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1 **Report**

2 **A homozygous activating *ATAD1* mutation impairs postsynaptic AMPA receptor trafficking**
3 **and causes a lethal encephalopathy with congenital stiffness**

4 Running title: *ATAD1* and lethal encephalopathy

5

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54

55 **Abstract**

56 Members of the AAA+ superfamily of ATPases are involved in the unfolding of proteins and
57 disassembly of protein complexes and aggregates. *ATAD1* encoding the ATPase family, AAA+
58 domain containing 1-protein Thorase plays an important role in the function and integrity of
59 mitochondria and peroxisomes. Postsynaptically, Thorase controls the internalization of
60 excitatory, glutamatergic AMPA receptors (AMPA) by disassembling complexes between the
61 AMPAR-binding protein, GRIP1, and the AMPAR subunit GluA2. Using whole-exome
62 sequencing, we identified a homozygous frameshift mutation in the last exon of *ATAD1*
63 [c.1070_1071delAT; p.(His357Argfs*15)] in three siblings who presented with a severe, lethal
64 encephalopathy associated with stiffness and arthrogyriposis. Biochemical and cellular
65 analyses show that the C-terminal end of Thorase mutant gained a novel function which
66 strongly impacts its oligomeric state, reduces stability or expression of a set of Golgi,
67 peroxisomal and mitochondrial proteins and affects disassembly of GluA2 and Thorase
68 oligomer complexes. *Atad1*^{-/-} neurons expressing Thorase mutant^{His357Argfs*15} display reduced
69 amount of GluA2 at the cell surface suggesting that the Thorase mutant may inhibit the
70 recycling back and/or reinsertion of AMPARs to the plasma membrane. Taken together, our
71 molecular and functional analyses identify an activating *ATAD1* mutation as a new cause of
72 severe encephalopathy and congenital stiffness.

73 Key words: *ATAD1*, encephalopathy, AMPA receptor trafficking

74

75 **Introduction**

76 Early-infantile onset encephalopathies come with an urgent need for a proper diagnosis as
77 immediate therapeutic decisions have to be made. The majority of these disorders has a
78 genetic etiology and follows a Mendelian inheritance pattern. Thus, whole-exome sequencing
79 (WES) is the method of choice to elucidate the related gene in extremely rare forms of early-
80 onset encephalopathies that can lead to early death (Cartault *et al.*, 2012, Kevelam *et al.*,
81 2013).

82 The AAA+ family is a large enzymatic group of ATPases associated with various cellular
83 activities that induce conformational changes in a wide range of substrate proteins. These
84 ATPases have been involved in various human diseases such as peroxisome biogenesis
85 disorders, early-onset torsion dystonia linked to *DYT1*, *SPG4*- and *SPG7*-related hereditary
86 spastic paraplegia and a specific form of inclusion-body myopathy (Hanson and Whiteheart,
87 2005). One of them, the AAA+ ATPase Thorase encoded by *ATAD1* plays a critical role in
88 regulating the surface-expression of AMPARs (alpha-amino-3-hydroxy-5-methylisoxazole-4-
89 proprionate receptors), thus regulating synaptic plasticity, learning and memory (Ahrens-
90 Nicklas *et al.*, 2017). Here, we report the genotype-phenotype relationship in three infants
91 exhibiting severe lethal encephalopathy with neonatal stiffness and arthrogryposis resulting
92 from a homozygous activating *ATAD1* mutation.

93

94 **Material and Methods**

95 **Exome sequencing and sequence data analysis**

96 Written informed consent was received from participants prior to inclusion in the study.
97 Targeted enrichment and massively parallel sequencing were performed on genomic DNA.

98 Enrichment of the whole exome was performed according to the manufacturer's protocols
99 using the Nextera Enrichment Kit (62 Mb) (Illumina) for patients 1 and 2 and their mother
100 (Kortüm *et al.*, 2015). Captured libraries were then loaded onto the 2500 platform (Illumina).
101 Trimmomatic was employed to remove adapters, low quality (phred quality score < 5) bases
102 from the 3' ends of sequence reads (Bolger *et al.*, 2014). Reads shorter than 36 bp were
103 subsequently removed. Further processing was performed following the Genome Analysis
104 Toolkit's (GATK) best practice recommendations. Briefly, trimmed reads were aligned to the
105 human reference genome (UCSC GRCh37/hg19) using the Burrows-Wheeler Aligner (BWA
106 mem v0.7.12). Duplicate reads were marked with Picard tools (v1.141). GATK (v3.4) was
107 employed for indel realignment, base quality score recalibration, calling variants using the
108 HaplotypeCaller, joint genotyping, and variant quality score recalibration. AnnoVar (v2015-03-
109 22) was used to functionally annotate and filter alterations against public databases
110 (dbSNP138, 1000 Genomes Project, and ExAC Browser). Exonic variants and intronic
111 alterations at exon-intron boundaries ranging from -10 to +10, which were clinically associated
112 and with allele frequencies <0.5% without homozygous carriers in public databases, were
113 retained.

114

115 **Variant validation**

116 Sequence validation and segregation analysis for all candidate variants were performed by
117 Sanger-sequencing. Primer pairs designed to amplify selected coding exons and exon-intron
118 boundaries of candidate genes and PCR conditions are available on request. Amplicons were
119 directly sequenced using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems) and
120 an automated capillary sequencer (ABI 3500; Applied Biosystems). Sequence

121 electropherograms were analyzed using the Sequence Pilot SeqPatient software (JSI medical
122 systems).

123

124 **RNA isolation, cDNA synthesis and sequencing**

125 Total RNA was extracted (RNeasy Mini kit, Qiagen) from cultured primary fibroblasts obtained
126 from patient 1 and a control individual. 1 µg total RNA was reverse transcribed (Superscript™
127 III RT, ThermoFisher) using random hexamers as primers, and 1 µl of the reverse transcription
128 reaction was utilized to amplify a 669-bp *ATAD1* cDNA fragment encompassing the
129 c.1070_1701delAT variant (forward primer 5'-ATGATGAAAGCTCAGTTTATGAGTC-3', reverse
130 primer 5'-GGAACAGTTGAATCCAGCCT-3'). The PCR product was directly sequenced.

131

132 **Antibodies and plasmids**

133 All antibodies were acquired commercially: Thorase (mAb, Neuromab, RRID: AB_2564836),
134 GluA2-N/C (mAb, Millipore-Chemicon, RRID: AB_2113875 and RRID: AB_2247874) and GRIP1
135 (pAb, Millipore-Chemicon, RRID: AB_11210079), Tomm20, Cox 4, Hexokinase 1, GOS28, PEX26
136 and VDAC1 (Cell Signaling Tech.), actin-HRP were purchased from GE healthcare (Amersham).
137 N-Methyl-D-aspartate (NMDA) was purchased from Sigma-Aldrich. Plasmids are described in
138 "Recombinant protein expression and ATPase activity assays".

139

140 **Protein expression and measurement of oxygen consumption rate in patient cells**

141 Patient-derived and control fibroblast cells were maintained in Dulbecco's Modified Eagle
142 Medium (DMEM, Corning Cellgro) plus 10% (v/v) bovine serum (FBS) and 1% (v/v) Penicillin-
143 Streptomycin (Corning Cellgro), at 37°C with a 5% CO₂ atmosphere in a humidified incubator.

144 Thorase expression and comparative immunoblot analyses were performed using cell lysates
145 from patient and control fibroblasts. Protein concentrations were determined by BCA protein
146 assay (Pierce™, Thermo Scientific) and 20 µg were resolved on SDS-PAGE. The immunoblot
147 membranes were stained with Ponceau stain to assess loading of proteins. Immunoblot
148 analyses were performed using antibodies to COX 4, Hexokinase 1 (HXK1), PEX26, VDAC1 and
149 actin (GE Healthcare) as control for loading. Band intensities were quantified using ImageJ
150 (NIH) and normalized to actin. The values obtained from ImageJ were further analyzed to
151 determine significant differences using GraphPad Prism software (GraphPad).

152 The patient-derived and control fibroblast cells cultured on glass coverslips were fixed by
153 replacing the media with PBS containing 4% PFA and incubated for 10 mins. The cells were
154 washed three times with PBS and then were permeabilized with 0.5% Triton X-100 in blocking
155 buffer (5% goat serum in PBS) for 30 mins. The blocking buffer was replaced with PBS
156 containing Tomm20, GOS28 or PEX26 antibodies (1:1000 dilution) at 4°C overnight. Cells were
157 washed three times with PBS, and incubated with fluorescent secondary antibodies (Alexa
158 Fluor 488, Life Technologies) for 2 hr. Coverslips were washed twice follow by 5 mins
159 incubation with PBS containing DAPI to stain the nuclei and then washed three times with PBS.
160 The coverslips were mounted on glass slides with Immu-mount (Thermo Scientific) and
161 imaged using Zeiss LSM Confocal microscope.

162 Mitochondrial oxygen consumption rate (OCR) was assessed in patient-derived and control
163 fibroblast cells in an XF24 Extracellular Flux Analyzer (Seahorse Bioscience), as described
164 previously (Chen *et al.*, 2014). Fibroblast cells (~0.5 X 10⁶ per well) culture media was replaced
165 with XF24 Dulbecco's Modified Eagle Medium (DMEM) containing 10 mM glucose, 2 mM L-
166 glutamine (Life Technologies) and 2 mM sodium pyruvate (Life Technologies). OCR was

167 measured at 37°C with 1-min mix, 1-min wait, and 5-min measurement. The OCR was then
168 analyzed in the presence of oligomycin, carbonilcyanide m-cholorophenylhydrazone (CCCP)
169 and rotenone after 30 mins incubation in a CO₂ free incubator to assess coupling of respiratory
170 chain and mitochondrial respiratory capacity. The OCRs were normalized relative to cell
171 number and basal respiration in each well and is presented as % change. The significant
172 differences in values obtained were analyzed using GraphPad Prism software (GraphPad).

173

174 **3D structure modeling**

175 The C-terminus of Thorase wildtype (Gln259-Asp361) and mutant^{His357Argfs*15} (Gln259-Gln370)
176 was modeled using the SWISS-MODEL (Arnold *et al.*, 2006) and confirmed by Phyre2 (Kelley
177 *et al.*, 2015) protein modeling, prediction and analysis software. All images obtained were
178 viewed and labeled with pdb viewer, Pymol. The 3D models are shown as obtained from the
179 SWISS-MODEL software without any modification.

180

181 **Recombinant protein expression and ATPase activity assays**

182 GST-Thorase was generated by cloning the coding sequence of *ATAD1* wildtype or
183 mutant^{His357Argfs*15} into pGEX6P1 (GE Healthcare) between *Bam*HI and *Xho*I. Thorase wildtype
184 or mutant^{His357Argfs*15} coding sequence were also cloned into FUGW (Adgene) between *Age*I
185 and *Bam*HI to generate GFP-tagged Thorase. GST-tagged fusion proteins were expressed in
186 *Escherichia coli* strain BL21-CodonPlus (DE3)-RIPL (Stratagene) and purified by using GSTrap
187 (GE Healthcare), respectively following the manufacturer's instructions. To obtain non-tagged
188 Thorase proteins, GST-Thorase bound to beads were treated with PreScission protease (GE
189 Healthcare) to cleave Thorase from GST. The eluted Thorase was further purified by size

190 exclusion chromatography using Superdex 200 10/300GL column (GE Healthcare). The purity
191 of the recombinant proteins was assessed by SDS-PAGE followed by Coomassie blue staining.
192 Immunoblotting was used to confirm the presence of proteins in the purified samples.
193 The ATPase activities of Thorase wildtype and mutant^{His357Argfs*15} were assessed by measuring
194 ATP hydrolysis and [α -³²P]-ATP binding. ATP hydrolysis measurements were carried out using
195 an ADP colorimetric assay kit (BioVision) according to the instructions from the manufacturer.
196 Approximately, 1.0 mg of purified non-tagged Thorase recombinant proteins were incubated
197 with 20, 40, 60, 80, 100 and 120 μ M ATP in 0.5 ml of ADP assay buffer (supplemented with 2
198 mM MgCl₂) at 37°C for 30 min. The amount of ADP formed due to ATP hydrolysis was then
199 determined to assess the ATPase activity of Thorase mutant^{His357Argfs*15} compared to wildtype.
200 The ATP binding was evaluated by a photo-labeling technique as described by Babst et al.
201 (Babst *et al.*, 1998). Approximately 2.0 mg of purified non-tagged Thorase proteins in 0.1 ml
202 nucleotide binding buffer (50mM Tris.Cl pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 5% glycerol)
203 containing 0.1 mM [α -³²P]ATP were incubated at 4°C for 1 hr. The mixtures were exposed to
204 UV light to cross-link the bound [α -³²P]ATP to Thorase, and SDS-PAGE sample buffer was
205 added to stop the reaction. The samples were resolved on SDS-PAGE, and then exposed to a
206 phosphor screen (Perkin Elmer). A scanning densitometer was used to quantify the amount of
207 ³²P protein labeling in the samples.

208

209 **GST-Thorase pull-down of GluA2-GRIP1 complex**

210 For the pull-down assay, purified GST-tagged Thorase proteins immobilized on glutathione
211 Sepharose beads were incubated with 1 mg of *Atad1* knockout whole brain lysates
212 (homogenized in nucleotide binding buffer with 1% Triton X-100) in the presence of 2 mM

213 ADP, ATP or non-hydrolyzable ATP γ S for two hrs at 4°C followed by 30 mins at 37°C. The beads
214 were thoroughly washed four times and the bound proteins were eluted in 1X SDS-PAGE
215 sample loading buffer followed by immunoblot analysis with mouse anti-Thorase, anti-GluA2
216 and anti-GRIP1 antibodies.

217

218 **Thorase oligomer assembling and disassembling assays**

219 Thorase mutant^{His357Argfs*15} oligomer formation was evaluated by chemical cross-linking using
220 glutaraldehyde as previously described (Babst *et al.*, 1998). Approximately 1.0 mg purified
221 Thorase proteins in nucleotide binding buffer with 2 mM ATP or non-hydrolyzable ATP γ S were
222 incubated at 4°C for 2 hrs (to allow oligomer assembling) followed by incubation at 37°C for
223 30 mins (to allow oligomer disassembling). The cross-linking reaction was stopped by addition
224 of glycine to a final concentration of 10 mM and samples were mixed with SDS-PAGE sample
225 loading buffer. The samples were resolved on 4-20% gradient SDS-PAGE (Invitrogen) and
226 immunoblotted with anti-Thorase antibody to evaluate the presence of Thorase oligomer
227 complex.

228

229 **Surface expression and NMDA-induced endocytosis of GluA2 assays**

230 The effects of the *ATAD1* mutation p.(His357Argfs*15) on GluA2 (encoded by *GRIA2*) surface
231 expression were examined in primary cortical neuron cultures prepared from embryonic day
232 15 embryos obtained from *Atad1* (+/-) x (+/-) breeding as previously described (Zhang *et al.*,
233 2011, Prendergast *et al.*, 2014). The neurons were infected with Thorase-GFP viruses 12 days
234 after plating. To estimate surface GluA2 surface expression in non-treated (control) and
235 NMDA-induced endocytosis, live neurons were incubated with 2 mg of mouse-monoclonal

236 anti-N-terminal GluA2 antibodies at 37°C for 1 hr in neuronal growth media. The neurons were
237 washed twice with fresh growth medium and then replaced with media containing Tyrodes'
238 buffer (119 mM NaCl, 5 mM KCl, 25 mM HEPES, 2 mM CaCl₂, 2 mM MgCl₂, 1 μM TTX + 100 μM
239 LY34195) with 20 μM CNQX followed by treatment with or without 100 μM NMDA for 5 min
240 at 37°C in Tyrodes' buffer containing 300 μM MgCl₂ and 10 μM glycine. The media was
241 replaced with 4% paraformaldehyde and 4% sucrose in PBS for 15 mins to fix the neurons. The
242 neurons were washed three times with PBS followed by treatment with 5% goat serum, 0.3%
243 Triton X-100 in PBS for 1 hr at 4°C and overnight incubation with rabbit-monoclonal anti-C-
244 terminal GluA2 antibody. The neurons were incubated with mouse-Alexa 555-conjugated and
245 rabbit-Alexa 350-conjugated secondary antibodies (Invitrogen) for 2 hrs after three washes
246 with PBS followed by three washes with PBS before imaging. Images were acquired by using a
247 Zeiss LSM 710 laser-scanning confocal microscope. The fluorescence intensities were
248 measured and the internalization index was calculated by intracellular fluorescence divided
249 by total fluorescence normalized to untreated neurons.

250 AMPAR surface expression was also evaluated using surface protein-crosslinking assay.
251 Primary cortical neuron cultures infected with Thorase-GFP viruses treated with/without
252 NMDA as described above (Zhang *et al.*, 2011, Prendergast *et al.*, 2014). Immediately after
253 NMDA treatment the medium was replaced with ice-cold artificial cerebrospinal fluid (ACSF)
254 containing 2 mM membrane-impermeant crosslinking agents, Bis(sulphosuccinimidyl)-
255 suberate [BS3, (Pierce Biotechnology)] (Conrad *et al.*, 2008) to selectively crosslink cell surface
256 proteins for 30 mins. The reaction was quenched by replacing the BS3 solution with ACSF
257 containing 0.1 M glycine (with 10 mins incubation) followed by three washes with ACSF
258 containing 0.1 M glycine. The neurons were suspended in lysis buffer (nucleotide binding

259 buffer with 1% triton X-100, and protease inhibitors) and the total protein concentrations
260 were determined. Equal amount of proteins were resuspended in 1X SDS-PAGE loading buffer,
261 resolved on 4-12% gradient SDS-PAGE and western immunoblotting were performed to
262 analyze the surface and intracellular pools of AMPA receptors using anti-GluA2, anti-Thorase
263 antibodies.

264

265 **Data analyses and statistics**

266 All experiments were repeated at least three times and quantitative data are presented as the
267 mean \pm standard error of the mean (SEM) performed by GraphPad prism6 software (Instat,
268 GraphPad Software). Statistical significance was assessed by one-way ANOVA. The significant
269 differences were identified by post-hoc analysis using the Tukey-Kramer post-hoc method for
270 multiple comparisons. Assessments were considered significant with as $p < 0.05$. Power
271 analysis and sample size calculation for all experiments were determined using G*Power 3.1
272 statistics software.

273

274 **Results and Discussion**

275 **Three siblings with severe encephalopathy and a homozygous *ATAD1* mutation**

276 The three siblings were born at term by cesarean section for fetopelvic disproportion with
277 normal growth parameters. Pregnancies were marked by maternal diabetes. Healthy parents
278 are first cousins and originated from southern Tunisia. The first patient (subject 1; IV:1; Fig.
279 1A) was a male who presented at birth with respiratory distress requiring assisted ventilation.
280 Generalized hypertonia with an exaggerated startle reflex, adducted thumbs, spontaneous
281 tremor and clonic movements were observed from day 1. EEG showed altered background

282 with slow and disorganized activity and multiple multifocal epileptic discharges. Examination
283 at two months showed major stiffness and distal arthrogryposis with fixed prone position of
284 upper limbs and bilateral camptodactyly. Deep tendon reflexes were brisk with extensor
285 plantar reflex. Visual contact was absent and narrow miosis unresponsive to light was noted.
286 Facial distinctive features including inexpressive facies, anteverted nares, high arched palate
287 and brachycephaly were observed. Kyphoscoliosis and benign umbilical hernia were also
288 noted. He had several episodes of pneumonia and died after multiple organ failure at the age
289 of 5 months. Abdominal ultrasound, eye fundus, spinal cord and brain MRI were normal.
290 Skeletal X-rays indicated dorsal scoliosis. A large metabolic screening and array CGH did not
291 show any abnormality. A muscle biopsy was performed and showed focal atrophy of both fiber
292 types with grouping suggesting an underlying neurogenic disorder. His younger brother
293 (subject 2; IV:2; Fig. 1A) presented at birth with respiratory distress, poor spontaneous
294 mobility and no visual contact. Examination showed generalized hypertonia with transient
295 tremor, bilateral adducted thumbs and clenched toes. He underwent surgery for bilateral
296 inguinal hernia at 2 months. Regular swallowing difficulties resulted in food misrouting
297 accidents with subsequent pneumonia. In contrast to his brother, a few intentional smiles
298 were noted before death, after a novel episode of aspiration pneumonia at the age of 3
299 months. The ultrasound follow-up of the third pregnancy indicated decreased fetal
300 movements during the third trimester. The girl (subject 3; IV:3; Fig. 1A) presented at birth with
301 transient respiratory distress requiring assisted ventilation. In her case, axial hypotonia
302 contrasted with limb hypertonia. Examination showed poor spontaneous mobility, distal
303 arthrogryposis with adducted thumbs, ulnar deviation and bilateral clubfoot. Eye contact was
304 present. She had gastro-esophageal reflux. Brain MRI performed at day 1 showed myelination

305 delay and a periventricular white matter hypersignal. EEGs were normal. She died at the age
306 of 6 months. The common clinical features of the siblings can be summarized as onset of
307 rigidity at birth, no achievement of developmental milestones and death within the first
308 months of life. Array CGH and Sanger-sequencing of *GLRA1* and *SLC6A5* mutated in
309 hyperekplexia (Tijssen and Rees, 1993, Carta *et al.*, 2012) and *SCN4A* mutated in congenital
310 paramyotonia (Koch *et al.*, 1991) did not identify any molecular alteration in the oldest sibling
311 (patient 1) (data not shown). The severe encephalopathy in the index patient remained thus
312 unexplained. Next, we performed WES in two affected siblings (subject 1/IV:1 and 3/IV:3) and
313 their healthy mother (III:3; Fig. 1A, B). Given parental consanguinity, analysis of WES data was
314 performed according to an autosomal recessive inheritance model. We identified five rare
315 homozygous variants [with an allele frequency <0.5% in population databases (dbSNP138,
316 1000 Genomes Project, Exome Variant Server, ExAC and gnomAD browsers) and no
317 homozygous carriers in the ExAC and gnomAD browsers] shared by the two affected siblings
318 and present in the heterozygous state in their healthy mother (Supplementary Table 1).
319 Segregation analysis excluded two of the variants (Supplementary Table 2), while the
320 remaining variants in *RNLS*, *CDH8* and *ATAD1* are not located within a region of significant
321 homozygosity as assessed by homozygosity mapper (Seelow *et al.*, 2009). However, *RNLS* and
322 *ATAD1* are located in a homozygous region of ~1.3 Mb on chromosome 10 (data not shown).
323 *In silico* pathogenicity assessment and splice-site tools predicted no deleterious effect on
324 protein function for the missense variant p.(Ile114Val) in *RNLS* (Supplementary Table 3) and
325 no pre-mRNA splicing alteration for the synonymous variant c.726T>C in *CDH8*
326 (Supplementary Table 4). In contrast, the 2-bp deletion c.1070_1071delAT in the last exon of
327 *ATAD1* was predicted to result in a frameshift with deletion of five amino acids and addition

328 of 14 unrelated *ATAD1* residues at the C-terminus [p.(His357Argfs*15)], possibly altering
329 protein function (Fig. 1C, Supplementary Table 3 and Supplementary Fig. 1). The
330 c.1070_1071delAT variant represents a very rare *ATAD1* allele, as it has an allele frequency of
331 0.00001221 in the gnomAD browser (3 heterozygotes in 245,646 alleles; Supplementary Table
332 1), in accordance with the rarity of the signs and symptoms presented by the three siblings.
333 *ATAD1* mRNA analysis from patient-derived fibroblasts demonstrated the presence of
334 transcripts harboring the -2 frameshift at codon 357 and 14 novel codons followed by the stop
335 codon TGA at their 3' end (Fig. 1C). This data indicates that mutated *ATAD1* mRNAs escape
336 nonsense-mediated mRNA decay.

337 *ATAD1* encodes the ATPase family, AAA+ domain-containing, member 1, also named
338 Thorase that is highly expressed in mouse brain and testis (Zhang *et al.*, 2011). Members of
339 the AAA+ superfamily of ATPases are involved in the unfolding of proteins and disassembly of
340 protein complexes and aggregates (Hanson and Whiteheart, 2005). In addition, Thorase is able
341 to maintain mitochondrial function through degradation of mislocalized tail-anchored
342 proteins to the outer mitochondrial membrane, thus playing an important role in
343 maintenance of mitochondrial function and integrity (Chen *et al.*, 2014, Okreglak and Walter,
344 2014). A similar caretaker function of Thorase has also been proposed for peroxisomes
345 (Grimm *et al.*, 2016). In addition, postsynaptically, Thorase controls the internalization of
346 excitatory, glutamatergic AMPARs by disassembling complexes between glutamate receptor
347 interacting protein (GRIP1) and the AMPAR subunit GluA2. Therefore, Thorase deficiency is
348 expected to impair function of mitochondria and peroxisomes and alter neurotransmission.
349 Accordingly, Thorase deficiency in mice leads to enhanced AMPAR density at the cell surface
350 that results in enhanced excitatory postsynaptic currents and impaired adaption to excitatory

351 stimuli. Although homozygous *Atad1* knockout mice are viable and do not show any obvious
352 gross malformation, approximately 80% die of a seizure-like syndrome (Zhang *et al.*, 2011).
353 The C-terminus of Thorase is evolutionarily highly conserved (Supplementary Fig. 1) and
354 involved in intra- and intermolecular contacts of oligomerized AAA+ ATPase complexes
355 (Grimm *et al.*, 2016). The *ATAD1* mutation identified here causes a deletion of the last five
356 residues of the C-terminus with addition of 14 novel amino acids (Fig. 1C and Supplementary
357 Fig. 1). To identify additional *ATAD1* variants in individuals with a phenotype similar to that of
358 the three siblings, two different patient cohorts were studied. First, we analyzed WES datasets
359 of 2,000 patients with epileptic encephalopathies and hereditary hyperekplexia. Second, 27
360 cases of glycinergic-negative hyperekplexia with parental consanguinity or atypical
361 degenerative phenotype with severe developmental outcomes were screened by Sanger-
362 sequencing. No further deleterious variant in *ATAD1* emerged from either datasets (data not
363 shown), suggesting that *ATAD1*-related congenital encephalopathy with hypertonic stiffness
364 is an extremely rare condition.

365

366 **Altered protein levels but functional mitochondria in fibroblasts from patients with the** 367 ***ATAD1* mutation p.(His357Argfs*15)**

368 *ATAD1* mRNA analysis from patient-derived fibroblasts suggested that the mutated *ATAD1*
369 mRNAs produce a protein with an altered C-terminal end. We examined the amount of *ATAD1*
370 protein in patient fibroblasts via immunoblotting, which confirmed that the C-terminally
371 altered *ATAD1* mutant is expressed in the patient cells (Fig. 2). Previous studies showed that
372 the deletion of *ATAD1* causes accumulation of peroxisomal biogenesis factor 26 (PEX26) and
373 Golgi SNARE 28 kDa (GOS28) in human cell lines (Chen *et al.*, 2014). In patient cells expressing

374 mutant Thorase, protein level of PEX26 was slightly lower compared to control cells (Fig. 3A,
375 B), however, we noticed variable expression of this and other proteins in control fibroblast
376 cells (Fig. 3A, B). In addition, cytochrome c oxidase subunit 4 (COX4), hexokinase 1 (HXK1), and
377 voltage-dependent anion channel 1 (VDAC1) were also reduced in patient fibroblasts (Fig. 3A,
378 B). Staining of patient and control cells for GOS28 and PEX26 to evaluate the distribution of
379 Golgi and peroxisomal proteins, respectively, showed that their levels were decreased in
380 patient cells when compared to healthy controls (Fig. 3C). Thus, the frameshift mutation in
381 *ATAD1* affects the stability or expression of GOS28 and PEX26. Interestingly, patient
382 fibroblasts exhibited normal tubular mitochondrial morphology when stained for the
383 mitochondrial protein TOMM20 (Fig. 3C). The patient fibroblasts also exhibited efficient
384 mitochondrial respiration (Cooper *et al.*, 2012, Chen *et al.*, 2014), similar to wild-type cells
385 (Supplementary Fig. 2). These results suggest that the *ATAD1* mutation has no significant
386 effect on mitochondrial function, despite reduction in the level of some mitochondrial
387 proteins.

388

389 ***ATAD1* mutation p.(His357Argfs*15) affects the oligomeric state of Thorase but causes no**
390 **defects in its ATPase activity**

391 The predicted 3D model of Thorase suggests that the *ATAD1* mutation p.(His357Argfs*15)
392 results in changes in the secondary structure at the C-terminus of Thorase (Fig. 3D). The wild-
393 type C-terminus (Ala349-Asp361) is predicted to form a helix, while in the Thorase
394 mutant^{His357Argfs*15} the helix is shortened and sandwiched by two loops to form a loop-helix-
395 loop (LHL) structure (Fig. 3D). To further examine the activity of the Thorase
396 mutant^{His357Argfs*15}, a recombinant expression vector encoding for it was generated. Purified

397 wild-type Thorase elutes as 70 kDa (dimer) on a size-exclusion column, while the
398 mutant^{His357Argfs*15} elutes at a higher molecular weight >400 kDa (Fig. 3E). Thus, the
399 p.(His357Argfs*15) mutation seems to lock the Thorase mutant in an oligomeric state. Both
400 wildtype and mutant migrated at ~36 kDa in SDS-PAGE as indicated by Coomassie staining and
401 immunoblot analysis, however, the mutant migrated higher than the wildtype due to the
402 elongated C-terminus (Fig. 3F). ATP binding assessment using UV light-induced cross-linking
403 (Harvey *et al.*, 2008) of radiolabeled [α -P³²]ATP bound to purified recombinant proteins
404 suggested that the Thorase mutant binds ATP similar to wildtype (Supplementary Fig. 2).
405 Similarly, ATP hydrolysis was not significantly affected in the mutant (Supplementary Fig. 2).
406 These results indicate that the p.(His357Argfs*15) mutation does not affect ATPase activity of
407 Thorase but strongly impacts its oligomeric state, most likely as a result of the LHL formation
408 at the C-terminus.

409

410 **The Thorase mutant^{His357Argfs*15} shows defects in the disassembly of AMPAR-GRIP1 and**
411 **Thorase oligomer complexes**

412 Since Thorase regulates AMPAR trafficking (Zhang *et al.*, 2011, Prendergast *et al.*, 2014), we
413 examined Thorase mutant^{His357Argfs*15} interactions with GluA2-GRIP1 complex and particularly
414 its disassembly. Purified GST-Thorase wildtype and mutant were immobilized on beads and
415 mixed with Thorase knockout (*Atad1*^{-/-}) whole brain lysates in the presence of ADP, ATP or
416 ATP γ S (Fig. 4A, B, C, D). Both wildtype and mutant bound efficiently to the GluA2-GRIP1
417 complex in the presence of non-hydrolyzable ATP γ S, which maintains Thorase in the
418 oligomeric substrate-bound state (Fig. 4A, B, C). However, in the presence of ATP, which can
419 be hydrolyzed and is required for the proper disassembly of protein complexes by Thorase,

420 wild-type Thorase disassembled the complex, whereas the disassembly was significantly
421 impaired by the Thorase mutant^{His357Argfs*15} (Fig. 4D). These data suggest that the
422 p.(His357Argfs*15) mutation affects Thorase-mediated AMPAR trafficking, likely by impairing
423 its transition from oligomeric to monomeric state. The formation of oligomeric complexes by
424 Thorase and other AAA+ ATPases is critical for their proper assembly and disassembly of
425 protein complexes (Fujiki *et al.*, 2008). Therefore, we attempted at determining whether the
426 defect in AMPAR complex disassembly by the mutant^{His357Argfs*15} results from improper
427 Thorase oligomer disassembly. Oligomeric formation and disassembly were evaluated by ATP
428 binding and glutaraldehyde cross-linking of protein complexes (Babst *et al.*, 1998). Purified
429 Thorase samples were mixed with ATP (at 4°C to prevent its hydrolysis or at 37°C to allow for
430 its hydrolysis) or non-hydrolysable ATPγS (Supplementary Fig. 2). In the presence of either ATP
431 at 4°C (ATP-4) or ATPγS Thorase formed large oligomeric complexes of molecular weights
432 greater than 250 kDa (Supplementary Fig. 2). While 71 ± 4.7% of Thorase wildtype formed
433 oligomers, 89 ± 6.3% of the mutants were found in the oligomeric state (ATP-4 in
434 Supplementary Fig 2). Upon ATP hydrolysis (ATP-37), 75.5 ± 4.4% of Thorase wildtype and 57.6
435 ± 7.6% of mutant disassembled oligomeric complexes (Supplementary Fig. 2). These results
436 indicate and further confirm that the p.(His357Argfs*15) mutation impairs normal
437 disassembly of Thorase oligomers. Although we observed a defect of 14-18% in oligomeric
438 disassembly in the Thorase mutant^{His357Argfs*15} compared to wildtype, this may be significant
439 enough to cause severe consequences in terms of clinical phenotype.

440

441 **Atad1^{-/-} neurons expressing the Thorase mutant^{His357Argfs*15} display reduced GluA2 surface**
442 **expression**

443 Since Thorase regulates surface expression of AMPARs the effects of the p.(His357Argfs*15)
444 mutation on AMPARs trafficking was evaluated. An antibody-feeding assay for endocytosis of
445 surface GluA2 receptors (Zhang *et al.*, 2011, Prendergast *et al.*, 2014) was performed in *Atad1*^{-/-}
446 primary cortical neurons expressing GFP-tagged Thorase wildtype or mutant^{His357Argfs*15}. Live
447 neurons were incubated with an anti-GluA2 N-terminal antibody followed by induction of
448 GluA2 endocytosis with N-methyl-D-aspartate (NMDA) (20 μM) and glycine (10 μM). In control
449 unstimulated Thorase mutant^{His357Argfs*15} cultures there was decreased surface expression of
450 GluA2 compared to Thorase wildtype cultures (Fig. 4E, F). In contrast, the ratio of surface
451 GluA2/intracellular GluA2 in NMDA- and glycine-stimulated Thorase mutant cortical cultures
452 remained similar to that of stimulated Thorase wildtype cultures (Fig. 4G, H). Thus, the
453 mutant^{His357Argfs*15}-expressing neurons exhibited significantly reduced surface GluA2 only
454 under unstimulated conditions. To further evaluate the effects of the p.(His357Argfs*15)
455 mutation in Thorase on AMPAR trafficking, GluA2 surface expression was assessed by a
456 bis(sulfosuccinimidyl)-suberate (BS3) cross-linking assay that allows for the quantification of
457 both surface and intracellular receptor pools (Conrad *et al.*, 2008). The results suggested
458 decreased levels of surface GluA2 in unstimulated mutant^{His357Argfs*15}-expressing cultures
459 compared to wildtype-expressing cultures (Fig. 4I, J). In response to NMDA, there was no
460 significant difference in internalization of surface GluA2 in the two cultures (Fig. 4I, J).
461 Together, the data suggest that the Thorase mutant^{His357Argfs*15} may inhibit the recycling back
462 and/or reinsertion of AMPARs to the surface following endocytosis resulting in a decrease in
463 the steady-state levels of these receptors at the cell surface.

464 Taken together, these results demonstrate that a homozygous frameshift mutation at
465 the 3' end of *ATAD1* leads to the production of Thorase protein with a novel function of its C-

466 terminal end. Interestingly, through a gain-of-function effect, an unusual mechanism in
467 autosomal recessive disease, the *ATAD1* mutation causes a congenital severe and lethal
468 encephalopathy associated with stiffness and arthrogryposis. Previously, we reported a
469 homozygous loss-of-function mutation (p.Glu276*) in *ATAD1* that underlies a neurological
470 disorder with remarkable clinical overlap to the phenotype reported here: patients showed
471 hypertonia, seizures, respiratory failure and early death (Ahrens-Nicklas *et al.*, 2017). In the
472 affected neonates, movement was precluded due to extreme hypertonia. Thus, stiffness was
473 common to both families (this report and (Ahrens-Nicklas *et al.*, 2017)), while clinical seizures
474 starting at birth were only present in infants with *ATAD1* nonsense mutation (Ahrens-Nicklas
475 *et al.*, 2017). Given that different *ATAD1* mutations have been identified in two separate
476 families with critical neurological phenotypes in neonates, *ATAD1* can be considered as an
477 important human disease gene. Our functional assays showed deficiency in AMPAR recycling
478 as the molecular mechanism associated with the disease. The gain-of-function mutation in
479 *ATAD1* decreases the population of excitatory postsynaptic AMPA receptors. However,
480 mitochondrial function does not seem to be affected by the *ATAD1* p.(His357Argfs*15)
481 mutation as patient-derived fibroblasts show normal mitochondrial morphology and
482 respiratory chain performance. In contrast, *Atad1* loss-of-function causes a decrease of many
483 mitochondrial proteins in mouse brain in which the Golgi protein GOS28 ectopically
484 accumulated. In addition, *Atad1*-deficient mouse embryonic fibroblasts show decreased basal
485 and mitochondrial respiration, severely fragmented mitochondria and mislocalization of
486 GOS28 and PEX26 in mitochondria suggesting significant mitochondrial impairment in this
487 mouse mutant (Chen *et al.*, 2014). These findings together with impaired AMPAR
488 internalization resulting in increased GluA1 and GluA2 surface levels in *Atad1* knockout

489 neurons suggest a combined effect of defects in both AMPAR trafficking and mitochondrial
490 function that might have contributed to the phenotype in *Atad1*^{-/-} mice (Zhang *et al.*, 2011,
491 Chen *et al.*, 2014). Similarly, we speculate that the distinct combination of functional
492 alterations arising from either gain- or loss-of-function *ATAD1* mutations may provide an
493 explanation for the discrete although overlapping phenotypes observed in the siblings
494 reported here and the previously described family.

495 Changes in AMPAR receptor surface expression is a known pathogenic mechanism in
496 encephalopathy; for example, a decrease in extrasynaptic AMPAR expression impairs synaptic
497 plasticity in a model of hepatic encephalopathy (Schroeter *et al.*, 2015). This disrupts the
498 efficacy of synaptic transmission and the fine balance between inhibitory and excitatory
499 signaling, which accounts, at least partially, for the encephalopathy. There are additional
500 possible targets of Thorase that contribute to the encephalopathy and neurologic phenotype
501 of patients with mutations in *ATAD1*. Consistent with this notion is the observation that the
502 AMPAR antagonist, perampanel, only partially rescued the phenotype of patients with a loss-
503 of-function mutation in *ATAD1* (Ahrens-Nicklas *et al.*, 2017). The findings from our study
504 establish an important avenue for clinicians to examine the role of *ATAD1* mutations in several
505 neurological diseases due to unknown cause.

506

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509

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513

514 **Conflict of interest**

515 Nothing to report.

516

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575

576 **Figure legends**

577 **Figure 1** DNA and RNA analysis in the family with three siblings carrying the homozygous
578 *ATAD1* mutation. **(A)** Pedigree of the family. **(B)** Partial sequence electropherograms
579 demonstrating the *ATAD1* c.1070_1071delAT [p.(His357Argfs*15)] mutation in the

580 homozygous state in leukocyte-derived DNA of the affected siblings (Subjects 1, 2 and 3). Their
581 healthy parents (Father and Mother) are heterozygous carriers of the mutation. (C) Partial
582 sequence electropherograms show the 2-bp deletion in *ATAD1* in fibroblast-derived cDNA of
583 one sibling (Mutant) in comparison to the cDNA sequence of a healthy individual (Wild-type).
584 Deleted bases are marked by parenthesis in the normal sequence. The encoded amino acid
585 residues are depicted below each sequence in the three-letter code and show the 14 novel
586 amino acid residues at the C-terminus of ATAD1 (highlighted in bold). *: stop codon.

587

588 **Figure 2** Mutant Thorase is expressed in patient-derived fibroblasts. (A) Immunoblot of lysates
589 obtained from patient and control fibroblasts. Expression of Thorase was monitored by using
590 anti-Thorase antibody, and anti-actin antibody was used to control for equal loading. As the
591 anti-Thorase antibody was generated against the C-terminus and this region contains a new
592 amino acid composition in the mutant, detection of Thorase in patient cells was difficult
593 (compare the clear band in control and the diffuse band in patient cells). (B) Optical
594 densitometry quantification of (A). Values represent the mean±SEM (n=3, n.s. $p>0.05$, Tukey's
595 multiple comparison tests).

596

597 **Figure 3** The *ATAD1* mutation p.(His357Argfs*15) leads to reduced amount of some
598 mitochondrial proteins in patient-derived fibroblasts and locks Thorase in the oligomeric state.
599 (A) Immunoblots of lysates obtained from patient and control fibroblasts. COX1, cytochrome
600 c oxidase subunit 1; HXK1, hexokinase 1; PEX26, peroxisomal biogenesis factor 26; VDAC1,
601 voltage dependent anion channel 1. (B) Optical densitometry quantification of (A). Values
602 represent the mean+SEM (n=3, n.s. $p>0.05$, * $p<0.05$, two-way ANOVA, Tukey's multiple

603 comparison tests). (C) Representative immunofluorescence images of the mitochondrial
604 morphology (TOMM20 staining) in control and patient fibroblasts. The cells were also stained
605 for Golgi (GOS28), peroxisomes (PEX26) and the nuclei with DAPI (blue). (D) Predicted 3D
606 structure of Thorase wildtype (green) and mutant^{His357Argfs*15} (red). (E) Size exclusive
607 chromatograph profile of purified recombinant Thorase. Wild-type Thorase appears as a
608 dimer (~70 kDa), whereas the Thorase mutant^{His357Argfs*15} appears as oligomer (>400 kDa). (F)
609 Purified proteins resolved on 10% SDS-PAGE stained with coomassie (left) and immunoblotted
610 with anti-Thorase antibody (right).

611

612 **Figure 4** *ATAD1* mutation p.(His357Argfs*15) affects GluA2-GRIP1 complex disassembly and
613 GluA2 surface expression. (A) Immunoblot analyses of GST-Thorase pulldown of the GluA2-
614 GRIP1 complex from Thorase knockout whole brain lysate in the presence of different
615 nucleotides (ADP, hydrolysable ATP; ATPγS, non-hydrolysable ATP). The samples were
616 incubated at 4°C for binding and then at 37°C for ATP hydrolysis to trigger the disassembly of
617 the protein complex. (B-C) The graphs represent normalized percent bound GluA2 (B) and
618 GRIP1 (C) in the GST-Thorase pulldown samples for (A). (D) Normalized percentage of GluA2
619 and GRIP1 disassembled from Thorase-GluA2-GRIP1 complex in (A). (E-F) Representative
620 immunofluorescence images of unstimulated and NMDA-induced endocytosis of GluA2 in
621 *Atad1*^{-/-} neurons expressing Thorase-GFP wildtype (WT) or the mutant^{His357Argfs*15} (Mutant). (G)
622 Normalized ratio of surface GluA2 (sGluA2) to internalized GluA2 (iGluA2) for (E-F). (H) GluA2
623 internalization index measured as the ratio of iGluA2 to the total GluA2 (iGluA2 plus sGluA2)
624 fluorescence intensities. (I) Immunoblot analyses of BS3-crosslinking of sGluA2 in *Atad1*^{-/-}
625 neurons expressing Thorase-GFP wildtype or mutant^{His357Argfs*15}. (J) The normalized optical

626 densitometry quantification of sGluA2 for (I). (mean±SEM of three experiments performed in
627 triplicate. n=3, ** p<0.05, * p<0.10, n.s p>0.10, ANOVA with Tukey-Kramer post-hoc test when
628 compared with wildtype).