.

- Page 6: "The lower EB3 radial speeds observed in the distal region may be attributed to the opposing force produced by the centripetal actin retrograde flow, which is highest in this region (Babich et al., 2012). " Retrograde flow could also explain the observed differences in the EB3 directionality.

We have now addressed this. We have included the following sentence at the end of the second paragraph (page 6). "The lower EB3 radial speeds, as well as the higher directionality change, observed in the distal region may be attributed to the opposing force produced by the centripetal actin retrograde flow, which is highest in this region (Babich *et al.*, 2012)."

Supplementary information:
- Add N data in Supplementary Tables captions

We have added this information in the Supplementary Tables captions.

Reviewer #2 (Remarks to the Author):

In this study Rey-Suarez et al. describe the interactions between the actin and microtubule cytoskeletons during immune synapse formation in T cells Briefly, the authors demonstrate that microtubule growth dynamics differ in the subdomains of the immune synapse, with microtubules displaying slower growth speeds and increased deformation in the dSMAC region. Through the use of inhibitors, the properties of the growing microtubules are shown to be impacted in different ways by the distinct actin-dependent systems that characterize the pSMAC and the dSMAC. Formin inhibtion moderately slows growth and reduces microtubule straightness, while Arp2/3 inhibition increases growth and increases microtubule straightness. Overall the work is nicely done and convincingly demonstrates the interconnectedness of these cytosksletal systems. The area is of interest as the interactions of these two cytoskeletal systems are not well characterized in the context of the immune synapse. In additional, the study develops broadly useful tools for the quantitative analysis of microtubule architecture.…. Although the work lacks functional data, the characterization of these cytoskeletal interactions lays the groundwork for such studies in the future.

We thank the reviewer for these comments. We are addressing the functional implications of these interactions in our ongoing work.

Figure 3A-B: Why is the narrow but dense lamellipodium at the boundary of the immune synapse in 3A not evident as an increase in F-actin intensity at R=1 in 3B? This is clear in 1A-B.

We found that the mask used to detect the edge of the cell overestimated the edge in some cells, generating zero intensity values over the last 10% of the radius. We have corrected this problem and now the narrow dense lamellipodium can be observed in the average profile.

Figure 4, text, page 8 (1): "The parameter b provides the correlation decay rate parameters c and d corresponds to the correlation amplitude and decay rate at later times" should probably be "The

parameter b provides the correlation decay rate AND parameters c and d correspond to the correlation amplitude and decay rate at later times" –

This typo has been fixed.

Figure 4, text, page 8 (2): "Notably, while the correlation decay time at the periphery was reduced for CK666 and SMIFH2 treated cells, in both cases the correlation decay time was still larger than that at the center, indicating that MT filaments are more dynamic at the cell periphery even with inhibition of either actin nucleator". If I am reading this correctly, this is the opposite of the finding. The sentence seems correct if referring to parameter b. It should probably read "Notably, while the correlation decay time at the periphery was INCREASED for CK666 and SMIFH2 treated cells, in both cases the correlation decay time was still SMALLER than that at the center, indicating that MT filaments are more dynamic at the cell periphery even with inhibition of either actin nucleator".

We thank the reviewer for catching this error and have now corrected this in the text.

Figure 5E & H: The color scales at the right of each image are presumably meant to convey a smooth color gradient corresponding to 'log curvature', but are instead displaying as a series of randomly colored horizontal bars on black bankground. Presumably blue indicates a lower 'log curvature' and teal-yellow-orange increasing 'log curvatures'. Please clarify.

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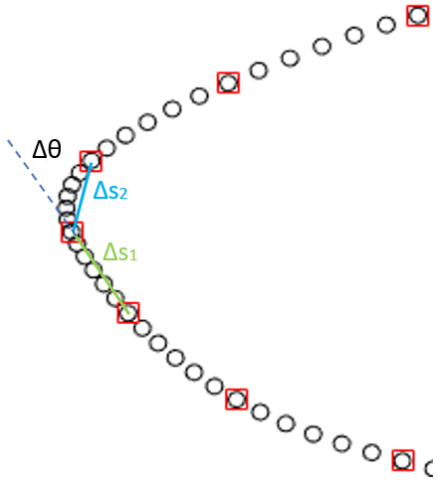
We appreciate this remark from the reviewer. We have now included a diagram (as Supplementary Figure 5D) to explain the cosine analysis better and expanded the description of the analysis in both the Results (page 10) and Methods section (page 15).

- For filament curvature, if the segment spacing is uniform, then wouldn't the segment lengths be constant, rendering curvature simply a measure of the angle between adjacent segments? And if the segment contour lengths differ from the inter-segment distances, then wouldn't the increased lengths be suggestive of more curvature, even though these deviations would lower the curvature metric?

The diagram below shows the original coordinates of a microtubule filament (black circles) and the coordinates used to generate the filament segments (red squares). The number of coordinates used to

make the filament segments is always the same. However, the segment distances are not equal (due to a higher sampling of the curved regions when compared to straight regions). Our analysis of filament curvatures is similar to that used by Gittes et al. (J. Cell Biol. year), Bicek et al. 2007, Brangwynne et al. 2008).



- For the cosine analysis, define the derivation of the angle theta beyond the "angle between segments". Is theta vectorial?

The angle theta for the cosine analysis is the angle between a line tangent to the first segment of the filament and a line tangent to each consecutive segment. We have now included a more detailed description and a diagram in Supplementary Figure 5D.

Supplementary Movie 4: The images of F-actin rather startlingly reveal a slowly contracting ring structure in the SMIFH2-treated cells on CD3+VCAM. In contrast, the actin structures in the equivalent DMSO-treated cell are more uniform and static. Ring structures of this sort are generally assumed to be formin-dependent and are not expected to assemble following SMIFH2 treatment. Is it possible that these are pre-existing actomyosin-arcs that are decoupled from the substrate by formin inhibition? In either case, it suggests that contractile rings have not been disabled by SMIFH2. Pre-treatment time may be the issue. This should be discussed.

The cell shown in Supplementary Movie 4 indeed did not exhibit complete disruption of arcs. However, this is not an entirely representative cell. We have now modified the movie to include a different cell in Movie 4 that clearly shows the disruption of arcs and is more representative. We noted that about two-thirds of the cells showed complete disruption of arcs (as observed in widefield TIRF) upon SMIFH2 application, for cells on anti-CD3+VCAM. This may be due to the effect of VCAM on actin arcs, making them more stable than on anti-CD3 alone. We do not think that incubation time is an issue, since Supplementary Movie 3 shows the disruption of arcs by SMIFH2 for cells activated on anti-CD3 only substrates. We have noted this in the Discussion on page 11.

Overall, the effects of the myosin inhibitor Y27632 exceed those of the formin inhibitor SMIFH2. Y27632 reduces peripheral microtubule curvature more effectively than SMIFH2 (5F & G). Similarly, Y27632

increases peripheral microtubule decay time, stabilizing MTs, to a greater extent than SMIFH2 (4G & H). And as noted for Supplementary Movie 4, SMIFH2 is not completely suppressing the contraction of actin rings. Is it possible that the length of SMIFH2 pretreatment in inadequate?

We would like to clarify that in Figure 4G-H, both SMIFH2 and Y27632 have similar effects on MT filament dynamics. As noted by the reviewer, in Figure 5F-G, we do observe a larger effect of Y27632 in reducing the curvature of MT filaments as compared to SMIFH2. As we pointed out above, the cell shown in Supplementary Movie 4 was activated on anti-CD3 and VCAM-1. However, the data showed in Figure 5 was obtained from cells activated on anti-CD3 antibody only, and therefore Supplementary Movie 4 cannot really be used to interpret the results of Fig. 5. We note that Supplementary Movie 3 shows the disruption of arcs in SMIFH2 cells on CD3 alone and therefore, believe that the pretreatment time is sufficient.

RE: Manuscript #E20-10-0685R
TITLE: "Actomyosin dynamics modulate microtubule deformation and growth during T cell activation"

Dear Prof. Upadhyaya:

Thank you for revising your manuscript in response to the referees' recommendations. I have read the revised manuscript carefully along with your responses to the referees and it is clear that you have satisfactorily addressed their concerns. I am pleased to accept your manuscript for publication in Molecular Biology of the Cell. Congratulations to you and your colleagues.

Sincerely,
Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Upadhyaya:

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.

Within approximately four weeks you will receive a PDF page proof of your article.

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Sincerely,

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