nature portfolio

Peer Review File

α-tubulin detyrosination fine-tunes kinetochore-microtubule attachments

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This paper addresses an important question about the role of microtubule Tyrosynation-Detyrosynation in cellular processes. While this modification on tubulin is highly abundant and has been identified many years ago, there is still no clarity on its exact biological functions. Thus, studies like this one are important and timely.

Overall, the paper is interesting and the experiments are convincing, but I was concerned that the authors seem to focus their line of reasoning too narrowly on the chromosome attachment and disregard other possible mechanisms that may account for the same defects. The writing is misleading in a few places, and I felt that it needs to be reemphasized throughout the paper.

1. The abstract is written misleadingly and makes an impression that this is a proteomics paper identifying and testing binding partners of Tyr- and De-Tyr tubulin. Such a study would have been interesting and important, but the authors in fact build most of their experiments around a different line of reasoning, attempting to address mitotic defects in cells with knockouts of TTL enzymes responsible for Tyr addition and VASH enzymes that mediate Tyr removal. The proteomics data appears secondary in this study, used mostly to identify a couple of candidate proteins for further testing. The abstract should be rewritten to adequately reflect this experimental design.

2. While VASH1/2 knockout, as expected, results in a dramatic reduction of De-Tyr tubulin, surprisingly the effects of TTL knockout on the levels of Tyr tubulin are much less consistent. In Fig. 1, no change could be seen in the levels of endogenous Tyr tubulin in TTL knockout. In Fig. 5, these levels do change, but overall the change is not that impressive. In extended data Fig. 1 the change is dramatic. I am aware that the effects of this knockout are more complex since Tyr is normally included into tubulin during translation, but these discrepancies don't make much sense. The authors should reconcile these data and perform quantifications of fold change of each tubulin variant versus total tubulin in each cell line.

3. The observed delay in chromosome alignment and metaphase onset in VASH1/2 KO expressing Tyr-tubulin is not sufficient to conclude De-Tyr is required for chromosome attachment. Other mechanisms affecting chromosome and spindle positioning could account for the same defects. These other mechanisms need to be discussed, and the authors' choice to further pursue this particular mechanism and discount others possibilities needs to be justified.

4. Following the same line of thought, the consequent experiments seem to be excessively focused on proving the chromosome attachment idea. The authors reason for this to be the case based on the increased width of the metaphase plate and impairments in sister kinetochore oscillations. This is a big leap in logic. None of these defects really show kinetochore attachments. The authors also bring up a possibility of motor protein regulation, but the effect they see can also be due to other factors – e.g., motor protein attachment to the microtubules, or changes in microtubule dynamics, to name a few. This needs to be better structured and justified. Sufficient reasoning backed with experimental data needs to be provided to exclude or identify other possible mechanisms.

Reviewer #2 (Remarks to the Author):

Tubulin modification might be an important regulation mechanism for kinetochore-microtubule attachment. However, it is largely unknown for relationship of tubulin modification. Then, authors of this paper focused on detyrosination/tyrosination of α-tubulin and generated knockout cell lines for enzymes, which govern tubulin detyrosination/tyrosination. In Vasohibin-1/2 (VASH1/2: carboxypeptidases)-knockout cells, detyrosinated tubulin was decreased, while in TTL (tubulin tyrosine ligase) knockout cells detyrosinated tubulin was increased. Mitotic duration became longer in VASH1/2 knockout cells, suggesting that detyrosinated tubulin contributes to proper mitosic progression. Authors also demonstrated that detyrosinated tubulin is mainly located on shorter microtubules, while tyrosination is mainly located on longer microtubules. Interestingly authors performed systematic proteomics approach using these knockout lines and identified proteins, which preferentially bind to tyrosinated or detyrosinated tubulins. KMN proteins including the Ndc80 complex preferentially bind to tyrosinated tubulin. Based on a single molecule imaging of the Ndc80 complex on purified tubulins, resident times of the complex on detyrosinated and tyrosinated tubulin are similar, however diffusion rate of Ndc80 on detyrosinated tubulin is higher, suggesting that α-tubulin detyrosination promotes diffusion of the Ndc80 complex on microtubules.

Tubulin modification is an interesting topic, however, it was not clear how such a modification is related with mitotic function. This paper tried to clarify a molecular basis of detyrosination/tyrosination of α-tubulin. Although there remained some ambiguous points for function of tubulin detyrosination/tyrosination, there are many of new data and suggestive idea, this reviewer supports with publication, if authors can address some concerns. Followings are my specific concerns.

1. In VASH1/2 knockout lines I agree with decrease of detyrosinated tubulin. In addition, exogenous expression of tyrosinated tubulin is also important. It may be good show actual proportion of detyrosinated/tyrosinated tubulin. Quantification values are informative.
2. In main Figure 2, VASH1/2 knockout showed longer mitotic time, suggesting that detyrosinated is required for proper chromosome segregation. However, cells with lagging chromosomes were increased in TTL KO cells, compared with VASH1/2 knockout cells. If authors explain such a phenotyope, more clearly, it would be better.
3. Proteomics experiments give us useful information. CKAP5 is known as Ndc80 binding protein, but they are enriched in different farction. Authors should mention about it.
4. Concerning Fig5 and Fig6 experiments, if they can binding affinity of the Ndc80 complex to detyrosinated or tyrosinated tubulin, it would be better.
5. In entire text, authors logic is slightly complicated, it is sometimes hard to follow their logic. Please try to revised text as simple as possible.

Reviewer #3 (Remarks to the Author):

Girao et al. established tubulin tyrosin ligase (TTL) and VASH1/2 KO lines to study how α-tubulin detyrosination/tyrosination impacts mitosis. The authors found that TTL KO cells, which express high levels of detyrosinated α-tubulin, have longer metaphase spindle length and an increased frequency of chromosome segregation errors, whereas VASH1/2 KO cells, which express low levels of detyrosinated α-tubulin, have shorter metaphase spindle length, chromosome alignment defects, altered sister kinetochore oscillations and increased time from NEB to metaphase. The authors observed decreased detyrosinated microtubules near the kinetochores of longer microtubules and increased detyrosinated microtubules near the kinetochores of shorter microtubules, attributing this to the different direction of their movement at their plus-ends. The authors conducted mass spectrometry analysis to determine proteins that are preferentially enriched to taxol-stabilized microtubules from mitotic cell extracts obtained from TTL or VASH1/2 KO cell lines. Among several candidates, the authors found that NDC80 preferentially associates with tyrosinated microtubules, whereas CLASP2 preferentially associates with detyrosinated microtubules.

This is an interesting study that used suitable models (TTL and VASH1/2 KO cells) to investigate how α-tubulin detyrosination/tyrosination impacts mitosis (if used with appropriate controls). Moreover, identifying proteins that preferentially bind to tyrosinated or detyrosinated microtubules to regulate cellular functions is significant. The model of kinetochore mobility regulation by microtubule detyrosination/tyrosination is interesting, although it requires further confirmation.
My major concern is the lack of clear mechanisms explaining most of the observed phenotypes. Moreover, most of the presented conclusions are not well-supported.
I hope addressing my below comments will help strengthen the manuscript.

Comments:
1- Lack of mechanism: Most of the experiments are descriptive. There are no direct experiments to explain or validate several phenotypes. For example:
• There are no direct experiments to explain how TTL KO increased metaphase spindle length and chromosome segregation errors or how VASH1/2 KO decreased metaphase spindle length.
• The authors found NDC80 to be preferentially associated with tyrosinated microtubules and CLASP2 to be preferentially associated with detyrosinated microtubules. However, there are no follow-up experiments to determine their role in relation to tyrosinated/detyrosinated microtubules (e.g., using TTL and/or VASH1/2 KO cells).

2- Several conclusions are not well-supported. For example:
• α-tubulin tyrosination/detyrosination and Kinetochore-microtubule attachments: Page 5 line 17 - page 6 lines 3: because VASH1/2 KO cells displayed delayed chromosome alignment and delayed anaphase onset, the authors concluded that “α-tubulin detyrosination mediated either by VASH1/2 or MATCAP is required to establish functional kinetochore-microtubule attachments necessary for timely SAC satisfaction”. The authors cannot provide this strong conclusion from the provided data. Also, throughout the manuscript, there are no experiments to “directly” assess kinetochore-microtubule attachments. This needs to be done.
• CENP-E and MCAK: The authors concluded that “α-tubulin detyrosination promotes the formation of

functional kinetochore-microtubule attachments necessary for normal metaphase chromosome dynamics and timely SAC satisfaction, independently from its role in CENP-E-mediated alignment of polar chromosomes and the regulation of MCAK activity". There are no data in "this manuscript" supporting all these conclusions. Moreover, there are no experiments targeting MCAK in this study.

3- Different distribution of tyrosinated and detyrosinated α-tubulin near oscillating leading vs. trailing kinetochores:

• The authors assessed the accumulation of detyrosinated α-tubulin on kinetochore microtubules to build their model. However, based on the quality of the representative images (Fig. 3a,b), it seems very difficult to quantify detyrosinated α-tubulin levels on kinetochore microtubules and to carry out an accurate analysis. Please provide separate images for detyrosinated α-tubulin channel in Fig. 3a,b and include images for tyrosinated α-tubulin staining.

• It could be challenging but providing time-lapse live imaging of this process (i.e., the different distribution of tyrosinated and detyrosinated α-tubulin near oscillating leading vs. trailing kinetochores) is necessary to support the authors' conclusion.

• The authors proposed that shorter K-microtubules associate with leading kinetochores are enriched with detyrosinated α-tubulin, whereas longer K-microtubules associate with trailing kinetochores are enriched with tyrosinated α-tubulin. How did the authors exclude the possibility that some of kinetochores that are associated with relatively longer microtubules (previously trailing Kinetochores) are in the middle of their corrective movement (i.e., become leading kinetochores) and, expectedly, should be associated with detyrosinated microtubules?

4- It is unclear why the authors expressed exogenous GFP-tagged detyrosinated or tyrosinated α-tubulin in all the groups to carry out the phenotypic analysis of TTL and VASH1/2 KO cell lines and to visualize microtubules (Page 4, line 12-15). Expressing detyrosinated or tyrosinated GFP-α-tubulin can influence the results in these cell lines, making the results difficult to interpret. Indeed, expressing detyrosinated GFP-α-tubulin showed different effect than expressing tyrosinated GFP-α-tubulin on TTL KO cells as shown in Extended Data Fig. 3b. Moreover, Fig. 1b should include a control group without expressing detyrosinated or tyrosinated GFP-α-tubulin.

5- Although TTL and VASH1/2 KO cells represent suitable models to study how α-tubulin detyrosination/tyrosination impacts mitosis, rescue experiments need to be conducted to validate these systems. For example, the authors may express VASH2/SVBP to rescue the phenotypes of VASH1/2 KO cells.

6- In Fig. 5, the authors conducted a mass spectrometry analysis to determine proteins that preferentially associate with tyrosinated vs. detyrosinated microtubules from only two cell lines (TTL or VASH1/2 KO cell lines). This experiment needs to be done with a proper control group (WT cell line). Also, the authors need to discuss the possibility that this experiment may not provide a clear answer to the "direct" association of identified proteins to tyrosinated or detyrosinated microtubules. For example, this association might be indirect. Indeed, α-tubulin detyrosination/tyrosination highly affects other microtubule post-translational modifications such as acetylation which could be the reason of this preferential association.

6- TLL KO cells express high detyrosinated α-tubulin levels and low tyrosinated α-tubulin levels. Throughout the manuscript, it is unclear whether the obtained findings are due to the presence of high levels of detyrosinated microtubules (i.e. the direct effect of abnormally increased detyrosinated microtubules) or due to the requirement of tyrosinated microtubules (i.e. absence of tyrosinated microtubules).

7- RNAi-mediated knockdown of MATCAP has no additive effect on detyrosinated α-tubulin levels in VASH1/2 KO cells (Extended Data Fig. 1c). However, in some experiments MATCAP RNAi resulted in stronger phenotypes in VASH1/2 KO (such as in Extended Data Fig. 2a,b) or had no additive effect on VASH1/2 KO (such as in Fig. 2C). This discrepancy raises the doubt that some of the phenotypes of VASH1/2 KO and/or MATCAP RNAi might not be due to decreased α-tubulin detyrosination.

8- Quantifications:
- All western blot data lack quantifications.
- Please provide the number of examined cells in each figure legend.
- Fig. 1c: Please provide quantifications for detyrosinated α-tubulin / α-tubulin ratio.
- Please check the statistical analysis between groups in several quantifications. For example, in Extended Data Fig. 2b, given the wide data distribution (big error bars) and the very mild difference in spindle length (less than 1 μm), please check if there is a significance difference between the "Control Tyro GFP-α-tubulin" group and "Tyro GFP-α-tubulin TLL KO" group.
- Extended Data Fig. 2a: Is the scale bar (5 μm) correct? Based on this scale bar, it seems that the spindle length is less than 5 μm in VASH1/2 KO + MATCAP RNAi group which is different from the quantification in Extended Data Fig. 2b.
- Extended Data Fig. 7a: I cannot see a noticeable difference in the level of NDC80 between parental and VASH1/2 KO cells.

9- Page 7 and Extended Data Fig. 5a: What is the significance of assessing the metaphase plate width in CENP-E-inhibited cells if the analysis was done only on aligned chromosomes. Is this the case for all groups within this experiment?

10- There are other studies indicating that tight regulation of tyrosinated and detyrosinated microtubules are critical for proper kinetochore-microtubule attachments. Please cite those studies.

Reviewer #4 (Remarks to the Author):

This manuscript entitled "α-tubulin detyrosination regulates kinetochore-microtubule attachments" by Girão et al describes the role of detyrosination during mitosis. The study shows kinetochore proteins differential binding to tyrosinated/detyrosinated microtubules. The authors have engineered cell lines with enzymes that modulate the tyrosinated/detyrosinated levels and mass spectrometry to identify the KMN network of kinetochore complex binds to the tyrosinated microtubules. While the experiments and methods used are of good quality, the model presented here contradicts the biochemical and cell biology data and does not take into account the global effects of modulating tyrosinated/detyrosinated

levels. To this reviewer the manuscript does not address the problem that the authors have claimed, I have detailed the following summary. In light of this, I do not recommend publication in its current form and a significant revision is necessary.

Some specific queries are outlined below;

1. In the CRISPR KO of VASH and TTL, the tyrosinated vs detyrosinated microtubule levels will be ideal to quantify. It must also be noted that the KO of either enzyme is equivalent to the overexpression of opposite enzymes and it is not clear why the authors chose only the CRISPR KO method, some key experiments need to be validated with overexpression of enzymes.
2. The authors have quantified the longer vs shorter microtubule Tyr/DeTyr levels. It is unclear how the demarcation of longer vs shorter criteria was chosen. Similarly, the percentage of Tyr/DeTyr levels including the free tubulin will make the conclusions valid.
3. From the mass spec data, the authors have chosen to focus on kinetochore proteins, however, the data points out that several signaling protein levels have been modulated due to KO of enzymes, perhaps the data points to a missing link of Tyr/DeTyr levels and global regulation of mitosis/cell division. While it is understood that it might be beyond the scope of this study, a discussion on this could be valuable to the community. Related to point no. 1 at least the key components of mass spec data can be validated with overexpression of VASH/TTL enzymes.
4. In the in vitro work, the authors purified only NDC80 as a case study to demonstrate the effect of Tyr/deTyr levels. From their data, only a small increase in the diffusion of NDC80 can be observed with deTyr microtubules. The authors provide a model that KMN network loading is aided by the tyrosinated microtubules and then the discussion of kinetochore is facilitated by the deTyr microtubules. The current data is insufficient to make such an argument, for example, the overall KMN network might have different binding/diffusion constants with Tyr microtubules or vice versa. Several important assessments are needed here before arriving at such a conclusion; A) Titration of Tyr/deTyr levels microtubule on the microtubules will provide information about the threshold for such a switch. B) Earlier studies have shown NDC80 interaction with CTTs (from cryoEM structural work of NDC80:MT), so it is known that PTMs in CTT might affect the KMN network. Here the binding constants of NDC80 or KMN titration against Tyr and deTyr microtubules will strengthen the mass spec data. C) Chimeric microtubules having Tyr and DeTyr within the same microtubule have been used to show the effects of tyrosination on dynein motility, a similar approach can be applied here to show such small changes.
5. The authors in the abstract have used ‘preference’ and ‘preferential binding’, there is no data to support preference, so I suggest avoiding using such anthropogenic terms to describe differential binding between two microtubule states.

Point-by-point response to the Reviewers (our answers in blue)

Reviewer #1 (Remarks to the Author):

This paper addresses an important question about the role of microtubule Tyrosynation-Detyrosynation in cellular processes. While this modification on tubulin is highly abundant and has been identified many years ago, there is still no clarity on its exact biological functions. Thus, **studies like this one are important and timely**.

Overall, **the paper is interesting and the experiments are convincing**, but I was concerned that the authors seem to focus their line of reasoning too narrowly on the chromosome attachment and disregard other possible mechanisms that may account for the same defects. The writing is misleading in a few places, and I felt that it needs to be reemphasized throughout the paper.

R: We thank the reviewer for recognizing the importance and interest of our study, as well as our experimental approaches. We thank him/her for guiding us through aspects that require additional clarification and assertiveness.

1. The abstract is written misleadingly and makes an impression that this is a proteomics paper identifying and testing binding partners of Tyr- and De-Tyr tubulin. Such a study would have been interesting and important, but the authors in fact build most of their experiments around a different line of reasoning, attempting to address mitotic defects in cells with knockouts of TTL enzymes responsible for Tyr addition and VASH enzymes that mediate Tyr removal. The proteomics data appears secondary in this study, used mostly to identify a couple of candidate proteins for further testing. The abstract should be rewritten to adequately reflect this experimental design.

R: We agree with the reviewer and have re-written the abstract to better reflect the experimental design and original goals of this work.

2. While VASH1/2 knockout, as expected, results in a dramatic reduction of De-Tyr tubulin, surprisingly the effects of TTL knockout on the levels of Tyr tubulin are much less consistent. In Fig. 1, no change could be seen in the levels of endogenous Tyr tubulin in TTL knockout. In Fig. 5, these levels do change, but overall the change is not that impressive. In extended data Fig. 1 the change is dramatic. I am aware that the effects of this knockout are more complex since Tyr is normally included into tubulin during translation, but these discrepancies don't make much sense. The authors should reconcile these data and perform quantifications of fold change of each tubulin variant versus total tubulin in each cell line.

R: The differences are essentially due to loading of different amounts of total protein in each gel and the resolution of different gels used to monitor different proteins. It is also much easier to detect changes in the usually much less abundant detyrosinated tubulin, than on the levels of tyrosinated tubulin, which constitutes the major tubulin pool. Given that different antibodies for each tubulin are used in these experiments, it makes no sense to quantify one form relative to the other, since even minor differences in exposure times would completely skew the results. The only meaningful comparison is between a given tubulin type in the different experimental conditions (assuming of

course an equivalent loading of total protein per condition). This quantification for all tubulin types is now provided for all western blots using whole cell extracts.

3. The observed delay in chromosome alignment and metaphase onset in VASH1/2 KO expressing Tyr-tubulin is not sufficient to conclude De-Tyr is required for chromosome attachment. Other mechanisms affecting chromosome and spindle positioning could account for the same defects. These other mechanisms need to be discussed, and the authors' choice to further pursue this particular mechanism and discount others possibilities needs to be justified.

R: We agree with the reviewer and we have now significantly re-written this section to avoid ambiguity and account for all different possibilities, while better justifying our choices in terms of mechanism. We also note that our 3D tracking experiments, combined with immunofluorescence analysis with antibodies against MAD1 (a bona fide marker of unattached/incomplete attached kinetochores) that focused exclusively on cells that have already aligned their chromosomes, revealed that these attachments were nevertheless not completed or sustained, regardless of any other mechanism involved in chromosome or spindle positioning.

4. Following the same line of thought, the consequent experiments seem to be excessively focused on proving the chromosome attachment idea. The authors reason for this to be the case based on the increased width of the metaphase plate and impairments in sister kinetochore oscillations. This is a big leap in logic. None of these defects really show kinetochore attachments. The authors also bring up a possibility of motor protein regulation, but the effect they see can also be due to other factors – e.g., motor protein attachment to the microtubules, or changes in microtubule dynamics, to name a few. This needs to be better structured and justified. Sufficient reasoning backed with experimental data needs to be provided to exclude or identify other possible mechanisms.

R: The reviewer is correct that other factors might account for the observed increase in metaphase plate width and impaired kinetochore oscillations. We have ruled out the known impact of tyr-tub on the inhibition of CENP-E (which, on its own, does not cause alteration of plate width, and only prevents polar chromosomes to align) and Detyr-tub or Tyr-tub on their own do not impact microtubule dynamics (Chen…Roll-Mecak, Dev Cell, 2021). We also clarify in the main text that experimental modulation of detyr-tub levels does not significantly affect kMT dynamics upon bi-orientation (Ferreira et al., JCB, 2020). We now also excluded a possible impact on the kinesin-13 MCAK that would indirectly affect microtubule dynamics. In particular, we now include new data showing that either UMK57 (an MCAK agonist) or MCAK overexpression had no comparable impact on the frequency of Mad1+ kinetochores and plate width relative to VASH1/2 KO, suggesting that the resulting decrease in detyr-tub after VASH1/2 KO does not cause kinetochore-microtubule detachments due to MCAK hyperactivation. Importantly, as mentioned in our response to the previous point, by monitoring the fraction of kinetochores positive for Mad1, we probed for kinetochore-microtubule attachment status. The frequency of Mad1-positive kinetochores is clearly increased on aligned chromosomes in VASH1/VASH2 KO cells with lower detyr-tubulin, even after providing at least 6x the time that cells normally spend in metaphase and complete Mad1 removal from kinetochores. Together these data suggest that tubulin detyr levels impact kinetochore-microtubule attachments through other possible mechanisms that likely involve additional molecular players, including NDC80 and CLASP2.

Reviewer #2 (Remarks to the Author):

Tubulin modification might be an important regulation mechanism for kinetochore-microtubule attachment. However, it is largely unknown for relationship of tubulin modification. Then, authors of this paper focused on detyrosination/tyrosination of α-tubulin and generated knockout cell lines for enzymes, which govern tubulin detyrosination/tyrosination. In Vasohibin-1/2 (VASH1/2: carboxypeptidases)-knockout cells, detyrosinated tubulin was decreased, while in TTL (tubulin tyrosine ligase) knockout cells detyrosinated tubulin was increased. Mitotic duration became longer in VASH1/2 knockout cells, suggesting that detyrosinated tubulin contributes to proper mitosic progression. Authors also demonstrated that detyrosinated tubulin is mainly located on shorter microtubules, while tyrosination is mainly located on longer microtubules. Interestingly authors performed systematic proteomics approach using these knockout lines and identified proteins, which preferentially bind to tyrosinated or detyrosinated tubulins. KMN proteins including the Ndc80 complex preferentially bind to tyrosinated tubulin. Based on a single molecule imaging of the Ndc80 complex on purified tubulins, resident times of the complex on detyrosinated and tyrosinated tubulin are similar, however diffusion rate of Ndc80 on detyrosinated tubulin is higher, suggesting that α-tubulin detyrosination promotes diffusion of the Ndc80 complex on microtubules.

Tubulin modification is an interesting topic, however, it was not clear how such a modification is related with mitotic function. This paper tried to clarify a molecular basis of detyrosination/tyrosination of α-tubulin. Although there remained some ambiguous points for function of tubulin detyrosination/tyrosination, **there are many of new data and suggestive idea, this reviewer supports with publication**, if authors can address some concerns. Followings are my specific concerns.

R: We thank the reviewer for recognizing the interest of our study and supporting its publication. We hope the additional experiments and clarifications now fully address the reviewer's concerns.

1. In VASH1/2 knockout lines I agree with decrease of detyrosinated tubulin. In addition, exogenous expression of tyrosinated tubulin is also important. It may be good show actual proportion of detyrosinated/tyrosinated tubulin. Quantification values are informative.

R: As explained previously to reviewer #1, quantifying one tubulin type relative to the other is not informative and may even be misleading, since even minor differences in exposure times using different antibodies would completely skew the results. We use more meaningful comparisons between a given tubulin type in the different experimental conditions (of course with equivalent loading of total protein per condition). This quantification for all tubulin types is now provided for all western blots using whole cell extracts.

2. In main Figure 2, VASH1/2 knockout showed longer mitotic time, suggesting that detyrosinated is required for proper chromosome segregation. However, cells with lagging chromosomes were increased in TTL KO cells, compared with VASH1/2 knockout cells. If authors explain such a phenotyope, more clearly, it would be better.

R: The major difference is that longer mitotic time in VASH1/2 KO cells reflects incapacity to timely satisfy the SAC. Once satisfied, chromosome segregation (in anaphase) is actually normal (see

Extended Data Fig. 3b). On the other hand, TTL KO prevents proper activity of microtubule depolymerases involved in the correction of errors that satisfy the SAC, resulting in chromosome missegregation in anaphase, as shown before (Ferreira et al., JCB, 2020). This is now clarified in the main text.

3. Proteomics experiments give us useful information. CKAP5 is known as Ndc80 binding protein, but they are enriched in different farction. Authors should mention about it.

R: This is an interesting point that we completely overlooked and we thank the reviewer for bringing this to our attention. We now refer to the relevant literature and discuss that despite the fact that CKAP5 binds also to Ndc80, they might have a differential binding to Detyr vs/ Tyr MTs. In particular, Ndc80, which independently binds directly to microtubules, might simply work as a docking factor for other proteins that will have other preferences for Detyr vs. Tyr MTs, in order to establish a functional kinetochore-microtubule interface. Curiously, CLIP1/CLIP170 and CLASP2, which are known interacting proteins, also came out enriched in different fractions, suggesting a fine regulation that likely reflects additional preferences related with other aspects of microtubule structure (e.g. lattice vs. plus ends).

4. Concerning Fig5 and Fig6 experiments, if they can binding affinity of the Ndc80 complex to detyrosinated or tyrosinated tubulin, it would be better.

R: In response to the reviewer's suggestion, we have developed a novel protocol to prepare microtubules with different detyrosination levels in quantities sufficient for this assay. We now used microtubule pelleting assays with recombinant proteins to determine the respective Kds of Ndc80 and CLASP2 on detyrosinated vs. tyrosinated microtubules in vitro. We found that CLASP2 affinity to detyrosinated microtubules is about 25x higher when compared to tyrosinated microtubules, whereas Ndc80 has only 25% higher affinity to tyrosinated microtubules relative to detyrosinated ones. This is in agreement with our single-molecule data with Ndc80 indicating only a small difference for the residence time along the microtubule lattice is response to tubulin detyrosination. Importantly, these assays also revealed changes in the diffusion rate. We emphasize that binding affinity provides important but incomplete information about kinetochore-microtubule attachments because they require protein-microtubule gliding, not just binding. Thus, parameters, such as diffusion rate, are essential for understanding how kinetochore-microtubule interface operates, especially during metaphase oscillations. The implications of our affinity and diffusion findings are discussed in the main text.

5. In entire text, authors logic is slightly complicated, it is sometimes hard to follow their logic. Please try to revised text as simple as possible.

R: We have now extensively re-written the main text and hope to have clarified all critical points.

Reviewer #3 (Remarks to the Author):

Girao et al. established tubulin tyrosin ligase (TTL) and VASH1/2 KO lines to study how α-tubulin detyrosination/tyrosination impacts mitosis. The authors found that TTL KO cells, which express high levels of detyrosinated α-tubulin, have longer metaphase spindle length and an increased frequency of chromosome segregation errors, whereas VASH1/2 KO cells, which express low levels of detyrosinated α-tubulin, have shorter metaphase spindle length, chromosome alignment defects, altered sister kinetochore oscillations and increased time from NEB to metaphase. The authors observed decreased detyrosinated microtubules near the kinetochores of longer microtubules and increased detyrosinated microtubules near the kinetochores of shorter microtubules, attributing this to the different direction of their movement at their plus-ends. The authors conducted mass spectrometry analysis to determine proteins that are preferentially enriched to taxol-stabilized microtubules from mitotic cell extracts obtained from TTL or VASH1/2 KO cell lines. Among several candidates, the authors found that NDC80 preferentially associates with tyrosinated microtubules, whereas CLASP2 preferentially associates with detyrosinated microtubules.

This is an interesting study that used suitable models (TTL and VASH1/2 KO cells) to investigate how α-tubulin detyrosination/tyrosination impacts mitosis (if used with appropriate controls). Moreover, **identifying proteins that preferentially bind to tyrosinated or detyrosinated microtubules to regulate cellular functions is significant**. **The model of kinetochore mobility regulation by microtubule detyrosination/tyrosination is interesting**, although it requires further confirmation.

My major concern is the lack of clear mechanisms explaining most of the observed phenotypes. Moreover, most of the presented conclusions are not well-supported.

I hope addressing my below comments will help strengthen the manuscript.

R: We thank the reviewer for recognizing the interest of our study and powerful model systems and experimental approaches used therein that supported the proposed model. We hope the additional experiments, including additional in vitro reconstitutions and functional assays, now fully address the reviewer's concerns regarding the proposed mechanism.

Comments:

1- Lack of mechanism: Most of the experiments are descriptive. There are no direct experiments to explain or validate several phenotypes. For example:

• There are no direct experiments to explain how TTL KO increased metaphase spindle length and chromosome segregation errors or how VASH1/2 KO decreased metaphase spindle length.

• The authors found NDC80 to be preferentially associated with tyrosinated microtubules and CLASP2 to be preferentially associated with detyrosinated microtubules. However, there are no follow-up experiments to determine their role in relation to tyrosinated/detyrosinated microtubules (e.g., using TTL and/or VASH1/2 KO cells).

R: We thank the reviewer for drawing attention to these points. The reason why the spindle length phenotypes were not followed up was simply because these were confirmatory observations of known phenotypes and mechanism (detyrosination inhibits the microtubule depolymerizing activity of

kinesin-13 proteins like MCAK, whereas tyrosination promotes their activity) that have been previously reported by us and others. We now highlight these previous studies in the main text and allude to the precise mechanism. As for the preferential association of NDC80 and CLASP2 to tyr and detyr MTs, respectively, we draw the reviewer's attention to Figure 8, where the amounts of NCD80 and CLASP2 detected on spindle MTs were measured by immunofluorescence and the in vitro reconstitution data of NDC80 on detyr vs. tyr MTs in figure 7. Finally, we also extended our in vitro analysis of CLASP2 and NDC80 interaction with detyrosinated and tyrosinated microtubules using microtubule-pelleting assays, which allowed us to estimate the respective Kds. These data are now included in Figure 6.

2- Several conclusions are not well-supported. For example:

• α-tubulin tyrosination/detyrosination and Kinetochore-microtubule attachments: Page 5 line 17 - page 6 lines 3: because VASH1/2 KO cells displayed delayed chromosome alignment and delayed anaphase onset, the authors concluded that "α-tubulin detyrosination mediated either by VASH1/2 or MATCAP is required to establish functional kinetochore-microtubule attachments necessary for timely SAC satisfaction". The authors cannot provide this strong conclusion from the provided data. Also, throughout the manuscript, there are no experiments to "directly" assess kinetochore-microtubule attachments. This needs to be done.

• CENP-E and MCAK: The authors concluded that "α-tubulin detyrosination promotes the formation of functional kinetochore-microtubule attachments necessary for normal metaphase chromosome dynamics and timely SAC satisfaction, independently from its role in CENP-E-mediated alignment of polar chromosomes and the regulation of MCAK activity". There are no data in "this manuscript" supporting all these conclusions. Moreover, there are no experiments targeting MCAK in this study.

R: We already alluded to these points in our detailed response to points 3 and 4 by reviewer #1. In addition, and as suggested by the reviewer, we now provide new experiments targeting MCAK activity that are included in this revised version.

3- Different distribution of tyrosinated and detyrosinated α-tubulin near oscillating leading vs. trailing kinetochores:

• The authors assessed the accumulation of detyrosinated α-tubulin on kinetochore microtubules to build their model. However, based on the quality of the representative images (Fig. 3a,b), it seems very difficult to quantify detyrosinated α-tubulin levels on kinetochore microtubules and to carry out an accurate analysis. Please provide separate images for detyrosinated α-tubulin channel in Fig. 3a,b and include images for tyrosinated α-tubulin staining.

• It could be challenging but providing time-lapse live imaging of this process (i.e., the different distribution of tyrosinated and detyrosinated α-tubulin near oscillating leading vs. trailing kinetochores) is necessary to support the authors' conclusion.

• The authors proposed that shorter K-microtubules associate with leading kinetochores are enriched with detyrosinated α-tubulin, whereas longer K-microtubules associate with trailing kinetochores are enriched with tyrosinated α-tubulin. How did the authors exclude the possibility that some of kinetochores that are associated with relatively longer microtubules (previously trailing Kinetochores)

are in the middle of their corrective movement (i.e., become leading kinetochores) and, expectedly, should be associated with detyrosinated microtubules?

R: We totally agree with the reviewer and we now repeated the experiments using EB1 as a marker of polymerizing kinetochore microtubule plus-ends. The new data confirms our previous conclusion using a distinct experimental approach.

4- It is unclear why the authors expressed exogenous GFP-tagged detyrosinated or tyrosinated α-tubulin in all the groups to carry out the phenotypic analysis of TTL and VASH1/2 KO cell lines and to visualize microtubules (Page 4, line 12-15). Expressing detyrosinated or tyrosinated GFP-α-tubulin can influence the results in these cell lines, making the results difficult to interpret. Indeed, expressing detyrosinated GFP-□-tubulin showed different effect than expressing tyrosinated GFP-α-tubulin on TTL KO cells as shown in Extended Data Fig. 3b. Moreover, Fig. 1b should include a control group without expressing detyrosinated or tyrosinated GFP-α-tubulin.

R: As pointed out and recognized by reviewer #1, there are detyr and tyr forms of tubulin that result from translation of specific tubulin isotype genes and could mask the effects of the modifications when introduced post-translationally. This is why we tried to minimize the impact of translated forms by expressing modified detyr or tyr tubulin in the respective KO cells. Without expression of these translated forms, phenotypes are nearly absent, since KO cells rapidly adapt to the new condition (presumably by compensating via genetic expression). This adaptation mechanism is extremely interesting and something that we are currently pursuing in the lab, but it is simply too premature at this stage to draw any meaningful conclusion. Importantly, we rule-out the effect of the overexpression of tubulins by always expressing both forms in each KO line. Only the combined expression of a specifically modified tubulin in the correct genetic background originates a phenotype. This is now clarified in the main text.

5- Although TTL and VASH1/2 KO cells represent suitable models to study how α-tubulin detyrosination/tyrosination impacts mitosis, rescue experiments need to be conducted to validate these systems. For example, the authors may express VASH2/SVBP to rescue the phenotypes of VASH1/2 KO cells.

R: All KO lines used previously published and validated sgRNAs, including the requested rescue experiment in VASH1/2 KO cells (Nieuwenhuis, J. et al. Vasohibins encode tubulin detyrosinating activity. Science (New York, N.Y 358, 1453-1456 (2017). We highlight that we used non-clonal KO populations that likely result from slight distinct, yet highly specific, genetic lesions. We demonstrate KO efficiently by monitoring the levels of the target enzymes and/or the associated tubulin PTMs. Whenever antibodies were not available, as it was the case for VASH2, we now provide PCR analysis of the respective genomic locus that includes the CRISPR-targeted sequence (new Extended Data Fig. 1c).

6- In Fig. 5, the authors conducted a mass spectrometry analysis to determine proteins that preferentially associate with tyrosinated vs. detyrosinated microtubules from only two cell lines (TTL or VASH1/2 KO cell lines). This experiment needs to be done with a proper control group (WT cell line). Also, the authors need to discuss the possibility that this experiment may not provide a clear answer to the "direct" association of identified proteins to tyrosinated or detyrosinated microtubules. For

example, this association might be indirect. Indeed, α-tubulin detyrosination/tyrosination highly affects other microtubule post-translational modifications such as acetylation which could be the reason of this preferential association.

R: We agree that the interaction might be indirect and we now clarify this aspect in the main text. Importantly, our previous analysis with a parental WT line was not sufficiently robust to draw semi-quantitative conclusions about relative protein abundance when compared with either TTL or VASH1/2 KO lines. Because meaningful differences (as defined in the criteria described in the main text) were only obtained when the two most extreme conditions were compared, we adopted this approach.

7- TLL KO cells express high detyrosinated α-tubulin levels and low tyrosinated α-tubulin levels. Throughout the manuscript, it is unclear whether the obtained findings are due to the presence of high levels of detyrosinated microtubules (i.e. the direct effect of abnormally increased detyrosinated microtubules) or due to the requirement of tyrosinated microtubules (i.e. absence of tyrosinated microtubules).

R: These two possibilities are inherently linked since these are two variants of a common tubulin pool and, therefore, cannot be dissociated. Nevertheless, the impact of TTL or VASH1/2 KO in the levels of Tyr tubulin is minimal since it represents the largest fraction that normally exists in these cells and continues to be translated. Therefore, the observed phenotypes are likely due to changes in the detyrosinated tubulin pool, as this is the one that is rate-limiting and post-translationally introduced into polymerized microtubules.

8- RNAi-mediated knockdown of MATCAP has no additive effect on detyrosinated α-tubulin levels in VASH1/2 KO cells (Extended Data Fig. 1c). However, in some experiments MATCAP RNAi resulted in stronger phenotypes in VASH1/2 KO (such as in Extended Data Fig. 2a,b) or had no additive effect on VASH1/2 KO (such as in Fig. 2C). This discrepancy raises the doubt that some of the phenotypes of VASH1/2 KO and/or MATCAP RNAi might not be due to decreased α-tubulin detyrosination.

R: We are confident that the observed differences are due to the detection limit for detyr tubulin by western blotting that changes as a function of total protein loaded in the gel. Minor discrepancies between phenotypes might be due to different depletion efficiency of MATCAP by RNAi which varies between experiments.

9- Quantifications:

- All western blot data lack quantifications.

R: We now provide these quantifications, also as requested by Reviewers #1 and #2

- Please provide the number of examined cells in each figure legend.

R: We apologize for the oversight, we had not indicated this in some figure legends in the Extended Data because it was a sub-analysis of the same data set in the main figures (and referred as such). This is now corrected.

- Fig. 1c: Please provide quantifications for detyrosinated α-tubulin / α-tubulin ratio.

R: These are super-resolution images that were provided for qualitative appreciation only and are not suitable for quantitative purposes as they represent "nice and crispy" maximum intensity 3D projections. For quantifications on the levels of detyrosintaed and tyrosinated tubulin between experimental conditions, we refer to western blots, now properly quantified as requested.

- Please check the statistical analysis between groups in several quantifications. For example, in Extended Data Fig. 2b, given the wide data distribution (big error bars) and the very mild difference in spindle length (less than 1 µm), please check if there is a significance difference between the "Control Tyro GFP-α-tubulin" group and "Tyro GFP-α-tubulin TLL KO" group.

R: We verified our statistical comparisons between all groups, including Extended Data Fig. 2b (significant, $p<0.05$). The difference between spindle length is significant because of the large number of measurements, but likely not biologically relevant.

- Extended Data Fig. 2a: Is the scale bar (5 µm) correct? Based on this scale bar, it seems that the spindle length is less than 5 m in VASH1/2 KO + MATCAP RNAi group which is different from the quantification in Extended Data Fig. 2b.

R: The scale bar is correct and the example shown reflects those cases where the phenotype is more penetrant (please note a range between 6-13 micrometers in the VASH1/2 KO + MATCAP RNAi condition). Also, the examples shown in the figure are 2D projections and do not account for possible spindle tilting in 3D that was taken into account in the length measurements.

- Extended Data Fig. 7a: I cannot see a noticeable difference in the level of NDC80 between parental and VASH1/2 KO cells.

R: We now provide inverted grayscale images for the NDC80 and CLASP2 channels only, using both max and sum projections, the latter used for quantification purposes.

10- Page 7 and Extended Data Fig. 5a: What is the significance of assessing the metaphase plate width in CENP-E-inhibited cells if the analysis was done only on aligned chromosomes. Is this the case for all groups within this experiment?

R: Because CENP-E inhibition gives rise to perfect metaphase plates with the exception of just few chromosomes that remain in close proximity to the poles due to their dependence on CENP-E motor activity for alignment (and are known to have unattached kinetochores; see Barisic et al., NCB, 2014), we concentrated on any putative additional role of CENP-E in the regulation of chromosome positioning (and kinetochore-microtubule attachments) upon completing alignment, for a fair comparison with the other experimental conditions (and parental controls) that do not give rise to chromosomes at the poles. This is now clarified in the main text.

11- There are other studies indicating that tight regulation of tyrosinated and detyrosinated microtubules are critical for proper kinetochore-microtubule attachments. Please cite those studies.

R: We apologize, but we are not aware of such studies, although we cite several papers, including our own work, that investigate the role of this modification in other aspects of mitosis.

Reviewer #4 (Remarks to the Author):

This manuscript entitled "α-tubulin detyrosination regulates kinetochore-microtubule attachments" by Girão et al describes the role of detyrosination during mitosis. The study shows kinetochore proteins differential binding to tyrosinated/detyrosinated microtubules. The authors have engineered cell lines with enzymes that modulate the tyrosinated/detyrosinated levels and mass spectrometry to identify the KMN network of kinetochore complex binds to the tyrosinated microtubules. While **the experiments and methods used are of good quality**, the model presented here contradicts the biochemical and cell biology data and does not take into account the global effects of modulating tyrosinated/detyrosinated levels. To this reviewer the manuscript does not address the problem that the authors have claimed, I have detailed the following summary. In light of this, I do not recommend publication in its current form and a significant revision is necessary.

R: We thank the reviewer for recognizing the quality of our study, while identifying potential weaknesses that we hope to have now clarified with additional experiments and extensive re-writing of the main text.

Some specific queries are outlined below;

1. In the CRISPR KO of VASH and TTL, the tyrosinated vs detyrosinated microtubule levels will be ideal to quantify. It must also be noted that the KO of either enzyme is equivalent to the overexpression of opposite enzymes and it is not clear why the authors chose only the CRISPR KO method, some key experiments need to be validated with overexpression of enzymes.

R: We now quantify detyr and tyr tub levels, also as requested by all other reviewers. We have previously conducted experiments with overexpression of VASH1/2, which phenocopied TTL loss of function (Ferreira et al., JCB, 2020). We would like to avoid TTL overexpression, as this is known to impair tubulin polymerization (Szyk, A., Deaconescu, A. M., Piszczek, G. & Roll-Mecak, A. Tubulin tyrosine ligase structure reveals adaptation of an ancient fold to bind and modify tubulin. Nat Struct Mol Biol 18, 1250-1258 (2011). For these reasons, we thought the CRISPR method would be ideal and without indirect effects over tubulin polymerization.

2. The authors have quantified the longer vs shorter microtubule Tyr/DeTyr levels. It is unclear how the demarcation of longer vs shorter criteria was chosen. Similarly, the percentage of Tyr/DeTyr levels including the free tubulin will make the conclusions valid.

R: We agree with the reviewer and we now provide experiments using EB1 as a marker of polymerizing kinetochore microtubule plus-ends. The new data confirms our previous conclusion using a distinct experimental approach.

3. From the mass spec data, the authors have chosen to focus on kinetochore proteins, however, the data points out that several signaling protein levels have been modulated due to KO of enzymes, perhaps the data points to a missing link of Tyr/DeTyr levels and global regulation of mitosis/cell division. While it is understood that it might be beyond the scope of this study, a discussion on this could be valuable to the community. Related to point no. 1 at least the key components of mass spec data can be validated with overexpression of VASH/TTL enzymes.

R: We thank the reviewer for pointing out this often neglected pathways and we now refer to them in the main text, although we admit our ignorance and lack of expertise in exploring these results. The reasons not to use overexpression of tubulin modifying enzymes have been justified in our response to point #1 of this reviewer.

4. In the in vitro work, the authors purified only NDC80 as a case study to demonstrate the effect of Tyr/deTyr levels. From their data, only a small increase in the diffusion of NDC80 can be observed with deTyr microtubules. The authors provide a model that KMN network loading is aided by the tyrosinated microtubules and then the discussion of kinetochore is facilitated by the deTyr microtubules. The current data is insufficient to make such an argument, for example, the overall KMN network might have different binding/diffusion constants with Tyr microtubules or vice versa. Several important assessments are needed here before arriving at such a conclusion; A) Titration of Tyr/deTyr levels microtubule on the microtubules will provide information about the threshold for such a switch. B) Earlier studies have shown NDC80 interaction with CTTs (from cryoEM structural work of NDC80:MT), so it is known that PTMs in CTT might affect the KMN network. Here the binding constants of NDC80 or KMN titration against Tyr and deTyr microtubules will strengthen the mass spec data. C) Chimeric microtubules having Tyr and DeTyr within the same microtubule have been used to show the effects of tyrosination on dynein motility, a similar approach can be applied here to show such small changes.

R: The reviewer has a point and we now fully acknowledge the fact that the behaviour of NDC80 in vitro might be an underestimation of the full potential brought by the entire KMN network in cells, or even through other sub-complexes with ZWINT. This is now extensively discussed in the main text. Nevertheless, we think that working with “just” the NDC80 complex in vitro is a reasonable first approximation to the problem, given that it is the major microtubule binding component of the KMN complex. Importantly, we stress that our data do not indicate any switch-like effects of the NDC80 complex on the different types of microtubules (i.e. detyrosinated and tyrosinated), but rather these PTMs gradually tune NDC80 complex diffusion along microtubules. This is consistent with our estimate of the changes in binding affinity of the NDC80 complex in vitro, which are indeed very small. We also note that dynein walks processively for many microns and unidirectionally, whereas NDC80 molecules undergo Brownian motions for under 300 msec, making the suggested assays not practical.

5. The authors in the abstract have used ‘preference’ and ‘preferential binding’, there is no data to support preference, so I suggest avoiding using such anthropogenic terms to describe differential binding between two microtubule states.

R: We now largely adopted the suggested terminology.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all my comments to my satisfaction. I believe the manuscript is greatly improved with revision.

Reviewer #2 (Remarks to the Author):

This is a revised version of the MS describing tubulin detyrosination/tyrosination by Maiato and his colleagues. I found that authors performed additional experiments to address concerns from reviewers and they extensively revised text. I feel that the paper is improved well, and support with the publication.

Reviewer #3 (Remarks to the Author):

The authors did not highlight the changes in the revised manuscript or provide line numbers in their response letter, which made the revision process challenging. However, the authors have addressed almost my comments and significantly improved the manuscript. I still think that it is an interesting and important study. A few minor comments remain:

- The authors responded to my concerns in the response letter, but some of these responses were not adequately incorporated in the text. Specifically, comments # 4, 6 and 8.

- Comment #5: The validation of VASH1/2 KO cells in the previous publication (Nieuwenhuis et al. Science, 2017) was conducted in a different cell line (hap1 cells). The efficiency/specificity of the KO may vary with cell type. I still believe that performing a rescue experiment in the used cell line is important to strengthen the data (i.e. expressing VASH2/SVBP to rescue, at least, the main phenotype of VASH1/2 KO cells). Alternatively, the authors could "discuss" the specificity and efficiency of the KO by referring to the rescue experiment reported in Nieuwenhuis et al. (Science, 2017).

- Extended Data Fig. 2.: I still suggest replacing the representative image of VASH1/2 KO + MATCAP RNAi group. Based on the scale bar, the spindle length is ~5 um. Even in the presence of spindle tilting, this is much shorter than the average (~9.5 um). Selecting another representative image that falls within the “average” range would be more appropriate.

- Consistent with this study and previous publications, Akera et al. (PMID: 29097549) suggested that increased tubulin tyrosination at the spindle pole side facing the oocyte cortex is associated with microtubule detachment from large centromeres. Similarly, PMID: 36800430 showed that excessive tubulin tyrosination perturbs correct centromere-microtubule attachments in oocytes.

Reviewer #4 (Remarks to the Author):

The authors have sufficiently addressed my previous queries except for the following points;

In the previous reviewer report, quantification of tyrosinated vs detyrosinated microtubule levels and validation of KO results by overexpression (OE) of opposite enzyme was raised. While the authors have quantified the levels Tyrd/deTyr to some degree of satisfaction. The KO Vs OE remains still unaddressed, it is understood that the TTL OE leads to other effects, however the authors should address VASH OE validation, this becomes important if the subtle effects of VASH KO seen here are truly due to the targeting the detyrosinase enzyme or CRISPR off-target effects.

Similarly, I fails to understand how EB1 experiments addresses the following queries raised earlier. "The authors have quantified the longer vs shorter microtubule Tyr/DeTyr levels. It is unclear how the demarcation of longer vs shorter criteria was chosen. Similarly, the percentage of Tyr/DeTyr levels including the free tubulin will make the conclusions valid".

REVIEWER COMMENTS (our responses in blue)

Reviewer #1 (Remarks to the Author):

The authors have addressed all my comments to my satisfaction. I believe the manuscript is greatly improved with revision.

R: We thank the reviewer for recognizing our effort in improving the manuscript.

Reviewer #2 (Remarks to the Author):

This is a revised version of the MS describing tubulin detyrosination/tyrosination by Maiato and his colleagues. I found that authors performed additional experiments to address concerns from reviewers and they extensively revised text. I feel that the paper is improved well, and support with the publication.

R: We thank the reviewer for recognizing our effort in improving the manuscript.

Reviewer #3 (Remarks to the Author):

The authors did not highlight the changes in the revised manuscript or provide line numbers in their response letter, which made the revision process challenging. However, the authors have addressed almost my comments and significantly improved the manuscript. I still think that it is an interesting and important study.

R: We thank the reviewer for recognizing our effort in improving the manuscript and apologize for not highlighting the changes in the revised manuscript. This was due to the extensive re-writing of the main text, which made it difficult to track very specific changes.

A few minor comments remain:

- The authors responded to my concerns in the response letter, but some of these responses were not adequately incorporated in the text. Specifically, comments # 4, 6 and 8.

R: Regarding our response to original comment #4, which we reproduce here for clarity:

"As pointed out and recognized by reviewer #1, there are detyr and tyr forms of tubulin that result from translation of specific tubulin isotype genes and could mask the effects of the modifications when introduced post-translationally. This is why we tried to minimize the impact of translated forms by expressing modified detyr or tyr tubulin in the respective KO cells. Without expression of these translated forms, phenotypes are nearly absent, since KO cells rapidly adapt to the new condition (presumably by compensating via genetic expression). This adaptation mechanism is extremely interesting and something that we are currently pursuing in the lab, but it is simply too premature at this stage to draw any meaningful conclusion. Importantly, we rule-out the effect of the overexpression of tubulins by always expressing both forms in each KO line. Only the combined expression of a specifically modified tubulin in the correct genetic background originates a phenotype. This is now clarified in the main text."

We have incorporated it in the main text as following: "Previous RNAi-based studies that aimed at interfering with TTL function indicated that the α-tubulin detyrosination/tyrosination code played a critical role in error correction during mitosis by regulating the activity of microtubule depolymerizing enzymes at centromeres/kinetochores. HOWEVER, COMPENSATORY OR CELLULAR ADAPTATION MECHANISMS TO THE CHRONIC LOSS OF TTL APPEAR TO EXIST, POSSIBLY INVOLVING THE CONTRIBUTION OF GENETICALLY-ENCODED TYROSINATED α-TUBULIN. Indeed, IN CONTRAST TO TTL KO CELLS EXPRESSING TYROSINATED GFP-α-TUBULIN, TTL KO CELLS CONSTITUTIVELY EXPRESSING DETYROSINATED GFP-α-TUBULIN SHOWED ~2-FOLD INCREASE IN THE FREQUENCY OF CHROMOSOME SEGREGATION ERRORS, consisting mostly in transient anaphase lagging chromosomes, a minority of which resulted in the formation of micronuclei (Extended Data Fig. 3a, b and Supplementary Video 1). THUS, UNDER THE FORCED EXPRESSION OF DETYROSINATED α-TUBULIN, CELLS ARE ABLE TO COMPENSATE/ADAPT TO THE CHRONIC LOSS OF TTL."

We think that we cannot be clearer on this point.

Regarding our response to original comment #6, which we reproduce here for clarity: "We agree that the interaction might be indirect and we now clarify this aspect in the main text. Importantly, our previous analysis with a parental WT line was not sufficiently robust to draw semi-quantitative conclusions about relative protein abundance when compared with either TTL or VASH1/2 KO lines. Because meaningful differences (as defined in the criteria described in the main text) were only obtained when the two most extreme conditions were compared, we adopted this approach."

We have incorporated it in the main text both in the results section and discussion as following: "Overall, our results identify a large cohort of potential DIRECT and INDIRECT readers of the α-tubulin detyrosination/tyrosination code in mitosis, suggesting that this code regulates a broad range of mitotic processes, including spindle microtubule organization and dynamics, centriole assembly and the formation of a functional kinetochore-microtubule interface." / "subsequent in vitro reconstitution experiments with purified NDC80 complex revealed no significant preference for its association with tyrosinated microtubules, suggesting that enrichment of the NDC80 complex on tyrosinated spindle microtubules might be INDIRECT and/or INVOLVE ADDITIONAL FACTORS."

We now also highlight the reason for using TTL and VASH1/2 KO cell extracts as following: "Our choice for TTL KO or VASH1/2 KO mitotic cell extracts was based on the rationale that they constitute two extreme conditions where microtubules can be experimentally enriched for detyrosinated and tyrosinated α-tubulin, respectively, and thus more likely to reveal meaningful differences on enriched MAPs."

Regarding our response to original comment #8, which we reproduce here for clarity: "We are confident that the observed differences are due to the detection limit for detyr tubulin by western blotting that changes as a function of total protein loaded in the gel. Minor discrepancies between phenotypes might be due to different depletion efficiency of MATCAP by RNAi which varies between experiments."

We now highlight in the methods section the following: “Minor inter-experimental differences after MATCAP RNAi might be due to the detection limit of detyrosinated tubulin by western blotting that changes as a function of the total protein loaded in the gel, or due to different depletion efficiency between experiments.”

- Comment #5: The validation of VASH1/2 KO cells in the previous publication (Nieuwenhuis et al. Science, 2017) was conducted in a different cell line (hap1 cells). The efficiency/specificity of the KO may vary with cell type. I still believe that performing a rescue experiment in the used cell line is important to strengthen the data (i.e. expressing VASH2/SVBP to rescue, at least, the main phenotype of VASH1/2 KO cells). Alternatively, the authors could "discuss" the specificity and efficiency of the KO by referring to the rescue experiment reported in Nieuwenhuis et al. (Science, 2017).

R: We believe that the issue of efficiency is now clarified by providing either western blotting or PCR of the genomic locus for each target. As for the specificity issue, it is highly unlikely that a guide RNA results in on-target deletions in one cell line and off-target deletions in another cell line from the same species. Nevertheless, we now refer to the rescue experiment reported in Nieuwenhuis et al. (Science, 2017) in the methods section, as suggested by the reviewer. It reads as following: “Previous rescue experiments in human Hap1 cells validate specificity of the VASH1/2 sgRNAs (ref. 4).”

- Extended Data Fig. 2.: I still suggest replacing the representative image of VASH1/2 KO + MATCAP RNAi group. Based on the scale bar, the spindle length is ~5 um. Even in the presence of spindle tilting, this is much shorter than the average (~9.5 um). Selecting another representative image that falls within the “average” range would be more appropriate.

R: We have now replaced the original image by another image that better represents the average spindle length, as recommended by the reviewer.

- Consistent with this study and previous publications, Akera et al. (PMID: 29097549) suggested that increased tubulin tyrosination at the spindle pole side facing the oocyte cortex is associated with microtubule detachment from large centromeres. Similarly, PMID: 36800430 showed that excessive tubulin tyrosination perturbs correct centromere-microtubule attachments in oocytes.

R: We thank the reviewer for highlighting these studies. Due to space limitations, we now only cite PMID: 29097549 in our Discussion section. We believe that the findings reported in PMID: 36800430 reflect a role for tyrosinated tubulin in error correction (already extensively covered in the main text for somatic cells), rather than the establishment of attachments per se.

Reviewer #4 (Remarks to the Author):

The authors have sufficiently addressed my previous queries except for the following points;

In the previous reviewer report, quantification of tyrosinated vs detyrosinated microtubule levels and validation of KO results by overexpression (OE) of opposite enzyme was raised. While the authors have quantified the levels Tyrd/deTyr to some degree of satisfaction. The KO Vs OE remains still unaddressed, it is understood that the TTL OE leads to other effects, however the authors should address VASH OE validation, this becomes important if the subtle effects of VASH KO seen here are truly due to the targeting the detyrosinase enzyme or CRISPR off-target effects.

R: As explained to a similar point raised by reviewer #3, we believe that the issue of efficiency is now clarified by providing either western blotting or PCR of the genomic locus for each target. In addition, as we now indicate in the Methods section, previous rescue experiments in human Hap1 cells validate the specificity of the VASH1/2 sgRNAs (Nieuwenhuis et al, 2017). Most relevant, we do not think that the VASH OE experiment requested by the reviewer would add anything to the effects observed in the VASH KO, since VASH OE would only phenocopy the TTL depletion. As we refer in our original response, we have shown previously that VASH1/2 overexpression phenocopies TTL loss of function (Ferreira et al., JCB, 2020).

Similarly, I fails to understand how EB1 experiments addresses the following queries raised earlier. "The authors have quantified the longer vs shorter microtubule Tyr/DeTyr levels. It is unclear how the demarcation of longer vs shorter criteria was chosen. Similarly, the percentage of Tyr/DeTyr levels including the free tubulin will make the conclusions valid".

R: We no longer refer to longer vs. shorter microtubules in the manuscript. Instead, we use EB1 as a bona fide marker of growing microtubule plus-ends to quantify the respective tyr/detyr levels on growing or shrinking kinetochore microtubule plus-ends as a proxy for chromosomes moving away or towards the associated pole, respectively.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The authors have addressed all my comments and significantly improved the manuscript.

Reviewer #4 (Remarks to the Author):

The authors have addressed all the concerns and the MS is significantly improved and reads well. I do not have any further queries.