Elongator subunit 3 (ELP3) modifies ALS through tRNA modification

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative motor neuron disorder of which the progression is influenced by several disease-modifying factors. Here, we investigated ELP3, a subunit of the elongator complex that modifies tRNA wobble uridines, as one of such ALS disease modifiers. ELP3 attenuated the axonopathy of a mutant SOD1, as well as of a mutant C9orf72 ALS zebrafish model. Furthermore, the expression of ELP3 in the SOD1G93A mouse extended the survival and attenuated the denervation in this model. Depletion of ELP3 in vitro reduced the modified tRNA wobble uridine mcm5’s2U and increased abundance of insoluble mutant SOD1, which was reverted by exogenous ELP3 expression. Interestingly, the expression of ELP3 in the motor cortex of ALS patients was reduced and correlated with mcm5’s2U levels. Our results demonstrate that ELP3 is a modifier of ALS and suggest a link between tRNA modification and neurodegeneration.
Introduction

Amyotrophic lateral sclerosis (ALS) is an adult motor neuron disorder characterized by the degeneration of motor neurons in the spinal cord, brainstem and motor cortex, resulting in muscle weakness, atrophy and spasticity. It is a progressive disease and usually fatal within 5 years after the diagnosis. Mutations in a heterogeneous set of genes, such as C9ORF72, SOD1, TARDBP and FUS have been identified to cause the familial form that accounts for 10% of all ALS. Most patients (90%) have now known family history and are classified as ‘sporadic’. Currently, there is no cure for ALS (1). The phenotypic heterogeneity of familial and sporadic ALS (2) suggests the existence of modifiers that determine disease characteristics such as site and age of onset, progression rate and duration of disease. It is important to identify these modifiers as they may be targets for therapeutic intervention, even in the absence of a known cause for ALS. One such factor is subunit 3 of the elongator complex (ELP3). An association between genetic variation in ELP3 and sporadic ALS was revealed in a genome-wide association study (3), and thereafter ELP3 was identified as a modifier of survival in patients carrying the C9orf72 repeat expansion (4).

ELP3 is the enzymatic core of the elongator complex, comprised of six subunits (ELP1-ELP6). Elongator complex promotes the side chain modification of the wobble uridine base of tRNA (position U34), a process greatly increasing fidelity and efficiency of translation (5–11). Although modification of U34 is chemically complex and still incompletely understood (12), it is known that elongator catalyzes the initial step in the formation of 5-methoxycarbonylmethyl (mcm5) and 5-carbamoylmethyl (ncm5), two modifications of the tRNA wobble uridine (5,13). In yeast, ELP3 is essential for the efficient translation of stress response transcription factors such as Atf1 and Pcr1. The absence of ELP3 results in poor expression of these factors and is lethal after stress (9). Interestingly, overexpression of two tRNAs (lysine and glutamine) rescues this stress-dependent phenotype of ELP3-deficient yeast (6,9). In higher order organisms, the absence of ELP3 seems to mainly affect the nervous system. In Caenorhabditis elegans, eplc-3 deletion abolishes tRNA uridine modification and affects translation, resulting in lower levels of neuroepitope and reduction of acetylcholine in the synaptic cleft (8). In the mouse, ELP3 regulates neuronal migration and differentiation in the developing cerebral cortex (14,15). The role of ELP3 in humans is far from understood, but recessive mutations in subunit 1 (ELP1) cause familial dysautonomia (FD), a severe hereditary sensory and autonomic neuropathy (16). ELP1 is the scaffold subunit of the complex and whose loss-of-function mutations causing FD severely affect the assembly of the complex. Accordingly, the level of modified wobble uridines was found to be reduced in the brain of FD patients (17).

ELP3 has an N-terminal S-adenosyl-methionine (SAM) domain and a C-terminal histone acetyltransferase (HAT) domain. The SAM domain contains an iron-sulfur (Fe-S) cluster that can cleave SAM to form 5'-deoxyadenosine radical and methionine. It has been shown that the SAM domain, but not the HAT domain, is required to maintain the integrity of the yeast elongator complex (18). Deletion of ELP3 is associated with hypoacetylation of histones H3 and H4 in yeast (19), through which it is thought to regulate transcription elongation, although this has been questioned (6,20). In Drosophila, ELP3 acetylates the synaptic protein Bruchpilot, resulting in dysregulated glutamatergic vesicle release (21).

Several findings suggest that ELP3 affects the nervous system through a loss-of-function mechanism. In mice, it controls the production and migration of cortical neurons (14,15,22). In ALS, the genotype increasing the risk to develop the disease was associated with lower brain expression levels of ELP3 (23). In Drosophila, loss-of-function mutations induced aberrant axonal outgrowth and synaptic defects and in zebrafish decrease of ELP3 expression induced motor axonal abnormalities similar to those induced by mutant SOD1 and mutant TDP43 (3).

We hypothesized that low ELP3 expression renders motor neurons vulnerable to neurodegeneration while overexpression is neuroprotective. In this study, we investigated this hypothesis in humans and in different ALS models (zebrafish and mouse) and found that overexpression of human ELP3 was indeed protective in zebrafish models for ALS, an effect for which the SAM domain is necessary. Furthermore, ELP3 affected motor innervation, disease onset and survival in the mutant SOD1 mouse model for ALS. Lowering ELP3 decreased the levels of the modified tRNA nucleoside, 5-methoxycarbonylmethyl-2-thiouridine (mcm5’sU), both in the mouse and in NSC34 cells, while increasing ELP3 expression restored the levels of this modified uridine and attenuated mutant SOD1 aggregation in NSC34 cells. Finally, ELP3 levels were reduced in the motor cortex of ALS patients, which correlated with mcm5’sU levels. Altogether, our results suggest that ELP3 expression modifies motor neuron degeneration and may do so by altering tRNA modification in the central nervous system.

Results

Expression of ELP3 prevents axonopathy in two zebrafish ALS models

To elucidate the role of ELP3 in ALS, we first studied its effect in two zebrafish models for ALS: one for mutant C9orf72-induced ALS (C9ALS) and one for mutant SOD1-associated ALS (SOD1ALS). Injection of 90x(GGGGCC) repeat RNA, but not of 3x(GGGGCC) repeat RNA, as well as injection of mutant SOD1 RNA but not of wild-type RNA in zebrafish embryos induced a motor axonopathy, characterized by reduced axonal outgrowth and aberrant branching, as described before (23,24). Co-expressing human ELP3 RNA significantly prevented the toxic effect of the 90x(GGGGCC) repeat expansion, as it reduced the outgrowth failure by 63.6% and the number of affected embryos by 56.7% (Fig. 1A and B). Likewise, co-expression of ELP3 greatly reduced the toxicity of mutant SOD1 (SOD1A4V), reducing the outgrowth deficit by 56.8% and the number of embryos with aberrant branching with 63.5% (Fig. 1C and D). These results in two different zebrafish models suggest a protective role for ELP3 in ALS.

Ubiquitous, but not neuronal, overexpression of ELP3 attenuates the ALS-like phenotype in the SOD1G93A mouse

We next investigated whether a similar protection was seen in a rodent model for ALS, the SOD1G93A mouse. At symptomatic stage (post-natal day 140), spinal cord and brain ELP3 expression is reduced in this model by 30.2% and 20.0%, respectively (Supplementary Material, Fig. S1A and B). We next determined ELP3 levels in neurons versus glial cells in the SOD1G93A mouse. To do so, we laser-capture microdissected spinal motor neurons and the glia surrounding them in SOD1G93A mice (Supplementary Material, Fig. S1C and D). We found ELP3 levels to be reduced both in spinal motor neurons and in glia, by 31.4% and 52.4%, respectively.
respectively, compared to non-transgenic mice. Moreover, ELP3 was less abundant in glia than in neurons both in non-transgenic mice, 38.5%, and in SOD1^G93A mice, 57.5% (Fig. 2A). We then aimed to increase ELP3 levels in the SOD1^G93A mouse by overexpressing human ELP3. To this end, we crossed the ELP3 KI mouse with the CAGG-CreER mouse that ubiquitously expresses the Cre recombinase (generating the ELP3 KI/CAGG mouse, herein designated ELP3^^{KI/GAGG}). The ELP3^^{KI/GAGG} mouse was then crossed with the SOD1^G93A mouse, generating a triple transgenic mouse. Induction of human ELP3 expression was successfully achieved by treatment with tamoxifen at post-natal day 60 (Supplementary Material, Fig. S1E, G and K). The total ELP3 levels were increased by 1.27-fold to 1.50-fold in the spinal cord and brain of the ELP3^^{KI/GAGG} mouse, respectively (Supplementary Material, Fig. S1F and J). The levels of endogenous ELP3 and human SOD1 were not modified by the induction of human ELP3 expression (Supplementary Material, Fig. S1H, I, L and M).

We then evaluated the effect of ubiquitous ELP3 expression in the SOD1^G93A mouse. ELP3 did not affect disease onset (SOD1^G93A/ELP3 KI mean 92.3 ± 1.8 days; SOD1^G93A/ELP3 KI^^{GAGG} mean 97.1 ± 2.0 days; P = 0.1671), but prolonged survival (SOD1^G93A/ELP3 KI mean 152.0 ± 1.8 days; SOD1^G93A/ELP3 KI^^{GAGG} mean 160.7 ± 1.9 days; P = 0.006) (Fig. 2B and C). This survival benefit was associated with a protection of neuromuscular innervation of the gastrocnemius muscle. Expression of ELP3 reduced the number of denervated neuromuscular junctions (NMJs) of the gastrocnemius muscle by 23.6% (SOD1^G93A/ELP3 KI 78.4%; SOD1^G93A/ELP3 KI^^{GAGG} 59.9%; P < 0.0001) (Fig. 2D). Nonetheless, the number of surviving spinal motor neurons at post-natal day 150 was not affected (Fig. 2E).

In addition, we studied the effect of virally expressed human ELP3 in the mutant SOD1 mouse. AAV9 viral vector expressing either ELP3 or GFP was delivered intrathecally in SOD1^G93A neonates. We found the AAV9 viral vector to be transduced in the ventral spinal cord in a non-cell specific manner, targeting both motor neurons and astrocytes (Supplementary Material, Fig. S2A). Human ELP3 expression was detected in different tissues, such as brain, spinal cord and muscle, with variable transduction efficiency (Supplementary Material, Fig. S2B and C). Increasing the expression of ELP3 delayed disease onset by 12 days (SOD1^G93A:: AAV9-GFP mean 98.4 ± 2.6 days; SOD1^G93A:: AAV9-ELP3 mean 110.6 ± 3.0 days, P = 0.0029) and survival by 12.5 days (SOD1^G93A:: AAV9-GFP mean 147.1 ± 1.6 days; SOD1^G93A:: AAV9-ELP3 mean 154.9 ± 2.1 days, P = 0.0077) (Supplementary Material, Fig. S2D and E). Viral transduction with AAV-ELP3 decreased the number of denervated NMJs of the gastrocnemius muscle (SOD1^G93A:: AAV9-GFP 69.1%; SOD1^G93A:: AAV9-ELP3 53.0%, P < 0.0001). Similar to the results obtained in the SOD1^G93A/ELP3 KI mice, the number of surviving spinal motor neurons was not affected (Supplementary Material, Fig. S2F and G).

Because the mechanism of ALS is known to be non-motor neuron autonomous, at least in SOD1-related ALS, and because of our finding that ELP3 levels are affected both in motor neurons and in glia, we wondered whether increasing the ELP3 levels in the transgenic mice could be neuroprotective.
Figure 2. Overexpression of human ELP3 attenuates the ALS-like phenotype in the SOD1\textsuperscript{G93A} mouse. (A) ELP3 mRNA relative expression in neurons and glia from the lumbar spinal cord of symptomatic (post-natal day 140) SOD1\textsuperscript{G93A} mice. Levels were reduced by 31% and 52% in neurons and glia, respectively, compared to non-transgenic mice. Moreover, ELP3 was less abundant in glia than in neurons: 37.5% in non-transgenic mice and 57.5% in SOD1 G93A mice. Data represent mean ± S.E.M., one-way ANOVA, n = 4. ***P < 0.001, **P < 0.01. (B–E) Effect of ubiquitous tamoxifen-induced expression of human ELP3 in SOD1\textsuperscript{G93A} mice. Tamoxifen was administered at post-natal day 60. (B) Mean disease onset was not affected by ELP3 expression: SOD1\textsuperscript{G93A}/ELP3 KI 92.3 ± 1.8 days, n = 22 versus SOD1\textsuperscript{G93A}/ELP3 KICAGG 97.1 ± 2.0 days, n = 19. Data are mean ± S.E.M., log-rank test, P = 0.1671. (C) Mean survival was significantly prolonged by ELP3 expression: SOD1\textsuperscript{G93A}/ELP3 KI 152.0 ± 1.8 days, n = 35 versus SOD1\textsuperscript{G93A}/ELP3 KICAGG 160.7 ± 1.9 days, n = 19. Data are mean ± S.E.M., log-rank test, P = 0.006. (D) Relative quantification of the innervation of NMJs of the
levels only in motor neurons was sufficient to observe the protective effect found after ubiquitous expression. We therefore expressed ELP3 in neurons by the generation of a triple transgenic mouse, in which tamoxifen treatment resulted in the expression of ELP3 in Thy1-mediated CreER expressing neurons such as motor neurons (herein designated SOD1<sup>G93A</sup>/ELP3<sup>KI</sup> (Thy1<sup>CreER</sup>) (Supplemental Material, Fig. S1N–U). Interestingly, this selective induction of ELP3 expression did not affect disease onset or survival in the SOD1<sup>G93A</sup> mouse (Fig. 2F and G), likely because either the expression of ELP3 in motor neurons alone is not sufficient (or necessary) to prolong the survival of SOD1<sup>G93A</sup> mice, or the motor neuron death process affects the expression of ELP3 during its induction.

**ELP3 deletion is detrimental**

We next assessed the effect of reducing ELP3 expression in the SOD1<sup>G93A</sup> mouse. To do so, we generated an ELP3 knockout mouse, using the gene trapping technique (25). We found that absence of ELP3 is lethal around embryonic day 8, as we did not find gene-trap homozygous embryos after this stage (Supplemental Material, Fig. S3, Table S1), confirming that ELP3 is essential for mammalian embryogenesis, as reported (26). Reduction of ELP3 expression by 50% (Supplemental Material, Fig. S3A–C) did not affect embryogenesis or result in gross abnormalities in adulthood. Constitutive heterogeneous deletion of ELP3 accelerated disease onset (Fig. 3A) (109 days in SOD1<sup>G93A</sup> and 102 days in SOD1<sup>G93A</sup>/ELP3<sup>+/−</sup> mice; P = 0.0230), but did not affect survival (Fig. 3B) (157 days in SOD1<sup>G93A</sup> and 154 days in SOD1<sup>G93A</sup>/ELP3<sup>+/−</sup>; P = 0.27) in the SOD1<sup>G93A</sup> mouse. Motor neuron count and neuromuscular innervation (Fig. 3C and D) did not differ between the SOD1<sup>G93A</sup> and SOD1<sup>G93A</sup>/ELP3<sup>+/−</sup> mice, most likely a reflection of the limited effect size of reducing ELP3 by only 50%. Unfortunately, we were unable to evaluate the effect of ELP3 reduction by more than 50% in the adult SOD1<sup>G93A</sup> mouse, as both ubiquitous and neuron-specific conditional knockout of ELP3 in adult mice was rapidly fatal, a phenotype not affected by the presence of mutant SOD1 (Supplemental Material, Fig. S4). In summary, the hastening of disease onset due to partial loss of ELP3 in the SOD1<sup>G93A</sup> mouse hints for a deleterious effect of ELP3 reduction in this ALS model. Moreover, these results show that ELP3 is as essential in the adult organism as it is during development.

**The SAM domain mediates the protective effect of ELP3 in the zebrafish**

To gain insight into the molecular mechanism of ELP3, we evaluated which catalytic domain of ELP3 was mediating its protective effect. To do so, we mutated the SAM (C109/I125S) and the HAT (Y529A) domains and also deleted the entire HAT domain (Supplemental Material, Fig. S5A and B), to generate proteins with reduced or no enzymatic activity (27–29). We tested the protective effect of these constructs in the C9ALS and the SOD1<sup>44V</sup> zebrafish models. ELP3 lacking the HAT domain (ELP3<sup>G93A</sup>HAT<sup>−</sup>) prevented the motor axonopathy to the same extent as the full-length ELP3 (Fig. 4), as well as the ELP3<sup>G93A</sup>HAT<sup>−</sup> mutant (Supplemental Material, Fig. S5D). In contrast, ELP3 in which the SAM domain was inactivated (ELP3<sup>G93A</sup>Sam<sup>−</sup>) lost its protective activity in both models (Fig. 4).

As expected, the axonopathy induced by knockdown of ELP3 in zebrafish (3) could also be prevented by ELP3 overexpression (Supplemental Material, Fig. S5C). Of note, the SAM domain was necessary for this effect as well, as it was lost for ELP3 lacking an active SAM domain, but not for ELP3 lacking an active HAT domain (Supplemental Material, Fig. S5C). These results demonstrate that in this zebrafish model, the SAM domain, but not the HAT domain, is necessary for the protective effect of ELP3.

**ELP3 regulates wobble mcm<sub>5</sub>s<sub>2</sub>U tRNA modification and affects mutant SOD1 aggregation in vitro**

The modification of the wobble uridine in tRNA is the main function of ELP3. In FD, a disease caused by loss-of-function mutations in ELP1 resulting in loss of activity of the elongator complex in the nervous system, the mcm<sub>5</sub>s<sub>2</sub>U modification of tRNA is reduced (17). Interestingly, ablation of ELP3 in yeast and mice impaired proteome integrity and increases protein aggregation, by affecting codon translation rates due to defective wobble mcm<sub>5</sub>s<sub>2</sub>U tRNA modification (15,30). To gain insight into the molecular mechanism underlying the protective effect of ELP3 in ALS, we investigated the relation between ELP3 expression, wobble uridine modification and protein aggregation.

We first examined the effect of ELP3 depletion on mcm<sub>5</sub>s<sub>2</sub>U levels. In NSC34 cells, reduction of ELP3 expression by 83% dramatically decreased the levels of mcm<sub>5</sub>s<sub>2</sub>U by 60% (Fig. 5A and B). In the mouse, the lethal reduction of ELP3 levels by 90% [tamoxifen-treated ELP3<sup>lox/lox</sup>/CAGGCreER (KO) mice] reduced mcm<sub>5</sub>s<sub>2</sub>U levels in the spinal cord and brain by 72% and 75%, respectively, compared to control mice (Fig. 5C and D; Supplemental Material, Fig. S6A and B). These results confirm that ELP3 regulates the mcm<sub>5</sub>s<sub>2</sub>U wobble modification of tRNA in vitro and in vivo. In the wild-type mouse, the pool of tRNAs that should have this modification appears to be already fully modified, as conditional ubiquitous expression of human ELP3 did not increase the levels of mcm<sub>5</sub>s<sub>2</sub>U any further (Supplemental Material, Fig. S6C and D).

We next evaluated the effect of ELP3 depletion on protein aggregation. Silencing ELP3 in NSC34 cells increased the total amount of aggregating proteins by 28% (Supplemental Material, Fig. S7A and B). Moreover, it induced an increase of insoluble mutant human SOD1 (SOD1<sup>44V</sup>, 73.7%; SOD1<sup>G93A</sup>, 93.2%), but not of wild-type human SOD1 (Fig. 5E–G). In these conditions, exogenous expression of human ELP3 significantly reduced the amount of insoluble SOD1<sup>G93A</sup> to levels similar to control (Fig. 5H–J) and, concomitantly, it restored the levels of mcm<sub>5</sub>s<sub>2</sub>U by 45.5% (Fig. 5K). These results suggest that the protective effect of ELP3 in ALS may at least in part be mediated by affecting mutant SOD1 solubility through an effect on wobble mcm<sub>5</sub>s<sub>2</sub>U tRNA modification.
ELP3 levels correlate with tRNA mcm5s2U levels in the motor cortex of sporadic ALS patients

To investigate whether the levels of ELP3 and mcm5s2U tRNA are affected in ALS patients, we quantified the ELP3 mRNA in the motor cortex and the occipital cortex of sporadic ALS patients and compared it to controls. ELP3 expression was significantly reduced in the motor cortex, but not in the occipital cortex (Fig. 6A, Supplementary Material, Fig. S7C), a reduction that correlated with the levels of mcm5s2U, although mcm5s2U levels in sporadic ALS patients were not altered compared to controls (Fig. 6B and C). Together, these results suggest that ELP3 may act as a modifier of ALS through influencing tRNA modifications.

Discussion

Loss of ELP3/elongator complex function is associated with detrimental phenotypes in yeast, fly, zebrafish, mice and humans (3,12,15). We therefore hypothesized that restoring ELP3 levels may be protective in neurodegeneration. We found ELP3 to be protective in two zebrafish ALS models, as well as in the SOD1G93A mouse. In the zebrafish, expression of human ELP3 prevented the toxicity induced by both SOD1A4V expression and 90x(GGGGCC) RNA, a novel zebrafish model for C9orf72 ALS. This result prompted us to investigate the role of ELP3 in the SOD1G93A mouse, in which we found the expression of ELP3 to be reduced in the brain and spinal cord. We expressed human ELP3 ubiquitously in pre-symptomatic (post-natal day 60) mice and found the survival to be moderately increased. The fact that the total expression of ELP3 after ubiquitous induction of human ELP3 was also moderately increased (not more than 1.5-fold of the endogenous ELP3 expression) underscores the biological relevance of these results.

A similar effect on survival was observed when human ELP3 was expressed in SOD1G93A neonates after intrathecal delivery of AAV9 viral vectors, although this effect was largely due to its effect on disease onset. The explanation for this effect on onset versus disease duration is unclear, although the difference between transgenic expression at post-natal day 60 versus viral expression at post-natal day 1 is likely to contribute to it. Nevertheless, these two approaches suggest that ELP3 expression in the SOD1G93A mouse is protective both in the pre-symptomatic stage and in the symptomatic stage. The expression of ELP3 in the SOD1G93A mouse was also associated with less denervated NMJs of the gastrocnemius muscle, compared to control mice. Together with the fact that the number of spinal motor neurons was unaffected, it suggests that ELP3 exerts its protective effect through maintaining axon integrity, more so than through maintaining motor neuron survival, and highlight the role of the NMJs as the main factor of progression and severity in ALS (31). These results are consistent with the findings from Gould et al., which dissociate motor dysfunction from motor neuron death (32).

Ablation of Bax, a pro-apoptotic gene, in the SOD1G93A mouse, remarkably preserved almost completely the spinal motor neurons, but had limited effect in the reduction of the denervated NMJs and in increasing survival (32).

Moreover, we found that increasing ELP3 expression specifically in neurons in pre-symptomatic SOD1G93A mice, per se, was not sufficient to prolong their survival, suggesting a role for glial...
cells in the protective effect of ELP3. In agreement, the expression of ELP3 was reduced in SOD1 G93A mice not only in spinal motor neurons, but, and to a larger extent, also in the glia surrounding them in which expression was reduced by more than 50%. We therefore hypothesize that increasing ELP3 expression in one or more cell types present in the glia would be protective in the SOD1G93A mouse, realizing that it is possible that the selective expression of ELP3 in either astrocytes, microglia or oligodendrocytes may not be sufficient to see the protective effect, but that a combined expression of ELP3 in different cell types is required. This is concurrent with the protective effect observed after AAV9-mediated ELP3 expression in the SOD1G93A mouse.

To confirm the role of ELP3 in neurodegeneration, we also investigated the effect of ELP3 depletion. We hypothesized that if low levels of ELP3 were associated with neurodegeneration in the fish, fly and humans (3), a reduction of ELP3 levels would exacerbate the phenotype of the SOD1G93A mouse. Heterozygous deletion of ELP3 did worsen SOD1G93A mouse phenotype, although to a limited extent, also in the glia surrounding them in which expression was reduced by more than 50%. Selective neuronal ELP3 depletion in the adult mouse was equally rapidly fatal and was accompanied by a severe neurological phenotype. Deletion of the HAT domain (ELP3D_HAT) does not affect the protective effect of ELP3 (dashed lines), whereas mutation of the SAM domain (ELP3SAM) abolishes the protective effect of ELP3. 3x(GGGGCC) RNA and SOD1WT were used as controls. Data represent mean ± S.E.M., one-way ANOVA (A and C) or mean ± 95% CI, logistic regression (B and D). n ≥ 45. ***P < 0.001, **P < 0.01, *P < 0.05.

The molecular mechanism of the protective effect of ELP3 in ALS models was further investigated. ELP3 is the catalytic subunit of the elongator complex. In a recent study, it has been shown that defective wobble uridine tRNA modification results in slower codon translation rates which impair the proteasome, thus increasing protein aggregation (30). It is known that ELP3 plays a major role in this modification process (12). In agreement, we found that the levels of the mcm's3U tRNA modification were largely reduced after depletion of ELP3 not only in NSC34 cells, but also in mice. Moreover, we found protein aggregation to be increased upon ELP3 ablation in NSC34 cells, similarly to yeast (30). Importantly, ELP3 depletion from NSC34 cells increased the amount of insoluble mutant SOD1, but not of

Figure 4. SAM domain, not HAT domain, mediates ELP3 protective effect in the zebrafish. Effect of ELP3 mutants in the axonopathy induced by C9orf72-associated repeat RNA (A and B) or SOD1A4V RNA (C and D), assessed by quantification of motor axon length (A and C) and affected embryos/axonal branching quantification (B and D). Deletion of the HAT domain (ELP3D_HAT) does not affect the protective effect of ELP3 (dashed lines), whereas mutation of the SAM domain (ELP3SAM) abolishes the protective effect of ELP3. 3x(GGGGCC) RNA and SOD1WT were used as controls. Data represent mean ± S.E.M., one-way ANOVA (A and C) or mean ± 95% CI, logistic regression (B and D). n ≥ 45. ***P < 0.001, **P < 0.01, *P < 0.05.
Figure 5. Effect of ELP3 expression levels on wobble mcm5's2U rRNA modification and protein aggregation. (A) ELP3 mRNA relative expression in NSC34 cells, 72 h post-transfection. Levels of ELP3 were reduced by 85.0 ± 2.2% in cells transfected with ELP3-siRNA, compared to control cells. Data represent mean ± S.E.M., unpaired t-test, n = 5. ***P < 0.001. (B) mcm5's2U levels in NSC34 cells were reduced by 60.1 ± 2.1% in cells transfected with ELP3-siRNA, compared to control cells. t6A was used as
Interestingly, the mcm5s2U levels correlated with ELP3 levels, the ELP3/elongator complex. It is necessary for the observed neuroprotective function of mcm5s2U modification reduces the speed of codon translation equally. Since the yeast, loss-of-function mutations in both domains of ELP3 are independent of the HAT domain. This was unexpected as, in yeast, loss-of-function mutations in both domains of ELP3 equally affect the formation of modified wobble tRNA uridines (29). Our data suggest that at least in fish, the HAT domain is not necessary for the observed neuroprotective function of ELP3/elongator complex.

We next checked the levels of ELP3 and modified tRNA uridines in spinal cord of ALS patients. We found ELP3 to be reduced in the motor cortex of sALS patients, but not in the occipital cortex. Interestingly, the mcm5s2U levels correlated with ELP3 levels, indicating that mcm5s2U tRNA modifications may contribute to the pathogenesis of ALS.

Proteasome impairment is considered as one of the pathogenic factors at play in ALS. It can be triggered by different mechanisms, ER stress and a heat-shock response amongst others, resulting in the aggregation of susceptible proteins, such as SOD1, TDP43 and FUS (36). It is known that defective wobble mcm5s2U modification reduces the speed of codon translation rates (15,30), which in turn triggers proteome impairment (30). Therefore, we propose that ELP3 modifies the pathogenesis of ALS, by affecting the aggregation of susceptible proteins, through control of mcm5s2U wobble tRNA modification. Our data suggest an unexpected link between tRNA modification and motor neuron degeneration.

Materials and Methods

Plasmids, morpholinos and in vitro RNA transcription

The plasmid encoding human ELP3-FLAG under the control of a T7 promoter was purchased from Origene (Rockville, MD). The plasmids encoding ELP3 SAM (C109/I125) and ELP3 HAT (Y529A) were generated from the human ELP3-FLAG plasmid with QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). To generate the ELP3 HAT vector, the human ELP3-FLAG plasmid was digested with Xhol (Thermo Fisher Scientific, Waltham, MA) and the resulting fragment subcloned into the Xho-digested pCMV6-Vector, under control of a T7 promoter (Origene). To generate the AAV: ELP3-FLAG transfer plasmid, the human ELP3-FLAG plasmid was PCR-amplified with the primers 5′ GCTGCTCTAGAATGAGCCGACCGCGAAAGAG and 5′ GTAGCTGACTAGTCGGCGGCCTTATGAACCTTATCG and the resulting product ligated into XbaI/SalI (Thermo) digested AAV transfer plasmid (pZac 2.1 eGFP3 SEED). Human cDNA of SOD1WT, SOD1AV and SOD1GLA in the pClneo vector were kind gifts from R.H. Brown Jr (Harvard Medical School, Harvard, MA). SOD1 was cloned in the pBCM vector under control of a T3 promoter. The p9OHNR construct to generate the ~90 Ggggcc repeat RNA fragment was obtained as described (24). To synthesize RNA, all plasmids were linearized by restriction digestion, transcribed with the mMESSAGE mMACHINE T7 or T3 kit (Ambion, Huntingdon, UK) and the resulting RNA purified with the MEGAclear TM Kit (Ambion). RNA concentration was determined by spectrophotometry (Nanodrop, Thermo). The antisense morpholino (Mo) against the start codon (ELP3-Mo; 5′-TGCTTTCCCCATCTTTAGACACAACT-3′) of zebrafish ELP3 and the non-targeting control morpholino (Ctrl-Mo; 5′-CCCTTTAC

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**Figure 6.** ELP3 expression correlates with wobble mcm5s2U tRNA modification in sALS patients. (A) ELP3 mRNA relative expression in the motor cortex from sALS patients. Data represent mean ± S.E.M., unpaired t-test, n=8. *P<0.05. (B) mcm5s2U levels in the motor cortex from sALS patients. Data represent mean ± S.E.M., unpaired t-test, n=6. P>0.05. (C) Levels of ELP3 mRNA correlate with mcm5s2U levels. Linear regression, n=9, P=0.0142.
CTCAGTTACAATTTATA-3’ were designed and obtained from Gene Tools (Philomath, OR).

rAAV production

Recombinant aden-associated virus (rAAV) production was performed by the Neurobiology and Gene Therapy laboratory (Katholieke Universiteit Leuven, Belgium) as described (37). The plasmids used were the construct for the AAV2/9 serotype, the pAdvDeltaF6 adenoviral helper plasmid and the AAV transfer plasmid, encoding either human ELP3 (AAV: ELP3-FLAG) or eGFP (AAV: eGFP) under control of the cytomegalovirus (CMV) promoter. Genomic copy number (GC/ml) was determined by qPCR.

Animals

Adult zebrafish (AB strain) and embryos were maintained and staged under standard laboratory conditions (38). For the intrathecal delivery of rAAV vectors, mice neonates (P0.5) were cryoanesthetized and immobilized on a styrofoam board. A total of 1 x 10^9 Gc of rAAV vectors were injected in the spinal canal at the lumbar region, as reported previously (39,40), with a 32G Hamilton syringe (Hamilton, Reno, NV). The maximum volume injected was 7 µl. The dye lissamine green (Sigma, St. Louis, MO) was added to the rAAV vectors at a final concentration of 0.05% (w/v) in PBS to assess delivery accuracy. The procedure was considered successful when the rAAV vectors diffused along the spinal tract into the cerebellum/brain, and only neonates successfully injected were considered for this study. The human ELP3 conditional overexpressing mouse was generated by Ozgene (Perth, Australia). In brief, ELP3-FLAG cDNA preceded by a loxP site flanked stop cassette was inserted in the ROSA26 locus by homologous recombination (designated ELP3 KI mouse). The ELP3 conditional knockout mouse (designated ELP3lox/lox mouse) was generated by insertion of loxP sequences flanking the exon 2 of ELP3 (15). The SIGTR embryonic stem cell line AQP461 (Mutant Mouse Recourse and Research Center, USA), containing a gene-trap cassette at intron 1 of the ELP3 gene, was used to generate the constitutive ELP3 knockout mouse. Genotyping of the constitutive ELP3 knockout mice generated by heterozygous pairings was performed using the following primers: TRAP cassette, 5’ TTA TCG ATG AGC GTG GTG GTG TT ACG ATC CCC GGC AAA TAA TAT C, ELP3 exon 1-3, 5’ TGAGCGAAAAGAGAAGGGGAC, 5’ CGGATCAGG TCTCCGGGGC. Heterozygous mice were backcrossed for eight generations into a C57BL/6J background. The human mutant SOD1 with the gene-trap cassette was stably integrated into the genome by homologous recombination. Fusion of the exons into the promoter region was determined by a PCR procedure. The SIGTR embryonic stem cell line AQP461 (Mutant Mouse Recourse and Research Center, USA), containing a gene-trap cassette at intron 1 of the ELP3 gene, was used to generate the constitutive ELP3 knockout mouse. Genotyping of the constitutive ELP3 knockout mouse generated by heterozygous pairings was performed using the following primers: TRAP cassette, 5’ TTA TCG ATG AGC GTG GTG GTG TT ACG ATC CCC GGC AAA TAA TAT C, ELP3 exon 1-3, 5’ TGAGCGAAAAGAGAAGGGGAC, 5’ CGGATCAGG TCTCCGGGGC. Heterozygous mice were backcrossed for eight generations into a C57BL/6J background. The human mutant SOD1 with the gene-trap cassette was stably integrated into the genome by homologous recombination. Fusion of the exons into the promoter region was determined by a PCR procedure. The SIGTR embryonic stem cell line AQP461 (Mutant Mouse Recourse and Research Center, USA), containing a gene-trap cassette at intron 1 of the ELP3 gene, was used to generate the constitutive ELP3 knockout mouse. Genotyping of the constitutive ELP3 knockout mouse generated by heterozygous pairings was performed using the following primers: TRAP cassette, 5’ TTA TCG ATG AGC GTG GTG GTG TT ACG ATC CCC GGC AAA TAA TAT C, ELP3 exon 1-3, 5’ TGAGCGAAAAGAGAAGGGGAC, 5’ CGGATCAGG TCTCCGGGGC. Heterozygous mice were backcrossed for eight generations into a C57BL/6J background. The human mutant SOD1 with the gene-trap cassette was stably integrated into the genome by homologous recombination. Fusion of the exons into the promoter region was determined by a PCR procedure.

Histological analyses

To visualize NMJs, mice were deeply anesthetized with Nembutal 10%, the gastrocnemius muscle dissected and snap frozen in liquid nitrogen-cooled isopentane. Longitudinal cryosections of 20 µm (Slee cryostat, Mainz, Germany) were fixed with 4% formaldehyde (FA) in PBS for 10 min at room temperature, blocked with 10% normal donkey serum (Sigma) in PBS-0.1% Triton X-100 (PBS-T) for 1 h at room temperature and immunostained with rabbit anti-NF-200 (1:200, # N4142, Sigma) for 16 h at 4°C, followed by 1 h incubation at room temperature with Alexa Fluor 555 anti-rabbit antibody (1:500, Molecular Probes, Eugene, OR) and an axonal branch of motor neurons were quantified as described previously (23) using Lucia software (version 4.60, Laboratory Imaging, Prague, Czech Republic). A minimum of 15 fish per condition was analyzed per experiment.

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To visualize spinal cord neurons, mice were anesthetized with Nembutal 10%, transcardially perfused with PBS and subsequently with 4% FA in PBS. The lumbar spinal cord was dissected, fixed with 4% FA for 16 h at 4°C, dehydrated in 30% sucrose and snap frozen in liquid nitrogen-cooled isopentane. Two methods were followed. To measure spinal cord neurons, cross cryosections of 20 µm were stained with cresyl violet (Sigma), followed by immersion in a 70% ethanol/10% acetic acid solution and dehydration in an increased ethanol concentration series. Sections were then mounted with PerFix® (Histolab AB, Goteborg, Sweden) and neurons visualized with a Zeiss Imager M1 microscope. Images were obtained with an AxioCam MrC5 camera (Carl Zeiss) and the soma area of normal
appearing neurons in the ventral horn of the spinal cord was measured with the AxioVision 4.8 software (Carl Zeiss). Every 10th section for a total of 10 sections were analyzed per animal. To immunolabel spinal cord neuronal nuclei, cryosections of 20 μm were blocked with 10% normal donkey serum in PBS-T for 1 h at room temperature and immunostained with rabbit anti-Choline acetyltransferase (Chat, 1: 500) (#AB1144P, Millipore) for 16 h at 4°C, followed by 1 h incubation at room temperature with anti-rabbit Alexa 488. Slides were mounted using ProLong Gold antifade reagent with DAPI (Thermo). Images were obtained using a Zeiss Imager M1 microscope and an AxioCam MrC5 camera. For pathological analysis, mice were euthanized by CO2 asphyxiation followed by complete pathological examination as described previously (41). Briefly, samples were immersion fixed in 10% neutral buffered formalin (Sigma), routinely processed for paraffin embedding, sectioned at 5 μm and stained with Hematoxylin (Diapath, Martinengo, Italy) and Eosin (Diapath). Head and sternum were decalcified in a 14% solution of tetrasodium EDTA for 15 days before processing and paraffin embedding.

**Laser capture microdissection**

To microdissect spinal cord neurons, five non-transgenic and five SOD1G93A mice were anesthetized with Nembutal 10% and the lumbar spinal cord was dissected and embedded in optimal cutting temperature (OCT) compound. Cross cryosections of 20 μm, collected on 1.0 PEN slides (Zeiss), were stained with cresyl violet as described above. Per spinal cord, a total of 1500 motor neurons from the ventral horn, and the surrounding glia, were microdissected (PALM RoboSoftware, Zeiss) and collected in Adhesive Cap 500 opaque tubes (Zeiss).

**Gene expression analyses**

Total RNA was extracted from tissue with Trizol (Thermo) and precipitated with isopropyl alcohol according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA with SuperScript III Reverse transcriptase (Thermo) and random hexamer primers, according to the manufacturer’s instructions. Quantitative PCR was performed by the StepOnePlus™ (Life Technologies) with TaqMan® Fast Universal PCR Master Mix (Life Technologies). Relative gene expression was determined by the 2⁻ΔCt method and normalized to the average of the control group. Graphs represent the relative gene expression as calculated by the Pol2r2a expression. Determining relative expression by G6pdx or GAPDH confirmed the differences between the genotypes as detected with Pol2r2a. Digital droplet PCR was performed following Bio-Rad guidelines (Bio-Rad, Hercules, CA), using ddPCR Supermix (without dUTPs) for probes. Droplets were generated in a QX200 Droplet Generator and scanned with a QX200 Droplet Reader (Bio-Rad). Results were analyzed with QuantaSoft software from Bio-Rad. Gene expression assays were purchased from Life Technologies: Human EL3P, Hs00216429_m1 and Hs00986853_m1; mouse EL3P, Mm00804538_m1; Human SOD1, Hs00533490_m1; mouse Chat, Mm01221880_m1; mouse Slc1a2, Mm01275814_m1; Human Polr2a, Hs00172187_m1; mouse Polr2a, Mm00839502_m1; Human GAPDH, Hs99999905_m1 and mouse G6pdx, Mm00656735_g1. The Polr2a-HEX labeled Taqman assay Mm.PT.SB.13811327 for the digital droplet PCR was from integrated DNA Technologies (Leuven, Belgium).

**HPLC and UPLC quantification of tRNA modified uridines**

Total RNA was extracted from tissue with Trizol and precipitated with isopropyl alcohol according to the manufacturer’s instructions. Total tRNA was isolated using Nucleobond RNA/DNA 80 columns (Macherey-Nagel, Düren, Germany) and precipitated with isopropyl alcohol. Total tRNA was digested to nucleosides using nuclease P1 (Sigma) and bacterial alkaline phosphatase (Sigma) and analyzed as described earlier (42). T6A was used as internal control to normalize mcm^5^s^2^U quantification.

**Cell culture and transfections**

NSC34 cells (Mouse Motor Neuron-Like Hybrid Cell Line, Cedarlane Laboratories, Ontario, Canada) were cultured in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Greiner), 4 μg Glutamax (Invitrogen), penicillin (100 U/ml), streptomycin (100 μg/ml) and non-essential amino acids (1%). Cells were grown at 37°C in a humidified atmosphere with 5% CO2. Cells were transiently transfected with siRNA (mouse EL3P or mouse negative control, Ambion) and/or plasmids (indicated where appropriate) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions, and collected 48 or 72 h after.

**Protein analyses**

Mouse tissue was homogenized in T-PER buffer (Thermo Fisher Scientific) supplemented with protease inhibitors (Complete, Roche) by mechanical disruption for 30 s (MagNA Lyser, Roche). NSC34 cells were lysed in the same lysis buffer. To isolate protein aggregates, lysates were sonicated (five times, duty cycle 50%, level 4) (Branson, Danbury, CT) and analyzed for protein concentration using the BSA protein assay (ThermoScientific). Samples with equal amount of protein were centrifuged at 20 000g for 30 min at 4°C. The supernatant was collected as the soluble fraction (containing ‘s soluble SOD1’) and the pellet was washed once with 1 ml of ice-cold lysis buffer, centrifuged again at 20 000g for 30 min at 4°C. The pellet (detergent-resistant or insoluble fraction, containing ‘insoluble SOD1’) was then resuspended in 5% of the original lysis volume with 2× Laemmlli buffer (Bio-Rad) and sonicated (three times, duty cycle 50%, level 3). When isolation of protein aggregates was not performed, lysates were centrifuged at 11 000g for 15 min at 4°C and the supernatants analyzed for protein concentration. For the immuno-noprecipitation assay, lysate supernatants were incubated with anti-FLAG M2 agarose beads (Sigma) overnight at 4°C. The beads were washed five times with ice-cold PBS and the proteins were eluted using the 3× FLAG peptide, according to the manufacturer’s instructions.

Samples were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and either silver stained (Pierce Silver Stain Kit, Thermo) or transferred to a polyvinylidifluoride membrane using a semi-dry blotting apparatus (TE70XP, Hoefer, San Francisco, CA). After blocking with 5% (w/v) low-fat milk in Tris-buffered saline (10 mmol/l Tris, 150 mmol/l NaCl, pH 7.6) for 1.5 h, the membranes were incubated overnight at 4°C with the primary antibody (mouse anti-FLAG antibody, #F3165, Sigma; rabbit anti-SOD1, #AD1-SOD-100, Enzo Life Sciences, Farmingdale, NY; mouse anti-GAPDH, #AM4300, Ambion), washed in Tris-buffered saline 1% Tween-20 and incubated with HRP-conjugated secondary antibody (Dako). After incubation, membranes were washed, developed with.
enhanced chemiluminescence (ECL western blotting substrate, Thermo Scientific) and scanned using a LAS4000 Biomolecular imager (GE Healthcare, Uppsala, Sweden). Images were analyzed with ImageQuant TL software (GE Healthcare).

Nerve conduction studies
Mice were anesthetized with 1% isoflurane/O2 gas inhalation and placed on a heating pad to maintain body temperature. Nerve conduction was measured using subdermal needle electrodes (Technomed Europe) and a Medelec EMG monitor (Medelec Vickers, Sidcup, UK) compatible with Synergy software (version 20.1.0.100). Compound muscle action potentials (CMAPs) were measured by supramaximal stimulation (1 pulse/s, 0.1 ms stimulus duration), the stimulating electrode was placed at the sciatic notch and the recording electrode at the level of the gastrocnemius muscle. Sensory nerve action potentials (SNAPs) were measured by supramaximal stimulation (6 pulse/s, 0.1 ms stimulus duration) at the distal tip of the tail and were measured 4 cm proximal in the tail. For SNAP recordings, multiple traces were averaged (43).

Human samples
Samples were collected after obtaining informed consent from all human subjects. All experiments on human material were approved by the Ethical Committee of University Hospital Leuven, the London–Camberwell St. Giles Research Ethics Committee and the Institutional Review Board (IRB) of Harvard University.

Statistical analysis
Statistics were performed using Graphpad prism 7.01 software (Graphpad Software, San Diego, CA) or RStudio (RStudio Team, Boston, MA). Survival and disease onset were analyzed using the log-rank test. Zebrafish aberrant branching were analyzed using logistic regression. Two-way ANOVA was used for neuron and NMJ innervation counts. Zebrafish axonal lengths, mRNA expression levels, protein expression levels and mcms2U levels were analyzed by one-way ANOVA. Significance level was defined at 0.05. For multiple comparisons, Tukey’s post-hoc correction was applied. Error bars in graphs are S.E.M. (or mean with 95% confidence interval for affected embryos) of at least 3 independent experiments.

Supplementary Material
Supplementary Material is available at HMG online.

Author Contributions
A.B.A. performed most experiments, analyzed the data and wrote the manuscript. G.J., B.S., S.H., A.J., K.S., I.T., C.E., L.S. and L.R. performed some experiments. R.N. and M.T. are laboratory technicians and performed experiments. A.C. and L.N. provided the knockout mouse. J.R. and A.A.C. provided human samples. R.L., D.C., L.V.D.B., P.V.D. and A.B. supervised some experiments. W.R. supervised and wrote the manuscript. All authors contributed to the final manuscript.

Conflict of Interest statement. None declared.

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References

