JCB

α-tubulin detyrosination links the suppression of MCAK activity with taxol cytotoxicity

Danilo Lopes, Alexandre Seabra, Bernard Nunes de Almeida Orr, and Helder Maiato

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Re: JCB manuscript #202205092

Prof. Helder Maiato
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Porto 4200-135
Portugal

Dear Helder,

Thank you for submitting your manuscript entitled "A cancer tubulin code survey unveils a role for detyrosination in taxol response by suppressing MCAK". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. All of them appreciate the high quality of the work. However, reviewers 1 and 3 recommend re-framing the manuscript to focus explicitly on the tubulin modifications studied. Reviewer 2 requests additional experiments reinforcing the link between TTL and MCAK, which we also feel is appropriate. If you feel that you can address these and the other comments of the reviewers, we would be happy to consider a revised manuscript together with a point-by-point response to the reviewers' comments. Please also highlight all changes in the text of the manuscript.

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.

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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.

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.

Best regards,
Rebecca

Rebecca Heald, Ph.D.
Editor
The Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

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

In their manuscript, Lopes et al. aim at deciphering what they call the "cancer tubulin code". The tubulin code is a concept predicting that microtubule functions in cells can be modulated by a number of molecular players, including tubulin posttranslational modifications, which in turn specifies the functions and properties of microtubules. Given that microtubules are the key targets of chemotherapy in many different forms of cancer, it was for a long time expected (but never demonstrated) that modifications of microtubules (by the tubulin code) underlie the differential sensitivity of different subtypes of cancer to tubulin-targeting chemotherapy, or even to cancer agressivity.
While recent advances have provided evidence for central roles of tubulin posttranslational modifications in neuropathologies and ciliopathies, still very little is known about their role in cancer. The current paper now takes up the challenge to systematically characterise the state of tubulin modification in a large panel of well-characterised cancer cell lines from the NCI-60 cancer cell panel. The authors perform systematic immunoblot analyses of the entire cell panel with well-characterised antibodies to two key tubulin modifications, detyrosination (with the follow-up modification Δ2-tubulin) and acetylation. Quantifying modification levels allows them to establish a "PTM signature" for all of these cells. Strikingly, they find that tubulin modification patterns hugely vary between different cell lines. The authors next demonstrate that both tubulin modifications are uncoupled in cancer cell lines, which is important because it was often assumed that different tubulin PTMs are actually coupled, and can thus not control microtubule functions in an independent manner.
The authors then show that while tubulin acetylation correlates with taxol cytotoxicity in cancer cell lines, the modification is not causatively linked to this feature. Thus, tubulin acetylation could serve as a diagnostic, but not therapeutic tool in cancerology. By contrast, tubulin detyrosination does affect taxol sensitivity. The authors demonstrate that this effect is related to the action of the microtubule depolymeriser MCAK, which had previously been shown to be regulated by the tyrosination levels of microtubules. On the functional level, the authors show that detyrosination aggravates taxol-induced spindle defects, which they then demonstrate leads to massive cell death. This suggests that coupling taxol treatment of cancers with drugs inhibiting the tyrosinating enzymes TTL could be a viable therapeutic option in the future.
The manuscript represents a remarkable amount of systematic and solid work, and will therefore be a strong addition to the current literature. Given that only very few, mostly inconsistent studies have been published on the implications of tubulin modifications in cancer, this first systematic study sets a ground stone for new developments in the field.
However, the manuscript has a number of weaknesses that must be addressed before considering it for publication. Most importantly, the authors do not fulfil the promise of their title, which suggests that they can explain how the interplay of different tubulin modifications controls specific cellular processes linked to cancer. First, they studied only two selected tubulin modifications out of a much larger number, notably excluding a modification that has already been shown to work during cell division - glutamylation. Second, they cannot really explain why cancer cell lines have so heterogenous levels of the two modifications they study, which is of course an important discovery, but does not reveal what would be expected from a "cancer tubulin code". Reading the paper with the expectation to find a revelation about this code is a frustrating experience, and completely overshadows the really exciting findings the authors make on the role of detyrosination in cancer progression, taxol toxicity, and its effect on cell division and cell death.
It would be therefore strongly recommendable to re-orient the manuscript onto these exciting discoveries, which would imply substantial changes in title, abstract, introduction, and potentially a re-structuring of the result and discussion section. A refocussed manuscript would certainly be a strong candidate for publication in the Journal of Cell Biology.

Major points:

1) The authors write throughout their manuscript that they performed a "comprehensive analysis" of tubulin PTMs. This is

misleading, as they in fact just check acetylation and detyrosination, while there are many other tubulin PTMs. They certainly have their reasons for having done this choice, but they fail to clearly state it. In any case, the term "comprehensive" is misleading the way it is used here.

2) In the chapter on tubulin detyrosination, the authors never discuss another possibility how cancer cells could change their detyr-tubulin levels: by expressing (or changing expression levels) of the alpha-tubulin isotype TUBA4A, which is naturally detyrosinated. It would be highly insightful to determine the expression levels of this tubulin isotype in different cell lines, as this could provide important complementary insights into the mechanisms that underlie the observations of the tyrosination status of the microtubules in different cell lines.

3) Representation of immunoblots: bands are generally cut out too tightly, in some images the band is even partially cut (examples: Fig. 4A,C; Fig. 6A,B; Fig. S5B,C; Fig. S6F). This is bad practise, and must be corrected even if the raw data will be shown alongside. The blots should be cut out more generously, and molecular weight markers must be shown on all panels.

Minor points:

1) The authors talk sometimes about taxol sensitivity, and sometimes about taxol-mediated cytotoxicity. It might be helpful to clearly define these two terms for non-specialists.

2) Expression levels of tubulin-modifying enzymes are expected to be quite low. Can the authors comment on the reliability of the expression levels of carboxypeptidases VASH1 and VASH2 using mRNA levels obtained from the CellMiner database? Are the detection levels sufficiently highly expressed for giving reliable values? If they find that the values they analysed are indeed low, it is highly recommended to re-analyse expression of the key genes with Q-PCR.

3) Line 188, the authors write: "Altogether, these results reveal that high alpha-tubulin acetylation is a potential predictive biomarker for taxol cytotoxicity in cancer patients." It is difficult for the reader to understand what how confident we can be about the predictive value of acetylation. Could the authors make a more precise statement? By contrast, in line 313 of the discussion, the authors much bolder as they write "supporting the use of cancer tubulin PTM signatures as reliable biomarkers". To adjust the two statements, they might want to write "..supporting potential the use of ..."

4) In Fig 5A,B the authors test the role of acetylation in the turnover of metaphase spindles. Why do they chose to analyse only acetylation, given that the manuscript analyses both, acetylation and detyrosination throughout?

5) Line 321: "functional dissection of tubulin PTMs" should be "functional dissection of selected tubulin PTMs"

6) Line 321: the authors write "Our findings reveal a previously unanticipated variability..." Was this really unanticipated? Several past reports have already hinted at the possibility that cancer cell lines and/or cancer subtypes might very different from each other in terms of tubulin PTMs.

7) In the figures, numerical P-values could be indicated in plots instead of stars and n.s.

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

Lopes et al. performed a comprehensive analysis of the roles of a set of post-translational modifications (PTMs) of alpha-tubulin (acetylation, detyrosination and delta2 modification) in cell behavior and drug resistance of the NCI-60 cancer cell panel. The two main findings of this study are that tubulin acetylation correlates with taxol sensitivity (although it is not required for it) and that experimental increase in tubulin detyrosination enhances taxol sensitivity, likely by suppressing the activity of kinesin-13/MCAK and therefore aggravating mitosis-related problems. The authors also shed a new light on the correlation and interdependence between the studied PTMs, unexpectedly showing their frequent uncoupling. Furthermore, they show that acetylation does not affect microtubule dynamics, additionally supporting its reported role in supporting the resistance to mechanical breakage, rather than having a general microtubule-stabilizing effect. The study is well-designed and addresses an important question about the role and potential usage of tubulin PTMs in the context of cancer and related more personalized therapies. The presented findings may help in improving the taxol sensitivity and considering the tubulin code as a potential biomarker for taxol-based anti-cancer therapies. The extensive amount of the data collected on tubulin PTMs in a large number of cancer cells provided in this study will serve as a useful source for future studies addressing the association between the tubulin code and cancer. Prior to publication the following points may help improving the manuscript and strengthening the claims.

1) HL-60 and RPE-1 cells were included in each immunoblot as the reference cell lines. However, the levels of tubulin acetylation and detyrosination are inconsistent in several blots. For instance, tubulin acetylation is quite higher in HL-60 compared to RPE-1 in most of the blots, but it appears to be the opposite in the two last blots on the right in Fig. S1. Along the same line, tubulin detyrosination seems to be higher in HL-60 in most of the blots, however it appears higher in RPE-1 in the fourth blot on the left in Fig. S1, as well as in Fig. S7. Could the authors comment on this discrepancy?

2) The authors have reported a weak correlation between tubulin detyrosination and vasohibins expression (Fig. S3). Recently, another enzyme with tubulin detyrosinase activity, called MATCAP, has been reported (PMID: 35482892). Is there any correlation between tubulin detyrosination and the expression of MATCAP?

3) Whereas both TTL RNAi and MCAK RNAi were shown to enhance taxol sensitivity in HCT-116 cells, only TTL RNAi data was presented for T-47D cell line (Fig. S6). To strengthen the association between the increased detyrosination and its effect on MCAK activity, it would be useful to add MCAK RNAi data for T-47D cell line.

4) TTL RNAi-induced increase in tubulin detyrosination sensitized cells to taxol in the same manner as MCAK RNAi did, with no cumulative effect upon TTL and MCAK co-depletion (Fig. 6). This suggests that increased detyrosination induces enhanced taxol cytotoxicity by suppressing the microtubule depolymerase activity of MCAK. Could the effect of TTL depletion be overcome by MCAK overexpression and/or MCAK agonist UMK57?

5) TTL RNAi increased the frequency of spindle multipolarity (Fig. 7) and spindle multipolarity-related catastrophic exit from mitosis (Fig. 8) in taxol-treated HCT-116 cells. Although phase contrast-based live-cell imaging showed similar effect of TTL RNAi and MCAK RNAi, the impact of MCAK RNAi on spindle multipolarity is not shown. Given that spindle multipolarity seems to be the main cause of the synergistic effect of TTL-depletion and taxol treatment, and given that suppressed MCAK activity is proposed to underlie the TTL-depletion effect, it would be important to compare the spindle multipolarity phenotypes between TTL RNAi + taxol and MCAK RNAi + taxol treatments.

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

In this manuscript, the authors present a broad characterization of alpha-tubulin post-translational modifications (detyrosination, acetylation, delta-2) in the NCI-60 panel of tumor cell lines. Tubulin acetylation is demonstrated to correlate with taxol cytotoxicity, but does not play a causal role. Conversely, tubulin detyrosination is shown to have a causal role in taxol cytotoxicity that is linked to spindle multi-polarity and mitotic catastrophe. Overall, the studies are conducted carefully with the connection to tubulin detyrosination established clearly with parallel methods, as well as the exclusion of a causative role for tubulin acetylation. Given the recent discovery of the tubulin carboxypeptidase enzyme, the direct role of tubulin detyrosination in cell viability and chemotherapy cytotoxicity will be an advance for the field. However, there remain some limitations to the data and the authors' conclusions extend beyond the current data in places. The following recommendations are made to strengthen the manuscript and refine the conclusions:

1) Given the causative role established for tubulin detyrosination, the expression of VASH1 and VASH2 protein should be included to complement the characterization of TTL protein expression. While VASH1 and VASH2 mRNA data is shown in the correlation graphs (Fig.S3), the immunoblots for VASH1 and VASH2 protein should be added to Fig.S1 so a direct comparison can be made between all the protein modifications simultaneously. For example, while the correlation between DeTyr-tubulin and TTL protein levels remains relatively weak (Spearman r: -0.3079), it is possible that DeTyr-tubulin will either be more strongly correlated with VASH1/2 protein, or that cell lines with the combination of low TTL and high VASH1/2 will show greater elevation of DeTyr-tubulin.

2) On line 270, the authors conclusions extend beyond the available data. The authors state the following:

"Since α-tubulin detyrosination, TTL expression and taxol sensitivity did not correlate with MCAK expression (Fig. S4D-F), these results indicate that α-tubulin detyrosination enhances taxol cytotoxicity by suppressing the activity of the microtubule-depolymerizing enzyme MCAK."

However, a lack of correlation does not prove that MCAK suppression is causative in the increased cytotoxicity with detyrosination elevation via siTTL. It is possible that suppression of either TTL or MCAK is sufficient to increase DeTyr-tubulin, but that these are independent events. The lack of a cumulative effect could simply indicate that there is an upper threshold for DeTyr-tubulin. In the absence of further data directly implicating MCAK in mediating the TTL effect, this conclusion should be more limited. For example, a statement that "elevation of DeTyr-tubulin by reducing either TTL or MCAK expression can increase taxol cytotoxicity" is a more accurate reflection of the current data. Since it has been recently shown that expression of VASH1/VASH2 can induce apoptosis (PMID: 35406479), it remains possible that the elevation of DeTyr-tubulin helps increase taxol cytotoxicity, but this DeTyr-tubulin increase can be accomplished via many independent methods (siTTL, siMCAK, VASH overexpression), without these molecular regulators necessarily working in a coordinated manner, as the authors imply in the current text.

3) Similar to note#2, the title "A cancer tubulin code survey unveils a role for detyrosination in taxol response by suppressing MCAK" is implying that tubulin detyrosination suppresses MCAK protein. However, in Fig.6A, it is clear when tubulin detyrosination is elevated by siTTL and Taxol treatment (lane 4) there is no effect on MCAK protein expression. So the title should be corrected. One option is to simply add "activity" to the end of the title to specify the effect is not MCAK protein levels.

However, this poses the same risks of note#2, since the suppression of MCAK activity by DeTyr-tubulin has not been firmly established. A more accurate title is similar to the wording suggested in note#2, "Elevation of tubulin detyrosination by suppression of either TTL or MCAK can increase taxol cytotoxicity".

4) Figure 5 is performed in U2OS osteosarcoma cells, while the majority of other figures use the HCT-116 colon cancer cells (Fig.3,4,6,7,8). The EB3-GFP experiments should be replicated in HCT-116 cells to allow comparison with other figures, where HCT-116 cell line is used consistently (Fig. 3,4,6,7,8). The fact that the U2OS cells are also not part of the NCI-60 collection highlights the lack of comparability with the other experiments in the article.

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

In their manuscript, Lopes et al. aim at deciphering what they call the "cancer tubulin code". The tubulin code is a concept predicting that microtubule functions in cells can be modulated by a number of molecular players, including tubulin posttranslational modifications, which in turn specifies the functions and properties of microtubules. Given that microtubules are the key targets of chemotherapy in many different forms of cancer, it was for a long time expected (but never demonstrated) that modifications of microtubules (by the tubulin code) underlie the differential sensitivity of different subtypes of cancer to tubulin-targeting chemotherapy, or even to cancer agressivity.

While recent advances have provided evidence for central roles of tubulin posttranslational modifications in neuropathologies and ciliopathies, still very little is known about their role in cancer. The current paper now takes up the challenge to systematically characterise the state of tubulin modification in a large panel of well-characterised cancer cell lines from the NCI-60 cancer cell panel. The authors perform systematic immunoblot analyses of the entire cell panel with well-characterised antibodies to two key tubulin modifications, detyrosination (with the follow-up modification Δ2-tubulin) and acetylation. Quantifying modification levels allows them to establish a "PTM signature" for all of these cells. Strikingly, they find that tubulin modification patterns hugely vary between different cell lines. The authors next demonstrate that both tubulin modifications are uncoupled in cancer cell lines, which is important because it was often assumed that different tubulin PTMs are actually coupled, and can thus not control microtubule functions in an independent manner.
The authors then show that while tubulin acetylation correlates with taxol cytotoxicity in cancer cell lines, the modification is not causatively linked to this feature. Thus, tubulin acetylation could serve as a diagnostic, but not therapeutic tool in cancerology. By contrast, tubulin detyrosination does affect taxol sensitivity. The authors demonstrate that this effect is related to the action of the microtubule depolymeriser MCAK, which had previously been shown to be regulated by the tyrosination levels of microtubules. On the functional level, the authors show that detyrosination aggravates taxol-induced spindle defects, which they then demonstrate leads to massive cell death. This suggests that coupling taxol treatment of cancers with drugs inhibiting the tyrosinating enzymes TTL could be a viable therapeutic option in the future.

The manuscript represents a remarkable amount of systematic and solid work, and will therefore be a strong addition to the current literature. Given that only very few, mostly inconsistent studies have been published on the implications of tubulin modifications in cancer, this first systematic study sets a ground stone for new developments in the field.

However, the manuscript has a number of weaknesses that must be addressed before considering it for publication. Most importantly, the authors do not fulfil the promise of their title, which suggests that they can explain how the interplay of different tubulin modifications controls specific cellular processes linked to cancer. First, they studied only two selected tubulin modifications out of a much larger number, notably excluding a modification that has already been shown to work during cell division - glutamylation. Second, they cannot really

explain why cancer cell lines have so heterogenous levels of the two modifications they study, which is of course an important discovery, but does not reveal what would be expected from a "cancer tubulin code". Reading the paper with the expectation to find a revelation about this code is a frustrating experience, and completely overshadows the really exciting findings the authors make on the role of detyrosination in cancer progression, taxol toxicity, and its effect on cell division and cell death.

It would be therefore strongly recommendable to re-orient the manuscript onto these exciting discoveries, which would imply substantial changes in title, abstract, introduction, and potentially a re-structuring of the result and discussion section. A refocussed manuscript would certainly be a strong candidate for publication in the Journal of Cell Biology.

R: We thank the reviewer for the thoughtful analysis of our results in light of the current state-of-the, for recognizing the significance of our findings and for guiding us on how to best present these data. To directly reflect the general comments by the reviewer, we have extensively modified the title, abstract, introduction, results and discussion section to refocus the manuscript onto the role of tubulin detyrosination in taxol cytotoxicity through a mechanistic link with MCAK. We also downplayed the coverage of tubulin PTMs in our study and went from a comprehensive dissection into a pilot study of the cancer tubulin code, focusing on specific PTMs.

Major points:

1) The authors write throughout their manuscript that they performed a "comprehensive analysis" of tubulin PTMs. This is misleading, as they in fact just check acetylation and detyrosination, while there are many other tubulin PTMs. They certainly have their reasons for having done this choice, but they fail to clearly state it. In any case, the term "comprehensive" is misleading the way it is used here.

R: We agree that this was an overstatement and have now re-written the manuscript to reflect our focus on selected tubulin PTMs.

2) In the chapter on tubulin detyrosination, the authors never discuss another possibility how cancer cells could change their detyr-tubulin levels: by expressing (or changing expression levels) of the alpha-tubulin isotype TUBA4A, which is naturally detyrosinated. It would be highly insightful to determine the expression levels of this tubulin isotype in different cell lines, as this could provide important complementary insights into the mechanisms that underlie the observations of the tyrosination status of the microtubules in different cell lines.

R: This is an excellent point that escaped our attention and interpretations. Since expression data on TUBA4A is available for all cell lines of the NCI-60 panel through the CellMiner database, we have now investigated whether tubulin detyrosination levels determined by our

WB analysis correlate with TUBA4A mRNA levels. We found no correlation. This is now indicated in the main text and the results included in Figure S3N

3) Representation of immunoblots: bands are generally cut out too tightly, in some images the band is even partially cut (examples: Fig. 4A,C; Fig. 6A,B; Fig. S5B,C; Fig. S6F). This is bad practise, and must be corrected even if the raw data will be shown alongside. The blots should be cut out more generously, and molecular weight markers must be shown on all panels.

R: The reason for tight trimming of immunoblots was to avoid confusion with unspecific bands due to re-probing of the same membranes with different tubulin antibodies after incomplete antibody stripping. We have now provided more generous blots and included molecular weight markers in all of them, while indicating unspecific bands with an asterisk (*) due to incomplete stripping of the membranes, alongside with uncropped immunoblots for all the panels provided as SourceData files.

Minor points:

1) The authors talk sometimes about taxol sensitivity, and sometimes about taxol-mediated cytotoxicity. It might be helpful to clearly define these two terms for non-specialists.

R: We now adopted the term ‘cytotoxicity’ throughout the text.

2) Expression levels of tubulin-modifying enzymes are expected to be quite low. Can the authors comment on the reliability of the expression levels of carboxypeptidases VASH1 and VASH2 using mRNA levels obtained from the CellMiner database? Are the detection levels sufficiently highly expressed for giving reliable values? If they find that the values they analysed are indeed low, it is highly recommended to re-analyse expression of the key genes with Q-PCR.

R: Since TTL mRNA and protein levels do correlate, we had no reason to consider that the data on VASHs mRNA levels would not correlate with the respective protein levels. Moreover, the reviewer is probably referring to VASH1 and VASH2 expression levels in normal cells where they are indeed expected to be quite low. However, this is not the case in cancer cells and the high variability found among the NCI-60 panel is a good indication that differences in VASH1 and VASH2 mRNA levels can be reliably detected.

3) Line 188, the authors write: "Altogether, these results reveal that high alpha-tubulin acetylation is a potential predictive biomarker for taxol cytotoxicity in cancer patients." It is difficult for the reader to understand what how confident we can be about the predictive value of acetylation. Could the authors make a more precise statement? By contrast, in line 313 of the discussion, the authors much bolder as they write "supporting the use of cancer tubulin PTM signatures as reliable biomarkers". To adjust the two statements, they might want to write "..supporting potential the use of ..."

R: We have re-written the text following the reviewer's suggestion.

4) In Fig 5A,B the authors test the role of acetylation in the turnover of metaphase spindles. Why do they chose to analyse only acetylation, given that the manuscript analyses both, acetylation and detyrosination throughout?

R: The role of detyrosination in the turnover of metaphase spindles was reported in a previous paper by our group and shown not to directly interfere with global spindle microtubule half-life (Ferreira et al., JCB, 2020). This is now cited in the appropriate section and discussed together with acetylation.

5) Line 321: "functional dissection of tubulin PTMs" should be "functional dissection of selected tubulin PTMs"

R: This is now re-written following the reviewer's suggestion.

6) Line 321: the authors write "Our findings reveal a previously unanticipated variability..." Was this really unanticipated? Several past reports have already hinted at the possibility that cancer cell lines and/or cancer subtypes might very different from each other in terms of tubulin PTMs.

R: We agree with the reviewer and have now re-written the text as "Our findings provide systematic evidence for high variability of selected tubulin PTMs among human cancer cells".

7) In the figures, numerical P-values could be indicated in plots instead of stars and n.s.

R: We have now replaced significance asterisks (*) by the respective (and more informative) P-values.

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

Lopes et al. performed a comprehensive analysis of the roles of a set of post-translational modifications (PTMs) of alpha-tubulin (acetylation, detyrosination and delta2 modification) in cell behavior and drug resistance of the NCI-60 cancer cell panel. The two main findings of this study are that tubulin acetylation correlates with taxol sensitivity (although it is not required for it) and that experimental increase in tubulin detyrosination enhances taxol sensitivity, likely by suppressing the activity of kinesin-13/MCAK and therefore aggravating mitosis-related problems. The authors also shed a new light on the correlation and interdependence between the studied PTMs, unexpectedly showing their frequent uncoupling. Furthermore, they show that acetylation does not affect microtubule dynamics, additionally supporting its reported role in supporting the resistance to mechanical breakage, rather than having a general microtubule-stabilizing effect. The study is well-designed and

addresses an important question about the role and potential usage of tubulin PTMs in the context of cancer and related more personalized therapies. The presented findings may help in improving the taxol sensitivity and considering the tubulin code as a potential biomarker for taxol-based anti-cancer therapies. The extensive amount of the data collected on tubulin PTMs in a large number of cancer cells provided in this study will serve as a useful source for future studies addressing the association between the tubulin code and cancer. Prior to publication the following points may help improving the manuscript and strengthening the claims.

R: We thank the reviewer for recognizing the breadth and utility of our study.

1) HL-60 and RPE-1 cells were included in each immunoblot as the reference cell lines. However, the levels of tubulin acetylation and detyrosination are inconsistent in several blots. For instance, tubulin acetylation is quite higher in HL-60 compared to RPE-1 in most of the blots, but it appears to be the opposite in the two last blots on the right in Fig. S1. Along the same line, tubulin detyrosination seems to be higher in HL-60 in most of the blots, however it appears higher in RPE-1 in the fourth blot on the left in Fig. S1, as well as in Fig. S7. Could the authors comment on this discrepancy?

R: Since RPE-1 cells were used in all immunoblots as internal control, cell extracts had to be produced on different occasions, leading to some variability. Tubulin PTM levels are also generally very low in RPE-1 cells, which might cause higher variability due to longer exposure times. In any case, all quantifications were performed relative to HL60, which is more consistent between experiments.

2) The authors have reported a weak correlation between tubulin detyrosination and vasohibins expression (Fig. S3). Recently, another enzyme with tubulin detyrosinase activity, called MATCAP, has been reported (PMID: 35482892). Is there any correlation between tubulin detyrosination and the expression of MATCAP?

R: We thank the reviewer for the suggestion. Since this was a very recent finding we had not included it in our original analysis, but this is now provided in the revised manuscript (Figure S3). We found no correlation between tubulin detyrosination and MATCAP mRNA levels. Unfortunately, available MATCAP antibodies do not work by immunoblot, which prevented us to validate MATCAP expression at the protein level.

3) Whereas both TTL RNAi and MCAK RNAi were shown to enhance taxol sensitivity in HCT-116 cells, only TTL RNAi data was presented for T-47D cell line (Fig. S6). To strengthen the association between the increased detyrosination and its effect on MCAK activity, it would be useful to add MCAK RNAi data for T-47D cell line.

R: We performed the requested experiment and confirmed an identical outcome between taxol+TTL RNAi and taxol+MCAK RNAi for the T-47D cell line. This is now shown in Figures 7D,E.

4) TTL RNAi-induced increase in tubulin detyrosination sensitized cells to taxol in the same manner as MCAK RNAi did, with no cumulative effect upon TTL and MCAK co-depletion (Fig. 6). This suggests that increased detyrosination induces enhanced taxol cytotoxicity by suppressing the microtubule depolymerase activity of MCAK. Could the effect of TTL depletion be overcome by MCAK overexpression and/or MCAK agonist UMK57?

R: This is an excellent point and we have shown previously that indeed UMK57 treatment partially rescues chromosome missegregation errors caused by TTL depletion (Ferreira et al., 2020). However, when we tried to rescue TTL depletion + taxol with UMK57 at the recommended concentration (250 nM) and time limits (48h) (Orr et al., 2016), no significant rescue was found (and also no significant improvement when UMK57 was used together with taxol alone). We suspect that simultaneous addition of UMK57 (which promotes microtubule destabilization) along with taxol (which promotes microtubule stabilization) for additional 48 hours after TTL depletion somehow cancels the effect of UMK57 or is insufficient to revert the accumulated errors over 72h of TTL depletion. Treating cells with UMK57 longer than 48 hours is not a possibility, since it has been reported that cells develop adaptive resistance to UMK57 (Orr et al., 2016). Likewise, MCAK overexpression would have to be performed upon transient transfection to avoid deleterious effects, which might not be sufficient to rescue the accumulated phenotype over 72 hours after TTL depletion.

5) TTL RNAi increased the frequency of spindle multipolarity (Fig. 7) and spindle multipolarity-related catastrophic exit from mitosis (Fig. 8) in taxol-treated HCT-116 cells. Although phase contrast-based live-cell imaging showed similar effect of TTL RNAi and MCAK RNAi, the impact of MCAK RNAi on spindle multipolarity is not shown. Given that spindle multipolarity seems to be the main cause of the synergistic effect of TTL-depletion and taxol treatment, and given that suppressed MCAK activity is proposed to underlie the TTL-depletion effect, it would be important to compare the spindle multipolarity phenotypes between TTL RNAi + taxol and MCAK RNAi + taxol treatments.

R: This was probably the most important point that we addressed in this revision. We are therefore highly indebted to this reviewer for suggesting investigating spindle multipolarity in MCAK RNAi + taxol, which made us re-interpret the TTL+Taxol data and more clearly isolate the main cause underlying the synergistic effect between high tubulin detyrosination and taxol. In short, and in contrast with TTL RNAi + taxol treatment, MCAK RNAi + taxol did not aggravate spindle multipolarity beyond taxol treatment alone at the used concentrations (please see new Figure 8B, C). While we do not dispute that high tubulin detyrosination might affect other targets involved in bipolar spindle assembly, these results, combined with an identical and not cumulative effect of MCAK and/or TTL depletion in taxol cytotoxicity, as well as an identical cell fate in mitosis and in the subsequent interphase, without a marked increase in multipolar cell divisions, strongly suggest that it is not the aggravation of spindle multipolarity per se that accounts for the enhanced taxol cytotoxicity. Instead, given the previous implication of both TTL and MCAK in mitotic error correction, these new data can be better explained by a key role of chromosome missegregation in the promotion of taxol

cytotoxicity. This is further supported by a recent work that showed that the extent of spindle multipolarity does not predict patient response to taxol, whereas pre-existing or experimental induction of chromosomal instability independently of spindle multipolarity were sufficient to increase paclitaxel efficacy and patient response (Scribano et al., 2021). In line with these findings and the new interpretation of our data, we now show that combined treatments with a CENP-E inhibitor, that compromises faithful chromosome segregation by affecting a completely different mechanism (polar chromosome alignment) than the one regulated by MCAK (error correction), can be synergistic with either TTL or MCAK depletion and further enhance taxol cytotoxicity relative to TTL or MCAK depletion alone (please see new Figure 6G-I). For these reasons, in the revised manuscript, we now propose that the enhanced cytotoxicity caused by TTL or MCAK depletion is mainly due to the promotion of chromosome missegregation events.

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

In this manuscript, the authors present a broad characterization of alpha-tubulin post-translational modifications (detyrosination, acetylation, delta-2) in the NCI-60 panel of tumor cell lines. Tubulin acetylation is demonstrated to correlate with taxol cytotoxicity, but does not play a causal role. Conversely, tubulin detyrosination is shown to have a causal role in taxol cytotoxicity that is linked to spindle multi-polarity and mitotic catastrophe. Overall, the studies are conducted carefully with the connection to tubulin detyrosination established clearly with parallel methods, as well as the exclusion of a causative role for tubulin acetylation. Given the recent discovery of the tubulin carboxypeptidase enzyme, the direct role of tubulin detyrosination in cell viability and chemotherapy cytotoxicity will be an advance for the field. However, there remain some limitations to the data and the authors' conclusions extend beyond the current data in places.

R: We thank the reviewer for recognizing the advances provided in our study for the field.

The following recommendations are made to strengthen the manuscript and refine the conclusions:

1) Given the causative role established for tubulin detyrosination, the expression of VASH1 and VASH2 protein should be included to complement the characterization of TTL protein expression. While VASH1 and VASH2 mRNA data is shown in the correlation graphs (Fig.S3), the immunoblots for VASH1 and VASH2 protein should be added to Fig.S1 so a direct comparison can be made between all the protein modifications simultaneously. For example, while the correlation between DeTyr-tubulin and TTL protein levels remains relatively weak (Spearman r: -0.3079), it is possible that DeTyr-tubulin will either be more strongly correlated

with VASH1/2 protein, or that cell lines with the combination of low TTL and high VASH1/2 will show greater elevation of DeTyr-tubulin.

R: This would have been a nice addition but unfortunately all available antibodies against VASH1/2 proteins that we tried did not work well by immunoblot. This could either be due to the poor quality of the antibodies or the relatively low expression of the enzymes that preclude an efficient immunodetection. Similar attempts were performed to investigate protein levels of the recently identified MATCAP detyrosinase, but they were equally unsuccessful. To mitigate these limitations, we now extended our mRNA expression analyses to include comparisons with MATCAP, as well as the naturally detyrosinated α-tubulin isotype TUBA4A.

2) On line 270, the authors conclusions extend beyond the available data. The authors state the following:

"Since α-tubulin detyrosination, TTL expression and taxol sensitivity did not correlate with MCAK expression (Fig. S4D-F), these results indicate that α-tubulin detyrosination enhances taxol cytotoxicity by suppressing the activity of the microtubule-depolymerizing enzyme MCAK."

However, a lack of correlation does not prove that MCAK suppression is causative in the increased cytotoxicity with detyrosination elevation via siTTL. It is possible that suppression of either TTL or MCAK is sufficient to increase DeTyr-tubulin, but that these are independent events. The lack of a cumulative effect could simply indicate that there is an upper threshold for DeTyr-tubulin. In the absence of further data directly implicating MCAK in mediating the TTL effect, this conclusion should be more limited. For example, a statement that "elevation of DeTyr-tubulin by reducing either TTL or MCAK expression can increase taxol cytotoxicity" is a more accurate reflection of the current data. Since it has been recently shown that expression of VASH1/VASH2 can induce apoptosis (PMID: 35406479), it remains possible that the elevation of DeTyr-tubulin helps increase taxol cytotoxicity, but this DeTyr-tubulin increase can be accomplished via many independent methods (siTTL, siMCAK, VASH overexpression), without these molecular regulators necessarily working in a coordinated manner, as the authors imply in the current text.

R: This is an interesting point that assumes that either TTL or MCAK depletion increases the levels of detyrosinated tubulin relative to control levels. However, this is clearly not the case and even more so in cells treated with taxol (please see Figure 6A). We therefore favour the alternative explanation that detyrosinated tubulin improves taxol cytotoxicity by impairing MCAK activity, which is supported by extensive previous evidence, including direct in vitro reconstitution assays (Peris et al., 2009 and Surajjudin et al., 2014) and by the identical and non-cumulative effect of TTL and/or MCAK depletion in taxol cytotoxicity. We have nevertheless toned-down our claims and have extensively re-written the main text to adopt a more conservative position.

3) Similar to note#2, the title "A cancer tubulin code survey unveils a role for detyrosination in taxol response by suppressing MCAK" is implying that tubulin detyrosination suppresses MCAK protein. However, in Fig.6A, it is clear when tubulin detyrosination is elevated by siTTL and Taxol treatment (lane 4) there is no effect on MCAK protein expression. So the title should be corrected. One option is to simply add "activity" to the end of the title to specify the effect is not MCAK protein levels. However, this poses the same risks of note#2, since the suppression of MCAK activity by DeTyr-tubulin has not been firmly established. A more accurate title is similar to the wording suggested in note#2, "Elevation of tubulin detyrosination by suppression of either TTL or MCAK can increase taxol cytotoxicity".

R: There are at least three independent studies (Peris et al., 2009; Sirajuddin et al., 2014; Ferreira et al., 2020) that provided compelling evidence both in vitro and in cells that detyrosinated tubulin is a potent inhibitor of MCAK activity. This, together with the similar outcome of TTL or MCAK depletion in taxol cytotoxicity and the lack of a cumulative effect when TTL and MCAK are co-depleted, supports the most straightforward interpretation that we currently favour in our paper. Nevertheless, we concede that in the present work a stand-alone demonstration of MCAK inhibition by detyrosination is not provided besides the genetic evidence. We have therefore re-written the title to tone-down this claim and avoid mentioning a direct causal effect of detyrosinated tubulin on MCAK activity in taxol cytotoxicity.

4) Figure 5 is performed in U2OS osteosarcoma cells, while the majority of other figures use the HCT-116 colon cancer cells (Fig.3,4,6,7,8). The EB3-GFP experiments should be replicated in HCT-116 cells to allow comparison with other figures, where HCT-116 cell line is used consistently (Fig. 3,4,6,7,8). The fact that the U2OS cells are also not part of the NCI-60 collection highlights the lack of comparability with the other experiments in the article.

R: This is an important point. The reason for using U2OS cells was because stable expression of PA-GFP-tubulin was only available in this cell line. For consistency and comparative purposes, we had originally performed EB3-GFP measurements also in U2OS cells. Nevertheless, we fully agree with the reviewer and have now repeated the EB3-GFP experiments in HCT-116 as requested.

November 3, 2022

RE: JCB Manuscript #202205092R

Prof. Helder Maiato
i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto
Rua Alfredo Allen, 208
Porto 4200-135
Portugal

Dear Prof. Maiato:

Thank you for submitting your revised manuscript entitled "α-tubulin detyrosination links the suppression of MCAK activity with taxol cytotoxicity". The reviewers have now assessed your revised manuscript and, as you can see, they are satisfied with revisions. Thus, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

Scale bars must be present on all microscopy images, including inset magnifications.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

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. Please, indicate whether N refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments).

If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please, see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

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

Sincerely,

Rebecca Heald, Ph.D.
Editor
The Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

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

The authors have carefully addressed all my comments and suggestions. The manuscript could now be considered for publication.

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

The authors have properly addressed all my earlier concerns and I recommend the revised manuscript for publication in JCB.