

TBCE Mutations Cause Early-Onset Progressive Encephalopathy with Distal Spinal Muscular Atrophy

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1 **TBCE mutations cause early-onset progressive encephalopathy with distal spinal**
2 **muscular atrophy**

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47 **Abstract**

48 We report on an early-onset neurodegenerative encephalopathy with distal spinal
49 muscular atrophy caused by *TBCE* mutations, with Ile155Asn occurring at the
50 heterozygous/homozygous state in all affected subjects. Clinical features resemble the
51 phenotype of *pmn/pmnm* mice (*Tbce*^{Trp524Gly}) and are associated with reduced *TBCE* levels
52 and altered tubulin polymerization. While truncating *TBCE* mutations have been described
53 to perturb developmental processes, we document altered *TBCE* function as a cause of
54 neurodegeneration in humans.

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56

57 Tubulinopathies include a family of severe neurodevelopmental disorders caused by
58 mutations in multiple genes encoding tubulin isoforms (*i.e.*, *TUBA1A*, *TUBA8*, *TUBB2B*,
59 *TUBB3* and *TUBB5*), with defective neuronal migration and malformation of the cerebral
60 cortex representing major features¹. Truncating mutations in *TBCE* (tubulin-specific
61 chaperone E), one of the five tubulin-binding co-factors involved in tubulin folding and
62 polymerization, are known to cause two rare neurodevelopmental diseases,
63 hypoparathyroidism-retardation-dysmorphism (HRD) and Kenny-Caffey syndrome (KCS)².
64 More recently, microtubule-disrupting mutations in tubulin genes have been shown to
65 trigger neurodegeneration, as illustrated by *TUBA4A* and *TUBB4A* mutations underlying
66 amyotrophic lateral sclerosis³ and hypomyelinating leukodystrophy associated with
67 progressive spastic dystonic tetraplegia⁴, respectively. Similarly, a missense mutation in
68 *Tbce* has been associated with progressive distal motor neuronopathy in mice⁵, but no
69 similar phenotype has so far been reported in humans.

70 We used a trio-based whole exome sequencing (WES) strategy to delineate the
71 molecular basis of a novel recessive encephalopathy affecting two apparently unrelated
72 young subjects originating from the island of Ischia, Southern Italy (**Fig. 1a**). Both
73 individuals showed developmental delay without dysmorphism or growth defects, and
74 manifested early-onset, slowly progressive distal motor neuropathy resembling distal
75 spinal muscular atrophy, with bilateral foot drop associated to spasticity and cerebellar
76 ataxia. WES data were analyzed to identify genes shared by the probands having
77 functionally relevant rare/private variants (**On-line methods**), taking into account
78 recessive inheritance models (**Supplementary Table S1**). We identified only one gene,
79 *TBCE*, satisfying these filtering criteria. Subject 1544334 (Pt1) was homozygous for the
80 c.464T>A missense change (p.Ile155Asn), which was in line with the documented
81 consanguinity ascertained in the family; subject 2518864 (Pt2) was a compound
82 heterozygote for the same amino acid substitution and the truncating c.1076delC change

83 (p.Ser359fs*1). Sanger sequencing confirmed both mutations and segregation in both
84 families (**Supplementary Fig. S1**). The shared genomic region encompassing the
85 c.464T>A change covered 2.5 Mb (chr1:234529552-237060433). Mutations scan of the
86 *TBCE* coding sequence performed on 14 subjects with similar clinical features allowed to
87 identify a third family from the same geographical area having two monozygotic twins
88 homozygous for the c.464T>A change. All subjects exhibited early-onset, slowly
89 progressive spastic ataxia, distal spinal muscular atrophy, cerebellar atrophy, and
90 developmental delay (**Supplementary Table S2**). This phenotype is reminiscent of the
91 mutant *pnn/pnn* mouse with progressive motor neuronopathy, hind limb atrophy and
92 reduced brain size⁵. In these subjects, the neurodegenerative signs, normal
93 calcium/phosphate metabolism, and absence of repeated infections (**Supplementary**
94 **Information**) clearly differ from HRD and KCS.

95 *TBCE* is characterized by a CAP-Gly domain at the N-terminus, followed by nine
96 tandemly arranged leucine-rich repeats (LRRs), and a C-terminal ubiquitin-like domain
97 (**Supplementary Fig. S1**). Ile¹⁵⁵ is a highly conserved residue across orthologs
98 (**Supplementary Fig. S1**) located within the LRR domain. A homology model of the *TBCE*
99 regions spanning the nine LRRs (residues 123-
100 348) was generated, which fitted well with the established structure of these motifs⁶, and placed Ile¹⁵⁵
101 into the hydrophobic core
102 stabilizing the second LRR motif (**Fig. 1b**). The introduction of the hydrophilic asparagine
103 is predicted to perturb dramatically the structure of the motif and the overall folding of the
104 LRR domain. Since LRR domains are functional modules that mediate protein-protein
105 interaction, this amino acid substitution is expected to affect *TBCE* function or the overall
106 *TBCE* structure and protein stability. To verify the latter hypothesis, the level of the *TBCE*
107 protein was evaluated in primary fibroblasts obtained from Pt1 and Pt2. Western blot
108 analysis documented a strongly reduced amount of *TBCE* compared to control cells (**Fig.**
1c), suggestive of rapid degradation of the *TBCE* mutant. Of note, reduction in *TBCE*

109 levels was more pronounced in fibroblasts from Pt2, who was a compound heterozygote for
110 the 155Asn and Ser359fs*1 changes. In these cells, *TBCE* mRNA pool was reduced to
111 half of normal (as shown by real-time qRT-PCR) (**Fig. 1d**), and the mutant allele carrying
112 the frameshift variant was undetectable by Sanger sequencing of retro-transcribed RNA
113 (data not shown), indicating nonsense-mediated mRNA decay. A
114 similar reduction in *Tbce* levels was documented in fibroblasts (**Fig. 1e**) and motoneurons⁷ of the *pmn/p*
115 *mn* mouse. Remarkably, *TBCE* levels had been reported to be virtually absent in HRD and KCS⁸.

116 *TBCE* is required for the folding of α -tubulin, α/β -tubulin dimerization and
117 subsequent heterodimer polymerization into microtubules, which are major components of the neur-
118 onal cytoskeleton⁹. To explore the impact of the two *TBCE* mutations on
119 microtubule polymerization, we first determined the levels of total, soluble and polymerized
120 α -tubulin in patient and control fibroblasts, using biochemical fractionation. The level of
121 total α -tubulin was not affected. By contrast, the amount of polymerized α -tubulin was
122 significantly reduced compared to control cells (**Fig. 2a**), in line with what occurred in tail
123 fibroblasts of *pmn/pm* mice (**Fig. 2b**). We then determined the nucleation of microtubules
124 at the centrosome and at extracentrosomal sites. Centrosomes organize the spindle
125 microtubules during mitosis, and confocal microscopy analysis of synchronized fibroblasts
126 from patients with mutated *TBCE* alleles documented an abnormal mitotic morphology and
127 disorganized mitotic microtubules; spindles appeared asymmetric and unrefined, with
128 irregular aster structure (**Supplementary Fig. S2**). Defective nucleation of
129 extracentrosomal microtubules was also noticed. Specifically, we observed significantly
130 reduced levels of Golgi-associated detyrosinated α -tubulin in patient fibroblasts (**Fig. 2c**),
131 in line with the reported loss of Golgi-derived microtubules in motoneurons of *pmn/pm*
132 mice⁷.

133 To demonstrate altered microtubule dynamics, we treated the fibroblast cultures with
134 the microtubule-disrupting drug nocodazole, and analyzed microtubule regrowth after drug

135 washout. In control fibroblasts, microtubule began to polymerize efficiently as early as 10
136 minutes after nocodazole washout, and extended polymerized structures were well-formed
137 at 30 min (**Fig. 2d**). Differently, microtubule polymerization appeared markedly delayed
138 in patient fibroblasts; microtubules were also less abundant and strongly disorganized in
139 both early and late stages of re-polymerization. Such altered dynamics appeared
140 particularly pronounced in fibroblasts with compound heterozygosity for the Ile155Asn and
141 Ser359fs*1 changes, consistent with the more profound reduction of TBCE expression
142 levels.

143 Overall, we describe a new distal neuronopathy and spastic ataxia caused by biallelic
144 mutations in *TBCE* overlapping the phenotype of *pmn/pmn* mouse, a well-known model of
145 motor neuron disease. On the basis of the previously documented impact of abolished
146 TBCE function on developmental processes and growth, our findings demonstrate a
147 stringent requirement of TBCE functions specifically for neuronal survival and function.

148

149 **URLs**

150 Genome Analysis Toolkit, <https://www.broadinstitute.org/gatk/>; Gene,
151 <http://www.ncbi.nlm.nih.gov/gene/>; RCSB Protein Data Bank,
152 <http://www.rcsb.org/pdb/home/home.do>; Gromacs, <http://www.gromacs.org>; SWISS-
153 MODEL, <http://swissmodel.expasy.org/vmd>, <http://www.ks.uiuc.edu/Research/vmd/>;
154 MODELLER, <https://salilab.org/modeller/>.

155

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161

162 **Author contributions**

163 A.S., G.B., T.R. and E.P. designed and performed the biochemical and functional studies,
164 analyzed the data; S.B. performed mutation analyses; E.F., C.C., and S.C. carried out the
165 confocal laser scanning microscopy analysis of cultured human and mice fibroblasts; A.C.,
166 M.N. and V.C. performed WES data processing, analysis and validation. E. Bell. performed
167 the structural analyses. G.T., A.D'A., B.D., G.Z., D.D., D.M. and C.D.-V., clinically
168 characterized the affected subjects, collected biological specimens and studied
169 histopathology of muscle biopsies. M.T., G.H. and E.B. conceived the project, designed
170 and supervised the experiments, analyzed and interpreted the data, and wrote the
171 manuscript. All authors contributed to the final manuscript.

172

173 **Competing financial interest statement**

174 All authors state that they have no competing financial interests.

175

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187 **LegendstoFigures**

188

189 **Figure1. *TBCE* mutations cause early-onset encephalopathy with distal spinal**

190 **muscular atrophy. (a)** Clinical features of Pt1 (1) and Pt2 (4,5,6); muscle biopsy of Pt1

191 stained with ATPase 4.3 (2) and esterase (3) showing neurogenic abnormalities; brain

192 MRIs of Pt1 (7,8,9, at 2 years; 10,11,12, at 3 years) and Pt2 (13,14,15, at 1 year; 16,

193 17,18, at 2 years). Note the hypoplasia of corpus callosum, and cerebellar atrophy with prominent f

194 olia (12,17). **(b)** Homology model of the TBCE LRR domain. Ile¹⁵⁵ with

195 surrounding hydrophobic residues contribute to the hydrophobic core required for proper

196 folding of the domain. **(c)** Western blot (WB) analysis documenting reduced TBCE levels

197 in patient fibroblasts. Data are presented as means \pm s.d., * indicates $P < 0.05$. **(d)** Real-

198 time qRT-PCR showing markedly reduced expression level of *TBCE* mRNA in fibroblasts

199 from Pt2. * indicates $P < 0.05$. **(e)** WB analysis demonstrating reduced level of Tbc in the

200 *pnm/pnm* mouse (Tbc^{Trp524Gly}); recombinant protein (TBCE) is also reported.

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202

203 **Figure2. Defective microtubule polymerization in subjects with**

204 **homozygous/heterozygous TBCE p.Ile155Asn substitution, and *pnm/pnm* mice. (a)**

205 Western blot (WB) analyses showing total (T), soluble (S) and polymerized (P) α -tubulin

206 (above), detyrosinated tubulin (middle), and β -actin (below) in patient fibroblasts and

207 control cells (Ctrl1, Ctrl2). Diagrams (right) show reduced fractions of polymerized α -

208 tubulin and detyrosinated tubulin in patient fibroblasts. **(b)** WB analyses showing reduced

209 amount of polymerized α -tubulin (P) in fibroblasts from *pnm/pnm* mice compared to

210 fibroblasts from unaffected mice. **(c)** Immunofluorescence analysis documenting loss of

211 microtubules containing detyrosinated tubulin (detyr-Tub) in patient fibroblasts. Note that

212 detyrosinated microtubules (red) in control fibroblasts are closely associated with GM130-

213 stainedGolgimembranes(green).NucleiareDAPI-stained.Scalebar,10 μ m. (d)
214 Immunofluorescenceanalysesshowingreducedre-polymerizationofmicrotubules
215 followingnocodazole treatment infibroblastsfromPt1andPt2,andcontrolcells(Ctrl).
216 Scalebar,20 μ m.
217
218



