nature portfolio

Peer Review File

Metabolic regulation of cytoskeleton functions by HDAC6-catalyzed α-tubulin lactylation

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript entitled: "Metabolic regulation of cytoskeleton functions by HDAC6-catalyzed alpha-tubulin lactylation", submitted by Li and co-workers, describes a range of experiments investigation the cause and effects of tubulin lysine lactylation.
The central discovery that HDAC6, which is known hydrolase enzyme, acts as a lactyl transferase enzyme is intriguing and somewhat counter intuitive. However, reversibility in enzymatic activity depending on substrate/product concentration is of course well known from for example esterases.
The identity and the effects of the tubulin lactylation is convincing and interesting but the dactyl transferase activity part of the manuscript requires substantiation. When reporting a new enzymatic activity like this, the catalytic efficiency should be determined in a biochemical assay by measuring the kcat/Km in Michaelis-Menten experiments. The data supporting the claim in the manuscript is solely based on bands in Western blots. Further, the authors should provide all the details to understand the experiments performed, ideally also in figure captions, such as enzyme concentration, substrate concentration, lactate concentration, incubation time etc. Currently the figures are difficult to judge without having to dig through experimental procedures and the information does not seem to be sufficient there either.
Finally, the part on HDAC3 and HDAC8 lactyl transferase activity is too preliminary and missing proper control experiments to be included in the manuscript.

This reviewer finds that the manuscript could be reconsidered upon major revision.

Reviewer #2 (Remarks to the Author):

The work by Sun and colleagues describes for the first time that tubulin lactylation depends mostly on HDAC6 catalytic activity. HDAC6 and other protein family members lactylate tubulin proteins. Consequently, lactylation occurs when tubulins are deacetylated. The authors use several in vitro and in vivo approaches to show that lactylation depends mostly on HDAC6. The paper is well presented, it describes an interesting and novel biochemical event. The experiments are sound, and all the controls are in place. The work is original.

The authors affirm that lactylation is a post-translational modification connecting cell metabolism and cytoskeletal function. They modulate lactate production and mitochondrial respiration. How would lactylation change in hypoxia, a condition in which glycolysis is highly enhanced? And what about starvation? Do lactylation levels change globally?

Another critical point is the physiological relevance of lactylation in vivo. The authors nicely demonstrate an effect on neurite outgrowth and branching. HDAC6 inhibition has been previously shown to ameliorate spinal cord injury. How do the authors reconcile these opposite results? Considering that HDAC6 knockout prevents tubulin lactylation, what is the HDAC6 mouse phenotype in physiological or pathological conditions? What would be the outcome of spinal cord injury in vivo?

Lines 139-150 contrast with lines 164-164 regarding the role of MEC-17 on tubulin lactylation.

How do neurons respond to an increase in lactate? Isn’t it toxic at certain levels? Did the authors control for neuronal viability in Figure 6?

In Figure 1b, HDAC6 appears among the lactylated proteins. I am not asking for a biological meaning but to comment that the protein that primarily controls lactylation is lactylated as well.

Sirtuins are usually associated with longevity. In Extended Data Figure 4, Sirt2 catalyzes tubulin delactylation. Again, could the authors comment on the physiological role of alpha-tubulin lactylation?

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The manuscript entitled: "Metabolic regulation of cytoskeleton functions by HDAC6-catalyzed alpha-tubulin lactylation", submitted by Li and co-workers, describes a range of experiments investigation the cause and effects of tubulin lysine lactylation. The central discovery that HDAC6, which is known hydrolase enzyme, acts as a lactyl transferase enzyme is intriguing and somewhat counter intuitive. However, reversibility in enzymatic activity depending on substrate/product concentration is of course well known from for example esterases.

Response: We thank the reviewer for the comments and critiques. These suggestions have significantly improved the manuscript.

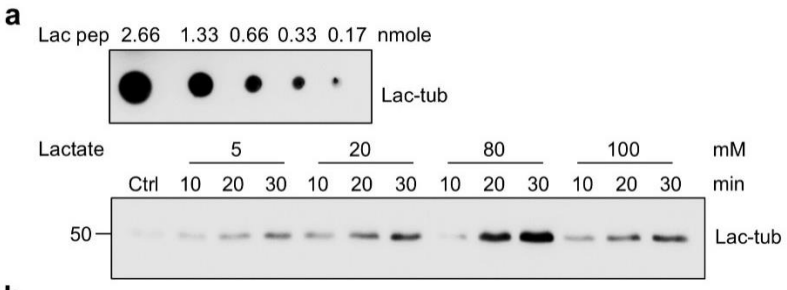
*1. The identity and the effects of the tubulin lactylation is convincing and interesting but the dactyl transferase activity part of the manuscript requires substantiation. When reporting a new enzymatic activity like this, the catalytic efficiency should be determined in a biochemical assay by measuring the **kcat/Km** in Michaelis-Menten experiments. The data supporting the claim in the manuscript is solely based on bands in Western blots.*

Response: We appreciate the constructive suggestions. To address these questions, we conducted additional experiments to measure the K_{cat}/K_m of HDAC6 in catalyzing α-tubulin lactylation. In the in-vitro lactylation assay, we used 4 μM purified HDAC6 as the enzyme, 2 μM purified tubulin heterodimers as the substrate, and various concentrations of lactate ranging from 5 mM to 160 mM. The reaction time points were set at 10, 20, and 30 minutes to determine the Km of HDAC6 for lactate.

Due to limitations in quantifying lactylated α-tubulin, we employed dot blot by using the synthesized peptide containing K40 lactylation to create a standard curve. The lactylated α-tubulin was revealed by immunoblot and the quantified by using the standard curve (shown in below). The results revealed that the Km of HDAC6 for **lactate** as substrate in catalyzing lactylation was 10.312±4.421 mM. Notably, the intracellular lactate concentration is as high as 2 mM to 30 mM. Compared to the K_m, the physiological lactate concentration is suitable for HDAC6-catalyzed protein lactylation. The turnover number (K_{cat}) of HDAC6 was 0.006±0.003 s^{-1} as shown in Fig. 3e. The relatively low catalytic efficiency may be due to HDAC6 low enzymatic activity, because previous studies have indicated that the K_{cat} of HDAC6 in catalyzing deacetylation is about 0.04/s[1].

Importantly, we discovered that HDAC6-catalyzed α-tubulin lactylation/delactylation is a reversible reaction. Notably, the critical concentration of lactate required to trigger HDAC6 as a lactyltransferase is about 1 mM (Fig. 3a). Considering that the intracellular concentration of lactate ranges from 2 to 30 mM, depending on cell types and metabolic status, we believe that HDAC6 functions as a lactyltransferase for α-tubulin.

These results have been provided in revised Fig. 3e and the manuscript (line 189-191, page 7).



b

	K_m for lactate (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}M^{-1}$)
α-tubulin K40	10.312±4.421	0.006±0.003	0.590

2. Further, the authors should provide all the details to understand the experiments performed, ideally also in figure captions, such as enzyme concentration, substrate concentration, lactate concentration, incubation time etc. Currently the figures are difficult to judge without having to dig through experimental procedures and the information does not seem to be sufficient there either.

Response: Thanks for the suggestion. We have added the enzyme concentration, tubulin concentration, lactate concentration and reaction time in the figure legends in each in-vitro assay (Fig. 3, Fig. 5, extended Data Fig. 1 and extended Data Fig. 4).

3. Finally, the part on HDAC3 and HDAC8 lactyl transferase activity is too preliminary and missing proper control experiments to be included in the manuscript.

Response: Thanks for the helpful suggestion. We think that demonstrating HDAC3's function as a lactyltransferase strengthens the idea that lactyltransferase activity may be conserved among HDAC family proteins.

To address the reviewer's concerns, we conducted additional experiments. First, we included the original mass spectrometry results (Extended Data Fig. 7a), which support the lactylation of EB3. Second, we observed that EB3 lactylation increased in cells overexpressing HDAC3 and decreased in HDAC3 KO cells (Extended Data Fig. 7b-e). Third, we found that the acetylation level of EB3 remained unchanged when HDAC3 or HDAC6 were overexpressed (Extended Data Fig. 7f, g). This suggests that the increase in EB3 lactylation by HDAC3 is not due to a decrease in acetylation. Fourth, we determined that deacetylase activity is necessary for lactyltransferase

activity. Evidence for this includes the abolishment of EB3 lactylation in cells treated with HDAC inhibitor TSA, or in cells expressing a deacetylase-dead mutant HDAC3-C145S (Extended Data Fig. 7h-k). Fifth, overexpression of HDAC3 led to increased global protein lactylation, while HDAC3 KO resulted in decreased global protein lactylation (Extended Data Fig. 7l, m). Similar results were observed for DCX lactylation (Extended Data Fig. 8).

Collectively, these results demonstrate that HDAC3 also functions as a lactyltransferase and likely has multiple substrates in cells. The lactyltransferase activity might be conserved in HDAC family proteins.

These results have been provided in the revised Extended Data Figures. 7 and 8, and the manuscript (line 339-349, page 10).

Reviewer #2 (Remarks to the Author):
The work by Sun and colleagues describes for the first time that tubulin lactylation depends mostly on HDAC6 catalytic activity. HDAC6 and other protein family members lactylate tubulin proteins. Consequently, lactylation occurs when tubulins are deacetylated. The authors use several in vitro and in vivo approaches to show that lactylation depends mostly on HDAC6. The paper is well presented, it describes an interesting and novel biochemical event. The experiments are sound, and all the controls are in place. The work is original.

Response: We thank the reviewer for the comments and critiques. These suggestions have significantly improved the manuscript.

1. The authors affirm that lactylation is a post-translational modification connecting cell metabolism and cytoskeletal function. They modulate lactate production and mitochondrial respiration. How would lactylation change in hypoxia, a condition in which glycolysis is highly enhanced? And what about starvation? Do lactylation levels change globally?

Response: We appreciate the constructive suggestions. To determine whether α-tubulin lactylation level is altered in hypoxia and glucose starvation, we performed additional experiments. In the hypoxia, the cells were incubated in 1% O_2 condition for 24 hours. Both global protein lactylation and α-tubulin lactylation were dramatically increased in cells after hypoxia (Extended Data Fig. 3k, l). In contrast, glucose depletion for 24 hours resulted in reduced global protein lactylation and α-tubulin lactylation (Extended Data Fig. 3m, n). These results further support that cell metabolism modulates α-tubulin lactylation.
These results have been provided in the revised Extended Data Fig. 3 and the manuscript (line 225-230, page 8).

2. Another critical point is the physiological relevance of lactylation in vivo. The authors nicely demonstrate an effect on neurite outgrowth and branching. HDAC6 inhibition has been previously shown to ameliorate spinal cord injury. How do the authors reconcile these opposite results? Considering that HDAC6 knockout prevents tubulin lactylation, what is the HDAC6 mouse phenotype in physiological or pathological conditions? What would be the outcome of spinal cord injury in vivo?

Response: Good point. We agree with the reviewer that the physiological relevance of α-tubulin remains to be elucidated in our future investigation.

Our study demonstrates that α-tubulin lactylation enhances neurite outgrowth and branching, as well as regeneration after injury in cultured neurons. However, HDAC6 inhibition has been shown to ameliorate spinal cord injury in vivo, presenting seemingly contradictory results. We believe our in-vitro experiments provide the insights into the functional roles of α-tubulin lactylation in neurons. To reconcile these findings, we offer the following explanations:

Firstly, axonal regeneration in vivo, particularly within the central nervous system, is highly complex. Neuronal regeneration capability and the surrounding cells, such as glial and endothelial cells, may be involved in axonal regeneration after HDAC6 inhibition[2]. Therefore, HDAC6 inhibition could affect other cell types in addition to neurons to promote axonal regeneration.

Secondly, HDAC6 serves as a deacetylase for multiple proteins, including α-tubulin, HSP90, cortactin, MIF and so on. HDAC6 inhibition may reduce α-tubulin lactylation, it also increases acetylation of many proteins. Our previous research demonstrated that HDAC6 deficiency and inhibition protects neurons in ischemic stroke through MIF acetylation[3]. Therefore, the protective effects of HDAC6 inhibition could result from increased acetylation of various proteins.

As suggested by the reviewer, we determined the axonal regeneration in HDAC6 KO mice, in CNS and PNS, respectively. We performed sciatic nerve (PNS) and optic nerve (CNS) injury experiments in HDAC KO mice. Axon regeneration was assessed 3 days after sciatic nerve injury and 14 days after the optic nerve injury. Although there was a slight increase in axon regeneration in HDAC6 KO mice, the results were not statistically significant (shown in below). This could be attributed to the multifaceted roles of HDAC6, which include lactyltransferase activity, deacetylase activity, and numerous non-enzymatic functions. Due to multiple functions of HDAC6, it is difficult to study physiological and pathological implications of tubulin lactylation in HDAC6 KO mice.

The HDAC6 KO mice survive well and seem to be normal in the physiology condition. However, in pathological conditions, particularly in neurodegenerative conditions,

HDAC6 KO or inhibition exhibits protective effects in models of AD, PD, ALS, and ischemic stroke models[4,5].

Thus, we propose that the physiological roles of α-tubulin lactylation likely involve neuronal morphogenesis during development, such as dendritic morphology, axon projection, or synapse formation. In pathological conditions, α-tubulin lactylation may be implicated in neurodegenerative disorders. Mitochondria deficits and lactate accumulation, frequently observed during neurodegeneration, might lead to elevated α-tubulin lactylation. Increased α-tubulin lactylation could reduce microtubule stability, impair axonal transport, and eventually result in neurodegeneration. Therefore, reducingα-tubulin lactylation by HDAC6 inhibition may be the therapeutic strategy for treating neurodegenerative diseases.

We have included a paragraph about the functional roles of tubulin lactylation in vivo in the discussion section in the revised manuscript (line 422-435, page 12).

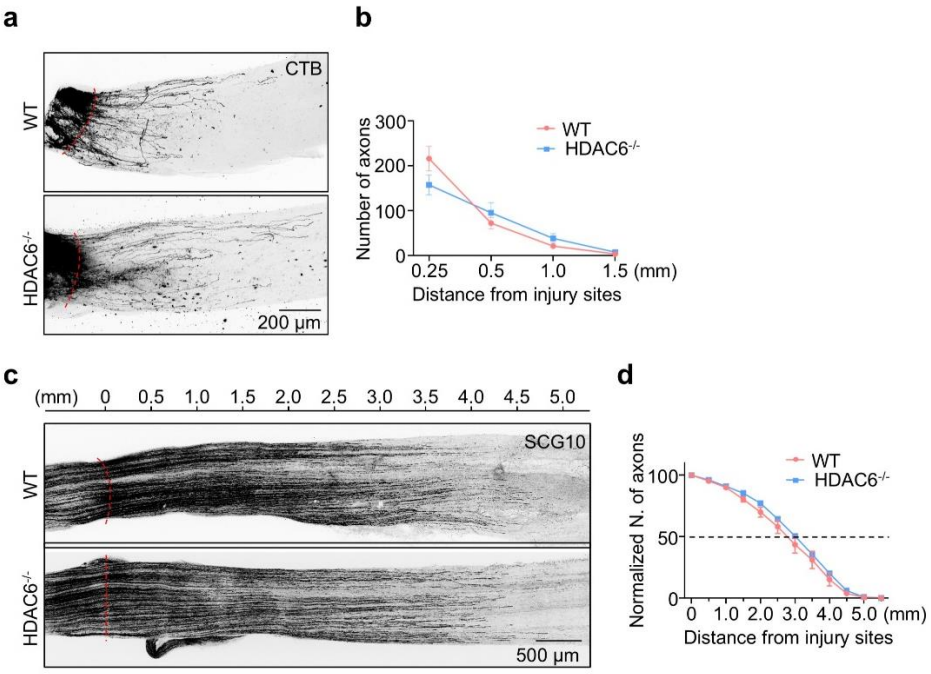


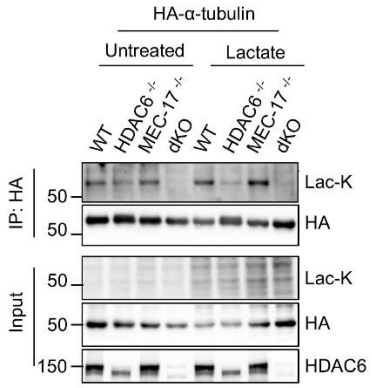
Figure. Axonal regeneration after injury in HDAC6 KO mice.
a, Representative images of longitudinal sections of optic nerves. CTB marks optic nerves and the red dashed line indicates the injured site.
b, Quantification of the number of regenerating axons as in **a.** (WT, n = 5; HDAC6 KO, n = 5)
c, Representative images of sciatic nerve regeneration in HDAC6 KO mice subjected to whole-mount immunostaining with SCG10 antibody. The red dashed lines mark the injury sites.
d, Quantification of the normalized mean numbers of regenerated axons in **c.** (WT, n = 4; HDAC6 KO, n = 6)

3. Lines 139-150 contrast with lines 164-164 regarding the role of MEC-17 on tubulin lactylation.

Response: Thanks for the suggestion.

We believe that the different contributions of MEC-17 in regulating α-tubulin lactylation may be due to variations in cell type and metabolic status. In HEK293T cells, MEC-17 deficiency resulted in a slight reduction in α-tubulin lactylation, but this reduction was not statistically significant (Fig. 2g, and extend data Fig.1i). In contrast, in the cortex, MEC-17 deficiency led to about 20% reduction in α-tubulin lactylation (Fig. 2i). This discrepancy may be attributable to the higher acetylation levels of α-tubulin in neurons, which suggests that the MEC-17 protein levels or enzymatic activity might be higher in the cortex compared to HEK293T cells.

Moreover, HDAC6 KO resulted in a large reduction in α-tubulin lactylation. Double KO of MEC-17 and HDAC6 abolished the α-tubulin lactylation in both the cortex (Fig. 2i) and HEK293T cells (shown in below). Therefore, our findings indicate that both MEC-17 and HDAC6 contribute to α-tubulin lactylation, with HDAC6 playing a major role and MEC-17 playing a minor role.



4. How do neurons respond to an increase in lactate? Isn't it toxic at certain levels? Did the authors control for neuronal viability in Figure 6?

Response: Thanks for the suggestion. To evaluate the potential toxicity of lactate on neurons, we treated primary cultured neurons with various concentrations of lactate for 72 hours and assessed cell viability using the CCK8 assay. As shown in Extended Data Fig. 6, lactate treatment had little effect on neuronal viability.

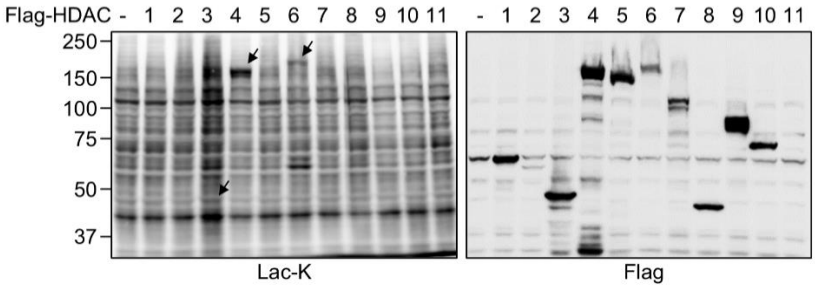
These results have been provided in the revised Extended Data Fig. 6 and the manuscript (line 316-319, page 10).

5. In Figure 1b, HDAC6 appears among the lactylated proteins. I am not asking for a biological meaning but to comment that the protein that primarily controls lactylation is lactylated as well.

Response: Good point. It is interesting that HDAC6 is also modified by lactylation, as reported in a recent reprint[6].

In fact, it is quiet common for the modification “writers” to catalyze modifications on themselves. For instance, the kinases (e.g. receptor tyrosine kinases) often autophosphorylate to initiate their kinase activity, and acetyltransferases can self-acetylate.

Interestingly, in addition to the mass spectrometry results, we also detected lactylation of HDACs in cell lysates from HEK293T cells overexpressing HDACs (shown in below). This suggests that HDACs undergo significant lactylation in cells. Therefore, we believe that HDAC lactylation could be self-catalyzed. This observation further supports the idea that HDACs could function as lactyltransferases.



6. Sirtuins are usually associated with longevity. In Extended Data Figure 4, Sirt2 catalyzes tubulin delactylation. Again, could the authors comment on the physiological role of alpha-tubulin lactylation?

Response: Thank you for your insightful comment. Sirtuins are NAD+-dependent deacetylases that have been associated with increased longevity. Studies have demonstrated that overexpression of Sirtuins, including Sirt2, extends lifespan across different species. Intriguingly, our research has identified that Sirt2 can catalyze the delactylation of α-tubulin, both in vitro and in vivo. We found that α-tubulin lactylation also enhances microtubule dynamics, both in purified tubulins and in neuronal axons. Our focus has been on the role of α-tubulin lactylation in neurite outgrowth during development and axonal regeneration after injury.

However, as the reviewer’s suggestion, it is crucial to consider that stable microtubules are essential for maintaining axonal structure and facilitating axonal transport, particularly during aging. In aging or neurodegenerative conditions,

mitochondrial dysfunction can lead to elevated lactate concentrations, subsequently increasing α-tubulin lactylation levels, which may impair cytoskeleton in axons and axonal transport. Therefore, Sirt2 overexpression, by reducing α-tubulin lactylation, may help stabilize microtubules and enhance axonal transport. The physiological roles of α-tubulin lactylation, especially in the contexts of aging and neurodegeneration, represent critical avenues for further investigation.

We have included a paragraph about the functional roles of tubulin lactylation in vivo in the discussion section in the revised manuscript (line 422-435, page 12).

References

1 Hai, Y. & Christianson, D. W. Histone deacetylase 6 structure and molecular basis of catalysis and inhibition. *Nat Chem Biol* **12**, 741-747 (2016). https://doi.org:10.1038/nchembio.2134

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3 Hu, J. X. *et al.* Macrophage migration inhibitory factor (MIF) acetylation protects neurons from ischemic injury. *Cell Death Dis* **13**, 466 (2022). https://doi.org:10.1038/s41419-022-04918-2

4 Dompierre, J. P. *et al.* Histone deacetylase 6 inhibition compensates for the transport deficit in Huntington's disease by increasing tubulin acetylation. *J Neurosci* **27**, 3571-3583 (2007). https://doi.org:10.1523/jneurosci.0037-07.2007

5 d'Ydewalle, C. *et al.* HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease. *Nat Med* **17**, 968-974 (2011). https://doi.org:10.1038/nm.2396

6 Cao, J. *et al.* Lactate-derived HDAC6 Lactylation as a new target for neuronal protection in cerebral ischemic reperfusion injury. *bioRxiv* (2022).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

This reviewer finds that the authors have addressed the concerns from all reviewers in a satisfactory manner and recommends acceptance of the manuscript for publication.

Reviewer #2 (Remarks to the Author):

The reviewers addressed my concerns, performed additional experiments that resulted in concordant results, and added pertinent comments on the physiological role of lactylation.