ATP-citrate lyase promotes axonal transport across species

Aviel Even1,10, Giovanni Morelli2,3,10, Silvia Turchetto2, Michal Shilian1, Romain Le Bail2, Sophie Laguesse2, Nathalie Krusy2, Ariel Brisker1, Alexander Brandis4, Shani Inbar1, Alain Chariot5, Frédéric Saudou6,7,8, Paula Dietrich9, Ioannis Dragatsis9, Bert Brone3, Loïc Broix2, Jean-Michel Rigo3, Miguel Weil1,11✉, Laurent Nguyen2,11✉

Microtubule (MT)-based transport is an evolutionary conserved process finely tuned by posttranslational modifications. Among them, α-tubulin acetylation, primarily catalyzed by a vesicular pool of α-tubulin N-acetyltransferase 1 (Atat1), promotes the recruitment and processivity of molecular motors along MT tracks. However, the mechanism that controls Atat1 activity remains poorly understood. Here, we show that ATP-citrate lyase (Acly) is enriched in vesicles and provide Acetyl-Coenzyme-A (Acetyl-CoA) to Atat1. In addition, we showed that Acly expression is reduced upon loss of Elongator activity, further connecting Elongator to Atat1 in a pathway regulating α-tubulin acetylation and MT-dependent transport in projection neurons, across species. Remarkably, comparable defects occur in fibroblasts from Familial Dysautonomia (FD) patients bearing an autosomal recessive mutation in the gene coding for the Elongator subunit ELP1. Our data may thus shine light on the pathophysiological mechanisms underlying FD.
Axonal transport is an evolutionary conserved process that delivers cargoes to distant subcellular compartments. It is supported by molecular motors (kinesins and dyneins) running along microtubule (MT) tracks and is particularly important for projection neurons that send axons to distant targets. MT-dependent transport contributes to neuronal development in growing dendrites and axons via slow axonal transport of cytoskeletal elements and, later, it sustains survival and homeostasis of neurons via fast axonal transport of organelles including mitochondria, lysosomes, and vesicles carrying various types of proteins (growth factors, synaptic proteins...). Axonal transport defect is a hallmark of several neurodegenerative disorders, whose disruption affects neuronal function and survival. This is exemplified by the loss of activity of the Elongator complex, which is associated with both neurodegeneration and axonal transport defects. This molecular complex, conserved from yeast to human, is composed of two copies of six conserved from yeast to human, is composed of two copies of six domains, a tRNA acetyltransferase and a S-adenosyl methionine binding domains. Elp1 is the scaffolding subunit of the complex, but disruption of any of the Elongator subunits leads to comparable phenotype in eukaryotes, suggesting that all subunits are essential for the integrity and activity of the complex. Elongator serves molecular functions in distinct subcellular compartments but predominantly accumulates in the cytoplasm where it promotes the formation of 5-methoxy carbonylmethyl (mcm5) and 5-carbamoylmethyl (ncm5) on side-chains of wobble uridines (U34) of selected tRNAs, thereby regulating protein translation. Convergent observations support a role for Elongator in intracellular transport in the nervous system as Elp3 is enriched at the presynaptic side of neuromuscular junction buttons in flies, where its expression is required for synapse integrity and activity. Elongator subunits are also detected in protein extracts from purified motile vesicles isolated from the mouse cerebral cortex, and they colocalize with the vesicular markers SV2 and RAB3A in human embryonic stem cells derived neurons. In humans, mutation of the gene coding for ELPL1, underlies Familial dystautonomia (FD), a devastating disease that mostly affects the development and survival of neurons from the autonomic nervous systems. Moreover, other neurological disorders are also associated with mutations affecting the expression or the activity of Elongator. Experimental data on animal models show that interfering with Elongator activity promotes early developmental and progressive neurodegenerative defects, including poor axonal transport and maintenance. At the molecular level, loss of Elongator correlates with poor acetylation of α-tubulin lysine 40 (K40) in neuronal microtubules. This post-translational modification (PTM) modulates axonal transport by facilitating the recruitment of molecular motors to MTs and the loading of motile vesicles on motors. The acetylation of MTs relies mostly on a vesicular pool regulating the stability of ATP-citrate lyase (Acly). This enzyme is enriched in vesicles and produces acetyl-coA, thereby providing acetyl groups for Atat1 activity towards MTs. Importantly, acetylation of MTs affects the pausing time both in anterograde and retrograde directions of lysosomes and mitochondria axons, which correlated with an increase of their pausing time, as compared to WT PNs (Supplementary Fig. 1b–h). These results confirmed that loss of Elongator activity leads to defects of α-tubulin acetylation and mitochondrial transport in cortical neurons. Since a vesicular pool of Atat1 promotes acetylation of α-tubulin, thereby controls axonal transport in cortical PNs, we postulated that Atat1 and Elongator might contribute to axonal transport via a shared molecular pathway. To test this hypothesis, we infected cortical PNs from E14.5 WT and Atat1 KO mice with lentiviruses expressing either sh-Elp3 or sh-Control (Supplementary Fig. 1i–j), and we cultured them in microfluidics devices for five days (Fig. 1b). Targeting Elp3 in WT neurons impaired lysosomes and mitochondria transport across PN axons. However, we did not observe an additive effect on MT acetylation or axonal transport kinetics upon reduction of Elp3 expression in Atat1 KO PNs (which show reduced acetylation of α-tubulin) (Fig. 1c–e, Supplementary Fig. 1k–o). Comparative observations were made in vivo in MNs of Drosofila melanogaster 3rd instar larvae obtained by crossing UAS:Elp1 or UAS:Elp3 RNAi flies with D42:Gal4 (further called Elp1 KD and Elp3 KD). The knockdown efficiency of the UAS:Elp1 and UAS:Elp3 in postmitotic neurons was validated in adult fly heads (Elav:Gal4 driver; Supplementary Fig. 1p–r). We first confirmed that the level of α-tubulin acetylation was reduced in Elp1 and Elp3 KD 3rd instar larval MNs, a defect genetically rescued by expressing human (h) Elp3 or by co-targeting the main α-tubulin deacetylase Hdac6 with RNAi (Elp1;Hdac6 KD, Elp3;Hdac6 KD) (Fig. 1f, Supplementary Fig. 1r). Moreover, knocking down both Elp3 and Atat1 (Elp3;Atat1 KD) did not further reduce the α-tubulin acetylation level, as compared to Elp3 KD alone (Fig. 1f; p = 0.259 and p = 0.775, respectively). In vivo time-lapse recording of the synaptotagmin-GFP axonal transport (SYT1-GFP) in MNs of anesthetized fly 3rd instar larvae (Fig. 1g) correlated with levels of α-tubulin acetylation (Fig. 1f). We observed a reduction of both the average and moving velocities of SYT1-GFP vesicles together with an extension of their pausing time both in Elp1 KD or Elp3 KD larvae when compared to controls, and with no cumulative defects in Elp3;Atat1 KD flies (Fig. 1h–j, Supplementary Fig. 1s). Moreover, knocking down Hdac6 fully rescued the observed axonal transport defects observed in Elp1/3 KD in 3rd instar larval MNs.
Interfering with Elongator activity affects codon-biased translation that can ultimately lead to protein aggregation, thereby blocking axonal transport. However, we did not detect significant protein aggregation in the axon of either cultured cortical neurons from Elp3 cKO mice (Supplementary Fig. 1t) or motoneurons from Elp1 or Elp3 KD 3rd instar larvae in vivo (Supplementary Fig. 1u), while axonal aggregates formed upon blocking proteasome activity with MG132 incubation. Altogether, these results suggest that the axonal transport defects observed in Elongator deficient neurons unlikely result from a local accumulation of protein aggregates.

Since impaired axonal transport in fly MNs leads to locomotion defects, we measured larvae crawling speed and adult flies climbing index as functional readouts of axonal transport activity in MNs. These parameters were affected upon depletion of either Elp1 or Elp3 (Fig. 1k–l), and were likely resulting from MT acetylation and axonal transport defects, as they were rescued by the knockdown of Hdac6 (Fig. 1k–l) and no morphological changes of neuromuscular junction synapses were observed (Supplementary Fig. 1v).

Elongator shares high amino acid similarity across species. Expression of human ELP3 in MNs of 3rd instar larvae of Elp3 KD flies (Elp3 KD + ELP3) rescued α-tubulin acetylation (Fig. 1f), axonal transport parameters (Fig. 1h–j, Supplementary Fig. 1s) and locomotion behavior defects (Fig. 1k–l), indicating that ELP3, and by extension Elongator, has a conserved evolutionary role from human to axonal transport.
molecular pathway to modulate MT acetylation and axonal transport.31,32

Loss of Elongator impairs the production of acetyl-CoA, thereby interfering with Atat1-mediated MT acetylation. Since Hdac6 knockdown rescued the axonal transport deficits observed in neurons lacking Elongator activity (Fig. 1h–i), we tested whether these defects may arise from a change of expression or activity of Hdac6 and/or Atat1, the enzymes that control α-tubulin deacetylation, respectively.38,39 Since no change in Hdac6 and Atat1 (isofrom #3 and #4)30 expression in cortical extracts from Elp3 cKO and WT littermate newborn mice (Fig. 2a–c) were observed, we measured the activity of these enzymes. The deacetylation activity of Hdac6 toward MTs, assessed in vitro by incubating free acetylated α-tubulin from bovine brains with cortical extracts from newborn Elp3 cKO or WT littermate controls38 was comparable between conditions (Fig. 2d). Atat1 activity was measured by using an in vitro α-tubulin acetylation assay.40 For this assay, pre-polimerized unacetylated MTs from HeLa cells were incubated with acetyl-CoA and P0 mouse brain extracts from Elp3 cKO or WT littermate controls (Fig. 2e) to assess the level of acetylation of α-tubulin. In striking contrast to brain extracts from Atat1 KO mouse pups, we did not observe any differences in MT acetylation levels between the cortical extracts from Elp3 cKO and WT mice upon addition of acetyl-CoA (Fig. 2e). Altogether, these results suggest that loss of Elongator activity: i) does not impair α-tubulin acetylation by changing the expression or activity of Hdac6 or Atat1; ii) may interfere with the metabolic production of acetyl-CoA. Accordingly, western blotting of E14.5 Elp3cKO cortical extracts showed that expression of Acyl-coenzyme A synthetase short-chain family member (Acsc2) and Acly, the enzymes that generate acetyl-CoA40, were significantly reduced, as compared to control extracts (Fig. 2f–h). Since α-tubulin acetylation is mainly driven by a vesicular pool of Atat1 in axons38, we performed sub-fractionation of cortical lysates (Fig. 2i; T: total, P1: nuclei, S2: cytosolic fractions (P3) (Fig. 2j). Moreover, proteomic analysis of cortical extracts from newborn mice confirmed the vesicular enrichment of several Elongator subunits, Atat1, Acly, but not Acsc2 (Supplementary Fig. 2a). Altogether, these data suggest that α-tubulin acetylation could be regulated by homeostatic changes in Acly-dependent acetyl-CoA concentration, as previously observed for histone acetylation.41 Specifically, we postulated that loss of Elongator could interfere with MT acetylation and axonal transport via reduction of Acly expression. To test this hypothesis, we performed an in vitro acetyl-CoA production assay (malate dehydrogenase coupled assay)31, where subcellular fractions of cortical brain extracts from Elp3 cKO and WT littermate newborns were mixed with Acly substrates (ATP, CoA, and citrate) (Fig. 2o). The vesicular fraction (P3), which is strongly enriched into Elongator subunits (Elpl and Elp3), Atat1 and Acly (Fig. 2j, Supplementary Fig. 2a), more efficiently produced acetyl-CoA (Fig. 2o–p). While cortical extracts from Elp3 cKO E14.5 mouse embryos showed no change of Acly transcription (Fig. 2q), we observed a reduction of Acly expression (Fig. 2f–g) together with a correlative decrease of MTs acetylation (Supplementary
Indeed, the analysis of *Etp3* cKO and WT newborn cortices show a robust decrease of Acly protein level in both the cytosolic and the vesicular fractions (*P*3, Fig. 2r–t). Comparable reduction of Acly (but not Atat1) was detected by mass spectrometry in the vesiculome of P0 Elp1 KO brains (Supplementary Fig. 2b–d). Moreover, we detected a reduction of acetyl-CoA level in cortical extracts from *Etp3* cKO mice, as compared to WT newborn littermates by using LC-MS metabolic analysis (Fig. 2u).

Altogether, these results support a molecular cooperation occurring at vesicles between the Elongator and Atat1 to drive MT acetylation via the regulated expression of the enzyme Acly, which provides acetyl-group donors to Atat1 for catalyzing α-tubulin acetylation in neurons.

Expression of Acly rescues both acetylation of α-tubulin and axonal transport defects in flies and mice that lack Elongator activity. In order to test whether Acly is required for proper axonal transport, we incubated WT mouse PNs with the Acly inhibitor hydroxycitric acid (HCA)\(^4\). This treatment reduced the level of α-tubulin acetylation in PNs axons (Fig. 3a). Moreover, incubation of
Fig. 2 Elongator deletion impairs tubulin acetylation and axonal transport via reduction of Acly-dependent acetyl-coA production. a–c Western blotting to detect and quantify Hdac6, Elp1, Elp3, Atat1 (isoform #3 and #4)20, and β-Actin in cortical extracts from newborn WT and Elp3 cKO mice. Histograms of proportion of Hdac6 b and Atat1 c expression to β-Actin. d In vitro deacetylation assay of endogenously acetylated bovine brain tubulin incubated for 4 h with extracts (S3) of cortices isolated from WT, Elp3 cKO, or without tissue extract (control). e In vitro acetylation assay of nonacetylated MTs from HeLa cells incubated for 2 h with Acetyl-CoA and extracts (P3) of brain cortices isolated from WT, Elp3 cKO or Atat1 KO mice. f–h Immunoblotting to detect Acss2, Acly, and α-Tub cortical extracts from E14.5 WT and Elp3 cKO embryos and histograms of proportion of Acss2 (g) and Acly (h) expression to α-Tub. i Experimental pipeline for subcellular fractionation of P0 mouse cortical extracts: T, total; S1, post-nuclear; P1, nuclear; S2, cytosol and vesicles; P3, vesicles. j Subcellular fractionation (T, S1, P1, S2, P3) of mouse brain cortex immunoblotted with specific antibodies to detect Acss2, Acly, Elp1, Elp3, Atat1 (isoform #3 and #4)20, Hdac6, synaptophysin (Syp), α-tubulin (α-Tub), and Histone H3 (H3H). k−l Histograms of proportion of Acss2 and Acly across subcellular fractions k or expressed as P3/S3 ratio l Immunoblammings of E14.5 mouse cortical neurons cultured for 5 DIV to detect Acly (purple) and α-tubulin (α-Tub, grey) m; Acly, Synaptophysin-1 (green) and α-Tub (n, top); Acly, Lamp-1-Green (green) and α-Tub (n, bottom). Scale bars are 20 μm (m), 10 μm (n). o–p Analysis of Acly activity by malate dehydrogenase coupled method performed in WT and Elp3 cKO brain cortex lysates. Histogram of relative Acly activity over assay (o) and of slopes (p) from the linear phase of the reaction. q qRT-PCR analysis of Acly mRNA in cortical brain extracts of Elp3 cKO or WT littermate mice. r–t Immunoblots and quantification of cytosolic (S3, s) and vesicular (P3, t) fractions of Acly protein from newborn WT and Elp3 cKO mice brain cortices. u LC-MS quantification of Acyl-CoA levels in WT and Elp3 cKO P0 mice brain cortex lysates. Description of graphical summaries within are histograms of means ± SEM. Significance was determined by: b, c, g, h, i, s, t two-sided t test, q, u two-sided Mann-Whitney test, d, e, p two-sided one-way analysis of variance (ANOVA), and o two-sided two-way ANOVA. Specifically, (b) p = 0.8411, t = 0.2093, df = 6; (c) p = 0.7505, t = 0.3248, df = 13; (d) p < 0.0001, F = 213.80; (e) p = 0.0001, F = 85.29; (g) p < 0.01, F = 17.48; (h) p = 0.0157, t = 2.776, df = 13; (l) p = 0.0013, t = 3.861, df = 17; (f) p = 0.056, t = 4.638, df = 5; (o) p < 0.0001, F = 38.78; (p) p < 0.0001, F = 1352; (q) p = 0.5714, U = 5; (s) p = 0.0145, t = 2.855, df = 12; (t) p = 0.0428, t = 2.470, df = 7; (u) p = 0.0159, U = 0. In addition, the post hoc multiple comparisons, to analyze statistical difference * of each condition compared to control for (d, e, p) are Holm-Sidak test, and are *p < 0.05, **p < 0.01, ***p < 0.001. Number of mice: WT n = 9; Elp3 cKO n = 6; (c) WT n = 4; Elp3 cKO n = 4; (d) Control n = 8; WT n = 4; cKO n = 4; (e) WT n = 5; Elp3 cKO n = 5; Atat1 KO n = 4; (g) WT n = 7; Elp3 cKO n = 8; (h) WT n = 11; Elp3 cKO n = 8; (i) WT n = 3 (Acly); (l) WT n = 4 (ACSS2); (o–p) WT n = 4 for 99 time points; (q) WT n = 5; Elp3 cKO n = 3; (s) WT n = 9; Elp3 cKO n = 5; (t) WT n = 4; Elp3 cKO n = 5; (u) WT n = 5; Elp3 cKO n = 4. e p = 0.031, t = 2.407, df = 13. Source data are provided with this paper.

HCA reduced average and retrograde moving velocities of lysosomes along axons, which correlated with an increase of their pausing times, as compared to vehicle treatment (Fig. 3b–d, Supplementary Fig. 3a). We next analyzed 3rd instar larvae from Acly flies13 whose MNs displayed reduced α-tubulin acetylation (Fig. 3e) together with impairment of SYT1-GFP vesicles transport (Fig. 3f–h, Supplementary Fig. 3b). Knocking down Hdac6 fully rescued the level of α-tubulin acetylation (Fig. 3e) and the axonal transport defects observed in Acly KD MNs (Acly KD;Hdac6 KD, Acly;Hdac6 KD) (Fig. 3f–h, Supplementary Fig. 3b). We next postulated that the impairment of α-tubulin acetylation and axonal transport observed upon loss of Elongator (Supplementary Fig. 1a–h) may arise from the reduced expression of Acly in PNs. To test this hypothesis, we expressed human ACly (which shares high amino acid homology with its murine and fly homologues; Supplementary Fig. 3c) in E14.5 control and Elp3 cKO MNs that were cultured in microfluidic devices for 5DIV. We showed that the level of MT acetylation (Fig. 3i) and the lysosomes and mitochondria transport defects were rescued upon expression of ACly (Fig. 3j–l, Supplementary Fig. 3d–g). Comparable data were obtained with Elp3 KD flies (that also show reduction of Acly level in the brain, Supplementary Fig. 3h) that express Acly (UAS:Elp3 RNAi and UAS:Acly, Elp3 KD + Acly) in MNs during development (3rd instar larvae), where both acetylation of α-tubulin and axonal transport of SYT1-GFP vesicles were rescued to control levels (Fig. 3m–p, Supplementary Fig. 3i). These data were also correlated with improvement of locomotion activities of Elp3 KD 3rd instar larvae and adult flies upon Acly expression (Supplementary Fig. 3j–k).

Altogether, these results suggest that reduction of Acly expression leads to MTs acetylation and axonal transport defects in Elongator deficient neurons.

Fibroblasts from Familial Dysautonomia patients show defects of α-tubulin acetylation and MT-dependent transport. In order to place our findings in a human pathological context, we analyzed primary skin fibroblasts from FD patients carrying the splice site IVS20 + 6T_C variant in ELPL114 that expressed barely detectable amount of ELPL1 proteins and low level of ELP3, resulting from its instability upon nonassemble of the Elongator complex (Supplementary Fig. 4a)22. We observed a reduction of acetylation of α-tubulin and MT-dependent transport defects in FD fibroblasts (Supplementary Fig. 4b–g), which were comparable to those observed in neurons from Elongator loss of function fly and mouse models. This modification was not resulting from an increased deacetylation activity of HDAC6 toward MTs in extracts fibroblasts nor an intracellular protein aggregation that would affect MT-dependent transport in FD fibroblasts (Supplementary Fig. 4h–i). Both the reduction of α-tubulin acetylation and the defects of MT-dependent transport in cultured FD fibroblasts were rescued by blocking tubulin deacetylation with the HDAC6 specific inhibitor, Tubastatin (TBA; 10μM during 30 min) (Supplementary Fig. 4c–g). Similarly to the animal models, FD fibroblasts express reduced amount of ACly proteins (Fig. 4a) and incubation with HCA for 8 h interfered in a dose-dependent manner with MT acetylation (Fig. 4b), in both control and FD fibroblasts, in line with the residual expression of ACly observed in FD fibroblasts (Fig. 4a). We next showed that over-expression of ACly in FD fibroblasts rescued the level of acetylation of α-tubulin (Fig. 4c), as well as the defects of MT-dependent transport of lysosomes (Fig. 4d–f, Supplementary Fig. 4j). Altogether, these results demonstrate that reduction of ACly in FD fibroblasts leads to MT acetylation and MT-dependent transport impairments; these molecular defects likely contribute to the pathological mechanisms underlying FD.

Loss of elongator activity interferes with Acly stability. Elongator is a tRNA modifier and its loss of activity may directly impair the translation of Acly mRNAs. To address this question, we performed a polysomal fractionation of cortical extracts of P0 Elp3 cKO and WT littermates to analyze the association of Acly mRNAs with ribosomes45 and found no differences between genotypes (Fig. 5a–b). These data were confirmed by labeling cultured Elp3 cKO an WT PNs with puromycin for 4 min, a tRNA analogue that incorporates into nascent peptides, causing their premature chain termination (reviewed in46). We visualized the de novo synthesis of Acly by combining anti-puromycin with anti-α-tubulin (α-Tub) and its loss of activity may directly impair the translation of Acly mRNAs. To address this question, we performed a polysomal fractionation of cortical extracts of P0 Elp3 cKO and WT littermates to analyze the association of Acly mRNAs with ribosomes45 and found no differences between genotypes (Fig. 5a–b). These data were confirmed by labeling cultured Elp3 cKO and WT PNs with puromycin for 4 min, a tRNA analogue that incorporates into nascent peptides, causing their premature chain termination (reviewed in46). We visualized the de novo synthesis of Acly by combining anti-puromycin with
anti-Acly antibodies and performing proximity ligation assay (PLA) to reveal Acly puro-PLA puncta in MNs (Fig. 5c–d). We showed comparable somatic and axonal Acly puro-PLA puncta in MNs from both genotypes (Fig. 5e–f). Together, these data suggest that loss of Elongator activity does not interfere with the translation of Acly mRNAs in cortical PNs.

We next tested whether the stability of ACLY might be impaired upon loss of Elongator activity. For this purpose, we cultured E14.5 WT and Elp3KO embryos transfected with Acly or control. Scale bar is 10 μm. **p < 0.0001, Kruskal–Wallis one-way ANOVA. Specifically, (a) p = 0.0166, t = 2.445, df = 81; (b) p = 0.0039, U = 6807; (c) p = 0.2524, U = 2152 and p = 0.0032, U = 4559 for anterograde and retrograde, respectively; (d) p < 0.0001, U = 6207; (e) p < 0.0001, K = 26.17; (f) p < 0.0001, K = 55.55; (g) p = 0.0130, K = 8.69 and p = 0.0004, K = 15.55 for anterograde and retrograde, respectively; (h) p < 0.0001, K = 93.1; (i) p = 0.0042, F = 4.969; (j) p < 0.0001, K = 28.69; (k) p = 0.001, K = 13.85 and p < 0.0001, K = 251.5; (l) p < 0.0001, K = 24.74; (m) p = 0.0003, F = 9.158; (n) p < 0.0001, K = 21.66; (o) p < 0.0001, F = 25.90, and p < 0.0001, F = 20.96 for anterograde and retrograde, respectively; (p) p < 0.0001, K = 22.35. In addition, the post hoc multiple comparisons, to analyze statistical difference of each condition compared to control (e) Kruskal–Wallis test, for (f, k), m) are Sidak’s test and are **p < 0.05, ***p < 0.001, and ****p < 0.0001.

**Fig. 3 Acly/Acly expression rescues α-tubulin acetylation and molecular transport defects upon loss of Elongator activity in mouse and fly neurons.** a Immunolabeling and quantification of acetylated α-tubulin (Ac α-Tub) and total tubulin (Tot α-Tub) levels in axons of DIV5 cultured cortical PNs isolated from E14.5 embryos and incubated with 10 mM hydroxy-citrate (HCA) during 8 h prior to fixation. Scale bar is 10 μm. b–d Time-lapse recording of axonal transport in corresponding PNs cultured for 5 DIV in microfluidic devices and treated with vehicle- or HCA-supplemented medium for 8 h showing (av.) velocity b, moving velocity c, and percentage of pausing time d of lysosomes (LysoTracker®). e Immunolabeling and quantification of acetylated α-tubulin (Ac α-Tub) and total tubulin (Tot α-Tub) levels in MN axons from 3rd instar larvae of Drosophila melanogaster control or expressing Acly RNAi (Acly KD) under a MN-specific driver (D42;Gal4). Scale bar is 10 μm. f–h Time-lapse recording of Synaptotagmin-GFP (Syt1-GFP) axonal transport in 3rd instar larvae MNs of control or Acly KD to analyze average (av.) velocity f, moving velocity g, and percentage of pausing time h. i Immunolabeling and quantification of acetylated α-tubulin (Ac α-Tub) and total tubulin (Tot α-Tub) levels in axons of cultured cortical PNs isolated from E14.5 WT and Elp3KO embryos transfected with Acly or control. Scale bar is 10 μm. j–l Cortical neurons isolated from E14.5 WT and Elp3KO embryos were transfected with control or Acly expressing constructs and cultured for 5 DIV in microfluidic devices to perform time-lapse recording of axonal transport and measure average (av.) velocity j, moving velocity k, and percentage of pausing time l. Description of graphical summaries here within are histograms of means ± SEM. Significance was determined by a two-sided t test, b–d—two-sided Mann–Whitney test, i, two-sided one-way analysis of variance (ANOVA), and e, f, g, h, j, k, l—two-sided Kruskal–Wallis one-way ANOVA. Specifically, (a) p = 0.0166, t = 2.445, df = 81; (b) p = 0.0039, U = 6807; (c) p = 0.2524, U = 2152 and p = 0.0032, U = 4559 for anterograde and retrograde, respectively; (d) p < 0.0001, U = 6207; (e) p < 0.0001, K = 26.17; (f) p < 0.0001, K = 55.55; (g) p = 0.0130, K = 8.69 and p = 0.0004, K = 15.55 for anterograde and retrograde, respectively; (h) p < 0.0001, K = 93.1; (i) p = 0.0042, F = 4.969; (j) p < 0.0001, K = 28.69; (k) p = 0.001, K = 13.85 and p < 0.0001, K = 251.5; (l) p < 0.0001, K = 24.74; (m) p = 0.0003, F = 9.158; (n) p < 0.0001, K = 21.66; (o) p < 0.0001, F = 25.90, and p < 0.0001, F = 20.96 for anterograde and retrograde, respectively; (p) p < 0.0001, K = 22.35. In addition, the post hoc multiple comparisons, to analyze statistical difference of each condition compared to control (e) Kruskal–Wallis test, for (f, k), m) are Sidak’s test and are **p < 0.05, ***p < 0.001, and ****p < 0.0001. a Number of axons: Vehicle n = 88; 10 mM HCA n = 151; 2 mice per group. b) Number of axons: Vehicle n = 133; 10 mM HCA n = 129. 2 mice per group. c) Number of vesicles: Vehicle n = 63 (anterograde), n = 113 (retrograde); 10 mM HCA n = 77 (anterograde), n = 105 (retrograde). d) Number of vesicles: Vehicle n = 133; 10 mM HCA n = 129. e) Number of axons: Control n = 9; Acly KD n = 9; Acly;Hdac6 KD n = 9. 6 larvae per group. f) Number of vesicles: Control n = 234; Acly KD n = 422; Acly;Hdac6 KD n = 182. 3 animals per group. g) Number of vesicles: Control n = 131 (anterograde), n = 134 (retrograde). h) Number of vesicles: Control n = 234; Acly KD n = 422; Acly;Hdac6 KD n = 182. 3 animals per group. i) Number of axons: WT + Control n = 28; Elp3 cKO + Control n = 25; Elp3 cKO + Acly n = 23. Number of mice per group: WT + Control n = 5; Elp3 cKO + Control n = 3; Elp3 cKO + Acly n = 5. j) Number of vesicles: WT + Control n = 441; Elp3 cKO + Control n = 429; Elp3 cKO + Acly n = 385. Number of mice per group: WT + Control n = 5; Elp3 cKO + Control n = 3; Elp3 cKO + Acly n = 5. k) Number of vesicles: WT + Control n = 108 (anterograde), n = 107 (retrograde). Number of mice per group: WT + Control n = 5; Elp3 cKO + Control n = 3; Elp3 cKO + Acly n = 5. l) Number of vesicles: WT + Control n = 108 (anterograde), n = 107 (retrograde). Number of mice per group: WT + Control n = 5; Elp3 cKO + Control n = 3; Elp3 cKO + Acly n = 5. i) Number of vesicles: WT + Control n = 441; Elp3 cKO + Control n = 429; Elp3 cKO + Acly n = 385. Number of mice per group: WT + Control n = 5; Elp3 cKO + Control n = 3; Elp3 cKO + Acly n = 5. m) Number of axons: Control n = 25; Elp3 KD n = 10; Elp3 KD + Acly n = 37. 3 mice per group. n) Number of vesicles: Control n = 304; Elp3 KD n = 78; Elp3 KD + Acly n = 181. Number of larvae per group: Control n = 5; Elp3 KD n = 10; Elp3 KD + Acly n = 10. o) Number of vesicles: Control n = 87 (anterograde), n = 141; Elp3 KD n = 99 (anterograde), n = 141 (retrograde); Elp3 KD + Acly n = 80 (anterograde), n = 81 (retrograde). Number of larvae per group: Control n = 5; Elp3 KD n = 10; Elp3 KD + Acly n = 10. p) Number of vesicles: Control n = 252; Elp3 KD n = 84; Elp3 KD + Acly n = 234. Source data are provided with this paper.
Fig. 4 ACLY expression rescues defects of α-tubulin acetylation and microtubule-dependent transport in human FD fibroblasts. a Immunoblotting of ACLY and β-ACTIN in cultured human primary fibroblast extracts from Control and FD patients. b Immunolabeling and quantification of acetylated α-tubulin and total α-tubulin levels in extracts from primary fibroblasts from healthy controls and FD patients transfected with control or ACLY expressing plasmids. Scale bar is 50 μm. d–f Time-lapse recording of MT-dependent transport of lysosome (LysoTracker®) in fibroblasts from Control or FD patients, transfected with Control or ACLY plasmids, to analyze average (av.) velocity (d), moving velocity (e) and percentage of pausing time (f). Description of graphical summaries here within are histograms of means ± SEM. Significance was determined by (a) two-sided t test, (b) two-sided two-way analysis of variance (ANOVA), (c) one-way ANOVA, and (d, e, f) two-sided Kruskal–Wallis test and are *p < 0.05, **p < 0.001, and ***p < 0.0001. a 5 human primary fibroblast lines per group. b Number of cells: Control n = 26 (Vehicle), n = 18 (3 mM HCA), n = 20 (10 mM HCA); FD n = 18 (Vehicle), n = 15 (3 mM HCA), n = 18 (10 mM HCA). c Number of fibroblast lines per group: Control n = 5; FD n = 4. d Number of cells: Control + EV n = 15; FD + EV n = 15; FD + Acly n = 15. d Number of vesicles: Control + EV n = 262; FD + EV n = 217; FD + Acly n = 143. e 5 human primary fibroblast lines per group. e Number of vesicles: Control + EV n = 133; FD + EV n = 68; FD + Acly n = 89. f 5 human primary fibroblast lines per group. Number of vesicles: Control + EV n = 251; FD + EV n = 217; FD + Acly n = 143. g 5 human primary fibroblast lines per group. Source data are provided with this paper.

Discussion

Here, we show that loss of Elongator activity impairs both the MT acetylation and bidirectional axonal transport in mouse cortical neurons in culture, as well as in fly motoneurons in vivo, ultimately resulting in locomotion deficits in flies. These defects are similar to those observed upon loss of Atat1 expression, the enzyme that catalyzes the acetylation of α-tubulin K40 residues in MTs. This shared phenotype prompted us to investigate a possible coordination between the Elongator and Atat1 in the control of MT-dependent axonal transport via α-tubulin acetylation. By combining conditional loss of function models with genetic experiments, we observed a molecular cooperation between Elongator and Atat1 and identified Acly as the common denominator, which provides acetyl groups from Acetyl-CoA to vesicular Atat1 and whose expression depends on Elongator activity. We showed that acute knockdown or pharmacological blockade of Acly impairs MT acetylation and axonal transport in fly and mouse, respectively. Here, the lower effect of HCA treatment on lysosome axonal transport may reflect a sub-therapeutic reduction of Acetyl CoA availability in mouse PNs, as patient fibroblasts and performed cycloheximide chase assay to measure ACLY half-life in several lines of control and FD fibroblasts. We showed a reduction of ACLY stability in FD fibroblasts as compared to control lines (Fig. 5g–h), a phenotype that may result from the disruption of an interaction between the Elongator and Acly, as suggested by co-immunoprecipitation experiments performed in HEK293 cells (Supplementary Fig. 5a). Altogether, these data suggest that loss of Elongator activity reduces Acly/ACLY stability, thereby affecting the local production of acetyl-CoA required by Acetyl-CoA to promote MT acetylation. Such regulation is likely to take place in motile vesicles (Fig. 5i).
compared to the one obtained in the genetic fly model. On the other hand, raising Acly level not only rescued axonal transport defects in both mouse and fly Elongator models but also improved locomotion activity of Elp3 KD larvae and flies. We found that loss of Elongator activity does not affect the transcription and translation of Acly but rather impairs Acly/ACYL stability. Our in vitro data suggest that Elongator may regulate Acly/ACYL protein stability via molecular interaction, as previously reported for other cytoplasmic proteins37 (Supplementary Fig. 5). Another non-mutually exclusive mechanism that may act downstream Elongator would involve the regulation of the acetyltransferase P300/CBP-associated factor (PCAF), which is known to increase Acly stability by promoting its acetylation47.

We previously showed that motile vesicles are the main driver of MT acetylation in axons30 and Elongator subunits and Atat1 have been detected in protein extracts from purified motile vesicles16,30. Here, we found that the vesicular fraction isolated from mouse cerebral cortical extracts does not only express Elongator subunits and Atat1, but it is also enriched with a functional pool of Acly (Fig. 2j–l). Therefore, we postulate that an Elongator/Acly/Atat1 (EAA) signaling pathway may directly act at vesicles to promote MT acetylation, further supporting their non-canonical role as a platform for local signaling and for...
regulating axonal transport in particular (Fig. 5i). Since these molecules are also detected in the cytosolic fraction together with Acss2 (S3, Fig. 2j–l), whose expression is reduced upon loss of Elongator (Fig. 2f, h), we cannot rule out the contribution of some cytosolic Acetyl-CoA generated by Acss2 in the vicinity of MTs, which would explain the remaining basal level of MT acetylation seen upon axonal transport blockade. 30. We observed comparable defects of α-tubulin acetylation and MT-dependent transport in fly, mouse, and human cells lacking Elongator activity, which, together with similar observations made in C. elegans, suggest a strong functional conservation of the EAA pathway across species.

A tight control of axonal transport is indeed very important to ensure the homeostatic activity of neurons by delivering cargos to distant regions, thereby controlling cytoskeleton maintenance as well as spreading long-range intracellular signaling that ensures cellular maintenance and function. Impairment of axonal transport is considered as an early pathological feature shared by several neurodegenerative disorders. 48,49. Along this line, our results obtained with FD fibroblasts support those previously described in neurons and suggest that loss of Elongator activity contributes to neurodegeneration by interfering at least with MT-dependent transport in FD patients via impairment of ALCY expression. We did not observe differences in MT deacetylation activity in FD and WT fibroblasts (Supplementary Fig. 4h), suggesting that reduction of MT acetylation in FD fibroblasts does not arise from changes of HDAC6 expression, in contrast to what others have reported.

More generally, α-tubulin acetylation not only modulates axonal transport but also provides resistance to mechanical bending to MTs. 50. Therefore, by controlling the cytoskeleton integrity and function, the EAA pathway likely acts as a key regulator of neuronal fitness whose progressive dysfunction may contribute to neuronal aging and even underlie neurodegeneration. Thus, targeting this molecular pathway may open therapeutic perspectives to prevent the onset or the progression of neurodegenerative disorders like FD characterized by poor axonal transport and degeneration.

Methods

Mice. All experiments performed in this study adhere to all relevant ethical regulations for animal testing and research. The animal work was approved by the ethical committee of the University of Liège under the license #18-2056. Brains were harvested from mice at P0–P2 or E14.5. FoxG1-Cre 2−/−Elp3 lous+ and Elp3 homo mice were time-mated to induce a conditional deletion of Elp3 in the forebrain progenitors. 33. Brains were harvested from 10–16 months old Elp1cKO and WT mice. 64. Atax1 1/− mice were crossed together to obtain WT and KO mice. 55. Mice were housed under standard conditions (animals were bred in-house and maintained with ad libitum access to food and constant temperature (19–22 °C) and humidity (40–50%) under a 7am–7pm light/dark cycle) and they were treated according to the guidelines of the Belgian Ministry of Agriculture in agreement with the European Community Laboratory Animal Care and Use Regulations (86/609/CEE, Journal Officiel des Comunautés Européennes L358, 18 December 1986). Neuronal cultures were prepared from dissected E14.5 mouse brain cortices, followed by mechanical dissociation in HBSS (Sigma-Aldrich, H6648) supplemented with 1.5% glucose. Cells were cultured at confluence (~70%) for 5 days with Neurobasal Medium (Gibco, Invitrogen, 21103049) supplemented with 2% B27 (Gibco, Invitrogen, 17504044), 1% Pen/Strep (Gibco, Invitrogen, 15140122), and 1% Glutamax (Gibco, Invitrogen, 35050061) at 37 °C. Drosophila melanogaster. Flies were kept at 25 °C in incubator with regular 12:12 light and dark cycle. All crosses were performed at 25 °C. After two days hatched first instar larvae were transferred to a 29 °C incubator until use. UAS-RNAi carrying lines were crossed with D42-Gal4-UAS-Syt1-GFP virgin females for axonal transport and immunohistochemistry, with D42-Gal4 for behavioral experiments, or with Elav-Gal4 for qPCR and WB analysis (See Table 1). All RNAi inserts sequences were validated by DNA sequencing.

Human primary fibroblasts. Fibroblasts were cultured on polystyrene culture plates (Corning) at 37 °C with 5% CO2 in DMEM (Gibco, Invitrogen, 11960092) medium supplemented with 10% Fetal Calf Serum (Gibco, Invitrogen, 10509006), 1 mM Sodium pyruvate (Gibco, Invitrogen, 11360070), 1 mM non-essential amino acids (Gibco, Invitrogen, 11400050). Primary fibroblasts from five FD patients and age-matched controls were purchased from Corell biobank (www.corell.org) (Table 2).

Immunofluorescence. E14.5 cortical neurons were plated on coverslips in 24 well plate at a density of 30,000 cells and cultured for 5DIV. Neurons were fixed by 4% PFA-succrose in PBS for 20 min at RT and washed with PBS + 0.3% Triton-X. After washing, neurons were incubated in blocking solution (PBS + 0.3% Triton-X + 10% normal donkey serum) for 1 h at RT. Following overnight incubation with primary antibodies in blocking solution at 4 °C, washing, and incubation with secondary antibodies (PBS + 0.3% Triton-X + 1% normal donkey serum) at RT for 1 h and washing, coverslips were then mounted on microscope slide using Mowiol. Images were acquired using a Nikon A1Ti confocal microscope (60x oil lens) or Airyscan super-resolution module on a Zeiss LSM 880 confocal (63x oil lens).

Larvae. Larvae were dissected in PBS to expose the brain and MNs. After dissection larvae were fixed with 4% PFA for 20 min at RT, washed with PBS + 0.2% (CSP staining) or 0.3% (α-Tubulin acetylation staining) TritoX-X and incubated in blocking solution PBS, 0.2% (CSP staining), or 0.3% (α-Tubulin acetylation staining) TritoX-X + 1% BSA for 30 min at RT. Following overnight incubation with primary antibodies at 4 °C, washing, and incubation with secondary antibodies at 4 °C at RT for 2 h, the larvae were mounted on microscope slide using Mowiol. Images were acquired using a Nikon A1Ti confocal microscope (60x oil lens).

Primary fibroblasts from FD patients or controls were plated at the concentration of 5000 cells in a 96-well plate (uClear, Grinere). Cells were fixed with 4% PFA for 10 min, washed, permeabilized, and blocked for 1 h at RT with 5% fetal bovine serum in PBS + 0.1% Triton-X. Following overnight incubation with acetylated α-tubulin (Sigma-Aldrich) and β-tubulin (cell signaling) antibodies, cells were washed with PBS + 0.05% Triton-X times and incubated with secondary antibodies for 1 h at RT. Finally, the cells were washed, and remained in PBS for image acquisition. Images were acquired using an iN Cell Analyzer 2200 fluorescent microscope (General Electric) using a 60x air lens.

Fluorescence intensity levels were measured by Fiji (https://imagej.net/Fiji/Downloads). To analyze α-tubulin and acetylated α-tubulin levels of mice cortical neuron axons and fly MNs, ROIs of 30 µm long axonal segments accounting for their full width were used. For human primary fibroblasts, ROIs of the entire cell were outlined. α-tubulin and acetylated α-tubulin levels were extracted from mean intensity levels. Background levels were subtracted and the ratio of acetylated α-tubulin/α-tubulin was calculated.
**Table 1** Drosophila melanogaster lines.

| Line            | Catalog number | Given name                  | Purchased from                      |
|-----------------|----------------|-----------------------------|-------------------------------------|
| Elav-Gal4       | BDSC 458       | Control                     | Bloomington Drosophila Stock Center |
| huD42-Gal4      | BDSC 8816      | Elp1                        | Bloomington Drosophila Stock Center |
| UAS:Syt-GFP     | BDSC 6925      | Elp1                        | Bloomington Drosophila Stock Center |
| UAS:RNAi Zp2    | VDRC CG10125   | Elp3                        | Bloomington Drosophila Stock Center |
| UAS:RNAi Elp1   | VDRC CG10535   | Atal1 KD                    | Vienna Drosophila Resource Center   |
| UAS:RNAi Elp3   | VDRC CG15433   | Hda6 KD                     | Vienna Drosophila Resource Center   |
| UAS:RNAi Atal1  | VDRC CG3967    | hElp3                       | Vienna Drosophila Resource Center   |
| UAS:Human Elp3  | DSBB 5118      | hACLY                       | Vienna Drosophila Resource Center   |
| UAS:Human ACLY  | VDRC CG8322    |                             | Vienna Drosophila Resource Center   |

**Table 2** Human fibroblast lines.

| Catalog number | Sex     | Age | Sample description          |
|----------------|---------|-----|-----------------------------|
| GM02036        | Female  | 11  | Apparently Healthy           |
| GM07492        | Male    | 17  | Apparently Healthy           |
| GM07522        | Female  | 19  | Apparently Healthy           |
| GM038          | Female  | 3   | Apparently Healthy           |
| GM05659        | Male    | 14  | Apparently Healthy           |
| GM02343        | Female  | 24  | Familial Dysautonomia        |
| GM04589        | Male    | 16  | Familial Dysautonomia        |
| GM04663        | Female  | 2   | Familial Dysautonomia        |
| GM04959        | Female  | 10  | Familial Dysautonomia        |
| GM04899        | Female  | 12  | Familial Dysautonomia        |

Plasmodids and drug treatments. For silencing of Elp1 in mouse cortical neurons we inserted sh-Elp3 5’GCACAGGGCGGACAGGUGU3’ or a control sequence sh-Control 5’-TAGGGCATAGATTGAGG-3’ previously described in35,36. The viral packaging vector is PSPAX2 and the envelope is VSVG. The lentiviral vector is pCDH-cmv-EF1-tcopGFP (CDS1181), and the promoter was replaced by a U6 promoter. For expression of human ACLY in E14.5 mouse cortical cells, neurons in suspension were transfected with pE6-EAcly (Addgene plasmid # 70765, a gift from Kathryn Wellen)34 or pE6 (control) and GFP (from VPG-1001, Lonza) using Mouse Neuron Nucleofector® Kit (VPG-1001, Lonza) according to the manufacturer’s protocol. For expression of human ACLY in cultured human primary fibroblasts, cells were transfected with pE6-EAcly (Addgene plasmid # 70765, a gift from Kathryn Wellen)34 or pE6 (control) and GFP (from VPG-1001, Lonza) using Lipofectamine® 2000 according to the manufacturer’s protocol. GFP-positive cells were used for analysis. Tubastatin A (TBA, 20 μM) or Hydroxy-citrate (HCA, 3 mM or 10 mM) were dissolved in Ultra-Pure Water (UPW) and added to cell cultures 2 h or 8 h, respectively, prior to recording.

Real-time quantitative PCR analysis (qRT-PCR). One mouse cortex or ten adult fly heads were collected in TRIzol Reagent (Ambion, Life Technologies) followed by RNA extraction performed following the manufacturer’s instructions. After DNase treatment (Roche), 1 μg of RNA was reverse transcribed with RevertAid Reverse Transcriptase (Fermentas). RT-qPCR was performed using Quanti Studio (Thermo) and TaqMan primers for mice, or a Light Cycler 480 (Roche) with Syber Green mix for Drosophila melanogaster. Analyses were done using the 2−ΔΔCT method35 with the primers listed in Table 3.

Protein aggregation assay. Cultured mice cortical neurons or human primary fibroblasts at 60% confluency were incubated with PROTEOSTAT® (Enzo Life-Sciences) to detect protein aggregates.

Locomotion activity and climbing assays. Larval crawling speed assays were performed by placing 3rd instar larvae in the center of 15-cm petri dishes coated with 3% agar, as previously published36. Velocities were extrapolated by measuring the distance traveled in 1 min. The climbing assay was performed as previously described in35, by measuring the average ratio of successful climbs over 15 cm for 10 adult flies.

Western blot. Mouse brain cortices, adult fly heads, or human primary fibroblasts were quickly homogenized on ice in RIPA buffer, or 320 mM sucrose, 4 mM HEPES buffer for subcellular fractions. Protease inhibitor cocktail (Roche, P8340 or Sigma-Aldrich, S8820) and 5 μM Trichostatin-A (Sigma-Aldrich, T8552) were added to inhibit protein degradation and α-tubulin deacetylation.

Immunoblotting was performed with the primary and secondary antibodies listed in Supplementary Table 1. We used 2 μg of protein lysate for α-tubulin acetylation analysis and 20–30 μg for all other proteins. Nitrocellulose membranes were imaged using Amersham Imager 600 (General Electric, 29083461) and band densitometry was measured using Fiji.

Subcellular fractionation. Subcellular fractionation of frozen mice brain cortex was performed as previously described35. Tissues or cells were homogenized in 320 mM sucrose, 4 mM HEPES, pH 7.4 (fractionation buffer). The homogenate was centrifuged for 10 min at 1000 × g to obtain a pellet (P1) (nuclear fraction) and a post-nuclear fraction (S1). The supernatant (S1) was centrifuged for 40 min at 12,000 × g to obtain large membranes (P2) and vesicle-enriched cytosolic fraction (S2). Finally, S2 was centrifuged again for 90 min at 120,000 × g using a TLA 120.1 fixed angle rotor in Optima TLX Benchtop Ultracentrifuge to obtain vesicle

Vesicular and mitochondrial transport recording in vitro and in vivo. Axonal transport in mice cultured cortical neurons was recorded in microfluidics devices, prepared as described in35. Briefly, air bubbles were removed from mixed sylgard 184 elastomer (VossChemie Benelux) using vacuum (1:5 ratio with curing agent) by centrifuging at 1000 × g for 5 min. The liquid was poured into the microfluidic device mold and was cured by 3 h incubation at 70 °C incubator. Molds were cut and washed twice with 70% ethanol, air dried in biological hood and placed on 35 mm glass-bottom dishes (MatTek, P35G-0-20-C) previously coated with 0.1 mg/mL poly-D-lysine (overnight incubation at 4 °C prior to washes and air drying). To increase the adhesiveness, the microfluidic chambers and dishes were heated to 70 °C before assembling. E14.5 mouse cortical neurons were isolated and resuspended at 40000 × 10^3 cells/mL for seeding at a 70% confluence into the microfluidic schannel. After plating, the axonal and somal compartments of the microfluidic devices were filled with culture medium supplemented with 50 ng/mL or 20 ng/mL BDNF (PeproTech, 450-02), respectively. Labeling was done on after 5DIV by adding 1 μM LysoTracker® Deep Red FM (ThermoFisher Scientific, L7528), MitoTracker® Green FM (ThermoFisher Scientific, M7514) or MitoTracker® Deep Red FM (ThermoFisher Scientifics, M22426) 30 min prior to time-lapse recordings. Axonal transport recording in Drosophila Melanogaster MNs were done on 3rd instar larvae expressing UAS:NR1 and Syt-GFP. Larvae were anaesthetized with ether vapors (8 min) and mounted dorsally on microscope slide using 80% glycerol. Recordings of mice cortical neurons and Drosophila melanogaster MNs were performed on an inverted confocal microscope Nikon A1Ti (60x oil lens) or Zeiss LSM-880 (Wellen)54 or pEF6 (control) and GFP (from VPG-1001, Lonza) using Lipofectamine® 2000 according to the manufacturer’s protocol. GFP-positive cells were used for analysis. To assay the subcellular localization of Acly in respect to lamp1-positive late endosomes/lysosomes in mouse cortical neurons, pCAG mEmerald-LAMP1 provided by F. Polleux (Columbia University, New York, USA) was transfected using Mouse Neuron Nucleofector® Kit (VPG-1001, Lonza) according to the manufacturer’s protocol. For expression of human ACLY in cultured human primary fibroblasts, cells were transfected with pE6-EAcly (Addgene plasmid # 70765, a gift from Kathryn Wellen)34 or pE6 (control) and GFP (from VPG-1001, Lonza) using Lipofectamine® 2000 according to the manufacturer’s protocol. GFP-positive cells were used for analysis. Tubastatin A (TBA, 20 μM) or Hydroxy-citrate (HCA, 3 mM or 10 mM) were dissolved in Ultra-Pure Water (UPW) and added to cell cultures 2 h or 8 h, respectively, prior to recording.
fraction (P3) and a cytosolic fraction (S3). The vesicle fraction was gently washed once with fractionation buffer, and resuspended by vigorous pipetting with the same buffer.

Mass spectrometry analysis. Pellets from three independent samples of pooled vesicles isolated from the brain cortex of WT or Elpi KD mice were solubilized using 5% SDS. The samples were dissolved in 10 mM DTT 100 mM Tris and 5% SDS, sonicated, and boiled at 95 °C for 5 min. The samples were precipitated in 80% acetone. The protein pellets were dissolved in 9 M Urea and 100 mM ammonium bicarbonate than reduced with 3 mM DTT (60 °C for 30 min), modified with 10 mM iodoacetamide in 100 mM ammonium bicarbonate (room temperature for 30 min in the dark), and digested in 2 M Urea, 25 mM ammonium bicarbonate with modified trypsin (Promega), overnight at 37 °C in a 1:50 (M/M) enzyme-to-substrate ratio. The resulting tryptic peptides were desalted using C18 tips (Harvard) and resuspended in 0.1% Formic acid. Samples were analyzed by LC-MS/MS using Q Exactive plus mass spectrometer (Thermo) fitted with a capillary HPLC (easy nLC 1000, Thermo). The peptides were loaded onto a homemade capillary column (20 cm, 75 micron ID) packed with Reprosil C18-Aqua (Dr Maisch GmbH, Germany) in solvent A (0.1% formic acid in water). The peptide mixture was re-equilibrated with a 5 (28–95%) linear gradient of solvent B (95% acetonitrile with 0.1% formic acid) for 60 min followed by a gradient of 28–95% for 15 min, and 15 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.15 µl/min. Mass spectrometry was performed in a positive mode using repetitively full MS scan followed by high collision-induced dissociation (HCD, at 35 normalized collision energy) of the 10 most dominant ions (+1 charges) selected from the first MS scan. The mass spectrometry data were analyzed using the MaxQuant software 1.5.1.2. (http://www.maxquant.org) using the Andromeda search engine, searching against the mouse uniprot database with a mass tolerance of 20 ppm for the precursor masses and 20 ppm for the fragment ions. Peptide- and protein-level false discovery rates (FDRs) were calculated using the UltimateXchange tool from the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021186.

In vitro α-tubulin assay. In vitro α-tubulin acetylation assay was performed as previously described38. Briefly, 96-well half-area plates (Greiner, 674061) were coated with 5 µg tubulin from HeLa cells or bovine brain (Cytoskeleton, catalog no. HTS02-A) in 25 µl of ultrapure water for 2.5 h at 37 °C, blocked (PBS + 3% BSA + 3% skim milk + 3% FBS) for 1 h at 37 °C, and washed with PBS + 0.05% Tween 20. Wells were incubated for 2 h at 37 °C with shaking at 140 x g with 25 µg of the total, S2, S3, or P3 fractions isolated from P0 mouse brain cortex previously incubated overnight at 4 °C with acetylated α-tubulin antibody (1:2000) in blocking buffer. Wells were then washed and incubated with trimethylboron/E (Merck Millipore, ES001) whose reaction was thereafter stopped with H2SO4. Following the manufacturer’s instructions, the HAT activity colorimetric assay kit (Sigma–Aldrich, EPI001) was used to measure CoA release.

α-Tubulin deacetylation assay. α-Tubulin deacetylation assay was performed as previously described in39. Briefly, 96-well half-area plates (Greiner, 674061) were coated with 1 µg tubulin in 50 µl of ultrapure water for 2.5 h at 37 °C, followed by blocking (PBS, 3% BSA, 3% skim milk, 3% fetal bovine serum) for 1 h at 37 °C, and washing with PBS + 0.05% Tween 20. 10 µg of cytosolic fraction isolated from newborn mice brain cortex in deacetylation buffer (50 mM Tris-HCl at pH 7.6, 120 mM NaCl, 0.5 mM EDTA) supplemented with protease inhibitor cocktail and phosphatase inhibitor (to avoid dephosphorylation of HDAc6) were added per well for incubation of 4 h at 37 °C with shaking at 120 x g. The wells were washed and incubated overnight at 4 °C with acetylated α-tubulin antibody (1:2000) in blocking buffer (PBS + 0.05% Tween 20 + 3%BSA). Wells were subsequently washed and incubated for 2 h at 37 °C with Peroxidase-conjugated Goat anti-mouse antibody (1:5000) in antibody blocking buffer. After the final washes, the peroxidase–isolate was probed with TMB/E (ES001, Merck Millipore), and the reaction stopped with H2SO4.

Acly activity assay. Acly activity assay was measured as previously described in41. Briefly, cells or brain extracts were disrupted by passing them 15 times in 25-gauge needle in 100 mM Tris-HCL buffer, supplemented with protease and phosphatase inhibitors. Sug of cells/brain lysate were added to the reaction mix (200 mM Tris- HCL, pH 8.4, 20 mM MgCl2, 20 mM sodium citrate, 1 mM DTT, 0.1 mM NADH (Sigma-Aldrich, N8129), 6 U/mL Malate dehydrogenase (Sigma-Aldrich, M1567), 0.5 mM CoA (Sigma–Aldrich, C3019) with or without ATP (Sigma-Aldrich, A1852). Acly activity was measured every 100 s for 4 h, in the volume of 50uL in a 384 well plate using 340 nm OD read. Acly specific activity was calculated as the change in absorbance in the presence or absence of ATP. For statistical comparison Acly activity was defined as the slope from the linear range of the reaction.

Acetyl-CoA sample preparation and LC-MS/MS analysis. Acetyl-CoA was extracted as previously described38. Briefly, cold methanol (300 µl −20 °C) was added to the cell pellets, and the mixture was shaked for 30 s (10 °C, 2000 r.p.m., Thermomixer C, Eppendorf). Cold chloroform (500 µl) −20 °C was added, the mixture was shaked for 30 s, and 200 µl of water (4 °C) added afterwards. After 30 s shaking and 10 min on ice, the mixture was centrifuged (21,000 × g, 4 °C, 10 min). The upper layer was collected and evaporated. The dry residue was re-dissolved in eluent buffer (500 µl) and centrifuged (21,000 × g, 4 °C, 10 min) before placing in LC-MS vials. Acetyl CoA was analyzed as previously described38. Briefly, the LC-MS/MS instrument consisted of an Acquity i-class UPLC system and Xevo TQ-S triple quadrupole mass spectrometer (both Waters) equipped with an electrospray ion source. LC was performed using a 100 x 2.1 mm i.d., 1.7-µm UPLC Kinetro XB-C18 column (Phenomenex) with mobile phases A (10 mM ammonium acetate and 5 mM ammonium hydrocarbonate buffer, pH 7.0, adjusted with 10% acetic acid) and B (acetonitrile) at a flow rate of 0.3 ml.min-1 and column temperature of 25 °C. The gradient was set as follows: 0–5.5 min, linear increase 0–25% B, then 5.5–6.0 min, linear increase till 100% B, 6.0–7.0 min, hold at 100% B, 7.0–7.5 min, back to 0% B, and equilibration at 0% B for 2.5 min. Samples kept at 4 °C were automatically injected in a volume of 5 µL. Mass spectrometry was performed in positive ion mode, monitoring the MS/MS transitions m/z 810.02 → 428.04 and 810.02 → 303.13 for acetyl-CoA. Spikes of defined amounts of AcCoA were added to the samples to confirm the absence of signal inhibition (matrix effect) in the analyzed extracts. Quantification of AcCoA was done against an external calibration curve with 1–1000 ng.mL−1 range of AcCoA concentrations using Targetlynx software (Waters).

Polysomal fractionation. Polysome-bound RNA was purified from the cortical extract of P0 pups according to Laguesse et al.35. Briefly, fresh mouse cortex was snap-frozen in 1.5 mL Eppendorf tube, pulverized in liquid nitrogen with a pestle, and kept on dry ice for 5 min. The powder was resuspended in 1 mL lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 5 mM MgCl2, 1% NP-40, 0.5% sodium deoxycholate, 40 mM dithiothreitol, 10 mM Ribonuclease Vinaydal Complex, 200 µg/mL cycloheximide and 400 µg/mL RNAsin). 200 µL of the homogenate was used to isolate total RNA using TRIzol reagent. The remaining 800 µL were centrifuged for 10 s at 12,000 x g to discard nuclei, and the supernatant was collected. Ribosomes were further released by adding 800 µL of 2x extraction buffer (200 mM Tris pH 7.5, 300 mM NaCl, and 200 µg/mL cycloheximide) and samples were centrifuged at 12,000 x g for 5 min at 4 °C to remove mitochondria and membranous debris. The resulting supernatant was loaded onto a 8 mL 15–45% sucrose gradient and centrifuged at 100,000 x g (at tmax), 4 °C for 2 h without brake, by using a SW50 rotor (Beckman Coulter). The four first 500 µL bottom fractions were collected and digested with protease K buffer (400 µg/mL Protease K, 10 mM EDTA, 1% SDS) at 37 °C for 30 min, to be thereafter extracted with phenol–chloroform. Polysomal fractions were assayed for purity by assessing the presence of 28S and 18S ribosomal RNA bands on an agarose gel, and by measuring absorbance at 254 nm, as previously described38.

Puro-PLA and immunostaining. Puro-PLA was performed as previously described41 to label newly synthesized proteins of interest. In brief, neurons cultured 5 days in vitro were incubated with 10 µg/mL puromycin for 4 min, quickly
washed with warm PBS, and fixed for 10 min in 4% PFA-sucrose. After fixation and permeabilization, the Duolink assay (DUO92007; DUO92004; DUO92002 Sigma) was performed as recommended by the manufacturer using the following primary antibodies: puromycin 1:75 (PMY-2A4 DSHB); Acly 1:100 (OAGA04026 Aviva Systems Biology). Following puro-PLA, cells were post-fixed for 10 min in PFA-sucrose, washed with PBS, blocked with 5% donkey serum in PBS, and labelled with antitubulin antibody 1:1000 (ab6160) in Duolink Antibody Diluent (Sigma) for 1 h. After incubation with fluorophore-labelled secondary antibody for 1 h and further washes, cells were mounted with Fluorescent Mounting medium (S3023 Agilent Dako). Images were acquired with a Zeiss LSM-880 microscope using x 40 objective. Images were processed with ImageJ (NIH).

Cycloheximide pulse-chase assay. Primary fibroblasts from FD patients or healthy controls were cultured in 6-well plates at 80% confluency before treatment with 50 μg/mL cycloheximide (CHX) (C7698 Sigma) for 0 h, 4 h, 6 h, 8 h. All samples were harvested simultaneously by washing the cells in each well with ice-cold PBS, adding in each well 150 μL lysis buffer (50 mM Tris-HCl, pH 8, containing 150 mM NaCl, 1% NP-40 supplemented with complete protease inhibitor cocktail (Roche)) and subsequent mechanical scraping. Following incubation on ice for 30 min, samples were centrifuged for 20 min at 13,000 x g to obtain supernatant. Protein concentration of each sample was measured by using the Protein Assay Kit (Pierce Biotechnology, Rockford) according to the manufacturer’s instructions. All samples were resuspended in SDS buffer before WB analysis.

Co-immunoprecipitation analysis. Co-immunoprecipitation experiments were performed in HEK293 cells (Merck Chemicals, Brussels). Pierce Crosslink immunoprecipitation kit (#26147) was used following the manufacturer’s instructions. For Elp3-FLAG and Myc-Acly analysis, 4 μg of Myc (Cell signaling), Flag (Sigma-Aldrich), or general mouse IgG (Millipore) were crosslinked using DSS fixed for 10 min in PFA–S-adenosylmethionine. Elongator subunit Elp3 contains a Fe4S4 cluster and binds RNA binding of holo-elongator. Structure-function relationship, and importance of the Elp3 subunit for the elongator-depleted cells: implication for familial dysautonomia. J. Biol. Chem. 279, 32087–32092 (2004).
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Author contributions
A.E., G.M., M.W., S.T. and L.N. designed the study. A.E., G.M. and S.T. performed and interpreted most experiments. M.S. and R.L.B. are to be considered as equal contributors. R.L.B. contributed to the Drosophila work with help of N.K.. M.S. contributed to cell culture and molecular work with L.B., Ar.B., S.J. and S.L. Alb. performed the LC-MS/MS analysis. P.D. and I.D. maintained Elp1 KD mice colonies and provided brain material. F.S., A.C., B.B and J.M.R provided guidance and help for experiments. M.W. and L.N. contributed to data interpretation; and A.E., G.M., S.T., M.W. and L.N. wrote the manuscript with input of all coauthors.

Competing interests
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Correspondence and requests for materials should be addressed to Miguel Weil or Laurent Nguyen.

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