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Author(s)	Tamura, Yuka; Sassa, Takayuki; Nishizawa, Takumi; Kihara, Akio
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1	Incomplete elongation of ultra-long-chain polyunsaturated acyl-CoAs by
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4	Yuka Tamura, <sup>a</sup> Takayuki Sassa, <sup>a</sup> Takumi Nishizawa, <sup>a</sup> Akio Kihara <sup>a</sup>
5	<sup>a</sup> Laboratory of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University,
6	Sapporo, Japan
7	
8	Running title: Pathogenesis of spinocerebellar ataxia type 34
9	
10	Address correspondence to Takayuki Sassa (tasasa@pharm.hokudai.ac.jp) and Akio Kihara
11	(kihara@pharm.hokudai.ac.jp)
12	

#### 13 ABSTRACT

14 Spinocerebellar ataxias (SCAs) are autosomal dominant diseases characterized by 15 cerebellar atrophy and ataxia. The SCA subtype SCA34 is caused by specific mutations in 16 the gene ELOVL4, which encodes a fatty acid (FA) elongase that synthesizes 17 ultra-long-chain (ULC;  $\geq$  C26) FAs. However, the pathogenesis and molecular mechanism 18 that confers dominant inheritance remains unknown. Here, a cell-based assay demonstrated 19 that each of the five known SCA34 mutants produced shorter ULC polyunsaturated 20 FA-containing phosphatidylcholines (ULC-PCs) than wild-type protein, in the following 21 order of severity: Q180P and T233M > W246G > I171T and L168F. Next, we generated 22 knock-in mouse embryonic stem cells that contained heterozygous Q180P, heterozygous 23 W246G, or homozygous W246G mutations. Neuronal differentiation-dependent production 24 of ULC-PCs was reduced in heterozygous Q180P and homozygous W246G cells relative to 25 control cells, and we observed shortening of the FA moiety in all mutant cells. This FA 26 shortening was consistent with our prediction that amino acid residues substituted by 27 SCA34 mutations are located in the transmembrane helices that interact with the  $\omega$ -end 28 region of the FA moiety of the substrate acyl-CoA. Hence, reduced levels and shortening of 29 ULC-PCs in neurons may cause SCA34, and incomplete elongation of ULC 30 polyunsaturated acyl-CoAs by mutated ELOVL4 may induce dominant inheritance.

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#### 32 **KEYWORDS**

33 ELOVL4, spinocerebellar ataxia, fatty acid, neuron, polyunsaturated fatty acid

#### 35 INTRODUCTION

36 The spinocerebellar ataxias (SCAs) are a group of cerebellar ataxias that are inherited in an 37 autosomal dominant manner and characterized by cerebellar atrophy and ataxia (1, 2). 38 Currently, there exist 49 SCA subtypes; for most of them, causative genes have been 39 identified (1, 3). Each causative gene exhibits a particular type of mutation, such as 40 expansion of CAG repeats encoding the polyglutamine tract, expansion of non-coding 41 repeats, missense mutations, or frameshift mutations (1). It is thought that these mutations 42 gain-of-function, negative, loss-of-function have dominant or (leading to 43 haploinsufficiency) effects. They cause various cellular abnormalities, including 44 transcriptional dysregulation, RNA toxicity, ion channel dysfunction, and mitochondrial 45 dysfunction. One type of SCA, namely SCA34, is caused by mutations in the fatty acid 46 (FA) elongase *ELOVL4*.

47 ELOVL4 is one of the seven FA elongases (ELOVL1-7) that exist in mammals (4, 5). 48 FAs are classified according to their chain-length; they can be categorized as long-chain 49 FAs (C11–C20) or very-long-chain (VLC) FAs ( $\geq$  C21). VLCFAs with C26 or more are 50 termed ultra-long-chain (ULC) FAs (4, 5). FAs are also classified according to their degree 51 of unsaturation and are categorized as saturated FAs (SFAs), monounsaturated FAs 52 (MUFAs), or polyunsaturated FAs (PUFAs). The long-chain FAs synthesized by FA 53 synthase in the cytosol or incorporated from dietary sources are subject to elongation via 54 the FA elongation cycle, which consists of four sequential reactions and takes place in the

55	endoplasmic reticulum (ER). ELOVLs catalyze the rate-limiting reaction: condensation of
56	an acyl-CoA-the activated form of FA-with a malonyl-CoA (6). Each ELOVL isozyme
57	exhibits characteristic substrate specificity toward acyl-CoAs with different chain-lengths
58	and/or degrees of unsaturation (7). Of the ELOVLs, only ELOVL4 is responsible for
59	generating ULCFAs, regardless of the saturation/unsaturation type (SFAs, MUFAs, and
60	PUFAs) (4, 5, 7). Thus far, five missense mutations—L168F
61	[NM_022726.4(ELOVL4):c.504G>C (p.Leu168Phe), rs587777598], I171T
62	[NM_022726.4(ELOVL4):c.512T>C (p.Ile171Thr), rs1554162301], Q180P
63	[NM_022726.4(ELOVL4):c.539A>C (p.Gln180Pro)], T233M
64	[NM_022726.4(ELOVL4):c.698C>T (p.Thr233Met), rs1554162016], and W246G
65	[NM_022726.4(ELOVL4):c.736T>G (p.Trp246Gly), rs1131692036]—have been identified
66	in ELOVL4 as mutations that cause SCA34. Gait ataxia, nystagmus, and dysarthria have
67	been reported as common neurological abnormalities in patients with these mutations (8-
68	17). Skin erythrokeratodermia was also observed in patients carrying one of the mutations
69	L168F, Q180P, and T233M (11, 12, 16), while retinitis pigmentosa was observed in a
70	subset of patients harboring the I171T mutation (17). The age of onset differs among the
71	mutations: 10-20 years for Q180P; 10-50 years for T233M and W246G; 30-50 years for
72	I171T; and 30-70 years for L168F. Recently, knock-in (KI) rats harboring the W246G
73	mutation have been generated (18). Assessment using the rotarod performance test revealed
74	that heterozygous and homozygous W246G KI rats exhibited impaired motor function

without apparent histological abnormalities, while homozygous W246G KI rats exhibited impaired synaptic plasticity in parallel and climbing fibers in the cerebellum (18). However, the effects of the SCA34 mutations on ELOVL4 enzymatic activity have not been examined, and the mechanism underlying the pathogenesis and autosomal dominant inheritance mode of SCA34 remains unclear.

80 Distinct sets of mutations in the *ELOVL4* gene are associated with two other hereditary 81 diseases: Stargardt disease 3 (STGD3) and ISQMR (ichthyosis, spastic quadriplegia, and 82 mental retardation) (19). STGD3 is a type of juvenile macular dystrophy without other 83 neurological or skin symptoms that is inherited in an autosomal dominant manner. Three 84 ELOVL4 mutations (frameshift mutations N264LfsX10 and N264TfsX9 and the nonsense 85 mutation Y270X) have been identified in STGD3 (20–22). It has been predicted that these 86 mutations produce mutant ELOVL4 proteins lacking the C-terminal of approximately 50 87 residues, including the ER retention motif. Several studies have demonstrated that these 88 mutant proteins exert dominant negative effects on wild-type (WT) ELVOL4 protein in the 89 retinal photoreceptor cells (23–26). Alternatively, the mutant proteins can be abnormally 90 transported to the outer segment of photoreceptors and phagocytosed by the retinal pigment 91 epithelium, where they cause non-cell-autonomous toxic effects (27, 28). ISQMR is an 92 autosomal recessive neurodevelopmental and cutaneous disorder characterized by skin 93 ichthyosis (scaly and dry skin), spastic quadriplegia, seizures, and intellectual disability. 94 Six ELOVL4 mutations have been identified in ISQMR (nonsense mutations Y26X, R70X,

95 and R216X; frameshift mutations I146YfsX29 and I230MfsX22; and the missense 96 mutation C163R) (29-31). R70X and C163R were found together in one compound 97 heterozygous patient, while the other mutations have been found in homozygous patients. 98 Except for C163R, all mutations produce a truncated ELOVL4 protein lacking more 99 C-terminal residues than STGD3 mutant proteins. The C163R mutation causes substitution 100 of the amino acid located near the catalytically essential histidine-motif (residues 158–162). 101 These findings, together with the recessive mode of inheritance, suggest that the ISOMR 102 mutations have loss-of-function effects. Since heterozygous carriers of ISQMR do not 103 exhibit symptoms (29-31), SCA34 is not caused by the simple haploinsufficiency of 104 ELOVL4. Other mechanisms may be responsible for rendering the disease dominant.

105 ELOVL4 is abundantly expressed in the retina, skin, testes, meibomian glands, and 106 brain (mainly in neurons) (32). In the retina, phosphatidylcholines (PCs) containing ULC 107 PUFAs (ULC-PCs) are present and important for visual function (33, 34). In the skin, ULC 108 SFAs/MUFAs are essential for the formation of the epidermal permeability barrier, as they 109 are components of  $\omega$ -O-acylceramides, which are specialized barrier lipids (4, 35, 36). In 110 the testes, ULC PUFAs are used to create glycosphingolipids and sphingomyelins and are 111 essential for spermatogenesis (37). In the meibomian glands, ULC SFAs/MUFAs are used 112 to create cholesteryl esters, wax monoesters/diesters, and (O-acyl)-ω-hydroxy FAs, which 113 are secreted as the constituents of tear film lipids and play an important role in preventing 114 dry eye disease (38–42). In the brain, ULC PUFAs are predominantly present in PCs, in which ULC PUFAs with four to six double bonds occupy the *sn*-1 position (43, 44).
However, the functions of ULC-PCs in the nervous system and their relationships with
SCA34 and ISQMR remain unexplored.

118 The aim of this study was to elucidate the pathogenesis of SCA34 and the molecular 119 mechanism that causes its dominant heritability. For this purpose, we deduced the positions 120 of substituted amino acid residues in ELOVL4 protein based on the recently resolved 121 crystal structure of ELOVL7 (45). We then examined the intracellular localization of 122 mutant proteins and measured the FA elongation activity of the mutant proteins in a 123 cell-based overexpression system. Furthermore, we generated mouse embryonic stem (ES) 124 cells carrying the Q180P or W246G mutation and measured the levels and FA composition 125 of ULC-PCs in them. Through these analyses, we obtained clues about the pathogenesis of 126 SCA34 and the molecular mechanism causing its dominant mode of inheritance.

#### 127 **RESULTS**

SCA34 mutations cause amino acid substitutions in the regions involved in the 128 129 binding of the FA moiety of acyl-CoA in ELOVL4 protein. It has been established that 130 five ELOVL4 missense mutations cause SCA34 (Fig. 1). However, the position of these 131 amino acid residues in the 3D structure of the ELOVL4 protein remains unclear. In this 132 study, we mapped the amino acid residues of ELOVL4 substituted in SCA34 onto the 133 recently revealed crystal structure of the human ELOVL7 protein (PDB: 6Y7F) (45), which 134 is one of the seven isozymes of the ELOVL family. The amino acid residues of ELOVL4 135 mutated in SCA34 were L168F, I171T, Q180P, T233M, and W246G, and correspond to 136 residues T157, F160, L169, I222, and F238 from ELOVL7, respectively (Fig. 1). These 137 five residues are not conserved but rather diverged among ELOVL isozymes. In the crystal 138 structure of ELOVL7, these amino acid residues were mapped in the transmembrane helix 139 (TMH) 4 (L168F and I171T), TMH5 (Q180P), TMH6 (T233M), and TMH7 (W246G) (Fig. 140 2). These residues were not located close to the catalytic site consisting of a histidine box, 141 but rather located at the  $\omega$ -end region of the FA moiety of the bound acyl-CoA analog (Fig. 142 2). Thus, the amino acid residues of ELOVL4 substituted in SCA34 constitute the substrate 143 binding site and may participate in the acquisition of substrate specificity in terms of the 144 FA chain length of acyl-CoAs.

146 SCA34 mutant proteins are localized in the ER. Although WT ELOVL4 is localized in 147 the ER, it has been reported that STGD3 mutations affect its subcellular localization (23, 148 27). On the other hand, the effect of SCA34 mutations on subcellular localization has not 149 been investigated. To address this, five SCA34 mutants and WT protein as a control were 150 transiently expressed in HeLa cells as 3×FLAG-tagged proteins. Subsequently, their 151 subcellular localization was examined using indirect immunofluorescence microscopy. 152 Consistent with a previous report (7), WT ELOVL4 protein was localized in the ER, as 153 demonstrated by its colocalization with the ER marker calnexin (Fig. 3). All five SCA34 154 mutant proteins were also localized in the ER, indicating that the SCA34 mutations do not 155 affect intracellular localization.

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157 SCA34 mutants produce shorter ULC lipids than WT ELOVL4. To investigate the 158 effect of SCA34 mutations on the ULCFA-producing activity of ELOVL4, we 159 overproduced 3×FLAG-tagged WT ELOVL4 or each of the SCA34 mutants in HEK 293T 160 cells, extracted the lipids from these cells, and measured the quantities of ceramides and 161 PCs via liquid chromatography (LC)-tandem mass spectrometry (MS/MS). As a control, 162 we also overproduced R216X ISQMR mutant, which lacks a C-terminus (one-third of the 163 protein) and therefore likely has no enzymatic activity. Since ceramides can contain 164 saturated, monounsaturated, or polyunsaturated ULCFA, measurement of ceramides is useful for monitoring the FA elongation activity of ELOVL4 towards each type of 165

166	acyl-CoA. To produce ULC polyunsaturated ceramides (Fig. 4A), HA-tagged ELOVL2
167	[synthesizes C24 PUFAs (7), the substrates for the synthesis of ULC PUFAs (Fig. 4B)] and
168	3×FLAG-tagged CERS3 [synthesizes ULC ceramides using ULC acyl-CoAs as substrates
169	(46, 47)] were co-overproduced with 3×FLAG-ELOVL4. In the immunoblot analysis, WT
170	ELOVL4 protein was detected as two bands, an upper glycosylated form and a lower
171	non-glycosylated form (Fig. 4C) (7). All five SCA34 mutant proteins were also detected as
172	two bands, with expression levels comparable to that of WT proteins. Two bands of the
173	R216X ISQMR mutant protein were observed (24 and 27 kDa), consistent with the large
174	C-terminus truncation. For the LC-MS/MS analysis, the levels of ceramides produced by
175	each ELOVL4 mutant were shown as a ratio to those produced by WT ELOVL4 and
176	displayed as a heat map (Fig. 4D). In cells expressing the R216X ISQMR mutant, the
177	ceramide composition was similar to that noted in cells transfected with an empty vector.
178	This composition included reduced levels of $\geq$ C28 saturated/monounsaturated ceramides
179	and $\geq$ C32 polyunsaturated ceramides, with concomitant increases in the shorter species
180	compared with WT ELOVL4-expressing cells (Fig. 4D and E; E indicates C32:6 and C38:6
181	ceramide levels as representatives). These results demonstrated that as expected, the R216X
182	mutant does not have activity. In cells expressing the SCA34 mutants, the levels of
183	numerous ceramide species containing $\geq$ C34 ULC PUFA were decreased, accompanied by
184	increases in the levels of $\leq$ C32 polyunsaturated ceramides (Fig. 4D). An exception to this
185	finding was the L168F mutant; its expression resulted in decreases in most $\geq$ C30 species,

187 strongest for the Q180P and T233M mutants, followed by W246G, I171T, and L168F.
188 Shortening was also observed for saturated and monounsaturated ceramides by many of the
189 SCA mutants. Nevertheless, shortening was not observed for saturated ceramides by the
190 Q180P mutant and for monounsaturated ceramides by the L168F and I171T mutants.

except C38:4 and C38:5. The chain-length-shortening effect of the SCA mutations was

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191 measure ULC-PCs (Fig. 4A), which are present in neurons, Next, to 192 3×FLAG-ELOVL4 WT or each of the SCA34 mutants were overexpressed together with 193 HA-ELOVL2 in HEK 293T cells. The expression levels of these proteins were confirmed 194 by immunoblot analysis (Fig. 4F). Lipids were extracted, and ULC-PCs were measured via 195 LC-MS/MS. Each PC species was expressed according to the sum of its chain length and 196 the number of double bonds in its two constituent FAs. Again, PC composition in cells 197 expressing the R216X ISQMR mutant was similar to that recorded in vector-transfected 198 cells (Fig. 4G). Numerous  $\geq$  C50 ULC-PC species were reduced and  $\leq$  C48 species were 199 increased compared with those measured in WT ELOVL4-expressing cells. In the cells 200 expressing the SCA34 mutants, the levels of PCs with  $\geq$  C52 decreased, whereas levels of 201 PCs with  $\leq$  C50 increased in most cases. The heatmap pattern of ULC-PCs for each SCA34 202 mutant was similar to that of polyunsaturated ceramides (Fig. 4D and G), confirming the 203 above observed activity of SCA34 mutants towards ULC polyunsaturated acyl-CoAs. 204 Comparison of the two heatmaps suggested, for example, that the C32:6 ceramide species corresponds to the C50:7 PC species. Therefore, the C50:7 PC species mainly consists of 205

C32:6 ULC PUFA and C18:1 FA (oleic acid) in the *sn*-1 and *sn*-2 positions, respectively
(Fig. 4A). Such PC species have been reported in human and rat brains (43, 44). These
results indicate that the SCA34 mutations cause incomplete elongation of ULC
polyunsaturated acyl-CoAs and shortening of the ULC PUFA-containing lipids.

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211 Neuronal differentiation increases ULC-PC levels. In the brain, ELOVL4 is 212 predominantly expressed in neurons (32), and ULC PUFAs synthesized by ELOVL4 213 mainly exist in PCs (43, 44). In the present study, we utilized neurons differentiated from 214 ES cells as model neurons. Mouse ES cells were subjected to differentiation into neurons 215 via the formation of embryoid bodies, treatment with retinoic acids, and growth in neuronal 216 differentiation medium (48). This protocol efficiently induced the differentiation of ES cells 217 into neurons, as demonstrated by their cell morphology and the expression of the neuronal 218 marker class III β-tubulin (Fig. 5A). To confirm *Elovl4* mRNA expression and ULC-PC 219 production in these cells, total RNA and lipids were extracted, and the levels of *Elovl4* 220 mRNAs and ULC-PCs were examined by quantitative RT-PCR and LC-MS/MS, 221 respectively. Both Elovl4 mRNA and ULC-PC levels increased as the cells differentiated 222 (Fig. 5B and C). Thus, neurons differentiated from ES cells are useful for the evaluation of 223 ELOVL4 activity.

225	Neurons with SCA34 mutations produce shortened ULC-PCs. To clarify the effects of
226	SCA34 mutations on the levels and composition of ULC-PCs, the Q180P and W246G
227	mutations were each introduced into the Elovl4 gene in mouse ES cells using
228	single-stranded oligodeoxynucleotide-mediated KI with a CRISPR/Cas9 system (49).
229	These mutations were selected as severe (Q180P) and moderate (W246G) examples in
230	terms of the age of disease onset (12, 50) and their shortening effect on ULC-lipids (Fig. 4).
231	A heterozygous KI clone, which mimics the dominant form of inheritance in SCA34, was
232	obtained for each mutation. A homozygous KI clone, which likely exhibits more changes
233	than the corresponding heterozygous KI clone, was obtained for the W246G mutation (Fig.
234	6A). These KI and control ES cells were differentiated into neurons, and the lipids prepared
235	after 1 and 5 days of differentiation were subjected to quantification of ULC-PCs by LC-
236	MS/MS. After 1 day of differentiation, the total levels of ULC-PCs in W246G homozygous
237	KI cells were reduced to 42% of those noted in control cells (Fig. 6B). On the other hand,
238	heterozygous Q180P and W246G cells exhibited reduction tendencies, which were not
239	statistically significant. The proportion of $\geq$ C52 ULC-PCs, which mainly consists of $\geq$ C34
240	ULC PUFAs, among the total ULC-PCs was lower in Q180P heterozygous and W246G
241	homozygous KI cells than in control cells. These findings were suggestive of ULC PUFA
242	shortening. The proportion of $\geq$ C52 ULC-PCs in W246G heterozygous KI cells was
243	slightly higher than that recorded in control cells at this early stage of differentiation. After
244	5 days of differentiation, total ULC-PC levels had reduced significantly in W246G

245 homozygous KI cells (reduction to 43%) and Q180P heterozygous KI cells (reduction to 246 44%). On the other hand, W246G heterozygous KI cells exhibited a reduction tendency, 247 which was not statistically significant. The proportions of  $\geq$  C52 ULC-PCs in all three KI 248 cells were lower than that measured in control cells. The observed reduction and shortening 249 of ULC-PCs in KI cells were not due to the reduced expression of *Elovl* genes involved in 250 the synthesis of ULC PUFA or defective differentiation into neurons. This conclusion is 251 based on a lack of reduced expression levels of Elovl4, Elovl2, Elovl5, and the neuronal 252 differentiation marker Tubb3 (encodes class III  $\beta$ -tubulin) in the KI cells after 5 days of 253 differentiation (Fig. 6C). Rather, increases in the levels of Elovl4 and Tubb3 in Q180P 254 heterozygous KI cells and in those of *Elovl2* in W246G heterozygous KI cells were 255 observed. These results indicate that polyunsaturated acyl-CoA elongation is incomplete in 256 neurons possessing SCA34 mutations and suggest that decreases and/or shortening of 257 ULC-PCs are associated with the pathogenesis of SCA34.

#### 259 **DISCUSSION**

260 Five ELOVL4 missense mutations have been identified in patients with SCA34 (12, 15, 50). 261 In the present study, all five amino acid residues substituted in SCA34 were mapped to 262 TMHs that interact with the  $\omega$ -end region of the FA moiety of the substrate acyl-CoA based 263 on the 3D structure of ELOVL7 (Fig. 2) (45). WT ELOVL4 protein exhibits activity for the 264 elongation of C24 or C26 acyl-CoA to become C36 or C38 (Fig. 4) (4, 51). This indicates 265 that its substrate-binding pocket has sufficient space to accommodate a C36 or C38 ULC 266 acyl-CoA. Each of the SCA34 mutations may alter the pocket structure so that it can only 267 accommodate shorter ULC acyl-CoAs, leading to incomplete elongation of acyl-CoAs (in 268 the case of polyunsaturated acyl-CoAs, up to C32; Figs 4 and 6). The effects of the Q180P, 269 T233M, and W246G mutations on the elongation of polyunsaturated ULC acyl-CoA were 270 greater than those of L168F and I171T (Fig. 4). There was thus a correlation between the 271 degree of ULC-PC shortening and the age of disease onset (see Introduction) (12, 50). The 272 Q180P mutation affected the elongation of polyunsaturated and monounsaturated ULC 273 acyl-CoAs, but did not affect saturated ULC acyl-CoAs (Fig. 4). The Gln180 residue is 274 present in TMH5, so its  $\alpha$ -helix structure is likely disrupted by the Gln-to-Pro substitution, 275 leading to a structural change in the substrate-binding pocket that alters the substrate 276 specificity depending on the saturation/unsaturation status.

In this study, we found that the levels of ≥ C52 PCs were lower in Q180P and W246G
heterozygous KI neurons compared with control neurons (Fig. 6B). In addition, Q180P

279 heterozygous KI neurons showed a decrease in total ULC-PC levels. These results suggest 280 that the shortening of ULC PUFAs and reduction in ULC-PCs in neurons are involved in 281 the pathogenesis of SCA34. The shortening of ULC PUFAs is particularly important, since 282 it was observed in all five SCA34 mutations (Fig. 4). The  $\geq$  C52 ULC-PCs may play an 283 important role in neuronal functions; hence, they cannot be replaced by shorter ULC-PCs. 284 Although the presence of ULC-PCs has long been recognized (33, 34), their function in 285 neurons remains unknown. Neurons like the Purkinje cells in the cerebellum have very 286 complex structures, including highly branched neurites and synapses. Given the special structure of ULC-PCs, with a ULC PUFA in the sn-1 position, they may be enriched in 287 288 membranes that make up complex structures like neurites and synapses, and the shortening 289 of ULC PUFAs may impair their function in these structures.

In this study, we also analyzed the R216X mutation, one of the causative mutations in recessively inherited ISQMR. In a cell-based assay, the R216X mutant did not exhibit activity for the production of ULC ceramides or ULC-PCs (Fig. 4). Thus, the R216X mutation has a loss-of-function effect, which is consistent with the recessive inheritance mode of ISQMR. Since the R216X mutant lacks TMH6 and subsequently the C-terminal region, it cannot form the substrate-binding pocket for acyl-CoAs.

Although the R216X ISQMR mutant did not exhibit activity, SCA34 mutants had residual activity. The neurological symptoms of ISQMR are severe and become evident within the first few months to 1 year after birth (29). In contrast, the symptoms of SCA34 are milder than those of ISQMR, with the onset of disease mostly observed in middle age
(12, 50). Thus, SCA34 pathology may develop due to long-term decreases and/or changes
in the quality (shortening) of ULC-PCs in neurons in the cerebellum, which may be more
susceptible than other brain regions.

303 We speculate that the cause of the dominant inheritance mode of SCA34 is the 304 substitution of highly functional  $\geq$  C52 ULC-PCs (ULCFA moiety, mainly  $\geq$  C34) for 305 weakly functional  $\leq$  C50 ULC-PCs (ULCFA moiety, mainly  $\leq$ C32) (Fig. 7). This 306 substitution may be related to the continuity of acyl-CoA elongation in the FA elongation 307 cycle involving ELOVL4. The FA elongation cycle consists of four sequential reactions 308 starting from condensation by ELOVLs, then reduction by 3-ketoacyl-CoA reductase, 309 dehydration by 3-hydroxyacyl-CoA reductases, and reduction by trans-2-enoyl-CoA 310 reductase (4, 5). These are all ER-resident proteins and may form complexes (4, 25, 52), 311 which enables efficient and sequential progress of reactions. Since ELOVL4 is the only 312 enzyme involved in the elongation of ULC acyl-CoAs from C24 or C26 to C34-C38, it is 313 reasonable to assume that the acyl-CoA synthesized in the previous cycle enters the next 314 cycle catalyzed by the same complex, rather than dissociate from the previous complex and 315 find and associate with another complex. Thus, one C24/C26 acyl-CoA may be elongated 316 repetitively to become C34-C38 by the same ELOVL4-containing complex (Fig. 7). 317 However, it is likely that C24/C26 acyl-CoA may be elongated only up to C32 by 318 FA-elongation complexes that contain an SCA34 mutant; they would then be released from the complex and used for PC synthesis. Although carriers that are heterozygous for the ISQMR mutation are predicted to have half the ELOVL4 activity of normal individuals, they do not develop symptoms (29–31). This may be because ULC acyl-CoAs are fully elongated by the remaining WT ELOVL4 (Fig. 7). In other words, SCA34 is induced by shortening of ULC PUFAs due to incomplete elongation reactions, rather than decreased activity of ELOVL4.

In summary, this is the first biochemical analysis of SCA34 mutations, and our 325 326 findings suggest that the molecular mechanism underlying the pathogenesis of SCA34 is a 327 decrease in the levels and/or shortening of ULC-PCs. Furthermore, the dominant 328 inheritance mode of SCA34 is caused by the substitution of  $\geq$  C52 ULC-PCs with  $\leq$  C50 329 ULC-PCs. Another SCA subtype, namely SCA38, is caused by mutations in ELOVL5 (53-330 55), which is responsible for the elongation of polyunsaturated acyl-CoAs from C18 to C22 331 (Fig. 4B) (4, 5, 7). This suggests a link between SCA34 and SCA38 and raises the 332 possibility that the neural symptoms in these disorders are caused by the same or a similar 333 molecular mechanism (that is to say, the shortening of ULC-PCs). Future research is 334 needed to investigate whether SCA38 and other SCA subtypes are also accompanied by 335 changes in the quantity or quality of ULC-PCs.

#### 336 MATERIALS AND METHODS

Amino acid mapping. Five amino acid residues of ELOVL4 that are substituted in SCA34
were mapped onto the crystal structure of human ELOVL7 (PDB ID: 6Y7F) using PyMOL
software (version 2.5.2; Schrödinger, New York, NY, USA).

340

341 Plasmids. The mammalian expression vectors pEFh-3×FLAG-1 (56) and pCE-puro 342 3×FLAG-1 (57) were used for the expression of N-terminally 3×FLAG-tagged proteins, 343 while the mammalian expression vector pCE-puro HA-1 (52) was used for the expression 344 of N-terminally HA-tagged protein. The pCE-puro 3×FLAG-CERS3 and pCE-puro 345 HA-ELOVL2 plasmids have been described previously (52, 58). The 346 pEFh-3×FLAG-ELOVL4 plasmid was constructed by transferring *ELOVL4* from pCE-puro 347 3×FLAG-ELOVL4 (59) to the pEFh-3×FLAG-1 vector. The ELOVL4 mutants were 348 generated via overlap extension PCR using pCE-puro 3×FLAG-ELOVL4 plasmid as a 349 template and primers (Table 1), followed by cloning into the pEFh-3×FLAG-1 vector to 350 produce pEFh-3×FLAG-mutated ELOVL4 plasmids.

The all-in-one CRISPR/Cas9 vector pYU751 consists of a WT *Cas9* nuclease, a guide RNA (gRNA) cloning cassette, *EGFP*, and the gene that encodes puromycin *N*-acetyltransferase. This vector was constructed from the GeneArt CRISPR Nuclease Vector with the OFP plasmid (Thermo Fisher Scientific, Waltham, MA, USA) by incorporating the puromycin *N*-acetyltransferase gene and substituting the *ORF* reporter with *EGFP*. To generate Q180P and W246G *Elovl4* KI mouse ES cells, a pair of
oligonucleotides (Table 2) encoding gRNAs targeting exon 4 (for Q180P) and exon 6 (for
W246G) of *Elovl4* were annealed and cloned into the *Bae*I site of pYU751, generating the
plasmids pUKA27 and pUKA16, respectively.

360

361 Cell culture and transfection. HEK 293T cells (RCB2202; Riken BioResouce Research 362 Center, Tsukuba, Japan) and HeLa cells (RCB0007; Riken BioResouce Research Center) 363 were cultured in Dulbecco's Modified Eagle's Medium containing 4,500 mg/L glucose 364 (D6429; Merck, Darmstadt, Germany) and 1,000 mg/L glucose (D6046; Merck), respectively, supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL 365 366 streptomycin. HEK 293T cells were grown on collagen-coated dishes. E14tg2a mouse ES 367 cells (AES0135; Riken BioResouce Research Center) were cultured in Glasgow's MEM 368 (Thermo Fisher Scientific) that was supplemented with 10% FBS, 0.1 mM MEM 369 Non-Essential Amino Acids Solution (Thermo Fisher Scientific), 1 mM sodium pyruvate 370 (Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol (FUJIFILM Wako Pure Chemical, 371 Osaka, Japan), 1,000 units/mL mouse Leukemia Inhibitory Factor (FUJIFILM Wako Pure 372 Chemical), 100 units/mL penicillin, and 100 µg/mL streptomycin, and were grown on a 373 0.1% gelatin-coated dish. All cells were cultured at 37 °C in 5% CO<sub>2</sub> and used in less than 374 20 passages. Transfections were performed using Lipofectamine Transfection Reagent with PLUS Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. 375

377 Indirect immunofluorescence microscopy. HeLa cells and differentiated ES cells were 378 subjected to indirect immunofluorescence microscopy as previously described (60). Rabbit 379 anti-FLAG polyclonal antibody (1:2,000 dilution) (61), mouse anti-calnexin monoclonal 380 PM060 antibody (RRID: AB 10597560; 1:400 dilution; Medical & Biological Laboratories, 381 Tokyo, Japan), and mouse anti-class III β-tubulin monoclonal MAB1195 antibody (RRID: 382 AB 357520; 1:500 dilution; R&D Systems, Minneapolis, USA) were used as primary 383 antibodies. Alexa Fluor 488-conjugated anti-mouse IgG antibody (RRID: AB 2534069) 384 and Alexa Fluor 594-conjugated anti-rabbit IgG antibody (RRID: AB 2534073; each 1:200 385 dilution; Thermo Fisher Scientific) were used as secondary antibodies, and DAPI (1 386 µg/mL) was added simultaneously. Cover slips were mounted with ProLong Gold Antifade 387 Reagent (Thermo Fisher Scientific) and observed using a Leica DM5000B microscope 388 (Leica Microsystems, Wetzlar, Germany).

389

**Immunoblotting.** Immunoblotting was performed as previously described (60), using rabbit anti-FLAG polyclonal antibody (1:1,000 dilution) (61) and mouse anti- $\alpha$ -tubulin monoclonal T9026 antibody (RRID: AB\_477593; 1:1,000 dilution; Merck) as primary antibodies and anti-rabbit and anti-mouse IgG, HRP-linked F(ab')<sub>2</sub> fragments (each 1:7,500 dilution; GE Healthcare Life Sciences, Little Chalfont, UK) as secondary antibodies. Chemiluminescence detection was performed using chemiluminescence solution; this 396 solution consisted of 100 mM Tris-HCl (pH 8.5), 0.2 mM *p*-coumaric acid (Merck), 2.5

mM luminol (FUJIFILM Wako Pure Chemical), and 0.002% hydrogen peroxide.

398

399 Generation of SCA34 KI ES cells. For homology-directed repair, the single-stranded 400 oligodeoxynucleotides (ssODNs) mElovl4Q180P ssODN and mElovl4W246G ssODN 401 were designed. Each ssODN contained the mutant sequence and bilateral homology arms 402 for homology-directed repair in ES cells (Table 3). For each ssODN, the non-target strand, 403 which was not complementary to the gRNA, was chosen to avoid heteroduplex formation 404 gRNA with (49). In the mutant sequence, mElovl4Q180P ssODN and 405 mElovl4W246G ssODN contained ApaI and SphI restriction enzyme sites, respectively; 406 these were introduced to examine the presence of mutations at the *Elovl4* locus. To obtain 407 Elovl4 Q180P KI cells and W246G KI cells, ES cells were transfected with pUKA27 and 408 mElovl4Q180P ssODN (for Q180P KI cells), pUKA16 and mElovl4 W246G ssODN (for 409 W246G KI cells), or empty pYU751 vectors (as controls). Twenty-four hours after 410 transfection, the cells were treated with 2  $\mu$ g/mL puromycin and incubated for another 24 h 411 to eliminate untransfected cells. The cells that survived were subsequently diluted and 412 cultured in the absence of puromycin for 9 days. Single colonies were isolated using 413 cloning rings, then expanded and stored. Genomic DNAs were extracted from the cells, and 414 the DNA fragments containing the target sites were amplified by PCR. The amplified DNA 415 fragments were digested with restriction enzymes (ApaI for Q180P and SphI for W246G) to

416 examine the presence of the mutations. Positive clones identified by restriction enzyme417 analysis were subjected to sequence confirmation by Sanger sequencing.

418

419 Neuronal differentiation of ES cells. Embryoid bodies were formed by growing ES cells 420 in embryoid body formation medium; this mixture consisted of equal volumes of Advanced 421 DMEM/F12 (Thermo Fisher Scientific) and Neurobasal Medium (Thermo Fisher 422 Scientific), supplemented with 10% KnockOut Serum Replacement (Thermo Fisher 423 Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 100 units/mL penicillin, 100 424 µg/mL streptomycin, and 0.1 mM 2-mercaptoethanol. Two days later, the embryoid bodies 425 were cultured in embryoid body formation medium that was supplemented with 5  $\mu$ M 426 retinoic acid. Three days later, differentiation of the embryoid bodies into neurons was 427 induced by replacing the medium with neuronal differentiation medium. This medium 428 consisted of a mixture of equal volumes of Advanced DMEM/F12 and Neurobasal Medium, 429 supplemented with 2% B-27 Supplement (Thermo Fisher Scientific), 2 mM L-glutamine, 430 100 units/mL penicillin, and 100 µg/mL streptomycin. On the following day, the embryoid 431 bodies were digested with 0.25% Trypsin/EDTA (Merck), dispersed in Leibovitz's L-15 432 Medium (Thermo Fisher Scientific) supplemented with 0.2 mg/mL DNase I (Merck), and 433 seeded onto a poly-D-lysine/laminin-coated dish in the neuronal differentiation medium for 434 an additional 4 days.

436 Lipid analyses. For the analysis of ceramides and PCs, HEK 293T cells were transfected
437 with the following combinations of plasmids: pEFh-3×FLAG-ELOVL4 (WT or one of the
438 mutants), pCE-puro 3×FLAG-CERS3, and pCE-puro HA-ELOVL2 plasmids for analysis
439 of ceramides; pEFh-3×FLAG-ELOVL4 (WT or one of the mutants) and pCE-puro
440 HA-ELOVL2 plasmids for analysis of PCs. Forty-eight hours after transfection, cells were
441 collected and subjected to lipid extraction.

For the extraction of ceramides from HEK 293T cells, the cells were suspended in 100 442 443 µL of water and mixed successively with 375 µL of chloroform/methanol/12 M formic acid 444 solution (100:200:1, v/v), 125  $\mu$ L of chloroform, and 125  $\mu$ L of water. After centrifugation, 445 the organic phase was recovered and treated with 71 µL of 0.5 M NaOH for 1 h at 37 °C to 446 hydrolyze ester bonds in the glycerolipids. The reaction mixture was neutralized via the 447 addition of 35.5 µL of 1 M formic acid and mixed successively with 135 µL of methanol 448 and 210 µL of water. After centrifugation, the organic phase was recovered and dried. For 449 the extraction of PCs from HEK 293T or ES cells, the cells were suspended in 100 µL of 450 water and mixed successively with 375 µL of chloroform/methanol/12 M formic acid 451 solution (100:200:1, v/v), 0.5 pmol of seven deuterium ( $d_7$ )-labeled internal standard 452 (15:0/18:1-d7-PC; Avanti Polar Lipids, Alabaster, USA), 125 µL of chloroform, and 125 µL 453 of water. After centrifugation, the organic phase was recovered and dried.

454 The dried lipids were suspended in a chloroform/methanol solution (1:2, v/v), and LC–
455 MS/MS analyses of ceramides and PCs were performed using an LC-coupled triple

456	quadrupole mass spectrometer Xevo TQ-S (Waters, Milford, MA, USA). Ceramides and
457	PCs extracted from HEK 293T cells were separated by LC using an ACQUITY UPLC
458	CSH C18 column (1.7 $\mu m$ particle size, 2.1 $\times$ 100 mm; Waters), and PCs extracted from ES
459	cells were separated by LC using a YMC-Triant C18 metal-free column (1.9 $\mu$ m particle
460	size, $2.1 \times 50$ mm; YMC, Kyoto, Japan). Lipid separation by LC was performed at a flow
461	rate of 0.3 mL/min for 25 min using a gradient system, in which mobile phase A and
462	mobile phase B were mixed as follows: 0 min, 40% B; 0-18 min, gradient to 100% B; 18-
463	23 min, 100% B; 23 min, return to 40% B; 23-25 min, 40% B. Mobile phase A consisted
464	of an acetonitrile/water solution (3:2, $v/v$ ) that contained 5 mM ammonium formate, while
465	mobile phase B consisted of an acetonitrile/2-propanol solution (1:9, $v/v$ ) that contained 5
466	mM ammonium formate. Separated lipids were ionized by electrospray ionization and
467	analyzed in positive ion mode. Quantitative analyses were performed in the multiple
468	reaction monitoring mode of the MS/MS using selected $m/z$ values and appropriate
469	collision energies and cone voltages (Tables 4 and 5). Data analyses were performed using
470	MassLynx software (Waters). Ceramides were quantified using the external standard curve
471	generated from C30:0 ceramide (Cayman Chemical, Ann Arbor, MI, USA) and expressed
472	as the proportion of each ceramide species to the sum of all measured ceramide species
473	listed in Table 4. PCs were quantified by calculating the ratio of the peak area of each PC
474	species to that of the internal standard (15:0/18:1- $d_7$ -PC).

476	Real-time quantitative RT-PCR. Total RNAs were isolated from ES cells using the
477	NucleoSpin RNA Kit (Takara Bio, Kusatsu, Japan), and cDNAs were synthesized from
478	total RNAs using the PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio). Real-time
479	quantitative RT-PCR was performed using the KOD SYBR qPCR Mix (TOYOBO, Osaka,
480	Japan), cDNAs, and specific forward and reverse primers for the respective genes (Table 6).
481	The reaction was performed on a CFX96 Touch real-time PCR detection system (Bio-Rad,
482	Hercules, CA, USA). The mRNA levels were normalized to that of Actb.
483	
484	Quantification and statistical analysis. Data are presented as means + SD. Tests for
485	normality of the data and outliers were not performed. Differences between groups were
486	evaluated using Dunnett's test in JMP 13 software (SAS Institute, Cary, NC, USA), and
487	p-values of less than 0.05 were considered to be statistically significant.
488	
489	Ethical approval. No ethical approval was required for this study.

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- 495

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733		

735 FIG 1 Multiple alignment of ELOVL1-7. Amino acid sequences of human ELOVL1-736 7 proteins were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). 737 The numbers in the right-hand margin refer to the adjacent amino acid residues. Amino 738 acid residues conserved in all seven isozymes are indicated by black boxes, while residues 739 conserved in five or six isozymes are indicated by gray boxes. SCA34-substituted residues 740 in ELOVL4 and corresponding residues in ELOVL7 are indicated by cyan and orange 741 boxes, respectively. The catalytically important histidine box is marked by a red rectangle. 742 743 FIG 2 Positions of the amino acid residues of ELOVL4 that are substituted in SCA34 744 mapped to the crystal structure of ELOVL7. Five amino acid residues of ELOVL4 that are 745 substituted in SCA34 (magenta) mapped on the ribbon diagram of the human ELOVL7 746 structure with the bound acyl-CoA analog containing the C20:0 FA moiety (PDB: 6Y7F) 747 (45). The catalytically important histidine box (H-box) is shown in lime green. 748 749 FIG 3 Localization of ELOVL4 SCA34 mutant proteins in the ER. HeLa cells were

transfected with a vector or each of the *3×FLAG-ELOVL4*-encoding plasmids: WT and five
SCA34 mutants (L168F, I171T, Q180P, T233M, and W246G). Twenty-four hours after

transfection, the cells were fixed and subjected to indirect immunofluorescence microscopy

vising anti-FLAG antibody (red) and anti-calnexin antibody (green), which mark the ER.
Nuclei were stained with DAPI (blue). Scale bar, 10 µm.

755

756 FIG 4 Incomplete elongation of ULC PUFAs by ELOVL4 SCA34 mutants. (A) 757 Structures of a representative ULC PUFA-containing ceramide (upper) and ULC-PC 758 (lower): a ceramide that contains C32:6 FA (upper) and a PC that contains FAs (lower) 759 making a total of C50:7 (C32:6 FA in the *sn*-1 position plus C18:1 in the *sn*-2 position). (B) 760 Schematic representations of PUFA elongation pathways (n-6 and n-3 series). Linoleic 761 acid (C18:2n-6) and  $\alpha$ -linolenic acid (C18:3n-3) are subject to a series of reactions, 762 including desaturation, elongation by ELOVL isozymes, and  $\beta$ -oxidation, and some of the 763 products are elongated to ULC PUFAs by ELOVL4. The letter E denotes ELOVL 764 isozymes and font size reflects the relative strength of their activities. (C-G) HEK 293T 765 cells were transfected with a vector or each of the  $3 \times FLAG$ -ELOVL4-encoding plasmids 766 (WT, L168F, I171T, Q180P, T233M, W246G, and R216X), together with 767 3×FLAG-CERS3- and HA-ELOVL2-encoding plasmids (C-E) or HA-ELOVL2-encoding 768 plasmid (F and G) for 48 h. (C and F) Total lysates prepared from the transfected cells were 769 separated by SDS-PAGE, followed by immunoblotting with anti-FLAG, anti-HA, and 770 anti- $\alpha$ -tubulin (for loading control) antibodies. (D, E, G) Lipids were extracted from the 771 transfected cells, and ceramides (D and E) or PCs (G) containing the indicated FA moiety 772 were analyzed by LC-MS/MS. PC species are expressed as the sum of the chain-length and

773	the number of double bonds of two constituent FAs. (D and G) Heatmaps presenting the
774	levels of ceramide (D) or PC (G) species in each kind of ELOVL4 mutant-expressing cell
775	relative to those in ELOVL4 WT-expressing cells ( $n = 3$ independent cell cultures). (E)
776	Percentages of the C32:6 and C38:6 ceramides among the total ceramides quantified in (D).
777	Values represent means + SD ( $n = 3$ independent cell cultures). Significant differences in
778	comparison to ELOVL4 WT-expressing cells (* $P < 0.05$ ; ** $P < 0.01$ ; Dunnett's test) are
779	indicated.

781 Differentiation of ES cells into neurons induces Elovl4 expression and ULC-PC FIG 5 782 production. ES cells were differentiated into neurons in neuronal differentiation medium for 783 5 days. The cells were harvested for RNA and lipid extractions prior to the initiation of 784 differentiation (day 0), on day 1, and on day 5. (A) On days 0, 1, and 5, bright-field images 785 of live cells were captured, then the cells were fixed and subjected to indirect 786 immunofluorescence microscopy using anti-class III β-tubulin antibody (green). Nuclei 787 were stained with DAPI (blue). Scale bars, 100 µm (day 0) and 25 µm (days 1 and 5). (B) 788 Total RNAs were prepared from the cells on days 0, 1, and 5, and were subjected to 789 real-time quantitative RT-PCR using specific primers for *Elovl4* or *Actb*. Values presented 790 are the mean (+ SD) levels of *Elovl4* mRNA relative to those of *Actb* (3 independent cell 791 cultures). (C) Lipids were extracted from the cells on days 0, 1, and 5, and ULC-PCs were

analyzed by LC–MS/MS. Values presented are mean (+ SD) levels of ULC-PCs (3
independent cell cultures).

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- 795

796 FIG 6 Incomplete elongation of ULC PUFAs in ELOVL4 Q180P and W246G KI 797 neurons. (A) The gene structure of Elovl4 and sequence chromatograms of the Q180P and 798 W246G mutations in control, heterozygous Q180P, heterozygous W246G, and 799 homozygous W246G KI ES cells. (B and C) Control and KI ES cells were differentiated 800 into neurons for 1 (B) and 5 days (B and C). (B) Lipids were extracted and the PC species 801 C44:5-C56:7, which are expressed according to the sum of their chain length and the 802 number of double bonds in their two constituent FAs, were analyzed by LC-MS/MS. 803 Values in the left panels on days 1 and 5 represent mean (+ SD) levels of PCs (3 804 independent cell cultures), with each PC species being color-coded. Values in the right 805 panels on days 1 and 5 represent the percentages of  $\geq$  C52 ULC-PCs among the sum of 806 C44:5–C56:7 PCs (3 independent cell cultures). Significant differences from control cells 807 (\*P < 0.05; \*\*P < 0.01; Dunnett's test) are indicated. (C) Total RNAs were prepared from 808 the cells and subjected to real-time quantitative RT-PCR using specific primers for Elovl2, 809 Elovl4, Elovl5, Tubb3, or Actb. Values presented are mean (+ SD) levels of each mRNA 810 relative to those of Actb and expressed as a ratio to the control cells (3 independent cell 811 cultures). Significant differences from control cells (\*P < 0.05; \*\*P < 0.01; Dunnett's test) 812 are indicated. Het, heterozygous; Homo, homozygous.

814 Model for the dominant mode of inheritance in SCA34. One C24/C26 FIG 7 815 acyl-CoA synthesized by ELOVL5 and ELOVL2 is elongated by the same WT or SCA34 816 mutant ELOVL4 protein (black arrows). In normal cases, in which both ELOVL4 alleles are 817 WT, C24/C26 acyl-CoAs are elongated to become C34–C38; this process produces highly 818 functional ULC-PCs. In patients with ISQMR, in whom both ELOVL4 alleles are ISQMR mutants, C24/C26 acyl-CoAs cannot be elongated by the ISQMR mutant protein (gray 819 820 arrows with crosses), and there is no production of  $\geq$  C28 ULC-PCs. In ISQMR carriers, in 821 whom one allele is WT and the other allele is an ISQMR mutant, all C24/C26 acyl-CoAs 822 are elongated by the remaining WT protein; thus, normal levels of highly functional 823 ULC-PCs may be produced. In patients with SCA34 in whom one allele is WT and the 824 other allele is an SCA34 mutant, a proportion of C24/C26 acyl-CoAs are elongated 825 normally to C34-C38 by the WT protein. The remaining proportion is elongated by the 826 SCA34 protein only up to C32, thereby producing weakly functional ULC-PCs. Gray 827 arrows indicate reduced elongation.

Primer name	Sequence
ELOVL4_F	5'- <u>GGATCC</u> ATGGGGCTCCTGGACTCGGAGCCGG-3'
	(BamHI)
ELOVL4_R	5'-TTAATCTCCTTTTGCTTTTCCATTTTTC-3'
E4_L168F-1	5'-TGTTTACCTTCTGGTGGATTGGAATTAAGTG-3'
E4_L168F-2	5'-CAATCCACCAGAAGGTAAACATCGTACAGTG-3'
E4_I171T-1	5'-TTGTGGTGGACTGGAATTAAGTGGGTTGCAG-3'
E4_I171T-2	5'-CTTAATTCCAGTCCACCACAAGGTAAACATC-3'
E4_Q180P-1	5'-GCAGGAGGACCAGCATTTTTTGGAGCCCAGTTG-3'
E4_Q180P-2	5'-AAAAAATGCTGGTCCTCCTGCAACCCACTTAATTC-3'
E4_T233M-1	5'-ATTGGGCACATGGCACTGTCTCTTTACACTG-3'
E4_T233M-2	5'-AGACAGTGCCATGTGCCCAATGGTCACATGG-3'
E4_W246G-1	5'-CTTCCCCAAAGGGATGCACTGGGCTCTAATTG-3'
E4_W246G-2	5'-CAGTGCATCCCTTTGGGGGAAGGGGCAGTCAG-3'
E4_R216X-1	5'-TTGGTGGAAATGATACCTGACTATGTTGCAAC-3'
E4_R216X-2	5'-GTCAGGTATCATTTCCACCAAAGATATTTCTG-3'

829 **TABLE 1** Oligonucleotides used for the generation of mutated *ELOVL4* plasmids.

830 The restriction enzyme *Bam*HI used for cloning into the expression vector is noted in

831 parentheses, with its sequence underlined.

832	TABLE 2	Oligonucleotides	used for the generati	on of CRISPR/Cas9 plasmids.
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Oligonucleotide name	Sequence	Plasmid
		name
mElov14_Q180P-gRNA_	5'-GAAAGGTGGGTCCCTCACCTGTTTT-3'	pUKA27
F1		
mElov14_Q180P-gRNA_	5'-AGGTGAGGGACCCACCTTTCCGGTG-3'	
R1		
mElovl4_W246G-gRNA	5'-AGAGCCCAGTGCATCCACTTGTTTT-3'	pUKA16
_F1		
mElovl4_W246G-gRNA	5'-AAGTGGATGCACTGGGCTCTCGGTG-3'	
_R1		

### 834 TABLE 3 ssODNs used for homology-directed repair

Elovl4 mutation	Sequence
Q180P	5'-CTAGGTAAACCTACTCCCACCACATGGGGGGTTCAGAC
	AGAAAGGTGGGTCCCTCACC <u>GGGGCCC</u> TCCAGCCACCCAC
	TTGATTCCAATCCACCACAGAGTGAACATGGTGCAGTGGT
	GGT-3' (ApaI)
W246G	5'-GTAGAAGTTGAGGAAGAGGAAGATGAAGCTGATGGCG
	TAGGCGATCAGAGCCCAGT <u>GCATGC</u> CCTTGGGGAAGGGG
	CAGTCGGTGTAGAGAGACAGTGCTGTGTGTCCGATGGTC
	ACGTGGAA-3' (SphI)

835 The restriction enzymes used for genotyping KI cells are noted in parentheses, with their

836 sequences underlined.

Acyl chain	Precursor ions $(Q1, m/z)$		Product ion	Collision
	$\left[M-H_2O+H\right]^+$	$\left[M + H\right]^+$	(Q3, m/z)	energy (eV)
C18:0	548.6	566.6	264.3	20
C20:0	576.6	594.6	264.3	20
C22:0	604.6	622.6	264.3	25
C24:0	632.6	650.6	264.3	30
C26:0	660.7	678.7	264.3	30
C28:0	688.7	706.7	264.3	30
C30:0	716.7	734.7	264.3	35
C32:0	744.8	762.8	264.3	40
C34:0	772.8	790.8	264.3	40
C18:1	546.6	564.6	264.3	20
C20:1	574.6	592.6	264.3	20
C22:1	602.6	620.6	264.3	25
C24:1	630.6	648.6	264.3	30
C26:1	658.7	676.7	264.3	30
C28:1	686.7	704.7	264.3	30
C30:1	714.7	732.7	264.3	35
C32:1	742.8	760.8	264.3	35
C34:1	770.8	788.8	264.3	40
C36:1	798.8	816.8	264.3	40
C38:1	826.8	844.8	264.3	40
C24:4	624.6	642.6	264.3	30
C26:4	652.7	670.7	264.3	30

838 TABLE 4 Selected m/z values and collision energies for the detection of ceramide species

839 in the LC–MS/MS analysis.

C28:4	680.7	698.7	264.3	30
C30:4	708.7	726.7	264.3	35
C32:4	736.8	754.8	264.3	35
C34:4	764.8	782.8	264.3	40
C36:4	792.8	810.8	264.3	40
C38:4	820.9	838.9	264.3	40
C30:5	706.7	724.7	264.3	35
C32:5	734.7	752.7	264.3	35
C34:5	762.8	780.8	264.3	40
C36:5	790.8	808.8	264.3	40
C38:5	818.8	836.8	264.3	40
C30:6	704.7	722.7	264.3	35
C32:6	732.7	750.7	264.3	35
C34:6	760.8	778.8	264.3	40
C36:6	788.8	806.8	264.3	40
C38:6	816.8	834.8	264.3	40

Sum of two acyl chains	Precursor ion $(Q1, m/z)$	Product ion (Q3, $m/z$ )
C33:1- <i>d</i> <sub>7</sub>	753.5	184.0
C44:5	892.8	184.0
C44:6	890.8	184.0
C44:7	888.7	184.0
C46:5	920.8	184.0
C46:6	918.8	184.0
C46:7	916.8	184.0
C48:5	948.8	184.0
C48:6	946.8	184.0
C48:7	944.8	184.0
C50:5	976.9	184.0
C50:6	974.9	184.0
C50:7	972.8	184.0
C52:5	1004.9	184.0
C52:6	1002.9	184.0
C52:7	1000.9	184.0
C54:5	1032.9	184.0
C54:6	1030.9	184.0
C54:7	1028.9	184.0
C56:5	1061.0	184.0
C56:6	1059.0	184.0
C56:7	1056.9	184.0

**TABLE 5** Selected m/z values for the detection of PC species in the LC–MS/MS analysis.

842 Collision energy and cone voltage were 30 eV and 15 V, respectively.

Primer name	Sequence
mElovl4-rt_F1	5'-ACGTGATCATGTACTCCTACTATGG-3'
mElovl4-rt_R1	5'-CCGTTCGATGAGATACCATTCGTGG-3'
mTubb3-rt_F3	5'-GAGGCCTCCTCTCACAAGTATGTGC-3'
mTubb3-rt_R3	5'-GTTGCCAGCACCACTCTGACCAAAG-3'
mElovl2-rt_F	5'-GCTGGTCATCCTGTTCTTAAACTTC-3'
mElovl2-rt_R	5'-TTATTGAGCCTTCTTGTCCGTCATG-3'
mElov15-rt_F2	5'-TCGGGTGGCTGTTCTTCCAGATTGG-3'
mElov15-rt_R	5'-AGGGAAGCTGTTGGTGTGTGTCCGTTG-3'
Mouse_Actb_primer	Mouse Housekeeping Gene Primer Set (Takara Bio)

**TABLE 6** Primers used for real-time quantitative RT-PCR.

ELOVL1       MERVYNLYQEVMKHADPRIGGYPLMGSPLL-MTSILLTYVF 41         SLOVL2       MEHLKAPDDEINAFLDNMFGPRDSRVRGWFMLDSYLP-TFPLTVMLLS       44         SLOVL3       MVTAMNVSHEVNQLFGPINFELSK-DMRPFFEEVMATSPIALILVL       45         SLOVL4       MGLLDSEPGSVLNVSTALMDTVFYRWSIADKRVENWFLDSTPTLSISTLYLF       55         SLOVL5       MEHLKAFDDEINAFLDNNFGPRDTVKGWFNLDNYIP-TFSVILLSISTLYLF       55         SLOVL6       MNMSVLTLQEVEFERVGFRENEAIGMNQENKKSFLFSALVAAF       43         SLOVL7       MARSDLTSRTVHLYDNWKDDAPRVEDWLLMSSPLPOTILLGFUVF       47         SLOVL1       VLSICPRIMARKFGLRGFMIVTFSLVALSLY			
ELOVL2       MEHLKAPDDEINAFLDNMFGPRDSKVKGWFMLDSXUP-TFPLTVMVLLS       44         ELOVL3       MUTAMNUSHEVNQLGPVNFEL-SK-DK-DWFFFEEVWATSPIALIVLV       44         ELOVL5       MEHFDASLSTVFKALLGPRDTEVKGWFLLDNYIP-TFICSVILL       44         ELOVL6       MENFDASLSTVFKALLGPRDTEVKGWFLLDNYIP-TFICSVILL       44         ELOVL6       MENFDASLSTVFKALLGPRDTEVKGWFLDNYIP-TFICSVILL       44         ELOVL7       MAFSDLTSRVKLYDNIKDADPRVEDWLLMSSPLP-OTILLGFUVF       47         SLOUL1       I-MIGNKYKKNPALSLRGTILLVNIFSLVALSLY	ELOVL1	MEAVVNLYQEVMKHADPRIQGYPLMGSPLL-MTSILLT <mark>y</mark> vyf	41
ELOVL3       MYTAMNUSHEUNQLFOPYNFELSK-DNRPFFEUWARSFFIALI [1]       47         ELOVL4       MGLLDSEPGSVLNVVSTALNDTVEFYRWEN IAKREWNPLMOSPWF-TLSISTLLF       45         ELOVL5       MHRSVLTLQEYEFRKOFNEN-ERIQWMCENNKSFFFSALMAF       43         ELOVL6       NMRSVLTLQEYEFRKOFNEN-ERIQWMLMSSPLF-QTILLGFUVF       44         ELOVL7       WAFSDLTSRTVHLYDWIKADAPRVEDWLMSSPLF-QTILLGFUVF       45         ELOVL1       VLSIGPRIMANKPFQLRGFMIVYNFSLVALSLY	ELOVL2	MEHLKAFDDEINAFLDNMFGPRDSRVRGWFMLDSYLP-TFFLTVM <mark>Y</mark> LLS	48
ELOVLAMGLLDSEPGSVLNVVSTALDTVË FYRMINSIADKRVENNPLMOSPWP-TLSISTLULL55ELOVLAMEHFDASLSTYFKALLGPRDTRVKGWFLLDNIP-TFICSVILLI44ELOVLAMAFSDLTSRTVILVDNIKDADPVEDWLLMSSPLP-QTILGFWVF47ELOVLAMAFSDLTSRTVILVDNIKDADPVEDWLLMSSPLP-QTILGFWVF47ELOVLAVLSLOPRIMANKFPOLRGFMIVVNFSLVALSLY	ELOVL3	MVTAMNVSHEVNOLFOPYNFELSKDMRPFFEEYWATSFPIALI <mark>y</mark> lvl	47
ELOUIS       MEHFDASLSTYFKALLOPRDTRVKGWPLIDNYFP-TFICSVILLI       44         ELOVIG       MNMSVLIQGYEFEKOFNENEAIQWMQENWKKSFLPSALMAF       45         ELOVID       MAFSDLTSRTVHLYDWNKKDADPRVEDWLMSSPLP-QTILLGFUVTF       45         ELOVID       VLSLGPRIMANRKPFQLRGFMIVUNFSLVALSLY	ELOVL4	MGLLDSEPGSVLNVVSTALNDTVEFYRWTWSIADKRVENWPLMOSPWP-TLSISTLVLLF	59
BIOVIG       MNMSULTLQEYEPEKQPNENEATQWMQENWKKSPIFSAL AAP         SLOVIG       MAFSDLTSRTVHLYDWNIKDADPRVEDWLMSSPLP-QYTLLGFYVF         SLOVI       VLSIGPRIMANKPFQLRGFMIVYNFSLVALSLY	ELOVL5	MEHEDASLSTYFKALLGPRDTRVKGWFLLDNYIP-TFICSVIVLLI	45
SLOVIT       MAPSDLTSRTUHLYDNWIKDADPRVEDWLLMSSPLP-QTILLGFUVTF         SLOVIT       MAPSDLTSRTUHLYDNWIKDADPRVEDWLLMSSPLP-QTILLGFUVTF         SLOVIT       VLSLGPRIMANNKPPQLRGFMIVVNFSLVALSLY	ELOVI.6	MNMSVI.TLOEYEFEKOFNENEATOWMOENWKKSFI.FSALVAAF	43
SLOVL1       VLSLGPRIMANKPF0LRGFNIVINFSLVALSLY	FLOVL7	MAESDLUS RUUHLVDNWIKDAD PRVEDWLLMSSPLP-OUTLLGEVVVF	47
ELOVL1       VLSLCPRIMANRKPFQLRGFMIVYNFSLVALSLYIVYEFLMSGWLSTYWRC       93         ELOVL2       I-WLGNKYKKNRPALSLRGILTLYNLGITLLSAYNLAELILSTWEGGNLCG       95         ELOVL2       I-WLGNKYKKNRPALSLRGILTLYNLGITLLSAYNLAELILSTWEGGNLCG       95         ELOVL2       I-WCGNKYKKNRPALSLRGILTLYNLGITLLSAYNLAELISTWEGGNLCG       95         ELOVL4       V-WLGPKWKNRPEPFQMRUVLIISVFCLAFFSLGPTLSLY			
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ELOVL7FGVKFAAGGLGTFHALLNTAVHVVMYSYYGLSALGPAYQKYLWWKKYLTSLQLVQFVIVA219ELOVL1LHISQYYFMSSCNYQYPVIIH-LIWMYGTIFFMLFSNFWYHSYTKGKRLPRAL265ELOVL2THTMS-AVVKPCGFPFGCLIFQSSYMLTLVILFLNFYVQTYRKKPMKKDMQEPP-270ELOVL3IVSILTYIWRQDQGCHTTMEH-LFWSFILY-MTYFILFAHFFCQTYIRPKVKAKTKSQ270ELOVL4GHTAL-SLYTDCPFPKWMHWALIAYAISFIFLFLNFYIRTYKEPKKPKAGKTA282ELOVL5IQTSC-GVIWPCTFPLGWLYFQIGYMISLIALFTNFYIQTYNKKGASRRKDHLKD268ELOVL6VVNYLVFCWMQHDQCHSHFQN-IFWSSLMY-LSYLVLFCHFFFEAYIGKMRKTTKAE265ELOVL7IHISQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFLHFWYRAYTKGQRLPKTV271ELOVL7-AG-KEVKNGFSKAYFTAANGVMNKKAQ296ELOVL4-MNGISANGVSKSEKQLMIENGKKQKNGKAKGD314ELOVL5HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD295ELOVL6KNCCKNKDN295ELOVL6KNCCKNKDN295ELOVL7KNCCKNKNN295ELOVL4MNGISANGVSKSEKQLMIENGKKQKNGKAKGD314ELOVL5HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD295ELOVL6KNCCKNKNN295ELOVL6KNC295ELOVL7KNCCKNKNN295ELOVL6KNC295ELOVL7KNCCKNKNN295ELOVL6KNC295ELOVL7KNCCKNKNN295ELOVL6KNC295ELOVL7KNCCKNKNN295ELOVL6KNC295ELOVL7KNCCKNKNN295EL	ELOVI.6	Y S Y K DM V A G G G WE - M T M N Y G V HA VM Y S Y YAL R A A G F R V S R K F A M F T WI. S O T T O M I. M G C	210
ELOVLILHISQYYFMSSCNYQYPVIIH-LIWMYGTIFFMLFSNFWYHSYTKGKRLPRAL265ELOVL1LHISQYYFMSSCNYQYPVIIH-LIWMYGTIFFMLFSNFWYHSYTKGKRLPRAL265ELOVL2THTMS-AVVKPCGFPFGCLIFQSSYMLTLVILFLNFYVQTYRKKPMKKDMQEPP-270ELOVL3IVSILTYIWRQDQGCHTTMEH-LFWSFILY-MTYFILFAHFFCQTYIRPKVKAKTKSQ270ELOVL4GHTAL-SLYTDCPFPKWMHWALIAYAISFIFLFLNFYIRTYKEPKKPKAGKTA282ELOVL5IQTSC-GVIWPCTFPLGWLYFQIGYMISLIALFTNFYIQTYNKKGASRRKDHLKD268ELOVL5IQTSC-GVIWPCTFPLGWLYFQIGYMISLIALFTNFYIQTYNKKGASRRKDHLKD268ELOVL6VVNYLVFCWMQHDQCHSHFQN-IFWSSLMY-LSYLVLFCHFFFEAYIGKMRKTTKAE265ELOVL7IHISQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFLHFWYRAYTKGQRLPKTV271ELOVL1-QQN-GAPGIAKVKAN276ELOVL2AG-KEVKNGFSKAYFTAANGVMNKKAQ296ELOVL3HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD295ELOVL6HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD295ELOVL7KNCECKNEDN295	ELOVI.7	<b>F</b> GUKEAAGGLGTEHALLNTAVHUVMYSYYGUSALGPAYOKYLWKKYLWSLOLVOEVIVA	210
ELOVL1LHISQYYFMSSCNYQYPVIIH-LIWMYGTIFFMLFSNFWYHSYTKGKRLPRAL265ELOVL2THTMS-AVVKPCGFPFGCLIFQSSYMLTLVILFLNFYVQTYRKKPMKKDMQEPP-270ELOVL3IVSILTYIWRQDQGCHTTMEH-LFWSFILY-MTYFILFAHFFCQTYIRPKVKAKTKSQ270ELOVL4GHTAL-SLYTDCPFPKWMHWALIAYAISFIFLFLNFYIRTYKEPKKPKAGKTA282ELOVL5IQTSC-GVIWPCTFPLGWLYFQIGYMISLIALFTNFYIQTYNKKGASRRKDHLKD268ELOVL6VVNYLVFCWMQHDQCHSHFQN-IFWSSLMY-LSYLVLFCHFFFEAYIGKMRKTTKAE265ELOVL7IHISQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFLHFWYRAYTKGQRLPKTV271ELOVL1-QQN-GAPGIAKVKAN279ELOVL2AG-KEVKNGFSKAYFTAANGVMNKKAQ296ELOVL4-MNGISANGVSKSEKQLMIENGKKQKNGKAKGD314ELOVL5HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD295ELOVL6VNCTCKNKDN295ELOVL7KNC705ELOVL4-MNGISANGVSKSEKQLMIENGKKQKNGKAKGD314ELOVL5HQNGSMAAVNGHTNSFSPLENNVKPRK-LRKD295ELOVL6KNC295ELOVL7KNC295ELOVL6295ELOVL7KNC295ELOVL6295ELOVL7KNC295ELOVL6295ELOVL7KNC295ELOVL7KNC295ELOVL6295ELOVL7KNC295ELOVL6295ELOVL7KNC295ELOVL7KNC295ELOVL7KNC295ELOVL7KNC295ELOVL6295			
ELOVL2       THTMS-AVVKPCGFPFGCLIFQSSYMLTLVILFLNFYVQTYRKKPMKKDMQEPP-270         ELOVL3       IVSILTYIWRQDQGCHTTMEH-LFWSFILY-MTYFILFAHFFCQTYIRPKVKAKTKSQ270         ELOVL4       GHTAL-SLYTDCPFPKWMHWALIAYAISFIFLFLNFYIRTYKEPKKPKAGKTA-282         ELOVL5       IQTSC-GVIWPCTFPLGWLYFQIGYMISLIALFTNFYIQTYNKKGASRRKDHLKD268         ELOVL6       VVNYLVFCWQHDQCHSHFQN-IFWSSLMY-LSYLVLFCHFFFEAYIGKMRKTTKAE265         ELOVL7       IHISQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFLHFWYRAYTKGQRLPKTV271         ELOVL1       -QQN-GAPGIAKVKAN       279         ELOVL2       -AG-KEVKNGFSKAYFTAANGVMNKKAQ       296         ELOVL4       -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD       314         ELOVL5       HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD       295         ELOVL6       KNC       295	ELOVL1	LHISOYYFMSSCNYOYPVIIH-LIWMYGTIFFMITSNTWYHSYTKGKRLPRAL	265
ELOVL3 IVSILTYIWRQDQGCHTTMEH-LFWSFILY-MTYFILFAHFFCQTYIRPKVKAKTKSQ 270 ELOVL4 GHTAL-SLYTDCPFPKWMHWALIAYAISFIFLFLNFYIRTYKEPKKPKAGKTA 282 ELOVL5 IQTSC-GVIWPCTFPLGWLYFQIGYMISLIALFTNFYIQTYNKKGASRRKDHLKD 268 ELOVL6 VVNYLVFCWMQHDQCHSHFQN-IFWSSLMY-LSYLVLFCHFFFEAYIGKMRKTTKAE 265 ELOVL7 IHTSQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFLHFWYRAYTKGQRLPKTV 271 ELOVL1 -QQN-GAPGIAKVKAN 279 ELOVL2AG-KEVKNGFSKAYFTAANGVMNKKAQ 296 ELOVL3 ELOVL4 -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD 314 ELOVL5 HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD 299 ELOVL6 KNC TCKNKDN	ELOVL2	THTMŚ-AVVKPCGFPFGCLIFOSSYMLTLVIIIJLNIYVOTYRKKPMKKDMOEPP-	270
ELOVL4       GHTAL-SLYTDCPFPKWMHWALIAYAISFIFLFLNFYIRTYKEPKKPKAGKTA       282         ELOVL5       IQTSC-GVIWPCTFPLGWLYFQIGYMISLIALFTNFYIQTYNKKGASRRKDHLKD       268         ELOVL6       VVNYLVFCWMQHDQCHSHFQN-IFWSSLMY-LSYLVLFCHFFFEAYIGKMRKTTKAE       265         ELOVL7       IHISQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFLHFWYRAYTKGQRLPKTV       271         ELOVL1       -QQN-GAPGIAKVKAN       279         ELOVL2      AG-KEVKNGFSKAYFTAANGVMNKKAQ       296         ELOVL3       ELOVL4       -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD       314         ELOVL5       HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD       299         ELOVL6       ELOVL7       KNC-TCKNKDN       299	ELOVL3	IVSILTYIWRODOGCHTTMEH-LFWSFILY-MTYFILJAHJFCÕTVIRPKVKAKTKSO	270
ELOVL5       IQTSC-GVIWPCTFPLGWLYFQIGYMISLIALFTNFYIQTYNKKGASRRKDHLKD       268         ELOVL6       VVNYLVFCWMQHDQCHSHFQN-IFWSSLMY-LSYLVLFCHFFFEAYIGKMRKTTKAE       265         ELOVL7       IHISQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFLHFWYRAYTKGQRLPKTV       271         ELOVL1       -QQN-GAPGIAKVKAN       279         ELOVL2       -AG-KEVKNGFSKAYFTAANGVMNKKAQ       276         ELOVL3       270       276         ELOVL4       -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD       314         ELOVL5       HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD       299         ELOVL6       265       265	ELOVL4	GHTAL-SLYTDCPFPKWMHWALIAWAISFIFUJLNJYIRTWKEPKKPKAGKTA	282
ELOVL6       VVNYLVFCWMQHDQCHSHFQN-IFWSSLMY-LSYLVLFCHFFFEAYIGKMRKTTKAE       265         ELOVL7       IHISQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFLHFWYRAYTKGQRLPKTV       271         ELOVL1       -QQN-GAPGIAKVKAN       279         ELOVL2      AG-KEVKNGFSKAYFTAANGVMNKKAQ       296         ELOVL3       210       270         ELOVL4       -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD       314         ELOVL5       HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD       299         ELOVL6       209       299         ELOVL7       KNC       CKNKDN       299	ELOVL5	IOTSC-GVIWPCTFPLGWLYFOIGWMISLIALFTNFYIOTWNKKGASRRKDHLKD	268
ELOVL7       IHISQFFFMEDCKYQFPVFAC-IIMSYSFMFLLLFUHFWYRAYTKGQRLPKTV       271         ELOVL1       -QQN-GAPGIAKVKAN       279         ELOVL2      AG-KEVKNGFSKAYFTAANGVMNKKAQ       296         ELOVL3       270         ELOVL4       -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD       314         ELOVL5       HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD       299         ELOVL6       200       314	ELOVI.6	VVNYLVFCWMOHDOCHSHFON-IFWSSLMV-LSYLVIDCHDFFEAVIGKMRKTTKAE	265
ELOVL1       -QQN-GAPGIAKVKAN       279         ELOVL2      AG-KEVKNGFSKAYFTAANGVMNKKAQ       296         ELOVL3       270         ELOVL4       -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD       314         ELOVL5       HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD       299         ELOVL6       200       265	ELOVI.7	THTS = -OFFFMEDGKYOFPVFAC = TIMSVSFMFLLIDELHEWYRAWTKGORLPKTV =	271
ELOVL1-QQN-GAPGIAKVKAN279ELOVL2AG-KEVKNGFSKAYFTAANGVMNKKAQ296ELOVL3270ELOVL4-MNGISANGVSKSEKQLMIENGKKQKNGKAKGD314ELOVL5HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD299ELOVL6265265ELOVL7KNCCKNKDN			273
ELOVL2AG-KEVKNGFSKAYFTAANGVMNKKAQ 296 ELOVL3 270 ELOVL4 -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD 314 ELOVL5 HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD 299 ELOVL6 265	ELOVL1	-QQN-GAPGIAKVKAN	279
ELOVL3 270 ELOVL4 -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD 314 ELOVL5 HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD 299 ELOVL6 265	ELOVL2	– – AG – KEVKNGFSKAYFTAANGVMNKK – – AO	296
ELOVL4 -MNGISANGVSKSEKQLMIENGKKQKNGKAKGD 314 ELOVL5 HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD 299 ELOVL6 ELOVL7 KNC TCKNKDN 281	ELOVL3	~	270
ELOVL5 HQNGSMAAVNGHTNSFSPLENNVKPRKLRKD 299 ELOVL6 265	ELOVL4	-MNGISANGVSKSEKOLMIENGKKOKNGKAKGD	314
ELOVL6 ΕΙΟΥΙ 7 ΚΝΟ ΠΟΚΝΚΡΝ 281	ELOVL5	HONGSMAAVNGHTNSFSPLENNVKPRKLRKD	299
	ELOVL6		265
	ELOVI.7		2.81





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Fig. 3

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