Non-catalytic allostery in α-TAT1 by a phospho-switch drives dynamic microtubule acetylation

Abhijit Deb Roy, Evan Gross, Gayatri Pillai, Shailaja Seetharaman, Sandrine Etienne-Manneville, and Takanari Inoue

Corresponding Author(s): Takanari Inoue, Johns Hopkins Medicine and Abhijit Deb Roy, Johns Hopkins University

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Monitoring Editor: Arshad Desai

Scientific Editor: Dan Simon

Transaction Report:

Revision 0

Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this study, the authors investigated the regulation of alpha-tubuin acetyltransferase.

3. Significance:

Significance (Required)

There is very little known about the regulation of alpha-tubuin acetyltransferase, and the results from this study showed that a phosphorylation-dependent nuclear export plays a critical role. The data is very clear and convincing. Overall, I think that the study is significant and well organized. I would suggest acceptance of publication of this study in an appropriate journal.

I would suggest to add time-dependent dynamic of LMB treatment and demonstrate the specificity of the antibody used for immunofluorescence (such as showing that it detects only a single band of the enzyme from extracts of the cells used).

The authors could also discuss whether such a mechanism is covered from worm to humans (e.g. by showing sequence conservation at the phosphorylation sites, NES and NLS).

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

In this manuscript, Roy et al propose a novel regulation of α-TAT1 and its activity through dynamic intracellular localization. The authors report signal motifs on the disordered C-terminus of α-TAT1 comprising of putative NES and NLS sites which govern the cellular localization. Using immunofluorescence, inhibitors and mutational analysis, the authors describe phosphorylation/dephosphorylation mediated regulation of these sites by CDK1, PKA and CK2 kinases and PP2A phosphatase. Finally, the authors suggest localization-mediated regulation of α-TAT1 governing cell cycle and DNA damage response. Although the proposed spatiotemporal regulation of α-TAT1 is an interesting finding, several concerns arise regarding the analysis and interpretation of the data.

Major points:

1)As cytosolic signal seem to be heterogenous within the representative images, quantification of α-TAT1 signal close to the perinuclear region might underestimate the nuclear enrichment phenotypes. Hence, a more precise way would be to quantify the whole cytoplasmic and nuclear signal to extract the nuclear/cytoplasmic ratios.

2)Although, quantification data shows that treatment with Exportin-1 inhibitor (LMB) results in enrichment of α-TAT1 in the nucleus, the representative images do not show any changes in the cytosolic α-TAT1 levels. Hence, to support the reduction in acetylated tubulin levels, the authors should show a corresponding reduction in total cytoplasmic levels. One way to show it could be using live-cell imaging before and after the addition of drug.

3)In figure 3b-d, although the catalytic dead mutant does not show any loss of nucleus exclusion as stated by the authors, it is clearly retained more in the cytoplasm compared to the wild-type. Concurrently, the catalytic domain alone is enriched in the nucleus and the C-terminus alone remains in cytoplasm. Furthermore, even after inhibition of exportin-1 using LMB, more of the C-terminal construct signal is in the cytoplasm than the nucleus. Taken together, the data implies a strong correlation between α-TAT1's catalytic activity and its nuclear localization which is left unexplored and should be discussed. Also, a representative image for the catalytic dead mutant is missing and should be presented as well.

4)Kinase pathways and subsequently their inhibition can have diverse impact on cellular functions. Thus the data regarding phospho-regulation of nuclear localization should be

strengthen, as in the current form they do not fully support the mechanism proposed by the authors.

-To support the main findings of this work, it would be important to show that α-TAT1 is indeed phosphorylated at the suggested sites either by in vitro phosphorylation assays or Phos-tag gel using appropriate mutant controls.

-As shown for exportin-1, the authors should show α-TAT1 interaction with importin and the interaction being regulated by phosphorylation using inhibitors and/or mutant constructs.

-The authors should discuss the proposed phosphorylation site near the NLS in the context of the known recognition sequences of the kinases proposed to phosphorylate α-TAT1.

5)In figure 5f, 5h and 5m, it would be helpful to provide the a-tubulin staining's of the corresponding images. Also, the authors should clarify why the basal acetylated tubulin/ α-tubulin ratio is much lower than what was reported in figure 2.

6)A recent preprint study (Vit G et al., 2021, Biorxiv) has reported that iHAP1 is not a PP2A activator, but a microtubule poison instead. Thus, the authors should consider showing a more direct evidence of dephosphorylation by PP2A to support the proposed mechanism.

7)The proposed model suggests that interaction of α-TAT1 with 14-3-3 should inhibit its interaction with importin, thereby limiting its nuclear localization. To substantiate this claim, the authors could complement the interaction analysis with pulldown assays showing the interaction between α-TAT1 and importin in the presence and absence of 14-3-3.

Minor points:

•The authors show that α-TAT1 localization is dynamic in nature and its nuclear localization changes temporally. As cell cycle-dependent nuclear localization was previously reproted by Nekooki Machida Y et al., 2018, the authors should discuss how their phosophorylation/dephosphorylation mediated regulation model fits with the previous study.

•Although the authors have sorted the rescue cells for comparable expression levels of different constructs, the images in supplementary figure S13 show some cells lacking mVenus signal in addition to different expression levels. It would be helpful to include FACS plots/western blots to compare the expression levels with the wild-type and mutant constructs along with the total acetylated tubulin levels.

•Previous report (Ryu N M et al., 2020) on α-TAT1's role on cell cycle and DNA damage showed that the catalytic activity (acetylation of microtubules) was important for both cell cycle regulation and DNA damage response. It would be helpful to discuss why the rescue with ST/A mutant which is able to restore acetylated tubulin levels comparable to that of the wild-type still has defects in both cell proliferation and DNA damage response.

•Several typos/mistakes should be corrected, e.g.:

Line 118-119, 556-558: repetitive statements
Line 146, 184, and 194: Wrong/missing figures referenced
Line 210: missing reference to the figure
Supplementary figure S4a: inconsistent coloring.

•The representative images shown for diffused and nucleus-enriched localization in Figure1b looks similar and does not show clear enrichment.

•Figure 3f and g should contain labeling for C-terminal construct.

•It would be helpful to show the nuclear/cytoplasmic ratio of α-TAT1 parallel experiments corresponding to the Figure 5b-e data.

•For the rescue experiments in Figure S12, the authors should add the mVenus channel images to differentiate between transfected and non-transfected cells.

3. Significance:

Significance (Required)

This study investigated the regulation of α-TAT1 function by characterizing its localization pattern. The authors identified novel NES and NLS in the disordered C-terminal region of α-TAT1 and propose a model where the NES and phospho-inhibited NLS regulate the dynamic localization and function of α-TAT1. Although this finding is interesting, the manuscript would benefit of adding additional data to better support the proposed model. A direct evidence for phosphorylation by the suggested kinases at the proposed sites would greatly increase the relevance of the data and would highly strengthen the proposed model. Data corresponding to α-TAT1 interaction with 14-3-3 is novel and interesting.

Revision Plan

Manuscript number: RC-2021-01133
Corresponding author(s): Takanari Inoue; Abhijit Deb Roy

1. General Statements

We thank the reviewers for their thoughtful and detailed comments. Both reviewers have commented on the significance and novelty of this study. Our goal here was to identify how α-TAT1 is regulated to dynamically mediate microtubule acetylation. Despite the long-term efforts, there are only a few reports on regulation of the catalytic activity of this enzyme, and it remains unknown as to how α-TAT1 achieves microtubule acetylation at the right place and the right time. With excitement, we have shown that it is intracellular localization of α-TAT1 that plays a key role in its function. We have further discovered a phospho-regulated nuclear transport motif in its intrinsically disordered C-terminus which allosterically, albeit non-catalytically, mediates α-TAT1 function downstream of specific kinases and phosphatases.

We have provided our responses to the reviewers' comments and revision plans below. Reviewers' comments are in bold. Underlines highlight specific action plans.

2. Description of the planned revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this study, the authors investigated the regulation of alpha-tubulin acetyltransferase.

Reviewer #1 (Significance (Required)):

There is very little known about the regulation of alpha-tubulin acetyltransferase, and the results from this study showed that a phosphorylation-dependent nuclear export plays a critical role. The data is very clear and convincing. Overall, I think that the study is significant and well organized. I would suggest acceptance of publication of this study in an appropriate journal.

I would suggest to add time-dependent dynamic of LMB treatment and demonstrate the specificity of the antibody used for immunofluorescence (such as showing that it detects only a single band of the enzyme from extracts of the cells used).

Both temporal changes in α-TAT1 localization upon LMB treatment and α-TAT1 antibody specificity have been shown in the original manuscript (**Fig.S6a, b** and **Fig. S4c**, respectively). The way we described these important data may have been insufficiently explicit to readers. Therefore, <u>we will improve the corresponding main text to make these points clear.</u>

Revision Plan

The authors could also discuss whether such a mechanism is covered from worm to humans (e.g. by showing sequence conservation at the phosphorylation sites, NES and NLS).

This is an important point as sequence conservation across animal species often implies the physiological significance of a phenomenon of interest. Thus, we performed sequence alignment of human and *C. elegans* α-TAT1 (Fig. A). We observed similarity in the N-terminal catalytic domain, but not so much in the C-terminal region. In particular, the worm α-TAT1 was predicted to have three 14-3-3 binding sites (red boxes), which may enable a similar regulatory role of spatial control of α-TAT1 function in worms as we have proposed. Interestingly, the worm α-TAT1 lacks the NES as well as NLS motif (underlined in black in the human sequence in Fig A), suggesting that regulation of worm α-TAT1 is less sophisticated than that of human. <u>We will discuss this analysis in Discussion section.</u>
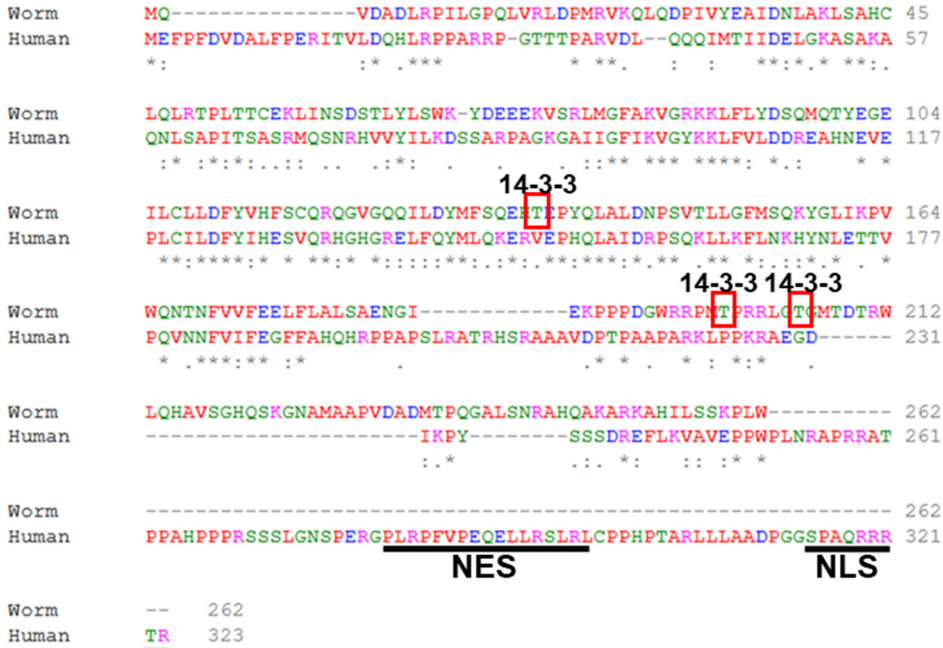

Fig A. Sequence alignment of human and C. elegans (worm) α-TAT1

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary:

In this manuscript, Roy et al propose a novel regulation of α-TAT1 and its activity through dynamic intracellular localization. The authors report signal motifs on the disordered C-terminus of α-TAT1 comprising of putative NES and NLS sites which govern the cellular localization. Using immunofluorescence, inhibitors and mutational analysis, the authors describe phosphorylation/dephosphorylation mediated regulation of these sites by CDK1, PKA and CK2 kinases and PP2A phosphatase. Finally, the authors suggest localization-mediated regulation of α-TAT1 governing cell cycle and

Revision Plan

DNA damage response. Although the proposed spatiotemporal regulation of α-TAT1 is an interesting finding, several concerns arise regarding the analysis and interpretation of the data.

Major points:

1)As cytosolic signal seem to be heterogenous within the representative images, quantification of α-TAT1 signal close to the perinuclear region might underestimate the nuclear enrichment phenotypes. Hence, a more precise way would be to quantify the

We agree with the reviewer that the cytosolic signal is heterogeneous, probably due to differences in cell morphology. That is the very reason why we have performed and presented three different analyses for all pertinent experiments in the original manuscript for evaluation of intracellular localization of α-TAT1. These are namely ratiometric, categorical and cross-correlational analyses. While the ratiometric analysis is expected to be subjective to a region of interest, the categorical and cross-correlational analyses are not, since the latter two account for "*the whole cell*". Importantly, we obtained consistent results among these three analyses throughout our work. <u>We will clarify in the main text that the two of our three analyses concern whole cells, thus non-subjective to heterogeneous cytosolic signals</u>.

Nevertheless, we became curious to test if and how the ratiometric analysis indicates difference between global whole cell (*WholeCell*) and local region of interest (*ROI*) (Fig. B). Here, we measured fluorescence intensity of mVenus-α-TAT1 in 24 single cells. Consistent with the reviewer's comment, the absolute values from the whole cell were higher than those from ROI-based analysis. However, their pattern of distribution showed a high level of correlation (0.908, right panel), indicating that the ROI-based ratiometric analysis is a valid parameter. <u>We will show Fig. B in the supplementary along with discussions.</u>
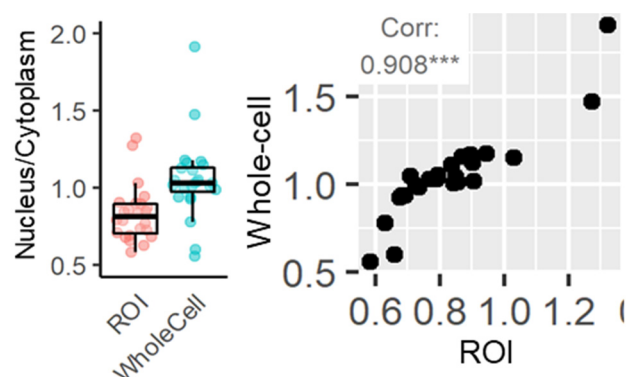

Fig B. Comparison of nucleus/Cytoplasm ratio based on ROI or whole nuclear and cytoplasmic signal (WholeCell).

2)Although, quantification data shows that treatment with Exportin-1 inhibitor (LMB) results in enrichment of α-TAT1 in the nucleus, the representative images do not show any changes in the cytosolic α-TAT1 levels. Hence, to support the reduction in acetylated tubulin levels, the authors should show a corresponding reduction in total cytoplasmic levels. One way to show it could be using live-cell imaging before and after the addition of drug.

Revision Plan

Temporal changes in mVenus-α-TAT1 on LMB treatment is shown in **Fig. S6a, b**. We agree that the representative images do not adequately visualize the changes in cytosolic fraction of α-TAT1. To better show the temporal changes in cytosolic fraction on LMB treatment, <u>we will add a pseudo-colored time-lapse image as shown below in Fig C. We will also quantify the temporal changes in cytosolic and nuclear fraction. We will also add a timelapse video of changes in mVenus-α-TAT1 localization with LMB treatment.</u>
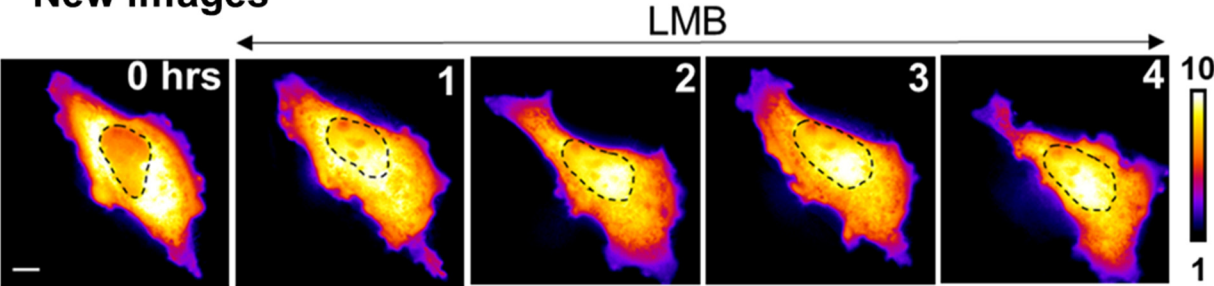
Current figure
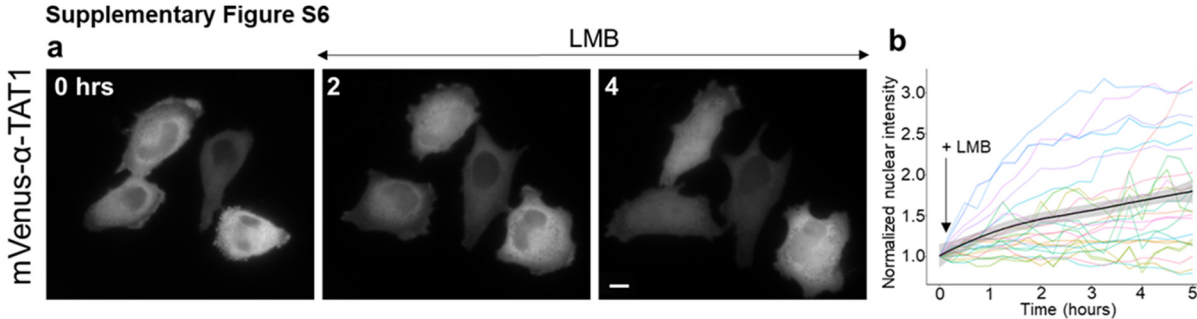
Fig C. Changes in mVenus-α-TAT1 localization on LMB treatment.

3) In figure 3b-d, although the catalytic dead mutant does not show any loss of nucleus exclusion as stated by the authors, it is clearly retained more in the cytoplasm compared to the wild-type. Concurrently, the catalytic domain alone is enriched in the nucleus and the C-terminus alone remains in cytoplasm. Furthermore, even after inhibition of exportin-1 using LMB, more of the C-terminal construct signal is in the cytoplasm than the nucleus. Taken together, the data implies a strong correlation between α-TAT1's catalytic activity and its nuclear localization which is left unexplored and should be discussed. Also, a representative image for the catalytic dead mutant is missing and should be presented as well.
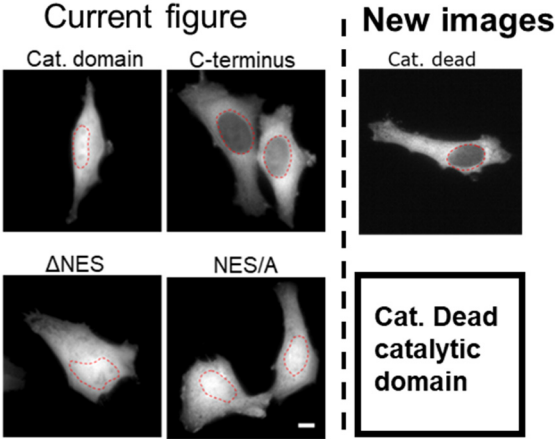

Fig D. mVenus-α-TAT1 domain localization

We will add a representative image for the D157N (catalytically dead mutant) as shown in Fig D (right panels). We agree with the reviewer that there may be a correlation between the catalytic activity and nuclear exclusion of α-TAT1. To address this point, we will compare intracellular distribution of WT catalytic domain and D157N catalytic domain (catalytically dead mutant) and add to the discussion.

4)Kinase pathways and subsequently their inhibition can have diverse impact on cellular functions. Thus the data regarding phospho-regulation of nuclear localization should be strengthen, as in the current form they do not fully support the mechanism proposed by the authors.

-To support the main findings of this work, it would be important to show that α-TAT1 is indeed phosphorylated at the suggested sites either by in vitro phosphorylation assays or Phos-tag gel using appropriate mutant controls.

To confirm phosphorylation of T322, we will perform western blots with Phos-tag gels with WT, T322A and ST/A mutants. Additionally, there is an antibody available against PKA substrates (RRXS*/T*), which matches the NLS sequence SPAQ**RRRT***. We will perform immunoprecipitation of mVenus-α-TAT1 (WT and T322A) with mVenus antibody and use the above phospho-specific antibody to confirm phosphorylation of T322 residue. If further needed, we will perform mass spectrometry to confirm phosphorylation of T322 residue.

-As shown for exportin-1, the authors should show α-TAT1 interaction with importin and the interaction being regulated by phosphorylation using inhibitors and/or mutant constructs.
We will perform co-IP of WT and ST/A mutant and test for importin binding. We will also perform live cell protein-protein interaction assay these mutants and importins.

-The authors should discuss the proposed phosphorylation site near the NLS in the context of the known recognition sequences of the kinases proposed to phosphorylate α-TAT1.
We will discuss phospho-regulation of the proposed NLS in the context of PKA, CDK1 and CK2 consensus recognition motifs along the lines as follows. T322 site (SPAQRRR**T***) matches the known recognition sites for PKA (RRXS*/T*). S319 matches the consensus motifs for CDK1 substrate S*/T*P and S*/T*XXR/K. Neither S319 nor T322 match CK2 consensus sequence S*/T*DXE. It is possible that CDK1 and CK2 facilitate phosphorylation of T322 by PKA or other kinases. Nevertheless, our data clearly show a role of CDK1 and CK2 in regulating α-TAT1 localization and function.

5)In figure 5f, 5h and 5m, it would be helpful to provide the a-tubulin staining's of the corresponding images.
We will add the corresponding α-Tubulin staining images for Fig. 5f, 5h and 5m.

Also, the authors should clarify why the basal acetylated tubulin/ α-tubulin ratio is much lower than what was reported in figure 2.
This is because the immunofluorescence assays for 5f, 5h and 5m were performed with secondary antibodies with different fluorophores than those for Fig.2. Additionally, the imaging conditions (illumination settings, exposure times etc) were not constant across experiments grouped differently. These conditions were constant in the data grouped together where the drug treatment, immunostaining and imaging were performed in parallel and thus may be compared.

6)A recent preprint study (Vit G et al., 2021, Biorxiv) has reported that iHAP1 is not a PP2A activator, but a microtubule poison instead. Thus, the authors should consider showing a more direct evidence of dephosphorylation by PP2A to support the proposed mechanism.
To circumvent possible off-target effects of iHAP1, we have already assessed involvement of PP2A using an alternative approach; overexpression of PP2CA (PP2A catalytic domain) was sufficient to promote nuclear localization of α-TAT1 (Fig. 5i, Supplementary Fig.S10b).

7)The proposed model suggests that interaction of α-TAT1 with 14-3-3 should inhibit its interaction with importin, thereby limiting its nuclear localization. To substantiate this claim, the authors could complement the interaction analysis with pulldown assays showing the interaction between α-TAT1 and importin in the presence and absence of 14-3-3.
We will perform a co-IP assay to detect binding between α-TAT1 and importins in the presence of pan-14-3-3 inhibitor R18, which inhibits nuclear exclusion of α-TAT1 as shown in Fig. 6b, c. If warranted, we will explore using Difopein (dimerized form of R18), a more potent inhibitor of 14-3-3s in these co-IP assays.

Minor points:

•The authors show that α-TAT1 localization is dynamic in nature and its nuclear localization changes temporally. As cell cycle-dependent nuclear localization was previously reproted by Nekooki Machida Y et al., 2018, the authors should discuss how their phosophorylation/dephosphorylation mediated regulation model fits with the previous study.
Since we observed differences in cell proliferation between WT and α-TAT1 KO cells, it stands to reason that α-TAT1 may regulate cell cycle or be differentially regulated in a cell-cycle dependent manner. While we have already cited the work on cell cycle dependent nuclear localization of α-TAT1 by Nekooki-Machida, Y. et al, we will add more discussion on their findings and ours.

•Although the authors have sorted the rescue cells for comparable expression levels of different constructs, the images in supplementary figure S13 show some cells lacking mVenus signal in addition to different expression levels. It would be helpful to include

Revision Plan

FACS plots/western blots to compare the expression levels with the wild-type and mutant constructs along with the total acetylated tubulin levels.
We will add western blots to compare expression levels of the rescue constructs and the corresponding microtubule acetylation levels.

•**Previous report (Ryu N M et al., 2020) on α-TAT1's role on cell cycle and DNA damage showed that the catalytic activity (acetylation of microtubules) was important for both cell cycle regulation and DNA damage response. It would be helpful to discuss why the rescue with ST/A mutant which is able to restore acetylated tubulin levels comparable to that of the wild-type still has defects in both cell proliferation and DNA damage response.**
We thank the reviewer for the thoughtful observation. It is indeed possible that cell proliferation and DNA damage response is regulated by a non-catalytic action of α-TAT1, which is mediated by T322 or its associated 14-3-3 proteins. We will specify this in the Discussion.

•**Several typos/mistakes should be corrected, e.g.:**
Line 118-119, 556-558: repetitive statements
Line 146, 184, and 194: Wrong/missing figures referenced
Line 210: missing reference to the figure
Supplementary figure S4a: inconsistent coloring.
We will correct these mistakes.

•**The representative images shown for diffused and nucleus-enriched localization in Figure1b looks similar and does not show clear enrichment.**
We agree that the images do not show sufficient contrast between the diffused and the nucleus-enriched localization. We will use pseudo-colored images to highlight the differences.

•**Figure 3f and g should contain labeling for C-terminal construct.**
We will add a label specifying that fig 3f and 3g are about α-TAT1 C-terminus localization.

•**It would be helpful to show the nuclear/cytoplasmic ratio of α-TAT1 parallel experiments corresponding to the Figure 5b-e data.**
We will add the nuclear/cytoplasmic ratios.

•**For the rescue experiments in Figure S12, the authors should add the mVenus channel images to differentiate between transfected and non-transfected cells.**
We will add the corresponding mVenus images.

Reviewer #2 (Significance (Required)):

This study investigated the regulation of α-TAT1 function by characterizing its localization pattern. The authors identified novel NES and NLS in the disordered C-terminal region of α-TAT1 and propose a model where the NES and phospho-inhibited

NLS regulate the dynamic localization and function of α-TAT1. Although this finding is interesting, the manuscript would benefit of adding additional data to better support the proposed model. A direct evidence for phosphorylation by the suggested kinases at the proposed sites would greatly increase the relevance of the data and would highly strengthen the proposed model. Data corresponding to α-TAT1 interaction with 14-3-3 is novel and interesting.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Upon receiving the reviewers' comments, we obtained new data and analyses. Instead of integrating them into the original manuscript, we chose to show them in this Revision Plan file as Figures A, B, C and D, for convenience of editors and reviewers. Therefore, no change is applied to the manuscript files.

4. Description of analyses that authors prefer not to carry out

April 7, 2022

Re: JCB manuscript #202202100T

Dr. Takanari Inoue
Johns Hopkins Medicine
855 N. Wolfe st.,
Rangos 476
Baltimore, MD 21205

Dear Dr. Inoue,

Thank you for submitting your manuscript entitled "Non-catalytic allostery in α-TAT1 by a phospho-switch drives dynamic microtubule acetylation." The manuscript has now been assessed by an expert reviewer, whose comments are appended to this letter. We invite you to submit a revision as outlined in your revision plan along with addressing the comments of Reviewer #3.

You will see that this reviewer finds your work interesting and suitable for JCB but also feels, and we agree, that the data linking nuclear localized α-TAT1 to proliferation and DNA damage response is not conclusive and suggests removing this from the manuscript. We also believe that more definitive insight into the non-catalytic roles of α-TAT1 in the nucleus would significantly enhance the impact of the study and would encourage you to add such data if you already have it or can do experiments in a reasonable timeframe.

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. Transfers 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,

Arshad Desai, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

Overall, this is an interesting paper and the reviewer comments are thoughtful and complete. In addition to the current reviewer suggestions, I have the following comments:

1) The authors frequently use the Pearson's R coefficient value to evaluate co-localization. While a Pearson's R value above threshold is reported, it is not clear exactly what this means, and whether there is a statistically significant cross-correlation between the evaluated signals. The use of this metric should be more clearly explained and justified.

2) Arguments regarding the importance of the described results regarding cell proliferation and the DNA damage response, and the results in Fig. 7, are not overly convincing. In addition, as noted by Rev #2, defects in cell proliferation and DNA damage response still occur in the ST/A mutant, even though it is able to restore acetylated tubulin levels to those comparable to that of the wild-type. While it is possible that cell proliferation and DNA damage response is regulated by a non-catalytic action of α-TAT1, this discussion is relatively speculative. My suggestion may be to remove Fig. 7 from the current manuscript, and save this data for another manuscript, in which the functional consequences for the cell of the currently described results can be more completely and specifically explored and described.

We thank all the reviewers for the constructive comments to improve our manuscript. Of note, we have reorganized the figures. For example, we consolidated supplementary items to five figures to conform to the journal guidelines. We also removed the original figure 7 where we characterized possible physiological roles of the aTAT1 regulation, based on a suggestion by one of the reviewers as well as by the editor. To circumvent possible confusion, we italicized the old figure numbers in this document, while new ones are shown in bold.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):
In this study, the authors investigated the regulation of alpha-tubulin acetyltransferase.
Reviewer #1 (Significance (Required)):

There is very little known about the regulation of alpha-tubulin acetyltransferase, and the results from this study showed that a phosphorylation-dependent nuclear export plays a critical role. The data is very clear and convincing. Overall, I think that the study is significant and well organized. I would suggest acceptance of publication of this study in an appropriate journal.

The authors are thankful for this reviewer who acknowledges the significance of our study.

I would suggest to add time-dependent dynamic of LMB treatment and demonstrate the specificity of the antibody used for immunofluorescence (such as showing that it detects only a single band of the enzyme from extracts of the cells used).

We understood that the reviewer suggests two separate experiments here. Although both temporal changes in α-TAT1 localization upon LMB treatment (*Fig.S6a, b*) and α-TAT1 antibody specificity based on immunofluorescence (*Fig. S4c*) were demonstrated in the original manuscript, this reviewer's comment made us realize that we need to describe these data better. The previous grayscale images (*Fig.S6a*) may not have sufficiently highlighted the changes in α-TAT1 localization on LMB treatment. So, we have added pseudo-coloured images (**Fig. 4h**) and moved their quantification to the main figure (**Fig.4i**). We have also explicitly stated the lack of α-TAT1 antibody staining for the KO cells (**Fig. S2e**) in the main text (**line 100**).

The authors could also discuss whether such a mechanism is covered from worm to humans (e.g. by showing sequence conservation at the phosphorylation sites, NES and NLS).

We performed sequence alignment of human and *C. elegans* α-TAT1 (**Fig. S1F**). We observed similarity in the N-terminal catalytic domain, but not in the C-terminal region. In particular, the worm α-TAT1 was predicted to have three 14-3-3 binding sites (red boxes), which may enable a similar regulatory role of spatial control of α-TAT1 function in worms as we have proposed. Interestingly, the worm α-TAT1 lacks the NES as well as NLS motif (underlined in black in the human sequence in **Fig. S1F**), suggesting that regulation of worm α-TAT1 is less sophisticated than that of human. We have mentioned this in the main text (**line 83, 366**).

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary:

In this manuscript, Roy et al propose a novel regulation of α-TAT1 and its activity through dynamic intracellular localization. The authors report signal motifs on the disordered C-terminus of α-TAT1 comprising of putative NES and NLS sites which govern the cellular localization. Using immunofluorescence, inhibitors and mutational analysis, the authors describe phosphorylation/dephosphorylation mediated regulation of these sites by CDK1, PKA and CK2 kinases and PP2A phosphatase. Finally, the authors suggest localization-mediated regulation of α-TAT1 governing cell cycle and DNA damage response. Although the proposed spatiotemporal regulation of α-TAT1 is an interesting finding, several concerns arise regarding the analysis and interpretation of the data.

Major points:

1) As cytosolic signal seem to be heterogenous within the representative images, quantification of α-TAT1 signal close to the perinuclear region might underestimate the nuclear enrichment phenotypes. Hence, a more precise way would be to quantify the whole cytoplasmic and nuclear signal to extract the nuclear/cytoplasmic ratios.

We agree with the reviewer that the cytosolic signal is heterogeneous, probably due to differences in cell morphology. That is the very reason why we have performed and presented three different analyses for all pertinent experiments in the original manuscript for evaluation of intracellular localization of α-TAT1. These are namely ratiometric, categorical and cross-correlational analyses. While the ratiometric analysis is expected to be subjective to a region of interest, the categorical and cross-correlational analyses are not, since the latter two account for "*the whole cell*". Importantly, we obtained consistent results among these three analyses throughout our work. We have clarified in the main text that the two of our three analyses concern whole cells, thus non-subjective to heterogeneous cytosolic signals (**line 88**).

Nevertheless, we tested if and how the ratiometric analysis indicates a difference between global whole cell (*WholeCell*) and local region of interest (*ROI*) (**Fig. S2b**). Here, we measured fluorescence intensity of mVenus-α-TAT1 in 24 single cells. Consistent with the reviewer's comment, the absolute values from the whole cell were higher than those from ROI-based analysis. However, their pattern of distribution showed a high level of correlation (0.908, right panel), indicating that the ROI-based ratiometric analysis is a valid parameter.

2) Although, quantification data shows that treatment with Exportin-1 inhibitor (LMB) results in enrichment of α-TAT1 in the nucleus, the representative images do not show any changes in the cytosolic α-TAT1 levels. Hence, to support the reduction in acetylated tubulin levels, the authors should show a corresponding reduction in total cytoplasmic

levels. One way to show it could be using live-cell imaging before and after the addition of drug.

We originally showed temporal changes in mVenus-α-TAT1 on LMB treatment (old *Fig. S6a, b*). However, as pointed out by the reviewer, the representative images did not adequately highlight the changes in cytosolic fraction of α-TAT1, primarily due to limited intensity contrast in these greyscale images. To better highlight temporal changes in the cytosolic fraction on LMB treatment, we have replaced old *Fig.S6a* with a "pseudo-colored" time-lapse image in **Fig. 2h**. Accordingly, we have also moved the quantification data on nuclear intensity from the supplementary (old *Fig. S6b*) to main figure **Fig. 2i**

3)In figure 3b-d, although the catalytic dead mutant does not show any loss of nucleus exclusion as stated by the authors, it is clearly retained more in the cytoplasm compared to the wild-type. Concurrently, the catalytic domain alone is enriched in the nucleus and the C-terminus alone remains in cytoplasm. Furthermore, even after inhibition of exportin-1 using LMB, more of the C-terminal construct signal is in the cytoplasm than the nucleus. Taken together, the data implies a strong correlation between α-TAT1's catalytic activity and its nuclear localization which is left unexplored and should be discussed. Also, a representative image for the catalytic dead mutant is missing and should be presented as well.

To address this point, we compared intracellular distribution of WT catalytic domain and D157N catalytic domain (**Fig. 3b, h, i**) and discussed this point (**line 147**). In short, we did not see a significant difference between the localization of WT versus D157N catalytic domains.
A representative image of the catalytic dead mutant D157N has been added (**Fig. 3b**).

4)Kinase pathways and subsequently their inhibition can have diverse impact on cellular functions. Thus the data regarding phospho-regulation of nuclear localization should be strengthen, as in the current form they do not fully support the mechanism proposed by the authors.

-To support the main findings of this work, it would be important to show that α-TAT1 is indeed phosphorylated at the suggested sites either by in vitro phosphorylation assays or Phos-tag gel using appropriate mutant controls.

Using an antibody against PKA substrates with a consensus sequence of RRXS*/T* which match the αTAT1 NLS sequence SPAQ**RRRT***, we performed western blot with GFP-αTAT1 and GFP-αTAT1(T322A) (**Fig. 4f**), and with GFP-αTAT1 in the presence or absence of RO-3306, an inhibitor for CDK1/2 (**Fig. S4f**). Compared to WT, we observed decreased signal for T322A mutant, or with treatment with RO-3306, thus confirming phosphorylation of the T322 residue.

-As shown for exportin-1, the authors should show α-TAT1 interaction with importin and the interaction being regulated by phosphorylation using inhibitors and/or mutant

constructs.

We performed co-IP of WT and T322A mutant with KPNA2, an importin subtype, and observed increased association between the T322A mutant and KPNA2 as compared to WT (**Fig. 4g**). Using the same T322A mutant, we also conducted chemically-induced assays that can assess protein-protein interactions in living cells. As a result, we not only confirmed association with KPNA2 but also observed interaction with KPNA4 and KPNA6 (**Fig.4h, i, j**). These experiments are discussed in the main text (**line 207**).

-The authors should discuss the proposed phosphorylation site near the NLS in the context of the known recognition sequences of the kinases proposed to phosphorylate α-TAT1.

As suggested, we discussed phospho-regulation of the proposed NLS in the context of PKA, CDK1 and CK2 consensus recognition motifs (**line 371**), which is copied below:
"Our data demonstrate that cytosolic localization of α-TAT1 is mediated by kinase and phosphatase action, possibly on Threonine-322 and Serine-315. T322 site (SPAQRRRT*R) matches the known substrate recognition site for PKA (RRXS*/T*), whereas S319 matches the consensus motif for CDK1 substrate (S*/T*P or S*/T*XXR/K). Neither S319 nor T322 match the consensus motif for CK2 substrate (S*/T*DXE). However, CK2 has been recently reported to bind to the C-terminus of α-TAT1, phosphorylate S236 and regulate its activity (You *et al.*, 2022). Nevertheless, our data demonstrate a role of CDK1 and CK2 in regulating α-TAT1 localization. It is possible that these kinases indirectly facilitate phosphorylation of T322 through PKA or some other kinase."

5)In figure 5f, 5h and 5m, it would be helpful to provide the a-tubulin staining's of the corresponding images.

We added corresponding α-Tubulin images (**Fig. 5f, 5h and 5m**).

Also, the authors should clarify why the basal acetylated tubulin/ α-tubulin ratio is much lower than what was reported in figure 2.

There were differences in the experimental conditions that could explain the ratio differences. These include secondary antibodies labeled with different fluorophores, as well as parameters for fluorescence microscopy (excitation light intensity, exposure time, etc.). Of note, the experimental conditions were kept identical among the data grouped together. We clarified these points in the main text (**line 272**).

6)A recent preprint study (Vit G et al., 2021, Biorxiv) has reported that iHAP1 is not a PP2A activator, but a microtubule poison instead. Thus, the authors should consider showing a more direct evidence of dephosphorylation by PP2A to support the proposed mechanism.

We thank the reviewer for bringing this latest information about the iHAP1 target specificity to our attention. In our original manuscript, we have assessed involvement of PP2A not only with iHAP1 but also with constitutively active PP2A (PP2CA, a PP2A catalytic domain) (*Fig. 5l, Fig. S10b*) where overexpression of PP2CA was sufficient to promote nuclear localization of α-TAT1. We believe that the experiment with PP2CA has complemented the iHAP1 experiment, despite the concern about its target specificity. Therefore, we kept these data as they were (now **Fig. 5l, S4j**), but also decided to cite the preprint in the main text (**line 286**).

7)The proposed model suggests that interaction of α-TAT1 with 14-3-3 should inhibit its interaction with importin, thereby limiting its nuclear localization. To substantiate this claim, the authors could complement the interaction analysis with pulldown assays showing the interaction between α-TAT1 and importin in the presence and absence of 14-3-3.

As suggested, we performed a co-IP assay and observed increased binding between αTAT1 and KPNA2 (one of the importin subtypes) in the presence of a pan-14-3-3 inhibitor, Difopein (**Fig.6j**). Independently, Difopein has been confirmed to inhibit nuclear exclusion of α-TAT1 (**Fig.6d**). Using the live-cell protein-protein interaction assay, we observed increased association of KPNA2 and KPNA6 with GFP-αTAT1 in the presence of Difopein (**Fig. 6k, S5d**).

Minor points:

•The authors show that α-TAT1 localization is dynamic in nature and its nuclear localization changes temporally. As cell cycle-dependent nuclear localization was previously reproted by Nekooki Machida Y et al., 2018, the authors should discuss how their phosophorylation/dephosphorylation mediated regulation model fits with the previous study.

Since we observed differences in cell proliferation between WT and α-TAT1 KO cells (old *Fig. 7b*), it stands to reason that α-TAT1 may regulate cell cycle or be differentially regulated in a cell-cycle dependent manner. While we have already cited the work on cell cycle dependent nuclear localization of α-TAT1 by Nekooki-Machida, Y. et al, we added more discussion (**line 378**).

•Although the authors have sorted the rescue cells for comparable expression levels of different constructs, the images in supplementary figure S13 show some cells lacking mVenus signal in addition to different expression levels. It would be helpful to include FACS plots/western blots to compare the expression levels with the wild-type and mutant constructs along with the total acetylated tubulin levels.

We added western blots to compare expression levels of the rescue constructs and the corresponding microtubule acetylation levels (**Fig 4k, S3g**).

•**Previous report (Ryu N M et al., 2020) on α-TAT1's role on cell cycle and DNA damage showed that the catalytic activity (acetylation of microtubules) was important for both cell cycle regulation and DNA damage response. It would be helpful to discuss why the rescue with ST/A mutant which is able to restore acetylated tubulin levels comparable to that of the wild-type still has defects in both cell proliferation and DNA damage response.**

Thanks for the thoughtful comments. Our results may indicate that cell proliferation and DNA damage response are regulated primarily by a non-catalytic action of α-TAT1, rather than by the catalytic action. While it is quite intriguing and important to experimentally explore their relative contributions, and/or to discuss their functional significance, we have decided to remove these data per suggestion by Reviewer #3 and the editor.

•**Several typos/mistakes should be corrected, e.g.:**
Line 118-119, 556-558: repetitive statements (Line 129, 641 in current manuscript)
Line 146, 184, and 194: Wrong/missing figures referenced
Line 210: missing reference to the figure
Supplementary figure S4a: inconsistent coloring.

We have corrected these errors. Please note that the line numbers are different in the revised manuscript.

•**The representative images shown for diffused and nucleus-enriched localization in Figure1b looks similar and does not show clear enrichment.**

We agree that the original images do not show sufficient contrast between the diffused and the nucleus-enriched localization. We thus added a different cell image that better represents the nucleus-enriched αTAT1.

•**Figure 3f and g should contain labeling for C-terminal construct.**

We have added a label specifying that Fig. 3f and 3g are about α-TAT1 C-terminus localization.

•**It would be helpful to show the nuclear/cytoplasmic ratio of α-TAT1 parallel experiments corresponding to the Figure 5b-e data.**

We have added the nuclear/cytoplasmic ratios (**Fig. S4.c, d, e, g**).

•**For the rescue experiments in Figure S12, the authors should add the mVenus channel images to differentiate between transfected and non-transfected cells.**

We have added mVenus images corresponding to the acetylated MT images (**Fig. S3i**).

Reviewer #2 (Significance (Required)):

This study investigated the regulation of α-TAT1 function by characterizing its localization pattern. The authors identified novel NES and NLS in the disordered C-terminal region of α-TAT1 and propose a model where the NES and phospho-inhibited NLS regulate the dynamic localization and function of α-TAT1. Although this finding is interesting, the manuscript would benefit of adding additional data to better support the proposed model. A direct evidence for phosphorylation by the suggested kinases at the proposed sites would greatly increase the relevance of the data and would highly strengthen the proposed model. Data corresponding to α-TAT1 interaction with 14-3-3 is novel and interesting.

Thank you for noting the novelty of our findings. We believe that we have now provided several lines of new evidence that strongly support our original findings of the phospho-regulated α-TAT1 localization.

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

Overall, this is an interesting paper and the reviewer comments are thoughtful and complete. In addition to the current reviewer suggestions, I have the following comments:

1) The authors frequently use the Pearson's R coefficient value to evaluate co-localization. While a Pearson's R value above threshold is reported, it is not clear exactly what this means, and whether there is a statistically significant cross-correlation between the evaluated signals. The use of this metric should be more clearly explained and justified.

We have added discussion on Pearson's R coefficient in the methods section (**line 620**). We have also cited the review article that we used as a reference for colocalization analysis (Dunn, Kamocka and McDonald, 2011). Pearson's R values are amenable to Student's T-tests, which we have used in this manuscript.

2) Arguments regarding the importance of the described results regarding cell proliferation and the DNA damage response, and the results in Fig. 7, are not overly convincing. In addition, as noted by Rev #2, defects in cell proliferation and DNA damage response still occur in the ST/A mutant, even though it is able to restore acetylated tubulin levels to those comparable to that of the wild-type. While it is possible that cell proliferation and DNA damage response is regulated by a non-catalytic action of α-TAT1, this discussion is relatively speculative. My suggestion may be to remove Fig. 7 from the current manuscript, and save this data for another manuscript, in which the functional consequences for the cell of the currently described results can be more completely and specifically explored and described.

As suggested by this reviewer (and backed up by the editor), we have removed *Fig.7* along with the corresponding descriptions in the main text.

June 27, 2022

RE: JCB Manuscript #202202100R

Dr. Takanari Inoue
Johns Hopkins Medicine
855 N. Wolfe st.,
Rangos 476
Baltimore, MD 21205

Dear Dr. Inoue,

Thank you for submitting your revised manuscript titled "Non-catalytic allostery in α-TAT1 by a phospho-switch drives dynamic microtubule acetylation." 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 limits: Articles may have up to 10 main text figures.

3) Figure formatting: 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 Figures 4f/g/k, 6j, S3g, S4f and a scale bar to S4h.

4) 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."

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

6) 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.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
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c. Temperature
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e. Fluorochromes
f. Camera make and model
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.).

8) 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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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Arshad Desai, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

The authors have greatly improved the revised manuscript. All my earlier concerns and comments have been properly addressed and therefore I support the acceptance of the manuscript for publication.

I would only like to add a small comment regarding the added discussion on the iHAP1 specificity. The experiment with constitutively active PP2A is appropriate to test the involvement of PP2A-mediated depohosphorylation. The added statement that iHAP may interfere with microtubule polymerization is also important. However, the main relevance of the detailed characterization of iHAP1 in the Vit et al. study (in the meantime published in EMBO J) for the set of experiments performed in this manuscript is that iHAP essentially has no effect on PP2A.

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

The authors have thoroughly addressed all of the reviewer concerns, and I recommend publication.