Elongator mutation in mice induces neurodegeneration and ataxia-like behavior

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Cerebellar ataxias are severe neurodegenerative disorders with an early onset and progressive and inexorable course of the disease. Here, we report a single point mutation in the gene encoding Elongator complex subunit 6 causing Purkinje neuron degeneration and an ataxia-like phenotype in the mutant wobbly mouse. This mutation destabilizes the complex and compromises its function in translation regulation, leading to protein misfolding, proteotoxic stress, and eventual neuronal death. In addition, we show that substantial microgliosis is triggered by the NLRP3 inflammasome pathway in the cerebellum and that blocking NLRP3 function in vivo significantly delays neuronal degeneration and the onset of ataxia in mutant animals. Our data provide a mechanistic insight into the pathophysiology of a cerebellar ataxia caused by an Elongator mutation, substantiating the increasing body of evidence that alterations of this complex are broadly implicated in the onset of a number of diverse neurological disorders.
Axatias are the most common neurological deficit resulting from cerebellar dysfunction\textsuperscript{1,2}. These are progressive and currently incurable disorders with gradual deterioration in signs and symptoms, most commonly due to neurodegeneration of an unknown etiology. Affected neurons are Purkinje neurons (PNs) and, rarely, granule neurons. Previous mouse models of cerebellar ataxia have provided insight into the neuropathology of the disease\textsuperscript{3}, however, the precise molecular mechanisms of neuronal loss remain largely unknown.

Using an ab initio N-ethyl-N-nitrosourea (ENU) mutagenesis screen, here we identify a yet uncharacterized Elp6 mutation, which perturbs the stability and function of the murine Elongator complex, and results in a severe ataxic phenotype, the wobbly mouse, with associated microgliosis and degeneration of cerebellar PNs. Elp6 is one of the six subunits (Elp1–6) of the highly conserved eukaryotic Elongator complex, which is organized in two subcomplexes, namely Elp123 and Elp456. It has been shown that all Elongator subunits equally contribute to the stability, integrity, and functionality of the complex in yeast\textsuperscript{4}. Involvement of the complex in various cellular processes, including transcription, cell motility, cytoskeleton organization, exocytosis and intracellular trafficking, has been highlighted by a number of reports\textsuperscript{5}. However, recent studies provide evidence that the above mentioned functions assigned to the complex are downstream effects of its master activity as a global translational regulator\textsuperscript{6,7}. In detail, Elongator-dependent modifications of uridines in the wobble position of tRNA anticodons seem to be of key importance for the fidelity and kinetics of translational elongation, which also guides and directs cotranslational folding dynamics\textsuperscript{8,9}. Over the past decade, a number of studies have showed that the Elongator complex is involved in various cellular activities that govern the development and maintenance of the nervous system\textsuperscript{10–14}. Moreover, several studies have linked the occurrence of specific mutations in Elongator subunits with the onset of various neurological disorders\textsuperscript{15–20}. Our results define a mechanism in the pathology of cerebellar ataxias whereby subtle deregulation of tRNA function caused by the Elp6 mutation, leads to protein misfolding, proteome aggregation, and consecutive neuronal death resulting in a severe manifestation of the disease.

Microgliosis following neuronal loss is a normal physiological response to injury, but when this usually transient event becomes chronic and self-propagating, it can lead to sustained neurodegeneration\textsuperscript{21,22}. Hence, microglia do not just provide neuroprotection, but can also promote neurotoxicity. Inflammases play a central role in microglia activation, being multi-protein complexes that sense various cellular and environmental stress signals\textsuperscript{23}. The NLRP3 inflammasome is expressed and functional in brain microglia\textsuperscript{24}, and associated with neurodegenerative disorders such as Alzheimer’s disease\textsuperscript{25}, Parkinson’s disease\textsuperscript{26}, multiple sclerosis\textsuperscript{27}, and prion-like diseases\textsuperscript{28} and is the only inflammasome known to be activated by misfolded proteins and their aggregates via a yet not fully defined mechanism\textsuperscript{29}. It has been suggested that the activation of the NLRP3 inflammasome occurs in response to infection or injury, and involves consequential NLRP3 oligomerization, which serves as a scaffold to nucleate an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) that further acts as a docking platform for pro-caspase-1. Ultimately, NLRP3 activation leads to activation of caspase-1, which itself promotes pro-interleukin-1β (IL-1β) processing and release of the mature cytokine IL-1β\textsuperscript{30}. Subsequently, cytokine release promotes inflammation and leads to further damage to neurons already primed for degeneration. Here, we demonstrate that blocking microglial priming by inhibiting the NLRP3 pathway can attenuate PN degeneration and the ataxic phenotype in wobbly mice.

**Results**

**Cerebellar ataxia and PN degeneration in wobbly mice.** The wobbly mouse was identified in a recessive ENU-mutagenesis screen on the basis of its wobbly gait. Ataxic symptoms commence at postnatal day (P) 60 in the form of loss of gait coordination and balance, reduced locomotor activity, and abnormal hindlimb clasping, which is commonly observed in mice with a neurodegenerative defect\textsuperscript{31} (Fig. 1a and Supplementary Fig. 1). The phenotype gradually becomes more pronounced and is most severely manifested by P100. The mutant mice also showed an impaired performance on the rotarod, balance beam and tests in the Catwalk system ( Supplementary Fig. 2). The defect was found to occur in a recessive manner, as the phenotype of heterozygous mice was indistinguishable from that of the wild-type animals (Fig. 1a and Supplementary Figs. 1 and 2). Notably, no differences were observed in the performance of male versus female wobbly animals in any of the performed tests (Supplementary Figs. 1 and 2). The mice show no alternations in overall life span, weight, and fertility.

Histological analyses at P60 revealed a specific and substantial loss of PNs in wobbly mice, as we were not able to detect any other changes in overall cerebellar morphology (Fig. 1b). Neuropathology was restricted to PNs in the cerebellum with other central nervous system structures and nonneuronal tissue being unaffected (Supplementary Fig. 3). The first histological signs of PN degeneration were already detectable at P40 and the cerebellum of mutant mice was completely depleted of PNs by P120 (Fig. 1c). Consistent with previous findings that Zebrin II can act as a neuroprotector\textsuperscript{32,33}, we found that PNs of wobbly mice that did not express this molecular marker were more susceptible to cell death than Zebrin II-immuno-positive cells (Supplementary Fig. 4a). Individual variations in the severity of the wobbly phenotype manifestation appeared due to different magnitudes rather than localization of PN loss, which was shown to be consistent across all cerebellar functional subdivisions, from anterior to posterior lobe, across vermis and hemispheres (Supplementary Fig. 4b–j).

To test the functional impact of an underlying mutation, electrophysiological recordings were obtained from PNs in wobbly mice at P21–P24, before motor symptoms were apparent. Already at this stage, PNs exhibited altered passive and active membrane properties (Fig. 1d–f and Supplementary Table 1), and an altered synaptic excitation/inhibition balance (Supplementary Fig. 5 and Supplementary Table 1). In summary, mutant PNs were less excitable showing increased resting membrane potential and action potential threshold and being able to generate significantly fewer action potentials. Parallel fiber stimulation failed to evoke excitatory synaptic currents (EPSCs) in more than 50% of PNs, while evoked synaptic inhibition was shown to be stronger.

**Elp6L126Q underlies wobbly mouse phenotype.** Whole-exome sequencing of wobbly mice identified a T/A substitution in the Elp6 gene (Fig. 2a), leading to a single amino acid L/Q substitution at position 126 in the protein (Elp6L126Q). To further define Elp6 function in vivo, we generated Elp6 knockout (KO) mice (Supplementary Fig. 6a). Consistent with previous studies that have identified developmental defects following Elp1 and Elp3 ablation\textsuperscript{34,35}, we found that a loss of Elp6 (Elp6\textsuperscript{−/−}) also results in early embryonic lethality (Supplementary Fig. 6b, c). To confirm that the Elp6L126Q allele drives the pathology observed in the wobbly mutant, we crossed Elp6\textsuperscript{+/−} to wobbly mice and screened


their progeny for main features of the phenotype. The compound heterozygous (Elp6^{L126Q/+}) animals expressed a more rapidly progressive phenotype than wobbly mice, demonstrating that Elp6L126Q likely functions as a hemimorph. Furthermore, Elp6^{L126Q/-} displayed a decreased survival rate, with 80% of animals not surviving beyond P40 (Fig. 2b). Histological and behavioral analyses of these mice demonstrated a full recapitulation of the wobbly phenotype (Fig. 2c, d). No phenotypic abnormalities were found in Elp6^{+/+} animals, indicating that one copy of wild-type Elp6 is sufficient for proper functioning of the Elongator complex.

In agreement with its here described role, in situ hybridization confirmed wide expression of the Elp456 subcomplex throughout the cerebellum (Supplementary Fig. 7). To establish whether Elp6L126Q-mediated degeneration was an intrinsic defect of PN neurons, a transgenic rescue of the wobbly phenotype was initiated by crossing wobbly mice to transgenic animals expressing the HA-tagged wild-type Elp6 driven by the PN-specific Pcp2 promoter (Pcp2-Elp6-HA; Fig. 3a). Ataxic phenotype and neurodegeneration in Pcp2-Elp6-HA; wobbly mice were fully rescued by the transgene (Fig. 3b–d), demonstrating that despite the widespread expression of the Elongator complex in the cerebellum, PN degeneration is mainly initiated cell-autonomously.

**Elp6L126Q negatively affects stability and function of the Elongator complex.** Taking advantage of the high sequence conservation of the Elongator subunits among eukaryotes, the recently determined crystal structure of the yeast Elp456 (yElp456) subcomplex, and the electron microscopy reconstruction of the fully assembled Elongator complex, we were able to assess the precise location of the mutated residue within the Elongator complex (Fig. 4a). Despite its detrimental effects, Elp6L126Q resides on the periphery of the complex, distant from known tRNA-, ATP-, SAM-, or acetyl-CoA binding sites and on the side of the Elp456 ring, which is located opposite from the enzymatically active Elp3 subunit and proposed tRNA binding and modification pocket. To understand the consequences of the Elp6L126Q mutation on the molecular level, we produced
recombinant mElp456 in bacteria and purified it to homogeneity. The three murine subunits, like yElp456, are able to form a dimeric Elp56 intermediate and a hexameric Elp456 assembly (Fig. 4b and Supplementary Fig. 8a). The introduced dimeric Elp56 intermediate and a hexameric Elp456 assembly demonstrate that the mutation in the Elp6 gene.

**Protein misfolding and aggregation in wobbly PN s.** Given that perturbation of wobble uridine modifications was shown to lead to ribosome pausing and protein misfolding and aggregation, we next performed ultrastructural analyses of degenerating neurons to screen for signs of proteotoxic stress. The analyses using transmission electron microscopy revealed extensive autophagy, apoptotic cell features, and an accumulation of electron-dense globular structures that likely represent protein inclusions. The three murine subunits, like yElp456, are able to form a dimeric Elp56 intermediate and a hexameric Elp456 assembly (Fig. 4b and Supplementary Fig. 8a). The introduced dimeric Elp56 intermediate and a hexameric Elp456 assembly demonstrate that the mutation in the Elp6 gene.

**NLRP3-mediated inflammation contributes to the wobbly pathology.** Next, we found prominent histopathological signs for substantial microgliosis (Iba1-marked glial population) (Fig. 6a–c) coupled to reactive astrogliosis (GFAP-marked glial population; Supplementary Fig. 9) in the cerebellum of wobbly mice. The appearance of these inflammatory markers is confined to the cerebellum and strictly associated with PN degeneration. Given that several studies in the past decade have demonstrated that neuroinflammation can be initiated by the inflammasome complexes in microglia activated by protein aggregates, we further checked for inflammasome activation in our mutant mice cerebella. Notably, the cerebella of the ataxic wobbly mice showed a strong upregulation of key inflammasome effectors, including cleaved caspase-1 and ASC (Fig. 6b–d). ASC was shown to be overexpressed and formed so-called specks, previously demonstrated to activate cytokine cascade.

Given the accumulation of protein aggregates in wobbly PNs and recent findings that the NLRP3 inflammasome can specifically act as a sensor for intracellular misfolded proteins, we used a potent and selective NLRP3-inhibitor MCC950 to test whether activation of the NLRP3 inflammasome contributes to the progression of neurodegeneration in wobbly mice. Treatment of wobbly mice with MCC950 significantly delayed...
the onset of ataxia (Fig. 7a) and decreased the rate of neurodegeneration (Fig. 7b), as a consequence of reduced inflammation and inflammasome activity (Fig. 7c–e). To verify these findings, we took a parallel genetic approach by crossing wobbly mice to NLRP3 null animals. Analysis of the ataxic and neurodegenerative features of the double mutant progeny and controls showed that NLRP3 deficiency in vivo mirrored the phenotype of MCC950-treated mutant mice (Fig. 8a, b), further confirming that the NLRP3-driven inflammatory response contributes to the progressive neuropathology in wobbly animals. Reduction of the neuroinflammation in NLRP3 KO; wobbly mice was shown to be even stronger than in MCC950-treated mice.
Discussion

Our data indicate a mechanistic route underlying neurodegeneration in cerebellar ataxias based on the perturbed function of the Elongator complex in the regulation of translation as a consequence of the destabilizing Elp6L126Q mutation. The mutation was identified in a mouse model for cerebellar ataxia, the wobbly mouse, which develops severe ataxic symptoms as a consequence of extensive Purkinje neurodegeneration. We characterized the ataxic wobbly phenotype in mice and found the induced neurodegeneration on the one hand to follow the pattern of neuroprotective Zebrin II expression and on the other hand to affect all cerebellar functional regions equally. We also showed that intrinsic cellular and synaptic changes of mutant PNs occur prior to clear pathologically recognizable degeneration, which is consistent with observations in other progressive neurodegenerative diseases, such as Alzheimer’s disease.

On the molecular level, Elp6L126Q destabilizes the assembly and integrity of the heterohexameric Elp456 subcomplex, which is ultimately necessary for tRNA binding and tRNA modification activity of the Elongator complex. Furthermore, we demonstrated that this mutation negatively affects the Elongator activity by detecting lower levels of tRNA modifications in cerebella of (Supplementary Fig. 10a), likely due to a relatively short half-life time of the administrated drug. Genetic ablation of caspase-1 function in wobbly mice not only reinforced the role of NLRP3 inflammatory cascade in these mutants, which is expected given that the inactivation of ncm5U, mcm5U, s2U, and m7G nucleosides in cerebellar lysates from wobbly animals in comparison to NLRP3 KO; wobbly mutants, which is expected a consequence of the destabilizing Elp6L126Q.

(A. P. K. and R. S. L. E. contributed equally to this work as first authors.)
**Fig. 5** Protein misfolding and ER-stress-induced apoptosis in wobbly PNs. a Electron micrographs and quantification of autophagic vacuoles (AV) in wobbly PNs at P40. Arrows indicate electron-dense protein aggregates and arrowheads point to protein aggregates in autophagic bodies, both are magnified in the lower panel (n = 3 animals per genotype; n = 5 cells per animal; representative images are shown). Statistical evaluation: two-tailed t test. Statistically significant difference is indicated (**P ≤ 0.001). Data represent mean ± SEM. b Immunofluorescence with antibodies to Pcp2, Hsp70, ubiquitin (Ub), caspase-3 (Casp-3), and CHOP on P40 wild-type and wobbly cerebella (n = 5 for each of the genotypes; representative images are shown). Scale bars: (a) 2 μm; (b) 50 μm.

**Fig. 6** Microgliosis and inflammasome activation in wobbly mice cerebella. a Immunofluorescence of wild-type and wobbly cerebella with Pcp2 and Iba1 antibodies. b Iba1 and ASC immuno-labeling of wobbly and control mice cerebellar sections. Arrows indicate ASC specks in microglia. White rectangles represent magnified areas. c Quantification of microgliosis in (a) and ASC specks in (b). d Western blot and quantification of cleaved caspase-1 (Casp-1) and ASC expression in P120 wobbly cerebellar brain lysates relative to control. For all experiments n = 5 for each of the genotypes; representative images and blots are shown. Scale bars: (a) 50 μm; (b) 10 μm. Statistical evaluation: (c) two-way ANOVA and Sidak’s multiple comparisons test; (d) two-tailed t test. Statistically significant differences are indicated (**P ≤ 0.01; ****P ≤ 0.0001). Data represent mean ± SEM.
mutant mice. Although reduction of tRNA modification levels is relatively modest, our data is in line with another report on the Elongator mutation that causes familial dysautonomia 15. In this rare disease, similar levels of reduction in tRNA modifications were observed (29–36% of reduction) in patient-derived samples. Together these observations indicate a scenario, where a certain level of reduction in modification levels causes severe cellular malfunctions, but still permits survival of the patients.

The Elongator-dependent mc35U and mc55U modifications have been shown to be of crucial importance for the fidelity and kinetics of protein synthesis and cotranslational folding dynamics 8,9. In concordance, we found defects in translational fidelity and protein folding in wobbly PNs. Hence, we show that the Elp6p126Q-mediated impaired function of the complex likely results in protein misfolding and aggregation that further induces ER-stress and subsequent apoptosis.

Protein aggregation is a common cause of neuronal death shared by various neurological disorders 34,35, as aggregated proteins commonly lose their physiological function and gain undesired toxic properties. As in vast majority of neurodegenerative diseases, cellular pathology is only observed in a specific neuronal subtype in wobbly mice, namely PNs. Our transgenic complementation study demonstrates that Elongator is a key regulator of PN integrity and although the observed pathological findings were found to be cell-intrinsic in ataxic mice, the cause of this selectivity remains elusive. In general, neurons are known to be highly sensitive to the presence of misfolded proteins given that they are postmitotic and cannot dilute toxic aggregates by cell division. PNs may be particularly sensitive to deleterious effects of these toxic species as they have an extraordinary high metabolic demand 11,16. Although codon-dependent regulation of translation by the Elongator complex has been previously reported 6, protein aggregates induced in yeast and worms by Elongator depletion show no specific accumulation of these Elongator-codon enriched proteins. Therefore, the slightly decreased tRNA modification levels primarily might have a large impact on the proper translation of individual trigger proteins, which nucleate and propagate the appearance of large aggregates and induce proteotoxic stress in the context of whole proteome, as suggested by previous studies 6. The study presented here adds to an emerging consensus that perturbations of the Elongator complex contribute to a range of neurological and neurodevelopmental disorders, including familial dysautonomia 15, amyotrophic lateral sclerosis 17,20, rolandic epilepsy 18, and intellectual disability 16,19. The mechanism by which specific mutations in different Elongator subunits cause different neuropathologies, remains intriguing and needs to be further clarified.

Fig. 7 MCC950 treatment delays the onset of the wobbly phenotype. a Analysis of the ataxic phenotype of wobbly and MCC950-treated wobbly and wild-type mice relative to wild-type controls (n = 6 (3 males and 3 females) for each of the genotypes). b Pcp2 and Iba1 immuno-staining and PN quantification in P120 wobbly mice with and without MCC950 treatment. White rectangles represent magnified areas. c Iba1 and ASC immunofluorescence on sagittal cerebellar sections of P120 MCC950-treated and untreated wobbly mice. d Quantification of microgliosis and ASC specks in (c). e Western blot analysis and densitometry of cleaved caspase-1 (Casp-1) and ASC in P120 MCC950-treated wobbly cerebellar brain lysates relative to the untreated wobbly controls. For (b–e) n = 5 for each of the genotypes; representative images and blots are shown. Scale bars: (b) left panel 500 μm; (b) middle and right panels, 100 μm; (c) 10 μm. Statistical evaluation: (a) two-way ANOVA and Sidak’s multiple comparisons test; (b, d, e) two-tailed t-test. Statistically significant differences are indicated ( * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001). Data represent mean ± SEM.
A number of studies in the past decade have demonstrated that neuroinflammation initiated by the activation of inflammasome complexes in microglia underlies a variety of neuronal phenotypes. Here, we have demonstrated that despite the fundamental mechanism of neuronal death being cell autonomous, activation of the NLRP3 inflammasome contributes significantly to the rate of neuronal loss in wobbly mice. Thus, PNs are both dying due to an intrinsic defect and being killed by an extrinsic inflammatory response. Gliosis is a common observation in a number of neurodegenerative conditions and our findings in ataxic mice experimentally confirm an increasing body of evidence proposing a common mechanism of NLRP3-mediated neuroinflammation to underlie and contribute to the pathogenesis of various neurodegenerative diseases. Whether NLRP3 activation is a direct consequence of the Elongator complex malfunction in wobbly mice or it is triggered by a release of danger-associated molecular patterns (DAMPs) from dying neurons, has to be investigated in the future. It has been previously established that NLRP3 activation is initiated by aggregated proteins, such as Lewy bodies in Parkinson’s disease, amyloid-β peptides in Alzheimer’s disease and prions in prion-related disorders. Thus, the NLRP3-mediated inflammatory cascade is likely to be triggered by the presence of protein aggregates in wobbly PNs. Previous studies demonstrated that the NLRP3 activation not only results in cytokine-induced neuronal damage, but also in poor microglial aggregate clearance resulting in accumulation of toxic aggregates that leads to further neuronal demise. In order to assess whether aggregates are responsible for the NLRP3-mediated neuroinflammation in cerebellar ataxia or the inflammatory response is induced by neuron-derived DAMPs, different ataxia mouse models with and without the involvement of proteinopathy need to be compared.

For cerebellar ataxia patients, prognosis is currently bleak and therapies in use are limited to symptomatic treatment with no pharmaceutical intervention available to ameliorate the disease pathology. Data presented here not only define a fundamental...
understanding of cerebellar neurodegeneration caused by the Elongator mutation, but also identify a potential therapeutic strategy to substantially delay the course of the disease. Hereditary ataxias display a variety of inheritance modes and the plethora of genetic pathways known to cause these conditions makes it difficult to conceive of a single pan-ataxia therapeutic approach. Though, several studies suggest that the majority of ataxias have some broad commonalities, such as protein aggregation, metabolic deficits, and perturbation in ion channel function[25]. Despite the potentially different biochemical bases that trigger neurodegeneration, a common feature of ataxias is the accompanying induction of neuroinflammation, and in particular microglial activation[20–22]. It is clear that the role of sterile inflammation in neurological diseases is a field that is generating great interest and our study provides the first instance that blocking inflammasome activation can significantly ameliorate neurodegeneration in ataxia by targeting the associated activated gliosis.

**Methods**

**Animals and genotyping strategies.** All animal experiments were approved by the University of Queensland and Molecular Biosciences Animal Ethics Committees (protocol license numbers IMR/098/14 and IMR/097/14). Mice were housed under a 12 h light cycle in a specific-pathogen-free climate-controlled facility with food and water provided ad libitum. Genomic DNA for genotyping purposes was obtained from tail tips using QuickExtract DNA extraction solution (Epicenter) as per the manufacturer's instructions. The genetic background of all animals (pro-nuclear injected) was wobbly, Elp6 KO, transgenic (Pcp2-Elp6-HA), NLPR3 KO and Casp-1 KO mice, was C57BL/6. No gender-related phenotypic differences were observed in all mice strains. Both sexes were found to be fertile with normal life span and body weight. The wobbly mutation arose from an ENU-mutagenesis phenotype-driven screen at the Australian Phenomic Facility, the Australian National University. This strain has been archived with the Australian Phenome Bank, ID 4118. Whole-exome sequencing revealed the homozygous Elp6L126Q mutation. A custom TaqMan SNP genotyping assay was used to genotype the animals through qPCR reaction based on allelic discrimination. Primers used were: forward 5′-AACCTGCGACACCCACGCTGCTC-3′, reverse 5′-GAGACACCCAGAAGCC-3′. The amplified product of 920 bp was further annealed with either VIC-labeled wild-type Elp6 sequence 5′-AGGACACCTTGAAAGCC-3′ or fluoroscein-labeled wobbly (Elp6L126Q) sequence 5′-AGGACACCCAGAAGCC-3′. For MCC950 treatment studies, wobbly mice were dosed orally via drinking water (0.3 mg/ml) from P21 until sacrificed at P120. The MCC950 dose to be used was established in a pilot study where we assessed the penetrance of the drug into the brain tissue of mice at levels above the IC50 of the drug (n = 3 for MCC950-treated and control animals). The concentration of the drug was measured in blood plasma and brain tissue upon transcardial perfusion with PBS.

**Tissue and embryo collection.** Experimental animals were anaesthetized using Dormitor (1 mg/kg, i.p.) and Zoletil (50 mg/kg, i.p.) and transcardial perfusion was performed with PBS, followed by 4% PFA solution. The brains were dissected and drop-fixed in 4% PFA at 4 °C for 12 h under constant agitation. The following day, brains were washed twice with PBS and left overnight in PBS. Brains were processed in the Leica TP1020 tissue processor over 15 h as per the user’s guide and subsequently embedded in paraffin and sectioned at 7 μm either in the transverse or sagittal plain using Leica RM2235 microtome. Sections were transferred to glass slides and dried overnight at 45 °C. Embryos were explanted and placed into cold PBS, followed by 4% PFA fixation for 6 h at 4 °C and subsequent series of PBS washing.

**H&E staining.** Following deparaffinization, slides were stained in Hematoxylin (Sigma Aldrich) for 3 min. The excess of Hematoxylin stain was removed by short immersion of slides in 1% HCl acid solution followed by another short immersion in 0.1% LiCO3 solution. Samples were then stained with Eosin Y solution (Sigma Aldrich) for 30 s and dehydrated using 70, 90, and 100% ethanol for 30 s each, followed by xylene for 10 min. Slides were mounted with Entellan mounting medium (ProSciTech) and dried for 1 h. Images were obtained using Olympus BX-51 upright bright-field microscope.

**Immunofluorescence.** Upon deparaffinization and hydration, the slides underwent heat-induced antigen retrieval using citrate buffer-based antigen unmasking solution (Abacus) at 100 °C for 10 min. Mouse on Mouse (M.O.M.) blocking reagents (Vector Laboratories) were used. Vimentin (1:200) (Abcam DO-1) and counterstained with DAPI (Sigma Aldrich). Images were captured using the Leica TCS SP8 confocal microscope and ImageJ. For immunofluorescence images, fields were imaged as single confocal planes using high magnification 63× oil immersion objectives. The confocal images were obtained from the region of interest, excluding background and including the entire tissue section (when a primary antibody was raised in mouse) or bovine serum albumin used to block unspecific binding of antibodies (when an antibody was not in other specie than mouse). Slides were incubated with primary antibodies: Pcp2 (1:100; sc-49072), Zeb2n (1:50; ab115212), Iba1 (1:400; ab5076), ASC (1:400; Ab177), Casp-3 (1:300; ab23002), Hsp70 (1:100; sc-6048), Ub (1:100; ab7780) and HA (1:100; ab9110), followed by incubation with an AF488, AF594, or AF647-labeled donkey anti-mouse, anti-rabbit or goat IgG antibody (1:250; Invitrogen) and counterstained with DAPI (Sigma Aldrich). Images were captured using Zeiss LSM 710 upright confocal microscope as Z-stacks and presented as the sum of the Z-projection. The number of Pcp2-labeled or Iba1-labeled cells were determined separately in every visible lobule of the vermis and in the hemispheres. For each mouse, three nonadjacent sections (separated by 70 μm) from the region of
vermis were analyzed and the mean value was recorded. Total of five mutant and five control animals were included in each of the studies.

**Electron microscopy.** Excised cerebellum were quickly trimmed and immediately immersed in 2.5% glutaraldehyde in cacodylate buffer for 4 h and then postfixed in reduced osmium, en-bloc stained with 2% uranyl acetate and dehydrated through ethanol solutions, before final embedding in Epon812 resin (ProSciTech). Ultra-thin sections were cut on a Leica UC6 Ultra microtome and viewed on a JEOL JEM-1011 electron microscope (JEOL Australasia Pty Ltd) at 80 kV. Images were captured using iTEM software (Soft Imaging System, Olympus).

**In situ hybridization.** Total RNA was extracted from granule neuron precursors isolated from P7 wild-type animals using the RNeasy mini kit (QIAGEN). In situ hybridization on P21 sagittal brain sections were analyzed and the mean value was recorded. Total of 33 modiﬁed ribonucleosides of which 5 compounds of interest were targeted for peak assignment, area calculation and normalization. Corresponding structures and molecular masses were obtained from Modomics database.

**Western blotting.** Mouse cerebellar whole tissue lysate was prepared by homogenization in radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were determined using BCA protein assay (Pierce). The XCell SureLock mini-cell electrophoresis system (Thermo Fisher Scientiﬁc) was used for SDS-PAGE and wet protein transfer. Proteins were transferred onto nitrocellulose membranes at 25 V for 90 min and detected by immunoblotting using relevant primary and horseradish peroxidase-conjugated secondary antibodies. Peroxidase activity was further detected using SuperSignal West Pico chemiluminescent reagent (Thermo Fisher Scientiﬁc). The membrane was exposed to an X-ray ﬁlm for 30 s–2 min prior to development using X-omat ﬁlm developer. Densimetric analysis of western blot images was performed using ImageJ software.

**Electrophysiology.** Whole-cell recordings were obtained from PNs in 300 μm-thick sagittal brain slices from P21–26 wobbly and wild-type mice. Mice were anesthetized with isoflurane, decapitated, and 300-μm-thick sagittal brain slices prepared in an ice-cold sucrose solution using a vibratome (Leica). Brain slices were continuously perfused with oxygenated aCSF (32 °C) and whole-cell patch-clamp recordings were performed as previously described. Spiking was evoked using current injections applied in increments of 20 pA from −60 to 340 pA. In the case of spontaneous excitatory postsynaptic current (sEPSC) measurements, peak amplitudes and half-widths were measured and compared with the previously published results. Microscale thermophoresis (MST) experiments were performed to determine binding affinities of Cy3-labeled trNA′s and trNA′s from *S. cerevisiae* (at concentration 210 and 55 nM) and purified Elp456 complexes. Proteins were titrated in a 1:1 dilution series in 20 mM HEPES pH 7.5, 50 mM NaCl, 2 mM DTT, 0.05% Tween, whereas labeled RNA concentrations varied from 20 to 95 °C (LED/excitation power setting 10%, temperature slope 2 °C/min). The fluorescence intensity was measured at probe speciﬁc excitation (470 nm) and emission (570 nm) wavelengths.

**Microscale thermophoresis.** Microscale thermophoresis (MST) experiments were performed to determine binding affinities of Cy5-labeled trNA′s and trNA′s from *S. cerevisiae* (at concentration 210 and 55 nM) and purified Elp456 complexes. Proteins were titrated in a 1:1 dilution series in 20 mM HEPES pH 7.5, 50 mM NaCl, 2 mM DTT, 0.05% Tween, whereas labeled RNA concentrations varied from 20 to 95 °C (LED/excitation power setting 10%, temperature slope 2 °C/min). The fluorescence intensity was measured at probe specific excitation (470 nm) and emission (570 nm) wavelengths.

**RNA modification analyses.** Total RNA was isolated from ~100 μg cerebellar tissue using TRIzol reagent (Life Technologies). After TRIzol addition, the tissue was homogenized with ceramic beads (Sapphire Bioscience) in tissue homogenizer (Progen) following the protocol for β-agarase I provided by manufacturer (NEB). After completion of gel digestion reaction, RNA was extracted with water-saturated phenol followed by repetitive chloroform extraction and precipitation by ethanol; 8–10 ng of RNA was recovered for each sample. RNA purity and quality was conﬁrmed using above mentioned PCR conditions. Primers were designed in the 3′UTR of the Elp4-6 cDNA: Elp4 forward 5′-CGACTCGATTTGCTTCAAGCAGTGTCCAGAC3′ and reverse 5′-GTTCTACACTCTATGGGGTG TGCATGCC-3′, Elp5 forward 5′-GCTATGCGCCAGGCTCTAGG-3′ and reverse 5′-CACA CATTCTCAGGTCGTGCATTGCCTGTCCTGG-3′, Elp6 forward 5′-GCTTCACCGGCTTGTTTTTGT-3′ and reverse 5′-GCTCCAGTGCCATGCTTTTG-3′. DIG RNA labeling kit (Roche) was used to synthesize the RNA probes and the probes were puriﬁed using the RNA cleanup protocol from the RNeasy mini kit (QIAGEN). In situ hybridization on P21 sagittal brain sections was carried out following the established protocol.

**Protein expression and puriﬁcation.** Coexpression constructs encoding mouse Elongator subunits were designed as previously described for Elp456. Constructs encoding truncations and mutations of mElp4, mElp5, and full-length mElp6 were created using standard quick-change protocol. mElp6 and mElp6L126Q were cloned into pETM11 using standard cloning procedures. All constructs were expressed in E. coli (BL21 pRARE) after transformation using electroporation. In detail, bacteria were grown in TB at 37 °C until an OD600 of ~1.2, followed by induction with 1 mM IPTG and subsequent incubation at 18 °C for 12–15 h. Bacteria were lysed in 50 mM HEPES (pH 7.5), 300 Mm NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 5% (v/v) glycerol, DNAse and protease inhibitors using a homogenizer. The soluble fractions were cleared by centrifugation (70,000×g for 45 min at 4 °C), and proteins were further puriﬁed using Ni-affinity chromatography, followed by size-exclusion chromatography on a 16/600 HLoad Superdex 200 pg column (GE Healthcare) and/or Superdex 200 Increased (10/300) in 20 mM HEPES (pH 7.5), 150 mM NaCl and 5 mM DTT. Respective fractions were analyzed by SDS-PAGE, pooled and concentrated.

**Thermal shift assay.** Thermal shift assays were performed to monitor protein unfolding using thermostability technology. Thermostability assays were conducted in the CFX96 Real-Time System C1000 Touch Thermal Cycler (Bio-rad). Protein concentrations of protein samples (1–0.25 mg/ml) were incubated with SYPRO Orange and 20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM DTT buffer. Samples were gradually heated from 4 to 98 °C at a heating rate of 0.2 °C/10 s. The fluorescence intensity was measured at probe speciﬁc excitation (470 nm) and emission (570 nm) wavelengths.

**Nanoflower scanning fluorimetry.** Nanoflower scanning fluorimetry (NFSF) experiments were performed to determine protein stability employing intrinsic tryptophan or tyrosine ﬂuorescence of puriﬁed Elp456 complexes. Proteins were diluted to concentration 100 μg/ml in 20 mM HEPES pH 7.5, 50 mM NaCl, 2 mM DTT, 0.05% Tween, whereas labeled RNA concentrations stayed constant. Samples were loaded into Monolith NT.115 HT Premium Coated Capillaries (NanoTemper Technologies) and measured using a Monolith NT.115 at room temperature (light-emitting diode (LED)/excitation power setting 20%, MST power setting 20%). Data was analyzed using MO. Affinity analysis software at the standard MST on time of 5 s.

**Data analysis and statistics.** Statistical analyses were performed using the GraphPad Prism software V6. To determine statistical signiﬁcance, the unpaired two-tailed t test was performed. For the simple composite phenotype scoring system, scores for cerebellar ataxia and for the quantitation of ASC specks were analyzed. For each of the different dependent variable in different age groups, Sidak’s test was used.
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Additional information

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