nature portfolio

Peer Review File

Doublecortin restricts neuronal branching by regulating tubulin polyglutamylation

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
Sebastien et al. investigated a tubulin posttranslational modification, polyglutamylation, and its developmental role on neuronal branching and migration associated with doublecortin.
In response to Reviewer 1, a substantial amount of data has been added to address the criticism, and most of the criticism has been addressed. Due to the lack of antibodies specific to TTLL and CCP, the authors could not show the localization of endogenous TTLL.

The new title adequately reflects the data presented and is more precise.

A few points remain, such as the referee's well-taken point on choosing lysosomal trafficking as an unusual cargo is unresolved in the revised manuscript. Other dyes are available to label mitochondria easily (mitotracker). Given that there is an effect on lysosomal trafficking in DCX KO neurons, mitochondrial or vesicular transport is also likely impaired and could impact overall neuronal homeostasis.
In addition, throughout the study, DIV 3 is chosen. Looking at neuronal morphology at a later developmental stage would strengthen the link between DCX and polyE, as it could be a transient phenomenon.
Only polyE reduction is confirmed in iPSC-derived neurons carrying human DCX mutations. Information regarding neurite number on patient neurons (at least in fixed cultures) is necessary to make claims about human disease.

Fluorescent images are only presented in inverted modes with separate channels. Merged, pseudocolored panels would help appreciate the up or downregulation of polyE or tubulin.
Sebastien et al. modulate glutamylation levels by overexpressing TTLL11, an elongating glutamylase. Effects of decreasing polyE levels by overexpressing a CCP in context of DCX KO are not investigated.

Minor points:
Figure 2 and elsewhere: only one scale bar per panel should be presented
Figure 3: a higher zoom or an inset showing the reduced tubulin staining on the tip would be helpful. The caption indicates it is Tubb3 staining, but on the panel it says tuba. Please clarify.
Figure 5 and 6: a higher zoom is necessary to show the PolyE levels more convincingly.
Figure 7: do tubulin levels change in U2OS cells?

Reviewer #5

(Remarks to the Author)
In their study, Sebastien et al. provide a comprehensive research study on the role of the microtubule-associated protein, doublecortin (DCX), in regulating neuronal branching and migration by promoting tubulin polyglutamylation. The authors utilize the CRISPR/Cas9 system to knock out the dcx gene in induced pluripotent stem cells (iPSCs) then differentiating them into cortical neurons. They find that DCX knockout (KO) neurons exhibit reduced nuclear movement velocities,

increased neurite branching, and impaired lysosome processivity. Interestingly, despite these changes, microtubule dynamics, as measured by EB comet dynamics, were unaffected in DCX-KO neurons, answering a long-standing question in the field about the role of DCX in microtubule polymerization. One of the most interesting findings in the paper is the significant reduction in α-tubulin polyglutamylation in DCX-KO neurons. This is the first study to link DCX and a tubulin posttranslational modification, highlighting a fascinating interplay between the MAP code and the tubulin code. The authors perform rescue experiments and find that polyglutamylation levels and neuronal branching are rescued by expressing DCX or TTLL11, an α-tubulin glutamylase. In addition, co-expression of DCX and TTLL11 in U2OS cells showed a synergistic increase in polyglutamylation. Finally, the authors produce neurons with lissencephaly-causing DCX mutations and find that these neurons also show reduced polyglutamylation levels. Overall, this study finds that DCX acts as a positive regulator of α-tubulin polyglutamylation, restricting neurite branching and maintaining microtubule homeostasis, which is crucial for proper neuronal migration and function. This is a beautiful study that has futhered our understanding of how the microtubule cytoskeleton is regulated in precise, synergistic ways by specific proteins during development. After seeing both the original and revised versions of the manuscript, I fully support publication in Nature Communications.

I have one suggested modification for Figure 2. The title states “DCX-KO neurons establish more branches through increased rates of initiation/retraction.” The word retraction should be deleted. The rates of initiation are significantly increased, but the rates of retraction are not. That is likely the reason for increased neurite number. Yet the title makes it sound like there are also increases in retraction rates, making it confusing to the reader.

Sébastien et al. Response to Reviews #2

Thank you for your continued interest and support for our manuscript. Below please find a point-by-point response to the concerns raised by Reviewers 4 & 5 in the 2nd round of peer review at *Nature* journals. As in our previous Response, reviewer comments are shown in blue and our response is shown in black.

REVIEWER #4

Reviewer #4 (Remarks to the Author):
Sebastien et al. investigated a tubulin posttranslational modification, polyglutamylation, and its developmental role on neuronal branching and migration associated with doublecortin. In response to Reviewer 1, a substantial amount of data has been added to address the criticism, and most of the criticism has been addressed. Due to the lack of antibodies specific to TTLL and CCP, the authors could not show the localization of endogenous TTLL.
The new title adequately reflects the data presented and is more precise.

Thank you for the positive evaluation of our new data, which indeed represented a considerable investment. We agree!

A few points remain, such as the referee's well-taken point on choosing lysosomal trafficking as an unusual cargo is unresolved in the revised manuscript. Other dyes are available to label mitochondria easily (mitotracker). Given that there is an effect on lysosomal trafficking in DCX KO neurons, mitochondrial or vesicular transport is also likely impaired and could impact overall neuronal homeostasis.

As discussed in our previous Response to Reviews, we did not pursue a comprehensive analysis of intracellular trafficking in our DCX-KO lines. We believe such an analysis warrants its own manuscript. Rather, our experiments with Lysotracker are intended to confirm, in our model system, the previously established concept that both DCX and polyglutamylation are regulators of trafficking (e.g., see Liu et al., *Mol Cell* 2012 for DCX and Magiera et al., *EMBO J* 2018 for polyE). Additional data on the transport of mitochondria or other cargos would make our results denser for the reader without changing the core discovery, which is the link between DCX and polyglutamylation.

Of course we are fascinated by this link, and by how DCX-KO is distinct from the direct perturbation of polyglutamylation (e.g. Sirajuddin et al., *Nat Cell Biol* 2014, Lessard et al., *J Biol Chem* 2019, and Bodakuntla et al., *EMBO J* 2021 for polyglutamylation and Deuel et al., *Neuron* 2006, Lipka et al., *EMBO J* 2016, Monroy et al., *Dev Cell* 2020, and Fu et al., *Elife* 2022 for DCX). Careful analysis of additional cargo types is absolutely a priority, and mitochondria are a clear place to start, as the reviewer points out.

Indeed, we have already started, and we have performed experiments with Mitotracker dye in control and DCX-KO neurons. As predicted, mitochondrial trafficking is disrupted (see Figure R1): the mitochondria show reduced processivity and more frequent switching of directions. These phenotypes are similar to the lysosome phenotypes. However, this project arm is the basis of new initiatives associated with new trainees, who will include the mitochondrial data along

with experiments in the pipeline on several other cargos. Thus, we request permission to reserve this data for a separate publication that will better serve the field, especially considering the substantial data already added in the first revision.

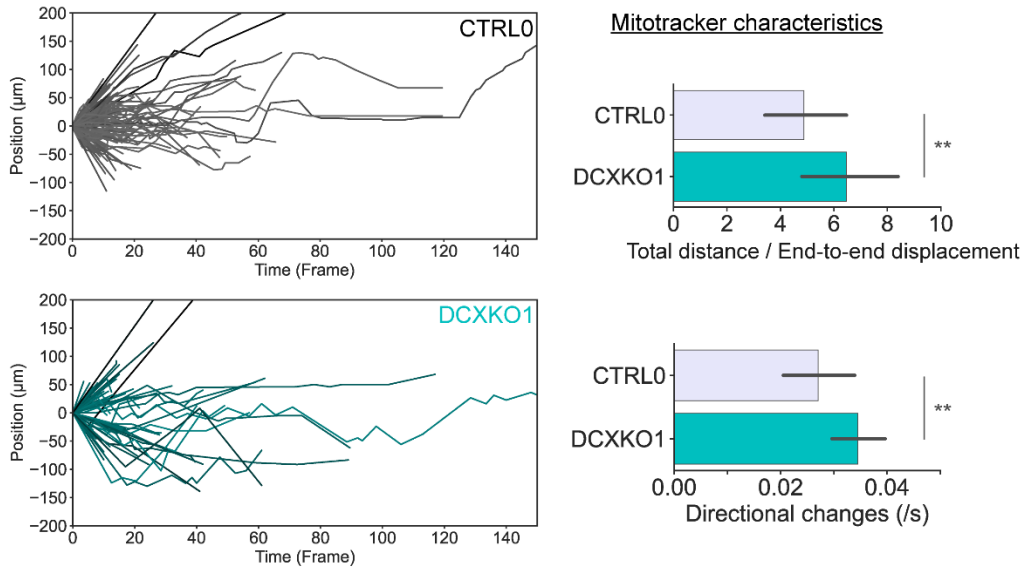

*Figure R1: (Left) Position plots for mitochondria visually detected on neurite kymographs during 150 frames for CTRL0 and DCXKO1 neurons. (Top right) Total distance / End-to-end displacement and anterograde to retrograde directionality switch per second (Bottom right). Statistical analysis was performed using the Student's t-test and revealed a statistically significant p value, p<0.001, **.*

In addition, throughout the study, DIV 3 is chosen. Looking at neuronal morphology at a later developmental stage would strengthen the link between DCX and polyE, as it could be a transient phenomenon.

First, the link between DCX and polyE has important implications to neuronal migration and morphology both if it is transient and if it persists into neuronal maturation. After all, DCX expression itself is transient, dropping significantly in brain tissue after the completion of neuronal migration and establishment of cortical layering. Many phenomena in development are transient but nevertheless essential.

Of course, we did explore developmental time frames when we initiated our study, and DIV3 was carefully chosen as the time point for comprehensive analysis. We found that DCX expression becomes detectable at DIV1 and remains observable until DIV14, which is the longest time point we have currently measured. As the cultures develop, the neurons develop larger and more complex arborizations, as expected; it quickly becomes challenging to assign any given neuronal process to its point of origin due to the density of the cultures. We chose DIV3 as the best time point because: (1) DCX expression is high and (2) neurites can be readily individuated and assigned to a point of origin. We have not gone deeper with other DIV's to avoid overwhelming

the reader and because analysis in more mature cultures would be difficult to interpret due to the technical challenges described above.

Despite the technical challenges of dense cultures, we *can* confirm that polyE levels remain low at later time points. As shown in Figure R2 below (Figure S9 in the manuscript), immunocytochemistry images at DIV7 and DIV14 show a clear reduction in polyE.

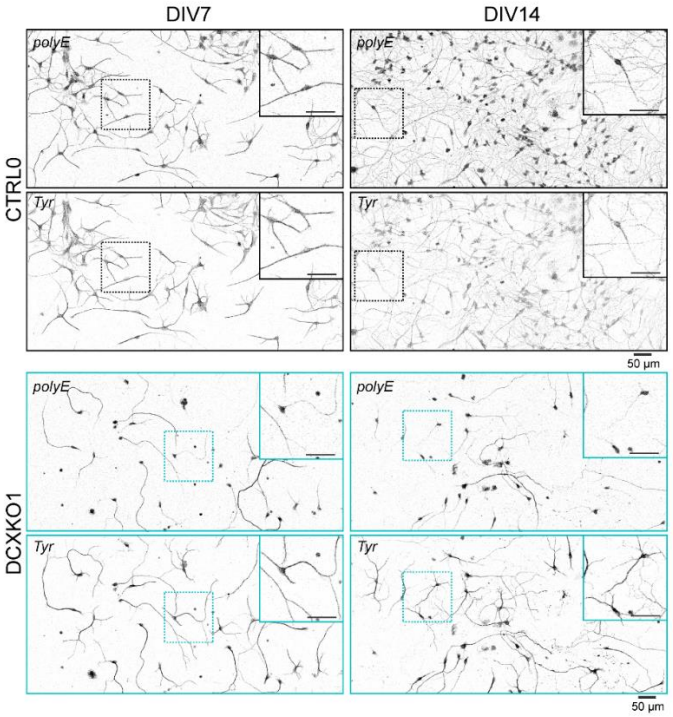

Figure R2: Representative images of CTRL0 and DCXKO1 neurons at DIV7 and DIV14, stained for polyglutamylation (polyE) and tyrosinated tubulin (Tyr). PolyE signal is low in DCXKO neurons while Tyr does not change between the two genotypes.

Only polyE reduction is confirmed in iPSC-derived neurons carrying human DCX mutations. Information regarding neurite number on patient neurons (at least in fixed cultures) is necessary to make claims about human disease.

As discussed in our previous Response to Reviews, the detailed analysis of multiple CRISPR'd cell lines in rigorously parallel conditions is technically challenging and beyond the scope of the current manuscript. We agree that we should be cautious in extrapolating from our iPSC cultures all the way out to a complex human disease. Thus, we have toned down our claims about the link between our results and X-linked lissencephaly.

In the introduction, line 103-104:

"Finally, we confirmed that polyglutamylation levels are reduced in 6 patient mutant knock-in neuronal models~~, linking our findings on the tubulin code to patient genotypes~~."

In the results section, line 341-343:

"To test ~~the relevance of our findings in the context of disease~~ whether polyglutamylation is perturbed by mutations in DCX, rather than gene knockout, we used our CRISPR/Cas9 approach to introduce point mutations into DCX found in patients with X-linked lissencephaly ~~causing patient mutations into DCX in iPSCs~~."

Fluorescent images are only presented in inverted modes with separate channels. Merged, pseudocolored panels would help appreciate the up or downregulation of polyE or tubulin.

We disagree, as the overlap between 2 channels largely depends on the reader's sensitivity to each color used. Our representation with separate channels is not subject to color sensitivity, as all channels are shown in black and white under strictly identical contrast conditions.

Sebastien et al. modulate glutamylation levels by overexpressing TTLL11, an elongating glutamylase. Effects of decreasing polyE levels by overexpressing a CCP in context of DCX KO are not investigated.

Respectfully, we do not see whether the experiment proposed by the reviewer will yield interpretable results. Polyglutamylation levels are already low in DCX-KO neurons. It's likely that the overexpression of a CCP will decrease the polyE levels further, but what does that tell us about DCX? Like TTLL's, the CCP family has some members that act primarily on chain initiation and some members that act primarily on chain length. A thorough exploration of the interplay between diverse TTLL's and CCP's is beyond the scope of this study.

Minor points:
Figure 2 and elsewhere: only one scale bar per panel should be presented

We prefer one scale bar per panel for the following reason: if every panel carries a scale bar, then the scale bar travels with the image if the figure is cropped, dissected, or only partially displayed. In our experience, figures are routinely cropped when transferred into PowerPoint presentations (e.g., for journal clubs). We agree that the repeated scale bars add some clutter, but on balance we believe it's worth it. We understand that not everyone will agree with this preference.

Figure 3: a higher zoom or an inset showing the reduced tubulin staining on the tip would be helpful. The caption indicates it is Tubb3 staining, but on the panel it says tuba. Please clarify.

Thank you for noticing this error, which we have fixed. We also added an inset at the neurite tip for better visualization of the staining (Fig. 3A).

Figure 5 and 6: a higher zoom is necessary to show the PolyE levels more convincingly.

As Figure 5 and 6 are already large, we included higher magnification images of the polyE signal in supplemental figure 8 (and here Figure R3) with insets showing the cell bodies and neurite tips.

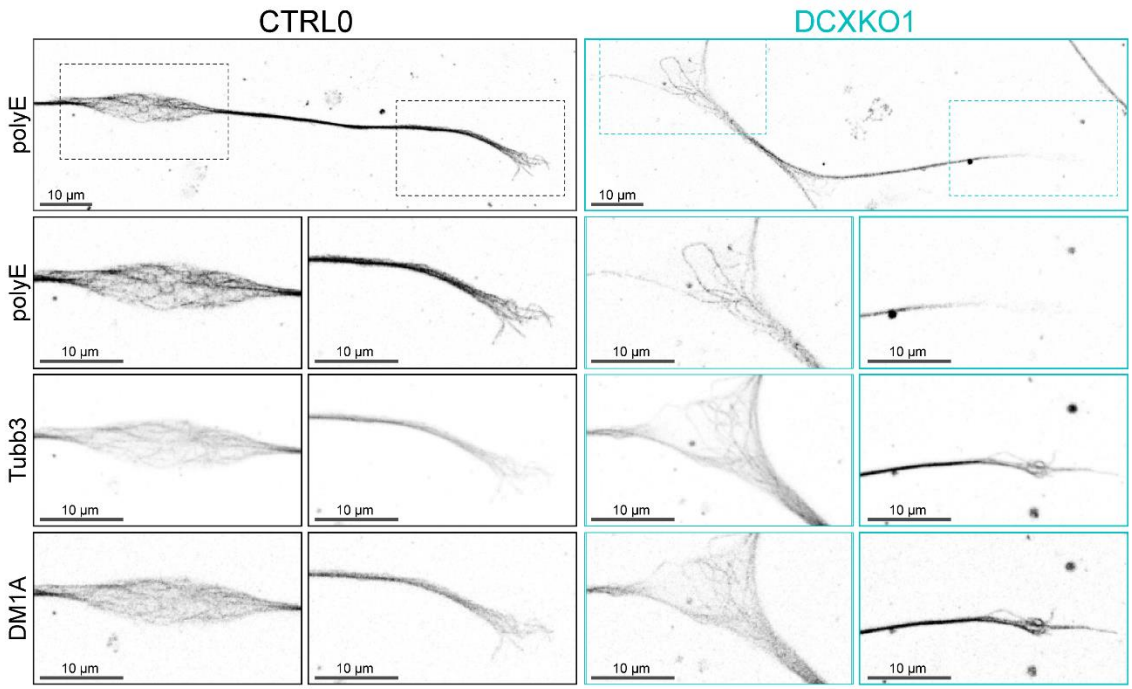

Figure R3: Representative images of CTRL0 and DCXKO1 neurons at DIV3, stained for polyglutamylation (polyE), α-Tubulin (DM1A) and β3-Tubulin (Tubb3).

Figure 7: do tubulin levels change in U2OS cells?

No, we do not have evidence of tubulin levels changing in U2OS cells (Immunoblot, Figure 7E). As shown in Figure 7D, the microtubule cytoskeleton and network organization is similar in all conditions in the U2OS cells.

REVIEWER #5

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Thank you for the positive feedback and support.

I have one suggested modification for Figure 2. The title states “DCX-KO neurons establish more branches through increased rates of initiation/retraction.” The word retraction should be deleted. The rates of initiation are significantly increased, but the rates of retraction are not. That is likely the reason for increased neurite number. Yet the title makes it sound like there are also increases in retraction rates, making it confusing to the reader.

We removed “retraction” from the figure title and the section title.