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## **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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46

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*<sup>Trp524Gly</sup>) 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.

55

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 features<sup>1</sup>. 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)<sup>2</sup>.  
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 sclerosis<sup>3</sup> and hypomyelinating leukodystrophy associated with  
67 progressive spastic dystonic tetraplegia<sup>4</sup>, respectively. Similarly, a missense mutation in  
68 *Tbce* has been associated with progressive distal motor neuronopathy in mice<sup>5</sup>, 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 dysmorphia 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 size<sup>5</sup>. 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**). Ile<sup>155</sup> 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 motifs<sup>6</sup>, and placed Ile<sup>155</sup>  
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 motoneurons<sup>7</sup> of the *pmn/p*  
115 *mn* mouse. Remarkably, *TBCE* levels had been reported to be virtually absent in HRD and KCS<sup>8</sup>.

116 *TBCE* is required for the folding of  $\alpha$ -tubulin,  $\alpha/\beta$ -tubulin dimerization and  
117 subsequent heterodimer polymerization into microtubules, which are major components of the neur-  
118 onal cytoskeleton<sup>9</sup>. To explore the impact of the two *TBCE* mutations on  
119 microtubule polymerization, we first determined the levels of total, soluble and polymerized  
120  $\alpha$ -tubulin in patient and control fibroblasts, using biochemical fractionation. The level of  
121 total  $\alpha$ -tubulin was not affected. By contrast, the amount of polymerized  $\alpha$ -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  $\alpha$ -tubulin in patient fibroblasts (**Fig. 2c**),  
131 in line with the reported loss of Golgi-derived microtubules in motoneurons of *pmn/pm*  
132 mice<sup>7</sup>.

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 re-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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160 E.B. and M.T.).



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. Ile<sup>155</sup> 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<sup>Trp524Gly</sup>); recombinant protein (TBCE) is also reported.

201

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)  $\alpha$ -tubulin

206 (above), detyrosinated tubulin (middle), and  $\beta$ -actin (below) in patient fibroblasts and

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

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

209 amount of polymerized  $\alpha$ -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 stainedGolgi membranes(green).NucleiareDAPI-stained.Scalebar,10 $\mu$ m. (d)  
214 Immunofluorescenceanalysisesshowingreducedre-polymerizationofmicrotubules  
215 followingnocodazole treatment infibroblastsfromPt1andPt2,andcontrolcells(Ctrl).  
216 Scalebar,20 $\mu$ m.  
217  
218



