JCB

# VASH1-SVBP and VASH2-SVBP generate different detyrosination profiles on microtubules

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*Corresponding Author(s): Marie-Jo Moutin, Univ. Grenoble Alpes, Inserm, U1216, CNRS, CEA, Grenoble Institut Neurosciences and Michel Steinmetz, Paul Scherrer Institute*

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## Transaction Report:

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

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July 8, 2022

Re: JCB manuscript #202205096

Dr. Marie-Jo Moutin
Univ. Grenoble Alpes, Inserm, U1216, CNRS, CEA, Grenoble Institut Neurosciences
Rue F Ferrini
Grenoble 38000
France

Dear Dr. Moutin,

Thank you for submitting your manuscript titled "VASH1-SVBP and VASH2-SVBP generate different detyrosination profiles on microtubules." The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the Reviewers are highly enthusiastic about your study and feel it provides a significant advance in our understanding of the isoform-specific functional properties in the Vasohibin protein family. Reviewer #1 raises concerns regarding the resolution of the structural model of the VASH2-tubulin interface and asks for a focused mutagenesis-based analysis to confirm the predicted binding sites. Reviewer #3 notes that the cell permeabilization assays seem to be from a single replicate and asks that you repeat and also quantify these. The rest of the comments seem to be fairly minor requests for additional information and clarifications that should not require additional experiments.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-

19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Tarun Kapoor, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

The authors expand on the current understanding of VASH-mediated tubulin de-tyrosination by comparing and contrasting the behavior of VASH1 and VASH2 in great detail. They begin by showing a clear difference in de-tyrosination patterns, where VASH1 acts "globally" along the entire length of the microtubule and VASH2 acts "locally" in discrete patches. Then, they determine a cryo-EM reconstruction of VASH2-SVBP bound to microtubules, indicating which regions bind to the microtubule. The authors then determined that the similar disordered C-terminal domains (CTDs) of both VASH1 and VASH2 increase residence time on microtubules relative to truncation mutants, likely due to interactions between basic CTD residues and acidic tubulin surfaces, but that the disordered N-terminal domains (NTDs) of VASH1 and VASH2 are quite different; the acidic NTD of VASH1 decreases residence time when compared to a truncated mutant or chimeric fusion while the basic NTD of VASH2 increases residence time. This leads to a model whereby the CTDs of both proteins briefly anchor the catalytic core and allow for better substrate interaction while the NTDs act as either a reciprocal detachment tool in the case of VASH1 or a second anchor in the case of VASH2. This model could explain the global and local detyrosination profiles of VASH1 and VASH2, respectively.

Overall, the paper represents an important comparison of VASH1 and VASH2 behaviors, as well as provides an improved cryo-EM reconstruction of VASH2-SVBP compared to a previous VASH1-microtubule reconstruction.

Major comment:
We appreciate the improved quality of the map for VASH2 on the microtubule surface. The authors indicate potential residues that mediate the interaction. However, if the authors want to show amino acid interaction at interfaces between VASH2 and tubulin, they need to show mutagenesis to demonstrate which site(s) are important for binding microtubules.

Minor comments:
We would like the authors to address how the interfaces between VASH2 and tubulin are depicted in the cryo-EM figures. In general, the authors should not be showing residues forming hydrogen bonds if there is no density present for this interaction. For example, VASH2 site 2 H257 shows very weak density. To help the reader appreciate the confidence, the authors should superimpose density over the model to help show whether density supports their amino acid placement.

Figure 3C, site 1: The position of αE434 is notably different in the submitted model and density map than in the model depicted here, and the resulting hydrogen bond is not found in the shared atomic model. Please address this discrepancy. Based on the density, the best model-map agreement likely points αE434 away from VASH2. Please address.

Figure 3C, site 2: The position of H257 is not well supported by density. In fact, there is a stronger density in the cryo-EM map to the other side of the residue. This puts the hydrogen bond here in question as well. Please consider the possibility of an alternate rotamer conformation here.

Figure 4C: The positions of VASH1 R234 and VASH2 R223 are not supported by the density for the cryo-EM maps for VASH1/2

bound to microtubules. The authors should remove these amino acids side chain depictions. The same is true for VASH1 V159, there is no density for this side chain in the VASH1 reconstruction on microtubules. Instead, the authors should focus their figure and discussion on the general position of helix 7.

Reviewer #2 (Comments to the Authors (Required)):

This study by Ramirez-Rios et al gets at a fundamental question of how tubulin-modifying enzymes generate different patterns of tubulin PTMs on cytoplasmic microtubule. The current work highlights how subtle differences in the microtubule-binding properties of 2 closely related microtubule detyrosination enzymes VASH1 and VASH2 can lead to distinct patterns of the same PTM on identical microtubules, and thus further expand the diversity of microtubule subpopulations in the cell. This is a beautiful body of work, which is also nicely presented. I support its publication.

In terms of specific key findings, the authors first show in a reconstituted system that VASH1 induces detyrosination of the entire lattice, while VASH2 does so over localized regions. Single molecule imaging shows that the VASH1-microtubule binding events are shorter-lived and more frequent than that of VASH2, and this difference is independent of the catalytic activity. Next, cryo-EM reconstructions of VASH2-SVBP bound to microtubules, and comparison to published VASH1-SVBP structures show that the microtubule-bound enzymes are rotated relative to each other and differ in their microtubule-binding interfaces. However, the 2 catalytic triads are positioned similarly with respect to H12 of alpha-tubulin, suggesting that the the catalytic domains of both enzymes should have similar activity, a hypothesis confirmed by subsequent microscopy assays. The authors then turn to disordered terminal regions of both proteins. They find that the N-terminal regions contribute to observed differences in microtubule-binding. Swapping the N-terminal regions of both proteins causes a switch in their microtubule-binding behavior and changes the detyrosination profile of VASH2 from local to global - a very cool result! Finally, the authors show that results are consistent with observations in cellular microtubules using permeabilized cells. I have a few minor suggestions and corrections (no additional experiments needed).

1) It is still confounding how the continuous microns-long patches of deTyr is established by VASH2 (Fig1). Shouldn't localized diffusion/activity coupled with random binding along the microtubule give rise to a more "spotty" pattern rather than these long stretches? In the single molecule experiments, does the binding of VASH2 promote preferential landing of other molecules in proximity? This information can be extracted from existing dataset. Please report and discuss.

2) Regarding the recruitment of VASH1 and VASH2 to distinct cellular microtubules:
Does the structure reveal if VASH1 and VASH2 could be recruited to different microtubule populations in the first place? For instance, if both enzymes are recruited to the same microtubule, wouldn't the global activity of VASH1 overwhelm the localized activity of VASH2? Further experiments are not required, but the authors could analyze both EM structures and check if:
(i) Sequence differences between different a-tubulin isoforms map on to the differential microtubule-binding surfaces of both proteins.
(ii) Would the microtubule-binding of both enzymes be sensitive to protofilament number, given that the binding sites straddle 2 protofilaments, and the binding orientation of both enzymes are different from each other?

3) Lines 302-304: "As the residues directly involved in microtubule-lattice binding are distinct and distal from the catalytic site, we may consider these as substrate recognition elements that confer specificity to the catalytic activity of the vasohibin family of tubulin carboxypeptidases."
Are the distinct microtubule-binding interfaces conserved among VASH1 homologs and among VASH2 homologs? Such surface conservation would make the claim about specificity in the whole family stronger.

4) Line 769: "The significance of ..... except for Figure 1C where the Conover-Iman test was employed."
Figure 1C does not contain statistical significance.

5) Line 258: The resolution of VASH2-SVBP was in the range of 3.3-4.8 Å becoming progressively higher in regions that are located closer to the microtubule surface.

Use "better" instead of higher for more clarity.

6) Line 188:
Use "Residence time" instead of run length

7) Line 653:
Typo: "prepa"?

Reviewer #3 (Comments to the Authors (Required)):

In their paper "VASH1-SVBP and VASH2-SVBP generate different detyrosination profiles on microtubules", Ramirez-Rios, Choi, Sanyal et al. dissect the differential properties of the a-tubulin detyrosinating enzymes VASH1 and VASH2. Like many cytoskeletal proteins (and indeed many proteins in general), the VASH family of enzymes has multiple isoforms, the functional relevance of which is generally not clear. Using primarily in vitro TIRF assays and primary-structure function analysis, the authors find that VASH1 globally detyrosinates microtubules, while VASH2 locally detyrosinates regions of microtubules. This is intuitively correlated with the biophysical properties of the enzymes (diffusivity and binding kinetics), which are analyzed at the single molecule level. Interestingly, the authors find this differential activity is primarily determined by the divergent disordered N-termini of the proteins, which feature opposite charges that likely mediate distinct interactions with the negatively charged C-terminal "tails" of tubulin subunits.
The authors additionally provide a cryo-EM reconstruction of the VASH2-SVBP complex bound to microtubules, which is compared with a previously reported structure of VASH1-SVBP bound to microtubules. This provides additional insight into the differential binding poses of the catalytic cores which work in concert with the disordered termini to determine the overall functional properties of the enzymes. Finally, they also show differential activity in a cell-based assay, supporting the physiological relevance of their observations.
Overall, I found this to be a highly thorough and complete mechanistic story that elucidates isoform-specific functional properties in the VASH family, achieving a level of understanding of isoform specificity that is broadly important for making progress in the field. This sets the stage for future work examining the interplay of these enzymes in vivo. I believe the paper's overall significance and consistently strong technical quality makes it suitable for publication in the JCB, pending minor revisions as detailed below:

Minor points:

1) The permeabilized cell assays presented in Fig. 7 appear to be a single trial. These experiments need to be performed with the same level of rigor (multiple independent trials, with quantification) as all other data presented in the paper to be suitable for publication.

2) The authors quote an overall resolution of 3.1 Angstroms for their cryo-EM reconstruction. However, it is apparent from examining the local resolution map that is provided that this resolution assessment is primarily driven by the well-ordered microtubule lattice, which is not the primary subject of this study. For readers to have a better sense of the resolution of the VASH molecule, it would be appropriate for the authors to also calculate an FSC for just the VASH-SVBP region of the map using a mask covering this region, and additionally quote this resolution value in the main text. Furthermore, the authors should include a model-map FSC curve (assessed at FSC 0.5), which is currently standard practice in the field.

3) In the section "Tyrosination has a little [sic] effect on the interaction of VASH-SVBP complexes with microtubules", the authors examine VASH1-SVPB and VASH2-SVBP under different ionic conditions. While I understand they had to do this to be able to perform single-molecule studies, this makes comparing the properties of VASH1 and VASH2 dubious (line 223). I thus recommend removing this and focusing on the internal comparisons of VASH1/2 under different conditions.

4) Is there a reason the authors did not cleave the HIS tags from their proteins before performing assays? Since the VASH flexible termini play a prominent role, and poly-HIS tags will impact the charge distribution, this should at a minimum be mentioned as a caveat.

Other small issues:

5) Line 59 should be, I believe, "kinesin motors"

6) Line 128, sfGFP should be defined at first usage

7) Line 186, "As the binding of microtubule-associated proteins to microtubules often involves electrostatic attractive interactions" should have a reference.

8) Lines 206-209, These sentences would be clearer if the order of tyrosinated, de-tyrosinated, and delta-2 tubulin was kept constant

9) Li et al. were able to visualize the C-terminal tail of alpha-tubulin in their VASH1 reconstruction when they low-pass filtered their reconstruction to lower resolution (~7A). It would be useful to see if this is also the case for VASH2 presented here.

10) Lines 361-364, "We thus examined the detyrosinating activity of VASH1 and VASH2 core domains...", this sentence and the following paragraph are confusing, as catalytically active and catalytically dead versions are presented in an interleaved fashion. This would be clearer if they were broken up into 2 separate paragraphs.

11) Line 474, "Indeed, by exchanging these regions, we were able to switch the microtubule-binding behavior and activity of one enzyme to that of the other one." This sentence is not strictly accurate, as only one of the swaps worked. Please be precise.

12) Line 653, "prepa" should be "prepared"

13) Lines 674-675, it is mentioned that new VASH2 and SVBP antibodies were generated in the course of this study. How was the specificity of these antibodies validated?

14) In the cryo-EM data collection section of the Methods, a "Hela sample" is mentioned. What does this mean?

15) Lines 719 - 721, The procedure for signal subtraction and the selection criteria used is not clear. Why would you subtract regions where VASH2 had bound? Please expand.

16) The authors mentioned fluorophore "quenching" several times in the manuscript, but I believe they mean fluorophore "bleaching".

17) Line 770-771: Why was the Conover-Iman test used instead of Dunn's test in only one case?

18) For Figures where individual channels are presented in addition to a merge, it would be clearer if the individual channels were shown in black and white, as magenta in particular is difficult to see on its own (Fig. 1A; 5A; 6G; 7; S2A,C).

19) Figure 3: I recommend moving the color key to the top of the Figure so it is clearly associated with A onwards (I missed this at first look).

20) Figure S5: The colors should be explained in the figure or its legend, and residues should be labelled.

We thank the referees for their constructive comments. We have revised our manuscript accordingly, with new experimental data (Figures 3DE & S3G), better analysis (Figures 7B), and other modifications (Figure S4). We believe our modifications based on the reviewers' comments have greatly strengthened our work.
Our detailed response to all comments can be found below, in blue.
Changes in text were highlighted in blue.

**Reviewer #1 (Comments to the Authors (Required)):**

The authors expand on the current understanding of VASH-mediated tubulin de-tyrosination by comparing and contrasting the behavior of VASH1 and VASH2 in great detail. They begin by showing a clear difference in de-tyrosination patterns, where VASH1 acts "globally" along the entire length of the microtubule and VASH2 acts "locally" in discrete patches. Then, they determine a cryo-EM reconstruction of VASH2-SVBP bound to microtubules, indicating which regions bind to the microtubule. The authors then determined that the similar disordered C-terminal domains (CTDs) of both VASH1 and VASH2 increase residence time on microtubules relative to truncation mutants, likely due to interactions between basic CTD residues and acidic tubulin surfaces, but that the disordered N-terminal domains (NTDs) of VASH1 and VASH2 are quite different; the acidic NTD of VASH1 decreases residence time when compared to a truncated mutant or chimeric fusion while the basic NTD of VASH2 increases residence time. This leads to a model whereby the CTDs of both proteins briefly anchor the catalytic core and allow for better substrate interaction while the NTDs act as either a reciprocal detachment tool in the case of VASH1 or a second anchor in the case of VASH2. This model could explain the global and local detyrosination profiles of VASH1 and VASH2, respectively.

Overall, the paper represents an important comparison of VASH1 and VASH2 behaviors, as well as provides an improved cryo-EM reconstruction of VASH2-SVBP compared to a previous VASH1-microtubule reconstruction.

Major comment:
We appreciate the improved quality of the map for VASH2 on the microtubule surface. The authors indicate potential residues that mediate the interaction. However, if the authors want to show amino acid interaction at interfaces between VASH2 and tubulin, they need to show mutagenesis to demonstrate which site(s) are important for binding microtubules.
To address this comment, we have cloned three constructs allowing expression of VASH2-SVBP double or triple mutants in which the VASH2 residues R134 and R137 (site 1), H257 and F259 (site 2), or R288, R291 and M595 (site 3) were mutated to alanines. We have analyzed the detyrosination activity and microtubule binding capacity of these mutants in cells. The new results, demonstrating the importance of each of the three sites, are presented in Figures 3DE and S3G (see also Results pages 10, lines 303-310, Material and Methods page 22, lines 707-731 and page 25, lines 835-838).

Minor comments:
We would like the authors to address how the interfaces between VASH2 and tubulin are depicted in the cryo-EM figures. In general, the authors should not be showing residues forming hydrogen bonds if there is no density present for this interaction. For example, VASH2 site 2 H257 shows very weak density. To help the reader appreciate the confidence, the authors should superimpose density over the model to help show whether density supports their amino acid placement.

This point is noted and illustrations depicting the interaction sites have been edited to display the superimposing EM density. We also removed the depiction of hydrogen bonds in cases where the density of side chain was poorly defined. See revised Figure 3C.

Figure 3C, site 1: The position of αE434 is notably different in the submitted model and density map than in the model depicted here, and the resulting hydrogen bond is not found in the shared atomic model. Please address this discrepancy. Based on the density, the best model-map agreement likely points αE434 away from VASH2. Please address.

Many thanks for pointing out this error. The difference in the position of αE434 between the submitted model and the figure was due to a mismatch in the version of the model used. As the submitted model is the final one, we have corrected the residues in the revised Figure 3C accordingly and removed αE434 interaction in the text (Results page 10, lines 294-296).

Figure 3C, site 2: The position of H257 is not well supported by density. In fact, there is a stronger density in the cryo-EM map to the other side of the residue. This puts the hydrogen bond here in question as well. Please consider the possibility of an alternate rotamer conformation here.

We appreciate the suggestion of the reviewer regarding the poor support for the position of the H257 side chain of VASH2 by the electron density. To address this issue, we have explored multiple rotamer conformations of H257; however, none of them produced a more satisfactory outcome. We have also attempted to adjust the S4-S5 loop of VASH2 to bring the H257 residue closer to the corresponding density but this then resulted in a poor fitting of the entire region of VASH2. One possible explanation for this electron density may be an alternative conformation of the αR390 side chain of α-tubulin, which may occupy this site to interact electrostatically with the main chain of H257. However, we feel that displaying a rotamer of H257 that would allow for a polar interaction with αH393 of α-tubulin (without explicitly depicting a hydrogen bond) is the best compromise at this stage. See revised Figure 3C.

Figure 4C: The positions of VASH1 R234 and VASH2 R223 are not supported by the density for the cryo-EM maps for VASH1/2 bound to microtubules. The authors should remove these amino acids side chain depictions. The same is true for VASH1 V159, there is no density for this side chain in the VASH1 reconstruction on microtubules. Instead, the authors should focus their figure and discussion on the general position of helix 7.

We initially included residues VASH1 R234 and VASH2 R223 to address and make a comparison with the microtubule-interacting residues identified by Li *et al.,* 2020. We agree with the reviewer that neither the electron density of VASH1 R234 nor the one of VASH2 R223 support the positions of the residue side chains. We thus removed these residues from the figure. The same point is also taken for VASH1 V159 that has been removed. See revised Figure 4C. As suggested by the reviewer, we now have focused our discussion on the general position of helix H7 of both enzymes (see Results page 11, lines 346-349).

**Reviewer #2 (Comments to the Authors (Required)):**

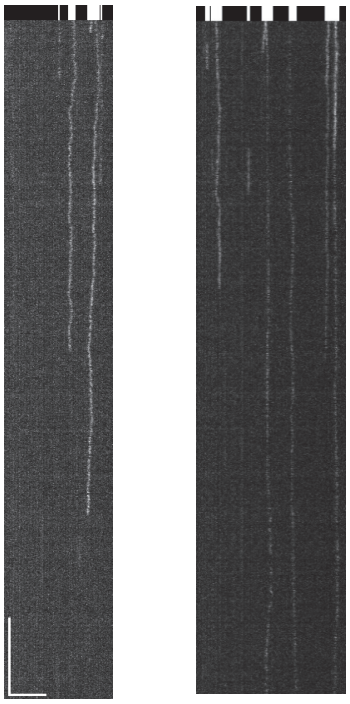
This study by Ramirez-Rios et al gets at a fundamental question of how tubulin-modifying enzymes generate different patterns of tubulin PTMs on cytoplasmic microtubule. The current work highlights how subtle differences in the microtubule-binding properties of 2 closely related microtubule detyrosination enzymes VASH1 and VASH2 can lead to distinct patterns of the same PTM on identical microtubules, and thus further expand the diversity of microtubule subpopulations in the cell. This is a beautiful body of work, which is also nicely presented. I support its publication.

In terms of specific key findings, the authors first show in a reconstituted system that VASH1 induces detyrosination of the entire lattice, while VASH2 does so over localized regions. Single molecule imaging shows that the VASH1-microtubule binding events are shorter-lived and more frequent than that of VASH2, and this difference is independent of the catalytic activity. Next, cryo-EM reconstructions of VASH2-SVBP bound to microtubules, and comparison to published VASH1-SVBP structures show that the microtubule-bound enzymes are rotated relative to each other and differ in their microtubule-binding interfaces. However, the 2 catalytic triads are positioned similarly with respect to H12 of alpha-tubulin, suggesting that the catalytic domains of both enzymes should have similar activity, a hypothesis confirmed by subsequent microscopy assays. The authors then turn to disordered terminal regions of both proteins. They find that the N-terminal regions contribute to observed differences in microtubule-binding. Swapping the N-terminal regions of both proteins causes a switch in their microtubule-binding behavior and changes the detyrosination profile of VASH2 from local to global - a very cool result! Finally, the authors show that results are consistent with observations in cellular microtubules using permeabilized cells.

I have a few minor suggestions and corrections (no additional experiments needed).

1) It is still confounding how the continuous microns-long patches of deTyr is established by VASH2 (Fig1). Shouldn't localized diffusion/activity coupled with random binding along the microtubule give rise to a more "spotty" pattern rather than these long stretches? In the single molecule experiments, does the binding of VASH2 promote preferential landing of other molecules in proximity? This information can be extracted from existing dataset. Please report and discuss.

This is a very interesting point. In response, we would first like to recall that the immunofluorescence experiments for measuring detyrosination activity were performed at a very low enzyme concentration (50 pM, as for TIRF assays). In the TIRF experiment at this concentration, single molecules of VASH2-SVBP bind and diffuse for long periods of time on microtubules (see Figure 1D). We have examined the covering of microtubules by VASH2 molecules by performing maximum binding projection (Z stacks). Examples can be seen below:



horizontal, 5 µm; vertical, 5 s.

Such analysis shows that micron-sized spots of VASH2 displacement are revealed on microtubules. These patches are in the same range to those observed for detyrosination in immunofluorescence assays under the same enzyme concentration conditions. This suggests that a single molecule is able to "surf" on microtubules over such a long distance and could be responsive for the micron-long patches of deTyr microtubules observed in immunofluorescence.

We have also followed the reviewer's suggestion by analyzing a set of 30 VASH2 kymographs for fluorescence intensity. We found that a mean of 15,7 % and 11 % of the binding events were due to two molecules that were in close proximity at 1 s and 5 s, respectively. This is consistent with the percentage of dimers (10-16 % at 50-100 pM enzyme) that were estimated from TIRF photobleaching experiments performed in the absence of microtubules to establish the single molecule conditions.

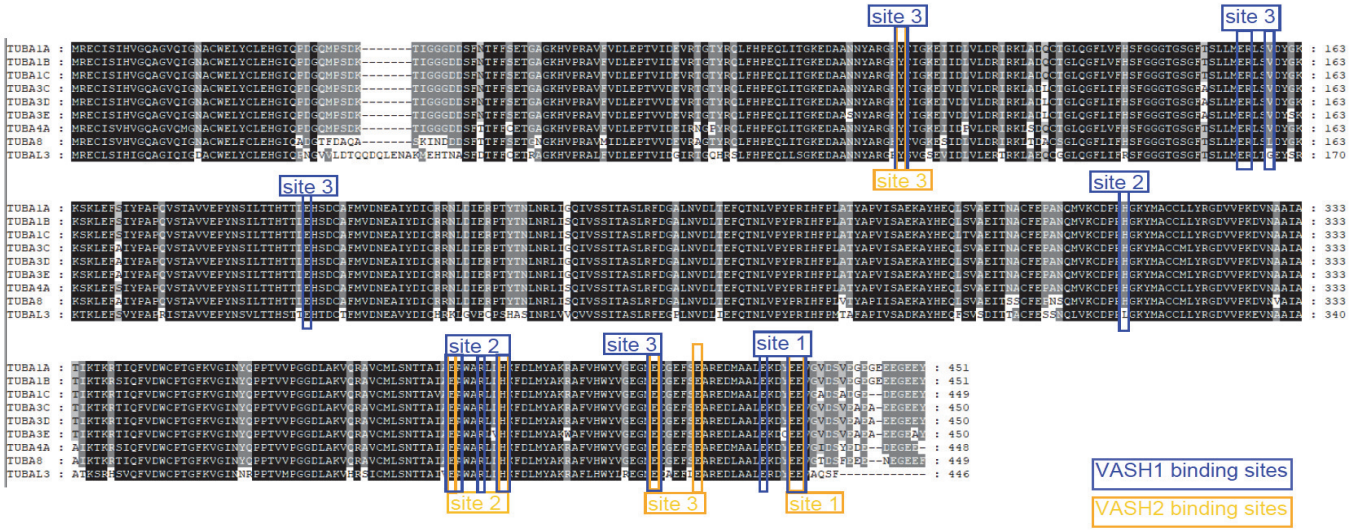
For all these reasons, we consider that VASH2 binding might not favor preferential landing of other molecules in proximity. However, to completely rule out this possibility, a full set of additional single-molecule TIRF experiments in the presence of GFP-labeled and unlabeled enzymes (to analyze cooperation/competition) would be required. Photobleaching of GFP fluorophore associated to VASH2 (as stated in Results page 6 line 180 and in Material and Methods page 24 line 806) might however make the study difficult.

Altogether, we prefer not to add any further conclusion concerning this point.

2) Regarding the recruitment of VASH1 and VASH2 to distinct cellular microtubules: Does the structure reveal if VASH1 and VASH2 could be recruited to different microtubule populations in the first place? For instance, if both enzymes are recruited to the same microtubule, wouldn't the global activity of VASH1 overwhelm the localized activity of VASH2? Further experiments are not required, but the authors could analyze both EM structures and check if:
(i) Sequence differences between different a-tubulin isoforms map on to the differential microtubule-binding surfaces of both proteins.

To address this important comment, we have made alignments of alpha-tubulin isoforms to examine the residue conservation of the VASH1- and VASH2-binding sites (see below). The alpha-tubulin

residues involved in VASH1 binding are well conserved with the exception of TUBAL3 (residue changes H309L and V159G) and TUBA8 (residue change V159L). It appears possible that the change in TUBAL3 alpha-tubulin residue H309 to L will result in a loss of an electrostatic attractive interaction with VASH1 E271 leading to a weakened VASH1-microtubule complex formation. Nevertheless, TUBAL3 is the only alpha-tubulin isoform with a shorter C-terminus (12 less amino-acids than TUBA1A/B), that probably does not reach the active site of VASH1-SVBP enzyme. In addition, this isoform lacks the two required glutamates preceding the cleavable aromatic residue (Landskron et al, Science 2022). For these reasons, TUBAL3 is probably not concerned by VASH1-SVBP activity. For alpha-tubulin residues interfacing VASH2, strict conservation was observed suggestive of comparable VASH2 interactions with all alpha-tubulin isoforms. Altogether, we think that there is no obvious alpha-tubulin isoform preference for VASH-SVBP activity.




(ii) Would the microtubule-binding of both enzymes be sensitive to protofilament number, given that the binding sites straddle 2 protofilaments, and the binding orientation of both enzymes are different from each other?

This is an interesting idea. Based on structural considerations, we expect that increasing or decreasing the number of protofilaments slightly “closes” or “opens”, respectively, both the VASH1 and VASH2-binding sites because of a change in the relative angle between neighboring protofilaments. However, we found it difficult to estimate meaningfully whether or not such relatively small binding-site changes would differently affect the affinity of VASH1 and VASH2 for microtubules. This could in principle be tested experimentally using buffer conditions that promote the formation of microtubules with different protofilament numbers, which could be an interesting analysis for a future study.

3) Lines 302-304: "As the residues directly involved in microtubule-lattice binding are distinct and distal from the catalytic site, we may consider these as substrate recognition elements that confer specificity to the catalytic activity of the vasohibin family of tubulin carboxypeptidases."

Are the distinct microtubule-binding interfaces conserved among VASH1 homologs and among VASH2 homologs? Such surface conservation would make the claim about specificity in the whole family stronger.

We thank the reviewer for this suggestion. Based on the sequence alignment of VASH1 and VASH2 homologs, the microtubule-binding interfaces are well conserved across the phylogenetic tree and could certainly be viewed as supporting evidence for the specificity of VASH binding interfaces for

microtubules. Alignment among vertebrates is shown in Figure S4A and mentioned on Results page 10, lines 316-318 of the revised manuscript. We have also added an analysis of the isoelectric point of VASH's domains among vertebrates, which support the conserved and differential model of interaction of the two VASH-SVBP enzymes with microtubules (Figure S4B and Results page 13 lines 421-422).

4) Line 769: "The significance of ..... except for Figure 1C where the Conover-Iman test was employed." Figure 1C does not contain statistical significance.
Sorry for the mistake, it was for Figure 1G. This is now corrected in Material and Methods page 25 line 824.

5) Line 258: The resolution of VASH2-SVBP was in the range of 3.3-4.8 Å becoming progressively higher in regions that are located closer to the microtubule surface.
Use "better" instead of higher for more clarity.
Sentences have been changed to be clearer, see Results page 9, lines 257-260.

6) Line 188:
Use "Residence time" instead of run length
This is now corrected (Results page 7, line 190).

7) Line 653:Typo: "prepa"?
"Prepa" was changed to "prepared" (Page 21 line 673).

**Reviewer #3 (Comments to the Authors (Required)):**

In their paper "VASH1-SVBP and VASH2-SVBP generate different detyrosination profiles on microtubules", Ramirez-Rios, Choi, Sanyal et al. dissect the differential properties of the a-tubulin detyrosinating enzymes VASH1 and VASH2. Like many cytoskeletal proteins (and indeed many proteins in general), the VASH family of enzymes has multiple isoforms, the functional relevance of which is generally not clear. Using primarily in vitro TIRF assays and primary-structure function analysis, the authors find that VASH1 globally detyrosinates microtubules, while VASH2 locally detyrosinates regions of microtubules. This is intuitively correlated with the biophysical properties of the enzymes (diffusivity and binding kinetics), which are analyzed at the single molecule level. Interestingly, the authors find this differential activity is primarily determined by the divergent disordered N-termini of the proteins, which feature opposite charges that likely mediate distinct interactions with the negatively charged C-terminal "tails" of tubulin subunits.
The authors additionally provide a cryo-EM reconstruction of the VASH2-SVBP complex bound to microtubules, which is compared with a previously reported structure of VASH1-SVBP bound to microtubules. This provides additional insight into the differential binding poses of the catalytic cores which work in concert with the disordered termini to determine the overall functional properties of the enzymes. Finally, they also show differential activity in a cell-based assay, supporting the physiological relevance of their observations.
Overall, I found this to be a highly thorough and complete mechanistic story that elucidates isoform-specific functional properties in the VASH family, achieving a level of understanding of isoform specificity that is broadly important for making progress in the field. This sets the stage for future work examining the interplay of these enzymes in vivo. I believe the paper's overall significance and consistently strong technical quality makes it suitable for publication in the JCB, pending minor revisions as detailed below:

Minor points:

1) The permeabilized cell assays presented in Fig. 7 appear to be a single trial. These experiments need to be performed with the same level of rigor (multiple independent trials, with quantification) as all other data presented in the paper to be suitable for publication.
The experiment was already done two times at 50 pM, which we unfortunately did not mention in the original version of our manuscript. To better address this comment, we did two other trials both at 50 and 200 pM of enzyme and quantified the results obtained. The new data are presented as Figure 7B and described on page 16, lines 502-504 of our revised manuscript.

2) The authors quote an overall resolution of 3.1 Angstroms for their cryo-EM reconstruction. However, it is apparent from examining the local resolution map that is provided that this resolution assessment is primarily driven by the well-ordered microtubule lattice, which is not the primary subject of this study. For readers to have a better sense of the resolution of the VASH molecule, it would be appropriate for the authors to also calculate an FSC for just the VASH-SVBP region of the map using a mask covering this region, and additionally quote this resolution value in the main text. Furthermore, the authors should include a model-map FSC curve (assessed at FSC 0.5), which is currently standard practice in the field.
In agreement with this statement, we have calculated the FSC for the VASH-SVBP region and have quoted the resolution value in the main text (Results page 9, lines 277-279). The model-map FSC curve assessed at FSC 0.5 has been included in the revised Supplementary Figure S3F.

3) In the section "Tyrosination has a little [sic] effect on the interaction of VASH-SVBP complexes with microtubules", the authors examine VASH1-SVPB and VASH2-SVBP under different ionic conditions. While I understand they had to do this to be able to perform single-molecule studies, this makes comparing the properties of VASH1 and VASH2 dubious (line 223). I thus recommend removing this and focusing on the internal comparisons of VASH1/2 under different conditions.
We agree with the reviewer that this was a bit questionable and have reorganized the paragraph (Pages 7-8, lines 212-228).

4) Is there a reason the authors did not cleave the HIS tags from their proteins before performing assays? Since the VASH flexible termini play a prominent role, and poly-HIS tags will impact the charge distribution, this should at a minimum be mentioned as a caveat.
The sequence added at the C-terminus in all constructs (including core domains) is TRTRPLEQKLISEEDLAANDILHHHHHH, with a myc epitope and 6His. This fragment has a pI of 6.60 due to the negatively charged residues present in the myc epitope. The pI of 6His alone is 7.60, which is not very basic compared to the C-termini of the VASHs. Therefore, the presence of the added sequence does not change much the global pI of the VASH proteins. We have mentioned the pI of the added sequence including myc and 6His tags under Material and Methods section (page 18, lines 585-586).

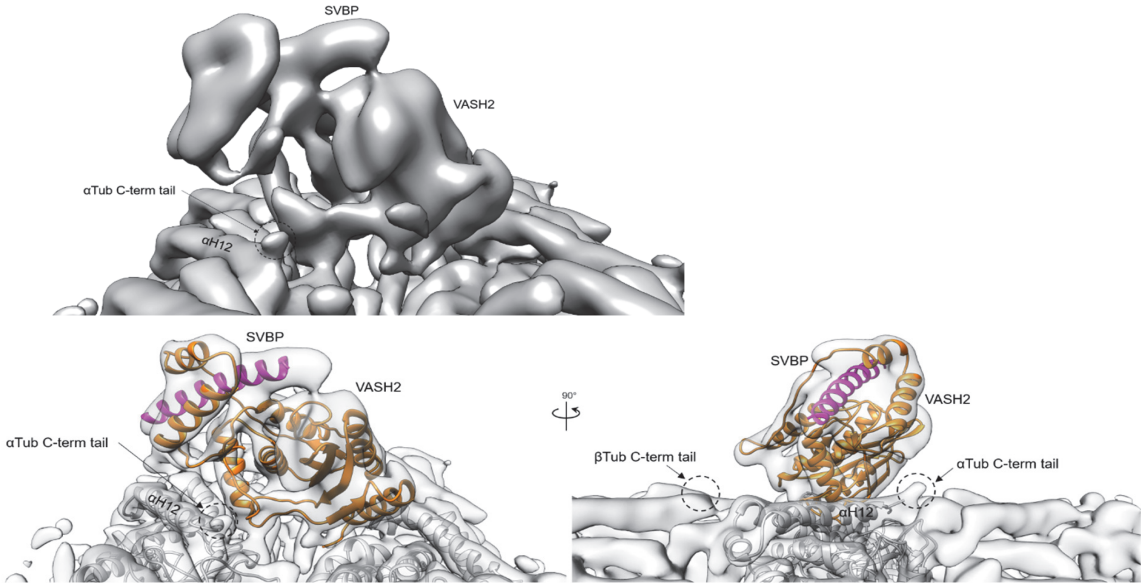
Other small issues:
5) Line 59 should be, I believe, "kinesin motors"
This is now corrected (Introduction page 3 line 60).

6) Line 128, sfGFP should be defined at first usage
This is now defined (Results page 5 line 128).

7) Line 186, "As the binding of microtubule-associated proteins to microtubules often involves electrostatic attractive interactions" should have a reference.
We have added references (Results page 7 line 188).

8) Lines 206-209, These sentences would be clearer if the order of tyrosinated, de-tyrosinated, and delta-2 tubulin was kept constant
This was corrected (Results page 7 line 210).

9) Li et al. were able to visualize the C-terminal tail of alpha-tubulin in their VASH1 reconstruction when they low-pass filtered their reconstruction to lower resolution (~7A). It would be useful to see if this is also the case for VASH2 presented here.
As suggested by the reviewer, we have applied a low-pass filter to our reconstruction. As highlighted in the figure panels below, we found a density corresponding to only a very short segment of the C-terminal tail of alpha-tubulin (notably not seen in beta-tubulin). We thus prefer not to draw any further conclusions concerning this alpha-tubulin segment.




10) Lines 361-364, "We thus examined the detyrosinating activity of VASH1 and VASH2 core domains...", this sentence and the following paragraph are confusing, as catalytically active and catalytically dead versions are presented in an interleaved fashion. This would be clearer if they were broken up into 2 separate paragraphs.
We agree with the reviewer that it could be more clear. As suggested, we have split this part into two separate paragraphs (Results page 12, lines 375-391).

11) Line 474, "Indeed, by exchanging these regions, we were able to switch the microtubule-binding behavior and activity of one enzyme to that of the other one." This sentence is not strictly accurate, as only one of the swaps worked. Please be precise.
We have clarified that the two swaps are working for microtubule binding and regarding activity only for VASH2 (Results page 15, lines 489-491).

12) Line 653, "prepa" should be "prepared"
We modified (Material and Methods page 21, line 673).

13) Lines 674-675, it is mentioned that new VASH2 and SVBP antibodies were generated in the course of this study. How was the specificity of these antibodies validated?
We have added sentences describing how peptide sequences were chosen and the antibodies tested (Material and Methods page 21, lines 693-700).

14) In the cryo-EM data collection section of the Methods, a "Hela sample" is mentioned. What does this mean?
The HeLa sample was explicitly mentioned to distinguish it from the bovine brain sample used for other experiments in our study. Because for the cryo-EM study we only used HeLa samples we removed "Hela" to exclude confusions.

15) Lines 719 - 721, The procedure for signal subtraction and the selection criteria used is not clear. Why would you subtract regions where VASH2 had bound? Please expand.
We now have better explained our image analysis procedure on Material and Methods page 23-24, lines 772-778 of the revised manuscript.

16) The authors mentioned fluorophore "quenching" several times in the manuscript, but I believe they mean fluorophore "bleaching".
We agree with the reviewer and changed quenching to bleaching (Results page 6, line 180 and page 13 line 415, Material and Methods page 24 line 806).

17) Line 770-771: Why was the Conover-Iman test used instead of Dunn's test in only one case?
We routinely used Dunn's test as implemented in GraphPad/Prism. In Figure 1G, based on the Kruskal-Wallis test, the null hypothesis of no difference between groups was clearly rejected. However the large gap between the VASH1 pair and the VASH2 pair causes the mean ranks of the two VASH1 to be relatively close to each other, even if the actual values differ. To avoid a type II error (false negative) in the post hoc pairwise comparison, we then turned to the Conover-Iman test, which is entirely relevant when the Kruskal-Wallis test rejects the null, and is known to be more powerful than the Dunn test. We could have used the Conover-Iman test for the other figures as well, but the Dunn's test was sufficient.
Please, note that the Conover-Iman test was also used in the new Figure 7B, for the same reason as in Figure 1G.
See the change in Material and Methods page 25 line 824.

18) For Figures where individual channels are presented in addition to a merge, it would be clearer if the individual channels were shown in black and white, as magenta in particular is difficult to see on its own (Fig. 1A; 5A; 6G; 7; S2A,C).
This has been addressed.

19) Figure 3: I recommend moving the color key to the top of the Figure so it is clearly associated with A onwards (I missed this at first look).
Done.

20) Figure S5: The colors should be explained in the figure or its legend, and residues should be labelled.

This has been addressed (see amended Figure S5).

_____

In addition, we have found errors in our original version that we have now corrected:

a) In Figure 1E, image and percentage of microtubule coverage have been corrected (see also Results page 6, line 168).
b) In Tables 1 and 3, there were errors in the link between the legend and the figures which are now corrected.

We sincerely apologize for these mistakes.

October 18, 2022

RE: JCB Manuscript #202205096R

Dr. Marie-Jo Moutin
Univ. Grenoble Alpes, Inserm, U1216, CNRS, CEA, Grenoble Institut Neurosciences
Rue F Ferrini
Grenoble 38000
France

Dear Dr. Moutin,

Thank you for submitting your revised manuscript entitled "VASH1-SVBP and VASH2-SVBP generate different detyrosination profiles on microtubules." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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2) Figures: Articles may have up to 10 main text figures. Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add MW markers to all blots in Figures 3D & S1B/C as well as scale bars to Fig. 1C and inset magnifications in S3G.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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5) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial please add a reference citation if possible.

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c. Temperature
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e. Fluorochromes
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g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations

involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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9) Video legends: Should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

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Sincerely,

Tarun Kapoor, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

No further suggestions - thank you for the revised manuscript.

Reviewer #3 (Comments to the Authors (Required)):

In their revision, Ramirez-Rios et al. have comprehensively addressed my comments, as well as those of the other reviewers. This strengthened an already outstanding manuscript, and I believe this should be accepted for publication without further delay.