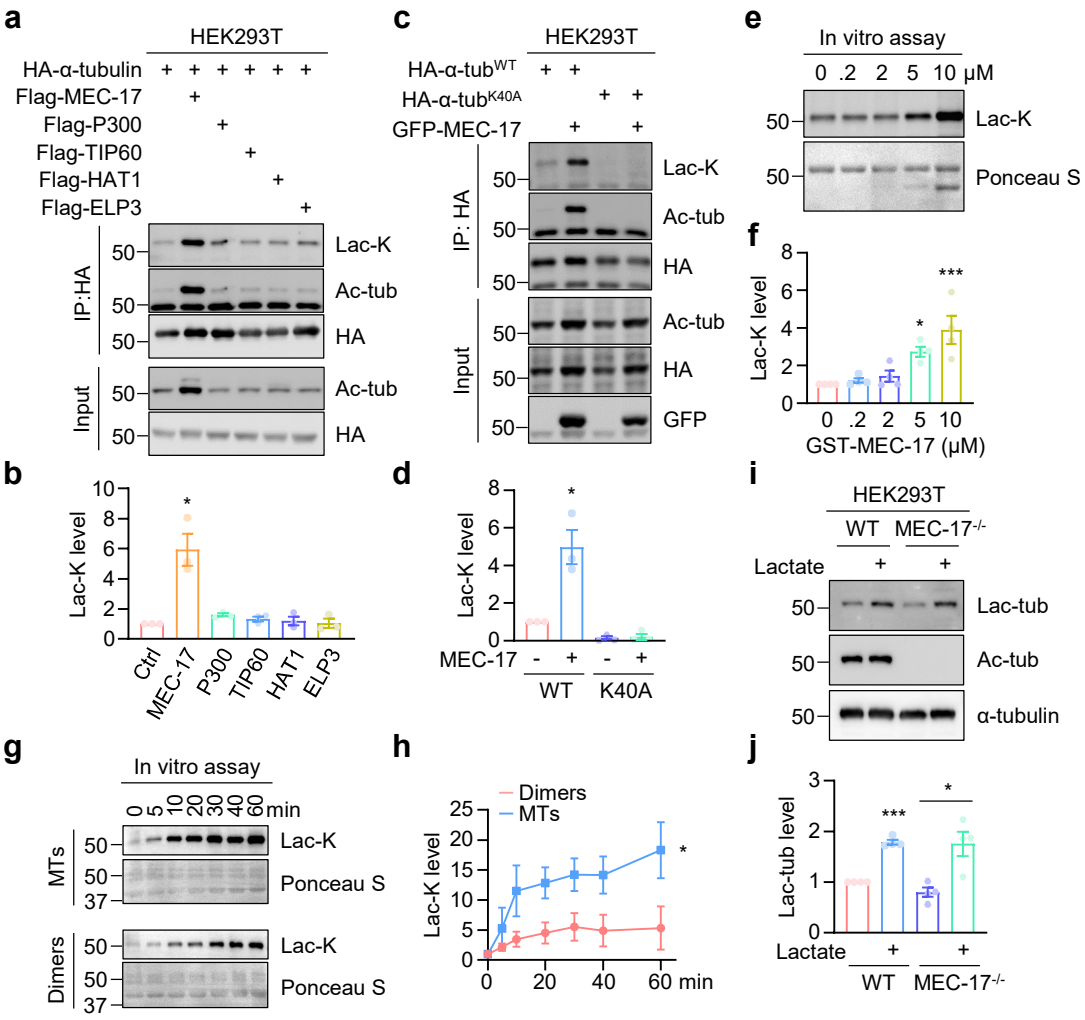



**Supplementary Figure 1. Regulation of α-tubulin lactylation by MEC-17.**

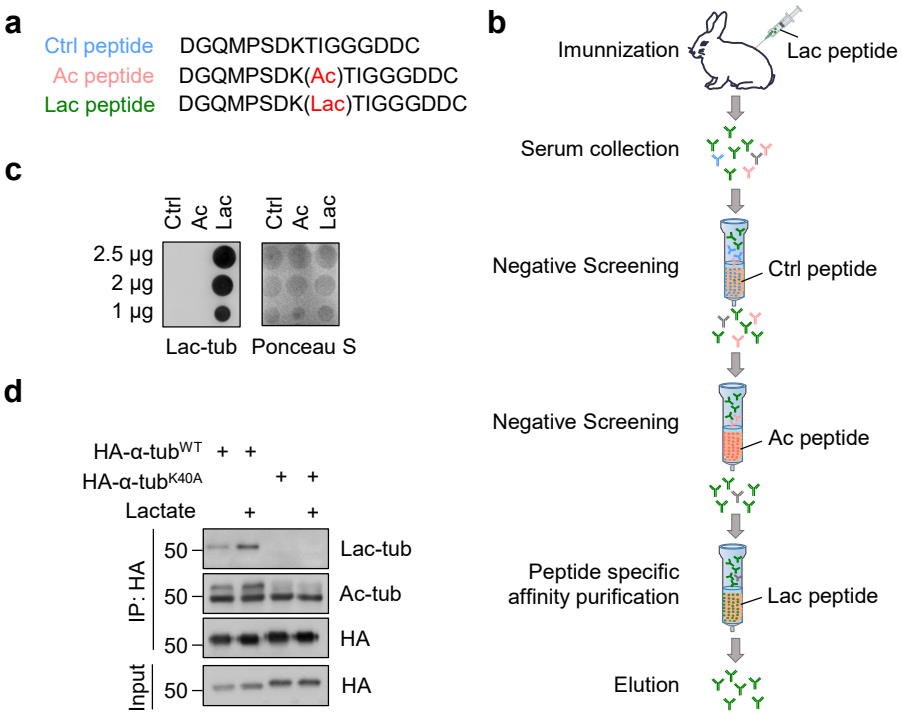
**a-b**, HEK293T cells were transfected with Flag-tagged acetyltransferases, together with HA-α-tubulin. HA-α-tubulin was immunoprecipitated with anti-HA antibody and α-tubulin lactylation was revealed by anti-Lac-K antibody. n = 3 experiments. Two-sided paired student's *t*-test, MEC-17 vs Ctrl, p = 0.0437.

**c-d**, MEC-17 increases α-tubulin lactylation on K40 residue. n = 3 experiments. Two-sided paired student's *t*-test, WT + MEC-17 vs WT, p = 0.0485.

**e-f**, In vitro assays using recombinant GST-MEC-17 and tubulins in the presence of Lac-CoA as a lactyl donor. 2 μM tubulin dimers were incubated with 10 μM Lac-CoA and the indicated concentrations of GST-MEC-17 at 37℃ for 1 hour and α-tubulin lactylation was revealed by immunoblot. n = 4 experiments. One-way ANOVA, 5 μM MEC-17 vs control, p = 0.0190; 10 μM MEC-17 vs control, p = 0.0003.

**g-h**, The catalytic preference of MEC-17 for microtubules over tubulin dimers. 7 μM GST-MEC-17 was incubated with 1 μM tubulin dimers or microtubules in the presence of 10 μM Lac-CoA at 37℃ for the indicated time points. n = 3 experiments. Two-way ANOVA, MTs vs Dimers, p = 0.0145.

**i-j**, α-Tubulin lactylation levels in MEC-17 KO cells. The cells were treated with or without 30 mM lactate for 16 hours. n = 4 experiments. Two-sided paired student's *t*-test, WT + Lactate vs WT, p = 0.0005; MEC-17$^{-/-}$ + Lactate vs MEC-17$^{-/-}$, p = 0.0159. Data are shown as mean ± SEM. *p < 0.05, ***p < 0.001. Source data are provided as a Source Data file.




**Supplementary Figure 2. Generation of α-tubulin K40 lactylation antibody.**

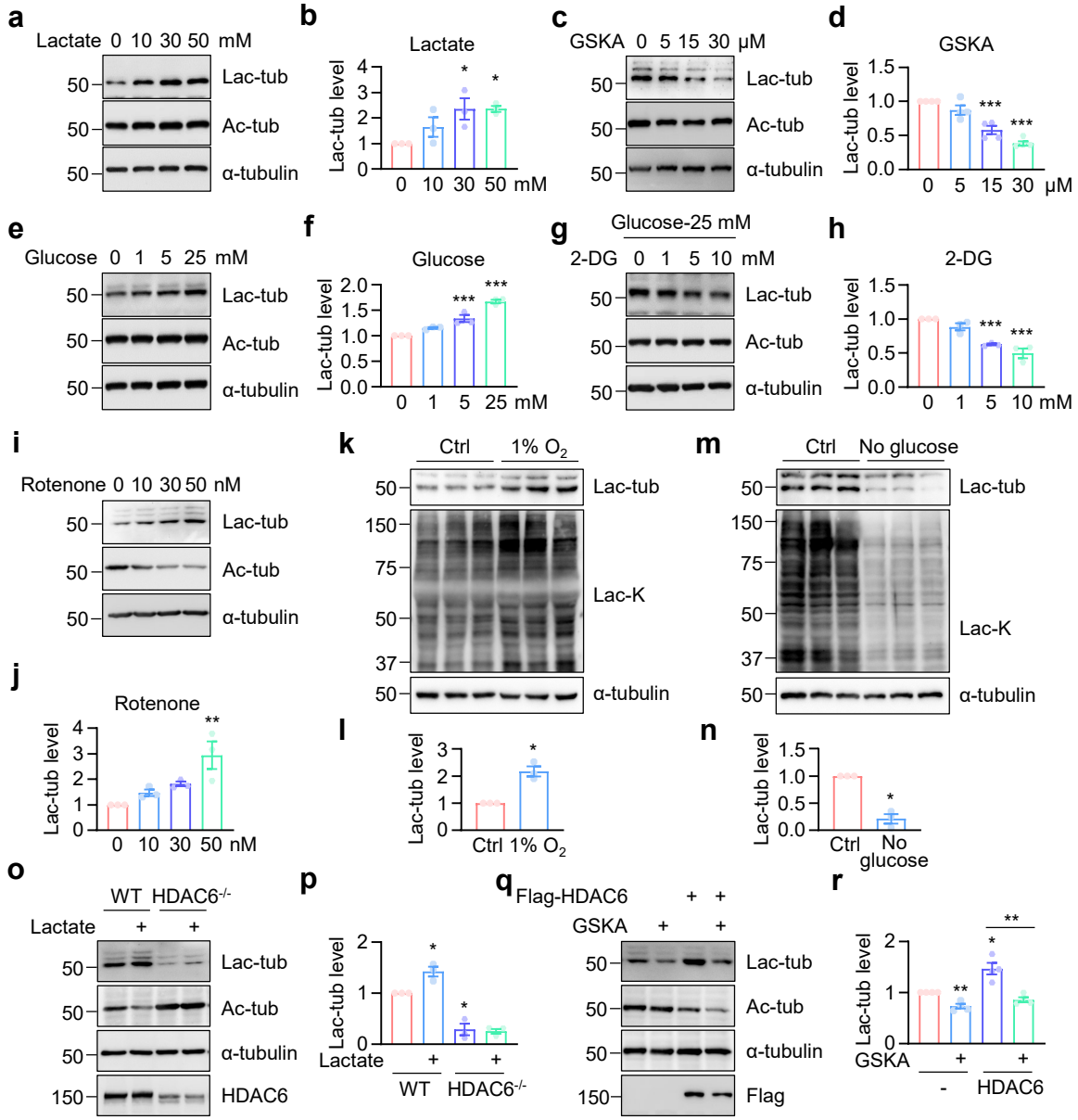
**a**, The synthesized peptides are unmodified or modified with K40 lactylation or acetylation.

**b**, Schematic diagram of generation of the α-tubulin K40 lactylation (lac-α-tubulin) antibody.

**c**, Dot blot assay using anti-lac-α-tubulin antibody and the synthesized peptides in **a**. n = 3 experiments.

**d**, The lac-α-tubulin antibody recognized α-tubulin lactylation in cells. Cells were treated with or without 30 mM lactate for 16 hours. n = 3 experiments.

Source data are provided as a Source Data file.




**Supplementary Figure 3. HDAC6 is required for lactate-induced α-tubulin lactylation.**

**a-h**, Immunoblots of cell lysates from HEK293T cells treated with different concentrations of glycolysis modulators, including lactate in **a-b** (n = 3 experiments), lactate dehydrogenase inhibitor GSKA in **c-d** (n = 4 experiments), glucose in **e-f** (n = 3 experiments), and 2-deoxy-D-glucose in **g-h** (n = 3 experiments). One-way ANOVA, for lactate, 30 mM vs 0 mM, p = 0.0255; 50 mM vs 0 mM, p = 0.0246. For GSKA, 15 μM vs 0 μM, p = 0.0002; 30 μM vs 0 μM, p < 0.0001. For glucose, 5 mM vs 0 mM, p = 0.0008; 25 mM vs 0 mM, p < 0.0001. For 2-DG, 5 mM vs 0 mM, p = 0.0010; 10 mM vs 0 mM, p < 0.0001.

**i-j**, Immunoblots of cell lysates from HEK293T cells treated with various concentrations of rotenone, an inhibitor of the mitochondria respiratory chain complex I. n = 3 experiments. One-way ANOVA, for rotenone, 50 nM vs 0 nM, p = 0.0032.

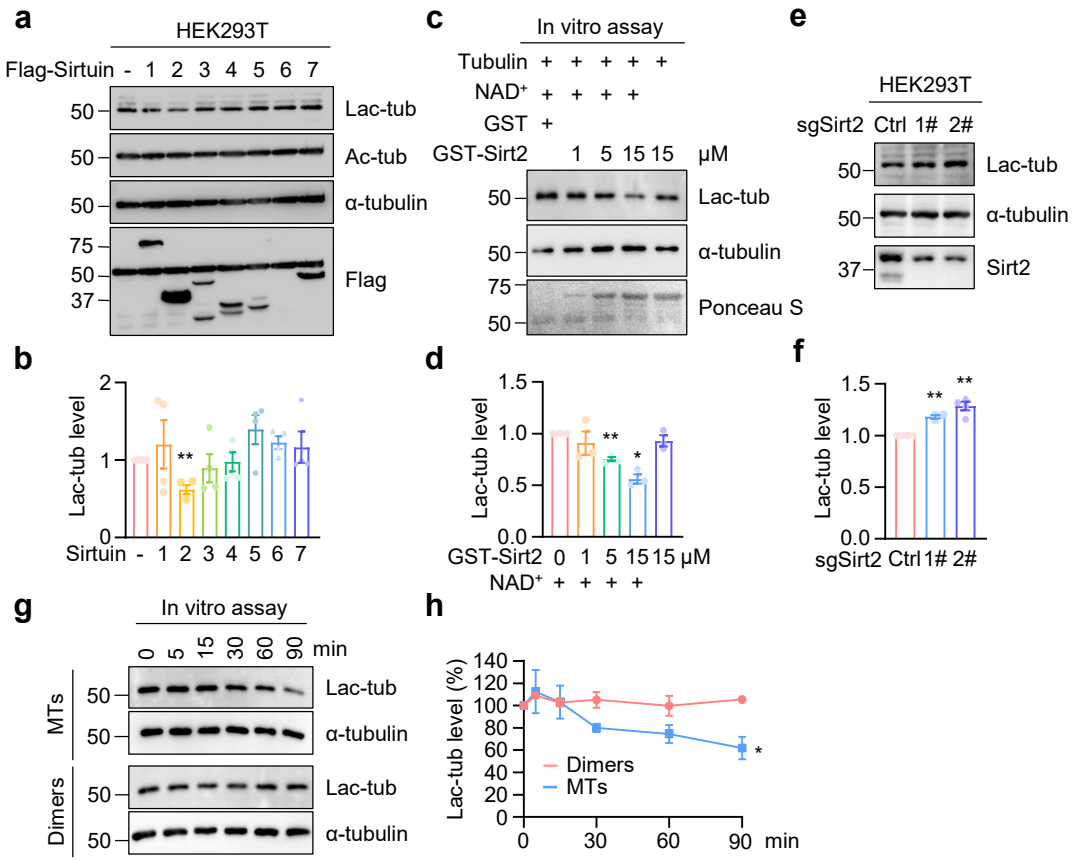
**k-l**, Both global protein lactylation and α-tubulin lactylation were dramatically increased in cells after hypoxia. The cells were incubated in 1% O2 condition for 24 hours and α-tubulin lactylation was revealed by immunoblot. n = 3 experiments. Two-sided paired student's *t*-test, p = 0.0244.

**m-n**, Glucose depletion resulted in reduced global protein lactylation and α-tubulin lactylation. The cells were cultured in glucose-depleted medium for 24 hours α-tubulin lactylation was revealed by immunoblot. n = 3 experiments. Two-sided paired student's *t*-test, p = 0.0122.

**o-p**, HDAC6 is required for lactate-induced α-tubulin lactylation in HEK293T cells. The cells were treated with or without 30 mM lactate for 16 hours. n = 3 experiments. Two-sided paired student's *t*-test, for Lac-tub, WT + Lactate vs WT, p = 0.0482; $HDAC6^{-/-}$ vs WT, p = 0.0239.

**q-r**, LDH inhibitor GSKA impairs HDAC6-induced α-tubulin lactylation in HEK293T cells. n = 4 experiments. Two-sided paired student's *t*-test, GSKA vs control, p = 0.0090; HDAC6 vs control, p = 0.0268; HDAC6 + GSKA vs HDAC6, p = 0.0040.

Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Source data are provided as a Source Data file.




**Supplementary Figure 4. Sirt2 catalyzes α-tubulin delactylation.**

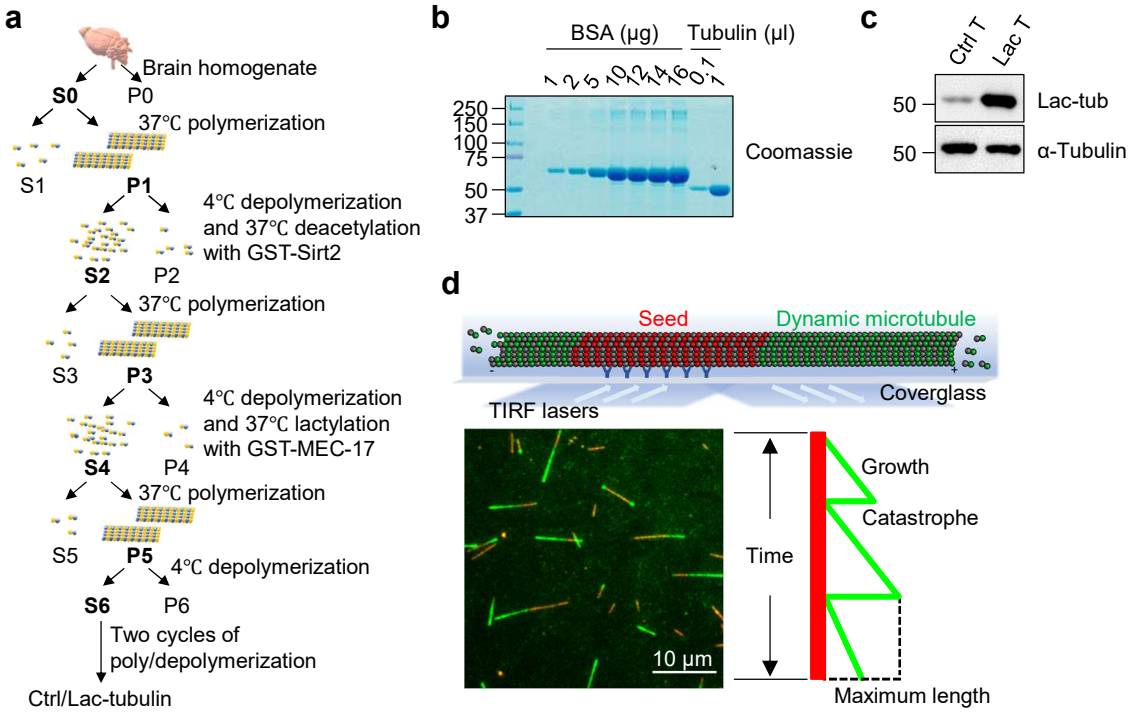
**a-b**, Screening for α-tubulin delactylase in cells. HEK293T cells were transfected with various sirtuin proteins, and α-tubulin lactylation was revealed by anti-lac-α-tubulin antibody. n = 4 experiments. Two-sided paired student's *t*-test, Sirt2 vs control, p = 0.0076.

**c-d**, The delactylase activity of Sirt2 for α-tubulin is dependent of NAD+. 1 μM tubulin dimers were incubated with 1 mM NAD+ and indicated concentrations of GST-Sirt2 at 37℃ for 1 hour. n = 3 experiments. Two-sided paired student's *t*-test, 5 μM Sirt2 vs control, p = 0.0073; 15 μM Sirt2 vs control, p = 0.0102.

**e-f**, Increased α-tubulin lactylation in Sirt2-deficient cells. n = 4 experiments. Two-sided paired student's *t*-test, for Lac-tub, sgSirt2-1# vs Ctrl, p = 0.0090; sgSirt2-2# vs Ctrl, p = 0.0067.

**g-h**, Sirt2 catalyzes α-tubulin delactylation with the preference for microtubules over tubulin dimers. 4 μM GST-Sirt2 was incubated with 1 μM tubulin dimers or microtubules, in the presence of 1 mM NAD+ at 37℃ for the indicated time points. n = 4 experiments. Two-way ANOVA, MTs vs Dimers, p = 0.0215.

Data are shown as mean ± SEM. *p < 0.05, **p < 0.01. Source data are provided as a Source Data file.




**Supplementary Figure 5. Preparation of lactylated α-tubulin.**

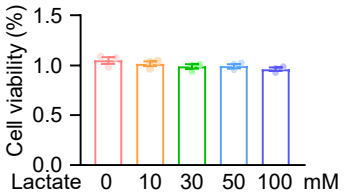
**a**, Schematic diagram of purification of tubulins from mouse brains.

**b**, Representative images of purified tubulins running in SDS-PAGE, revealed by Coomassie blue staining. n = 4 experiments.

**c**, Immunoblots of purified tubulins, revealed by anti-lac-α-tubulin antibody. n = 3 experiments.

**d**, Schematic of the in vitro microtubule reconstitution by TIRF microscopy. The red region indicates the GMPCPP-stabilized microtubule seeds, and the green region shows the dynamic microtubules growing from the seeds. Representative images of TIRF microscopy. Schematic diagram for the parameters of microtubule dynamics.

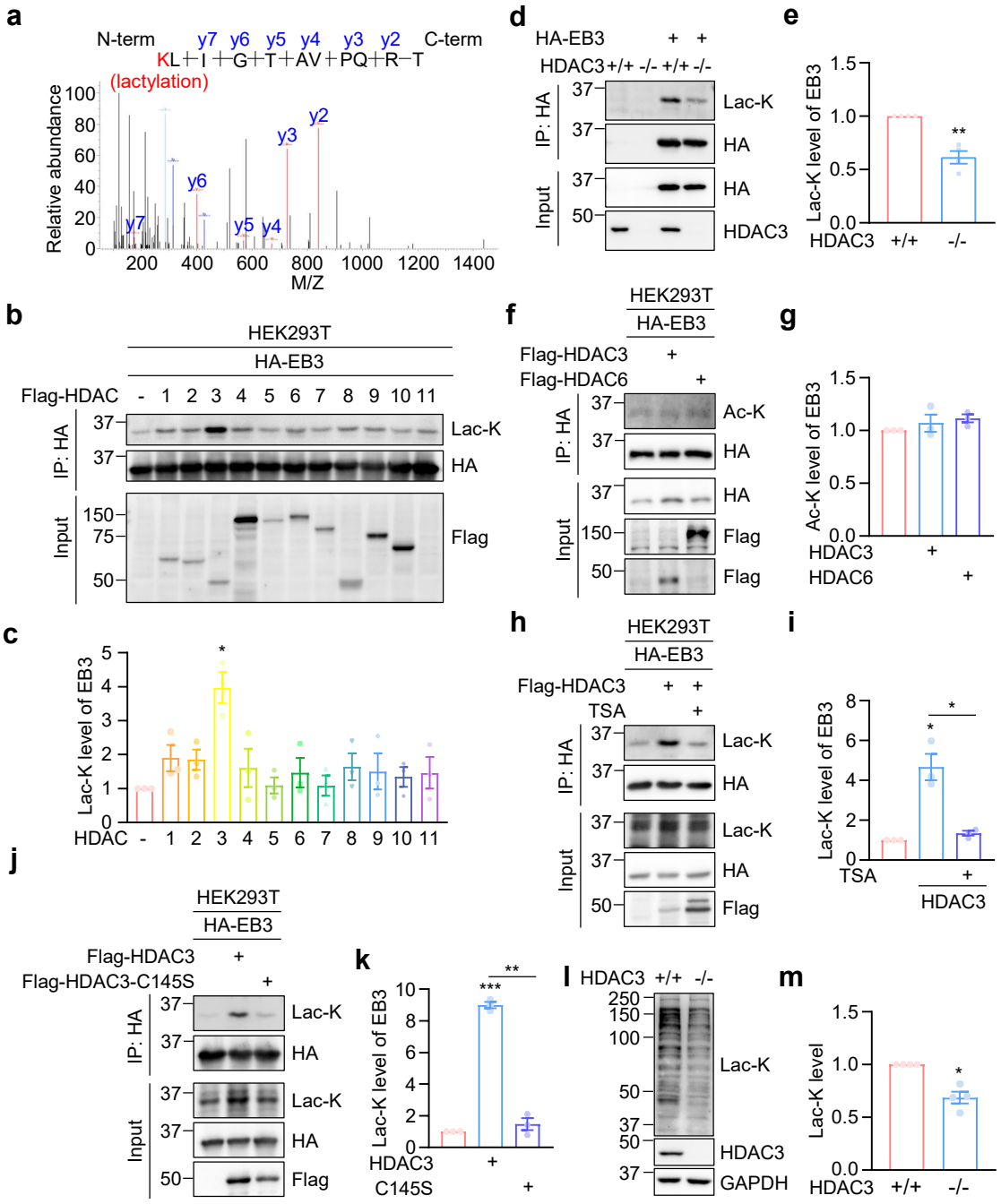
Source data are provided as a Source Data file.




**Supplementary Figure 6. Treatment of cultured neurons with various concentrations of lactate.**

Primary cultured neurons were treated with various concentrations of lactate for 72 h and cell viability was determined by using the CCK8 assay. n = 3 experiments.

Data are shown as mean ± SEM. One-way ANOVA. Source data are provided as a Source Data file.




**Supplementary Figure 7. EB3 lactylation is catalyzed by HDAC3.**

**a**, Mass spectrometry analysis showing lactylation at K151 on EB3 from cultured cortical neurons. Expected molecular weights of lactylated peptides from N terminus and C terminus are shown as peaks in red and blue, respectively.

**b-c**, HDAC3 overexpression leads to increased EB3 lactylation. HEK293T cells were transfected with Flag-tagged HDAC family proteins, together with HA-EB3. HA-EB3 was immunoprecipitated with anti-HA antibody and EB3 lactylation was revealed by anti-Lac-K antibody. n = 3 experiments. Two-sided paired student's *t*-test, HDAC3 vs control, p = 0.0229.

**d-e**, Reduced EB3 lactylation in HDAC3 KO cells. n = 4 experiments. Two-sided paired student's *t*-test, p = 0.0077.

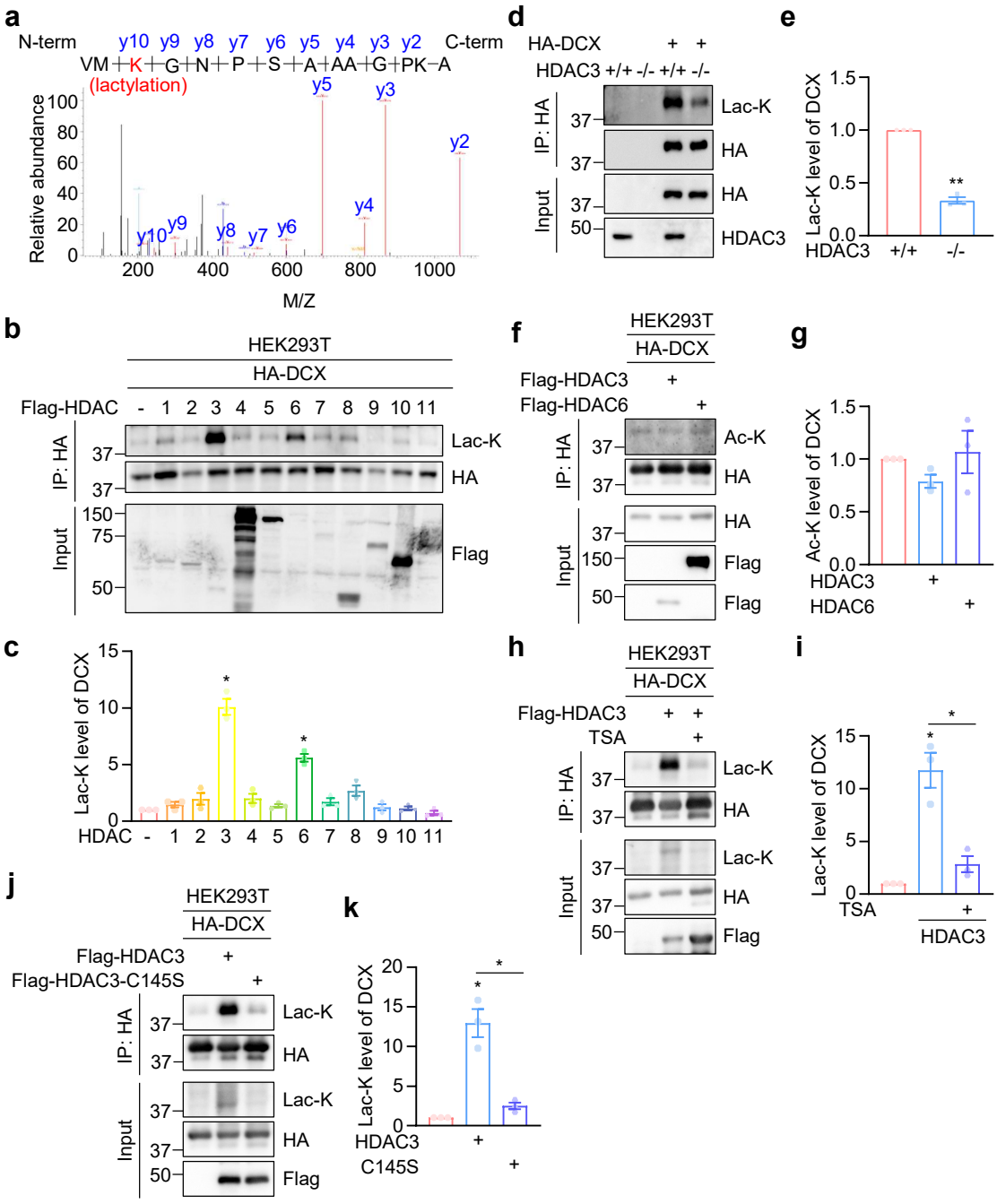
**f-g**, The acetylation level of EB3 remained unchanged when HDAC3 or HDAC6 were overexpressed. n = 3 experiments. Two-sided paired student's *t*-test.

**h-i**, HDAC inhibitor TSA attenuates HDAC3-induced EB3 lactylation in HEK293T cells. Cells were treated with 5 μM TSA for 12 h. n = 3 experiments. Two-sided paired student's *t*-test, HDAC3 vs control, p = 0.0312; HDAC3 + TSA vs HDAC3, p = 0.0497.

**j-k**, HDAC3-catalyzed EB3 lactylation requires its deacetylase activity. n = 3 experiments. Two-sided paired student's *t*-test, HDAC3 vs control, p = 0.0006; C145S vs HDAC3, p = 0.0020.

**l-m**, Reduced protein lactylation levels in HDAC3 KO cells. n = 4 experiments. Two-sided paired student's *t*-test, p = 0.0113

Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Source data are provided as a Source Data file.




**Supplementary Figure 8. DCX lactylation is catalyzed by HDAC3.**

**a**, Mass spectrometry analysis showing lactylation at K275 on DCX from cultured cortical neurons. Expected molecular weights of lactylated peptides from N terminus and C terminus are shown as peaks in red and blue, respectively.

**b-c**, HDAC3 or HDAC6 overexpression leads to increased DCX lactylation. HEK293T cells were transfected with Flag-tagged HDAC family proteins, together with HA-DCX. HA-DCX was immunoprecipitated with anti-HA antibody and DCX lactylation was revealed by anti-Lac-K antibody. n = 3 experiments. Two-sided paired student's *t*-test, HDAC3 vs control, p = 0.0058; HDAC6 vs control, p = 0.0054.

**d-e**, Reduced DCX lactylation in HDAC3 KO cells. n = 3 experiments. Two-sided paired student's *t*-test, p = 0.0023.

**f-g**, The acetylation level of DCX remained unchanged when HDAC3 or HDAC6 were overexpressed. n = 3 experiments. Two-sided paired student's *t*-test.

**h-i**, HDAC inhibitor TSA attenuates HDAC3-induced DCX lactylation in HEK293T cells. Cells were treated with 5 μM TSA for 12 h. n = 3 experiments. Two-sided paired student's *t*-test, HDAC3 vs control, p = 0.0228; HDAC3 + TSA vs HDAC3, p = 0.0246.

**j-k**, HDAC3-catalyzed DCX lactylation requires its deacetylase activity. n = 3 experiments. Two-sided paired student's *t*-test. HDAC3 vs control, p = 0.0210; C145S vs HDAC3, p = 0.0159.

Data are shown as mean ± SEM. *p < 0.05, **p < 0.01. Source data are provided as a Source Data file.