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KCTD7 defines a neurodegenerative disorder with autophagy-lysosome defect

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1 ***KCTD7* deficiency defines a distinct neurodegenerative disorder with a conserved**
 2 **autophagy-lysosome defect**

3
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For Peer Review

71 Abstract

72

73 Objective

74 Several small case series identified *KCTD7* mutations in patients with a rare autosomal
75 recessive disorder designated progressive myoclonic epilepsy (EPM3) and neuronal ceroid
76 lipofuscinosis (CLN14). Despite the name KCTD (potassium channel tetramerization domain),
77 KCTD protein family members lack predicted channel domains. We sought to translate insight
78 gained from yeast studies to uncover disease mechanisms associated with deficiencies in
79 *KCTD7* of unknown function.

80

81 Methods

82 Novel *KCTD7* variants in new and published patients were assessed for disease causality using
83 genetic analyses, cell-based functional assays of patient fibroblasts and knockout yeast, and
84 electron microscopy of patient samples.

85

86 Results

87 Patients with *KCTD7* mutations can exhibit movement disorders or developmental regression
88 before seizure onset, and are distinguished from similar disorders by an earlier age of onset.
89 Although most published *KCTD7* patient variants were excluded from a genome sequence
90 database of normal human variations, most newly identified patient variants are present in this
91 database, potentially challenging disease causality. However, genetic analysis and impaired
92 biochemical interactions with cullin 3 support a causal role for patient *KCTD7* variants,
93 suggesting deleterious alleles of *KCTD7* and other rare disease variants may be underestimated.
94 Both patient-derived fibroblasts and yeast lacking *Whi2* with sequence similarity to *KCTD7*
95 have impaired autophagy consistent with brain pathology.

96

97 Interpretation

98 Bi-allelic *KCTD7* mutations define a neurodegenerative disorder with lipofuscin and lipid
99 droplet accumulation but without defining features of neuronal ceroid lipofuscinosis or
100 lysosomal storage disorders. *KCTD7* deficiency appears to cause an underlying autophagy-
101 lysosome defect conserved in yeast, thereby assigning a biological role for *KCTD7*.

102

103

104

105 **Introduction**

106 Autism, schizophrenia, dystonia, epilepsy and other disorders have been linked to several
107 members of the gene family *KCTD* (potassium channel tetramerization domain).¹⁻⁴ All *KCTD* family
108 proteins have an N-terminal BTB domain that is most similar in sequence to voltage-gated potassium
109 channel tetramerization (T1/BTB) domains, hence the name *KCTD*.⁵ However, *KCTD* proteins
110 appear unlikely to form channels as they lack predicted transmembrane domains and any direct
111 interaction with potassium channels remains uncertain. Although *KCTDs* could indirectly alter
112 channel properties, the gene name is potentially misleading and has caused some diagnostic
113 challenges with implied treatments for channelopathies.⁶ Patients with *KCTD7* mutations have been
114 diagnosed with progressive myoclonic epilepsy (EPM3),^{4, 7-12} neuronal ceroid lipofuscinosis 14
115 (CLN14),¹³ or opsoclonus-myoclonus syndrome (OMS).¹⁴ We sought to further define the clinical
116 syndrome resulting from *KCTD7* deficiency, to distinguish deleterious from normal variants in the
117 general population, and to assign a function to the uncharacterized *KCTD7* protein based on insights
118 gained from our studies of the related protein in yeast, *Whi2*.

119 *KCTD* family proteins are relatively uncharacterized, but one common theme has begun to
120 emerge. A subset of *KCTD* family proteins may be components of cullin 3 (*CUL3*) ubiquitin ligase
121 complexes.¹⁵ *CUL3* uses BTB-containing adaptor proteins from other protein families to recruit cargo
122 proteins for ubiquitination. Several *KCTD* family proteins including *KCTD7* have been reported to
123 bind *CUL3*,^{13, 16} consistent with structure modeling for other *KCTDs*.¹⁵ In this capacity as potential
124 cargo adaptors, several other *KCTD* family proteins have been suggested to target specific cargo
125 proteins for degradation,¹ although most await confirmation. A similar role for *KCTD7* is consistent
126 with lysosome pathway defects in several other EPM and CLN disorders,^{17, 18} and other
127 neurodegenerative processes.¹⁹ However, the molecular and cellular consequences of *KCTD7*
128 deficiency are not known.

129 This project was prompted by our genome-wide yeast genetic screen that uncovered the
130 *KCTD*-like BTB-containing protein *Whi2* of *Saccharomyces cerevisiae*.⁵ Yeast *Whi2* is reportedly a
131 general stress response factor,²⁰ and has a contested role in mitophagy.^{21, 22} Yeast *Whi2* and
132 autophagy regulator *Atg6* (human Beclin 1) were identified in our screen for factors required to
133 respond to low amino acid availability,^{5, 23} a condition known to induce catabolic processes such as
134 autophagy in yeast and mammals.²⁴ Because yeast *Whi2* shares sequence similarity and a common
135 domain architecture with human *KCTD* proteins,⁵ we sought to gain new insight into the disease
136 mechanisms due to *KCTD7* mutations. We found that both yeast *Whi2* and human *KCTD7* are

137 required for normal autophagy in low nutrients, further supported by the accumulation of lipid bodies,
138 defective mitochondria and abnormal autolysosomes.

139

140 **Methods**

141 **Patient data collection**

142 Clinicians were provided a form for deidentified data (**Table S1**, IRB exempt status).

143

144 **Electron microscopy**

145 Patient fibroblasts were fixed in 2.5% glutaraldehyde (EM grade; Electron Microscopy Sciences,
146 Hatfield, PA) in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature, and processed
147 by the Yale University Electron Microscopy Core as described.²⁵

148

149 **PCR verification of *KCTD7* mutations in patient fibroblasts**

150 Genomic DNA for *KCTD7* exon-2 of primary human fibroblasts was amplified from extracted DNA
151 using 5' Primer (TGGCACCAATCAGACCCAGGGATTGAAGATGGAGCAGCCC) and 3'
152 Primer (CCCATTTATTAAATTCATCAATATGCTATCTCCTCTTCTAGG), separated on 1%
153 agarose gel, extracted using Qiagen Gel Extraction Kit, and sequenced at MacrogenUSA using the 5'
154 primer to verify patient mutations in these cell lines.

155

156 **Plasmids**

157 Wild type *KCTD7* ORF was PCR amplified (Invitrogen AccuPrime Pfx) from HEK293 cDNA
158 (Qiagen RT Kit) and cloned into a pSG5-derived vector to create N-terminal tagged proteins. Coding
159 changes were subsequently engineered into *KCTD7* and *CUL3* expression vectors and verified by
160 Sanger sequencing. *pr^{PGK}-GFP-ATG8* was engineered by replacing the *ATG8* 5' regulatory region
161 with the 5' regulatory region of *PGK*.

162

163 **Mammalian autophagy assays**

164 Primary patient fibroblasts (derived from 3 mm axilla skin punch) were shipped/received live and
165 maintained in DMEM, 10-20% FBS and pen/strep. Age- and passage-matched control human
166 fibroblasts GM05757 and 498 (Corriell Institute) were maintained in parallel and *KCTD7* expression
167 was assessed by qRT-PCR. Subconfluent cells, passage 4-8, were plated in 12-well dishes
168 (50,000/well). The next day were washed 1x with PBS before treating. For nutrient deprivation, cells
169 were switched to EBSS or to RPMI1640 without L-glutamine, amino acids or glucose (US

170 Biologicals) supplemented with 5% dialyzed FBS (Thermo) with or without amino acids (R7131
171 Sigma) and glucose (4.5 g/L, Gibco). For lysate preparation, cells were washed with PBS,
172 solubilized in lysis buffer (62.5 mM Tris-HCl, 2% SDS, 0.01% Bromophenol Blue, 10% glycerol,
173 2% β -mercaptoethanol) with protease inhibitor cocktail (Thermo Scientific) and heated to 100°C for
174 10 min before separation by 12% SDS-PAGE and transfer to PVDF. Immunoblots were performed
175 using antibodies for LC3B (CST #2775 or 3868, 1:1000) and actin (MP Biologicals #691001,
176 1:10,000) and visualized using a Bio-Rad ChemiDoc MP system and analyzed with ImageLab 5.0
177 software.

178

179 **Immunofluorescence microscopy**

180 Kyoto HeLa cells (ATCC) were grown on 12 mm glass cover slips in DMEM, 10% FBS plus
181 pen/strep, and transfected the next day with 150 ng total DNA using JetPRIME (VWR). At 16-18 h
182 posttransfection, cells were fixed 10 min with 4% formaldehyde (Polysciences, Warrington, PA),
183 permeabilized with 0.2% Triton X-100 in PBS, immunostained, mounted (Prolong Gold, Life
184 Technologies) and viewed with a Zeiss AxioImager M2 (60x Olympus objective), Hamamatsu Orca
185 R2 camera and Volocity Software, or an Applied Precision DeltaVision microscope and software
186 (60x Olympus objective) with Hamamatsu Photonics camera.

187

188 **Co-immunoprecipitations**

189 N-terminal 3HA/3Flag-KCTD7 and 6Myc-cullin 3 (CUL3) expression vectors were transiently
190 transfected into HEK-293 cells using BioT (Bioland Scientific) for 2 days. Cells were lysed in native
191 lysis buffer containing AEBSF, pepstatin, and leupeptin, rotated for 60 min at 4 °C, and remaining
192 insoluble material was removed by centrifugation. Samples were normalized (A280 nm) and rotated
193 at 4 °C for 2 h with pre-equilibrated EZview Red anti-HA or anti-FLAG affinity gel (Sigma). Beads
194 were washed 4 times in the same buffer and proteins eluted by boiling in SDS-PAGE sample buffer.
195 Whole-cell lysates (WCL) and anti-HA precipitates (IP) were separated by SDS-PAGE, transferred to
196 PVDF membranes and probed with antibodies against FLAG (F1804, Sigma), HA (12CA5, Roche),
197 or α -tubulin (HRP-66031, Proteintech) and HRP-conjugated secondary antibodies. Proteins were
198 detected with Pierce ECL Western Blotting Substrate, SuperSignal West Femto Maximum Sensitivity
199 Substrate and autoradiography.

200

201 **Yeast autophagy assays**

202 Yeast strains (BY4741) transformed with autophagy reporter plasmids were grown overnight in
203 synthetic SC_{CSH} medium, refed for 1 h in fresh SC_{CSH} medium at 1 OD₆₀₀/ml, washed once and
204 switched to low amino-acid medium SC_{ME} as described.⁵ Cells corresponding to 2 OD₆₀₀ units were
205 collected, lysed for immunoblot analysis.⁵

206

207 **Results**

208 To better define the disorder caused by *KCTD7* mutations, we identified 18 novel mutations
209 in 15 patients (11 families) from several sequencing centers and institutions (**Fig 1A**). A total of 30
210 novel *KCTD7* variants from 37 new and published patients can be grouped into three protein regions,
211 the N-terminal BTB domain, a C-terminal cluster and a less defined middle region (**Fig 1B**). All 37
212 patients have homozygous or compound heterozygous variants in *KCTD7* (23 missense, 3 stopgain
213 and 4 frameshifts). However, their association with disease does not constitute proof of pathogenicity
214 for each patient variant. To address this point, we first characterized the clinical syndrome.

215

216 ***KCTD7* mutations are associated with a progressive neurodegenerative disorder**

217 Overt seizures mark the recorded age of onset for 76% of *KCTD7* patients, many with
218 accompanying developmental delays and movement disorders, predominantly ataxia, tremors and
219 dyskinesia (**Fig 1A**). The remaining 24% first develop movement disorders or developmental delays
220 prior to seizure onset. Genetic testing for *KCTD7* variants at earlier ages could potentially identify
221 more patients with movement disorders before seizure onset. Other prominent clinical features
222 include the loss of normal developmental milestones achieved in early childhood, difficulty walking,
223 loss of speech and fine motor skills, and severe cognitive decline. While EEG findings are often
224 positive, brain MRI is typically normal at onset but may detect diffuse or focal brain atrophy after
225 disease progression, for example as observed for new patient-1 (T64A/R211X) but not for new
226 patient-2 (**Table S1**). Patient-2 underwent a complete corpus callosotomy at age 6.9 years, and was
227 seizure-free for at least 18 months, with some improvement in motor control. All patients progressed
228 to develop myoclonic epilepsy, and all with available data developed movement disorders. Most
229 become wheelchair-bound and non-verbal, and 6 of 37 died at ages 3-18 years (**Fig 1A**, asterisks).
230 The few ambulatory patients now in their 20's (patients-9, -10 and -19) have significant motor and
231 cognitive deficits and exhibit autism, obsessive compulsive disorder or schizophrenia, in addition to
232 epilepsy (**Table S1**).

233

234 **Early onset without retinal degeneration distinguishes *KCTD7* patients**

235 Distinguishing KCTD7/EPM3 patients from related disorders is the early age of onset,
236 consistent with published case reports (**Fig 2**). Average onset age for all 37 patients is ~17 months,
237 range 5-24 months except one of a sibling pair lacking C-terminal residue W289 with disease onset
238 reported at 36 months (patient-37) (**Fig 1A**). No gender bias is present for incidence (49% males)
239 although males tended to be diagnosed at a younger age (mean onset 15.1 mo males, 19.1 mo
240 females). This early onset age for KCTD7/EPM3 patients does not overlap with other early onset
241 myoclonic epilepsies (EPM1A, EPM2, EPM4), with the exception of infantile CLN1 (onset 6-24
242 months) caused by mutations in lysosomal enzyme PPT1 (**Fig 2**).^{26, 27} However, KCTD7 patients
243 uniformly lacked the characteristic CLN1-associated retinal abnormalities at onset and associated
244 blindness (**Fig 1A**).^{18, 26} These findings shift the age downward for considering the diagnosis of
245 EPM3, which is typically diagnosed in childhood or adolescence.

246 KCTD7 patients are also distinguished from other related disorders. They have more severe
247 cognitive decline and earlier onset than patients with a BTB domain variant in Kv3.1/ KCNC1 (onset
248 age 3-15yr),²⁸ but a later average onset than infantile spasms due to autosomal dominant mutations in
249 *DNMI* (onset typically 4-7 months) or autosomal recessive mutations in *TBC1D24* (GTPase-
250 activating ARF6-binding protein) that cause pleiotropic neurologic disorders with myoclonic seizures
251 (median onset 2-3 months, often at birth) (**Fig 2**).^{29, 30}

252

253 **Prevalence of patient *KCTD7* mutations in the general population**

254 To address disease causality of *KCTD7* variants found in new patients, family pedigrees were
255 constructed for the 15 new patients, revealing all variants were inherited (**Fig 3A**). For all patients
256 (except published patient-15), 57 unaffected family members with available data were either wild
257 type (2 of 10 sequenced siblings) or heterozygous for patient mutations (41 parents, 8 siblings, and 1
258 grandparent) based on sequence data, and 5 parents based on kinship records of consanguinity (**Table**
259 **S2**). The gene damage index GDI-Phred value for *KCTD7* (1.235, medium damage prediction) and
260 the selective pressure assessed by the McDonald-Kreitman neutrality index (0.004, moderate
261 purifying)³¹ are also consistent with a monogenic autosomal recessive disease with complete
262 penetrance. However, cautious causality assignments may still be warranted for specific *KCTD7*
263 variants given ~500-20,000 protein-altering variants per individual.³²

264 If all 30 *KCTD7* variants are disease-causing, each is expected to be rare in the general
265 population. In an effort to catalog normal genetic variation in healthy individuals, the exome
266 aggregation consortium ExAC database of ~60,000 unrelated healthy individuals excluded cancer
267 genomes and cohorts with severe pediatric diseases.³² Thus, most of the *KCTD7* patient variants

268 listed at ExAC (10 of 14) are from previously unpublished patients (**Table S2**). Among all *KCTD7*
269 variant alleles identified, T64A (patient-1, T64A/R211X) is the most frequent in ExAC, where it is
270 reported in 9 individuals (0.010% to 0.019% allele frequency in European and African populations,
271 respectively), and in 16 heterozygous individuals in the aggregate gnomAD database of ~130,000
272 individuals (**Fig 3B**). However, this frequency is still rare and to date no homozygotes for any amino
273 acid change in *KCTD7* has been reported in healthy individuals.³² Some disease variants reported to
274 cause other CLN disorders (e.g. autosomal recessive CLN1 and CLN6) were subsequently challenged
275 because of their prevalence in the ExAC database; for example CLN6 variant R252H.³³ However,
276 this CLN6 variant remains a disease candidate as none of the 21 normal individuals with this variant
277 is homozygous.³³

278

279 ***KCTD7* patient mutations cause altered protein behavior**

280 To acquire further evidence that the most prevalent *KCTD7* variant T64A (patient-1) is
281 pathogenic, we sought a functional assay. As *KCTD7* has no established biochemical activities, we
282 tested for altered subcellular localization as an alternative strategy. The N-terminal amino acids 1-149
283 of wild type *KCTD7* containing the BTB domain, when expressed in HeLa (or other) cells, forms
284 unusual flowing filament-like structures of unknown relevance in the cytoplasm and forms similar
285 smaller structures in the nucleus (**Fig 4A**). Taking advantage of these elaborate structures to
286 distinguish the effects of patient mutations, we found that expression of T64A(1-149) abolishes these
287 cytoplasmic structures and instead localizes predominantly in a nuclear ball-and-stick pattern (**Fig**
288 **4A**). Two other BTB domain mutations from published patients cause other distinguishable
289 morphologies. L108M (patients 11-13) increases the occurrence of mini-circles at filament termini
290 (**Fig 4A**, arrows), and D115Y (patient-15, lacking family genetics) causes massive filament-like
291 structures (**Fig 4A**), which is likely a cause or consequence of protein stabilization (**Fig 4B**). When
292 co-expressed with CUL3, an E3 ubiquitin ligase component and reported binding partner of
293 *KCTD7*,^{13, 16} both wild type and D115Y, and to a lesser extent L108M, were capable of recruiting
294 CUL3 from its more diffuse localization to *KCTD7* structures, except the T64A mutant that only
295 rarely co-localizes with CUL3 in fuzzy nuclear spots (**Fig 4C and D**). Thus T64A, as well as L108M
296 and D115Y are likely to alter *KCTD7* function, consistent with functional-effect prediction
297 algorithms PolyPhen2, SIFT and PROVEAN, and L108M and D115Y are predicted damaging by
298 two of these algorithms (**Table S2**).

299 Providing further evidence that patient mutations can alter interactions with CUL3, co-
300 immunoprecipitation assays revealed that the N-terminal BTB-containing region but not the C-

301 terminus of KCTD7 is required for CUL3 interaction, consistent with a role for KCTD7 as a CUL3
302 adaptor. Furthermore, the patient BTB domain mutations tested in full-length KCTD7 (R70W,
303 L108M and likely R84Q) impair binding to CUL3 (**Fig 4E**).

304 The only patients predicted to have functionally benign variants by PolyPhen2, SIFT and
305 PROVEAN also have less debilitating disease (sibling patients-9/-10, **Table S1**). However, their
306 nucleotide change corresponding to G105E (c.314G>A) is located at the exon2-intron junction and is
307 predicted to affect normal splicing (SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer
308 and Human Splicing Finder). Other KCTD7 patient variants have discordant functional predictions
309 between the different algorithms (**Table S2**), reinforcing that pathogenicity predictions are inherently
310 limited without 3D structures and functional biochemical assays.

311

312 ***KCTD7* heterozygosity in other neurological disorders**

313 We also identified 16 novel heterozygous *KCTD7* variants in 18 additional unrelated
314 individuals with phenotypes related to bi-allelic *KCTD7*EPM3 patients. These heterozygous patients
315 with predominantly unsolved disease etiologies (mean onset age 8.6 years) have neurological
316 phenotypes including developmental delays, seizures, disease progression, movement disorders
317 and/or intellectual disabilities (**Table S3**). Only one of these 18 variants occurs more frequently than
318 T64A in the population (Y86H occurs in 53 normal heterozygotes).³² Two of the 18 are also found in
319 EPM3 patients with bi-allelic *KCTD7* mutations, R121L and R153H. However, any role for
320 heterozygous *KCTD7* variants as genetic modifiers is not known.

321 Conversely, we cannot rule out the possibility that non-*KCTD7* variants act as genetic
322 modifiers of more complex traits affecting onset age or other variations between bi-allelic *KCTD7*
323 patients. Patient-9 (G105E/G114E) also has a heterozygous pathogenic variant in *GALC* that is
324 reported in patients with autosomal recessive, late onset neurodegenerative Krabbe disease, and a
325 heterozygous variant of unknown significance (VUS) in *ARID1A*, a conserved gene responsible for
326 autosomal dominant intellectual disability (Coffin-Siris syndrome). Patient-14 (R112C/R112C) has
327 mutations in three other genes linked to epilepsy, including a homozygous predicted damaging VUS
328 in the glutamate receptor *GRIN2A*. These and other noted variants in bi-allelic *KCTD7* deficient
329 patients (**Table S1**) are currently not predicted to be disease-related, but only on the basis that all
330 were inherited from one heterozygous parent and therefore insufficient to cause disease.

331

332 **Evidence for lysosome-pathway defects without characteristic neuronal ceroid lipofuscinosis**

333 Electron microscopy analysis of a frontal lobe brain biopsy from new patient-1
334 (T64A/R211X) at age 8 years revealed neuronal pathology (**Fig 5A**) that was absent in the brain of a
335 neurologically normal 6.7 yr child (not shown). The prominent brain pathology in patient-1 was the
336 electron-dense lipofuscin (lysosomes) (**Fig 5A**). Similar structures are commonly observed in normal
337 aging brain but not in children. A potential feature of KCTD7 brain lipofuscin is the late
338 autophagosome-like structures (**Fig 5B-D**, arrows) engorged with both electron-dense lysosomes and
339 electron-lucent structures presumed to be lipid droplets (**Fig 5A-D**, arrowheads). Soft lipid droplets
340 are compressed between lysosomes or bulging against a delimiting membrane (**Fig 5C and D**,
341 arrowheads). Others have observed similar structures in autophagy-deficient cells, and in cells
342 overfed with oleic acid.³⁴ The persistence of lipid droplets in brain tissue could potentially reflect an
343 underlying defect related to lipophagy, a form of autophagy that is required for utilization of lipid
344 stores as an energy source and that requires components of the autophagy pathway.³⁵

345 Bi-allelic *KCTD7* mutations that define a diagnosis of EPM3, also define the diagnosis of
346 neuronal ceroid lipofuscinosis 14 (CLN14) based on a study of patient-24 (R184C/R184C).¹³ That
347 study reported characteristic ultrastructural features of fingerprint-like profiles and granular
348 osmiophilic deposits (GROD) in fibroblasts and neurons from a skin biopsy and in lymphocytes.^{13, 36}
349 However, we did not observe these or other features considered to be characteristic of neuronal
350 ceroid lipofuscinosis, such as cytosomes, rectilinear profiles (RLP) or curvilinear morphologies
351 described for other CLN subtypes.³⁶ Clinical records indicate that new patient-1 also lacked such
352 ultrastructural features in lymphocytes, skin and rectal biopsy histology, recommended sites for
353 detection of neuronal ceroid lipofuscinosis.³⁶ Although detection can be challenging,³⁶ electron
354 microscopy and/or light microscopy of skin and muscle biopsies from published patients-4, 5, 11, 15,
355 26, and 30/31,^{7, 8, 11} as well as new patients-1, 20, 22 and 28 also lack these specific features (**Table**
356 **S1**). In addition, no storage material resembling other lysosomal storage disorders was detected in
357 neurons, vascular endothelial or smooth muscle cells of patient-1. Broader diagnostic criteria may be
358 needed to include KCTD7 as a CLN disorder.

359 Cultured skin fibroblasts naturally lack some of the lysosomal substrates enriched in the brain
360 such as specific glycosphingolipids, and are therefore not expected to reveal some brain pathologies.
361 However, fibroblast cultures are useful for evaluating other autophagosome-lysosome functions and
362 ultrastructure. In contrast to an age- and passage-matched control, prominent features of early-
363 passage skin-derived fibroblast cultures from patient-3 and patient-4 with BTB domain mutations
364 include the supernumerary lipid droplets often in close proximity to mitochondria and ER (**Fig 6A**).

365 Some lipid droplets appeared to be engulfed in membrane-bound structures (**Fig 6A**, arrowheads),
366 reminiscent of the lipid droplets in patient brain, possibly suggesting a stalled catabolic process.

367 Other prominent features that distinguish both patient fibroblasts from age- and passage-
368 matched control fibroblasts include hybrid structures resembling late single-membrane or partially
369 double-membrane autolysosomes.³⁷ These hybrid structures contain local electron-densities typical of
370 lysosomes (**Fig 6B**, black arrows) that are associated with larger autophagosome-like structures
371 containing residual undegraded material (**Fig 6B**, white arrows) and occasional electron dense lipid
372 whirls (**Fig 6B**). Similar hybrid structures are also characteristics of diverse neurodegenerative
373 disorders including mucopolipidosis type IV, Alzheimer's disease, CLN types and autophagy-deficient
374 cell lines.³⁷⁻³⁹

375 Although mitochondrial organelles were not sufficiently preserved in patient biopsy
376 preparations, most cultured fibroblasts from both patients contained a fraction of mitochondria with
377 internal, closed double membrane structures presumed to reflect cristae malformations (**Fig 6C**).
378 These malformations are somewhat reminiscent of mitochondria in mitofilin-deficient cells with
379 concentric cristae.⁴⁰ Ultrastructural abnormalities were prevalent in patient cells but absent in control
380 cells (**Fig 6D**). The apparent accumulation of several abnormal organelles in patient-derived
381 fibroblasts and in a brain biopsy is consistent with a defect in the phagolysosome pathway.

382

383 **Conserved autophagy defects as a potential mechanism of disease pathogenesis**

384 We initiated this KCTD7 project based on insights gained from studying the poorly
385 characterized yeast protein Whi2, which shares sequence similarity with KCTD7 and harbors a
386 homologous BTB structural domain.⁵ Yeast lacking *WHI2* are sensitive to multiple cell stresses and
387 fail to halt the cell cycle in response to low amino acid levels in the media, conditions known to
388 induce autophagy.^{5, 20, 23} Taken together with our ultrastructural findings in patient samples, we asked
389 if *whi2*-deficient yeast have a general autophagy defect using an established reporter for yeast Atg8,⁴¹
390 homolog of mammalian autophagy marker LC3. In low amino acid medium, the autophagy-
391 responsive *ATG8* promoter is induced to express the reporter, and during autophagy flux undergoes
392 lysosomal/vacuolar processing that cleaves the protease-sensitive Atg8 moiety from the more stable
393 GFP protein in wild type cells.⁴¹ In striking contrast to wild type, *whi2*-deletion strains are profoundly
394 defective for autophagy induction and flux (**Fig 7A**). Similar but less dramatic results were obtained
395 with an autophagy flux-specific reporter expressed by the constitutive *PGK* promoter, indicating that
396 Whi2 is required for normal autophagy induction and flux after switching to low amino acids (**Fig 7**
397 **B**). This autophagy defect was not due to a general defect in reporter expression or global protein

398 translation, as free GFP expressed via an autophagy-independent promoter (*ADHI*) was expressed
399 indistinguishably in wild type and *whi2*-deficient yeast (**Fig 7C**).

400 To extend these findings from yeast, the skin-derived fibroblast lines from patients-3 and -4
401 with BTB domain mutations were analyzed for autophagy defects. The accumulation of endogenous
402 lipidated/mature LC3-II following treatment with chloroquine to inhibit lysosome function that
403 otherwise degrades LC3-II.⁴² Consistently, LC3-II accumulated less efficiently in low-passage patient
404 fibroblasts versus age- and passage-matched controls following chloroquine treatment to assess basal
405 autophagy flux (**Fig 8A and B**), and when autophagy was induced by withdrawing amino acids and
406 glucose (**Fig 8B and C**). Autophagy induced by more severe starvation was also significantly lower
407 in patient fibroblasts assessed by conversion of LC3I to LC3II (**Fig 8E**). Thus, both *WHI2*-deficient
408 yeast and patient cells have a defect in autophagy based on Atg8/LC3 assays following nutrient
409 depletion. To confirm the origins of our fibroblast cell lines, genomic DNA isolated from patient and
410 control cells used in these studies was sequenced, revealing the expected variants for patients-3 and -
411 4.

412 The functional consequences of autophagy deficiency were assessed by determining the
413 effects of *KCTD7* on neurite outgrowth triggered by serum withdrawal in mouse neuroblastoma N2a
414 cells. The maturation of N2a cells to produce extensive neuron-like processes requires autophagy, as
415 knockdown of conserved Beclin 1/Atg6 blocks neurite extension.⁴³ We found that partial knockdown
416 of endogenous mouse *Kctd7* severely reduces neurite outgrowth triggered by serum withdrawal,
417 indicating a critical role for *KCTD7* in neurite maturation (**Fig 8F and G**).

418

419 **Discussion**

420 **Manifestations of *KCTD7* mutations**

421 Guided by our findings in yeast, we investigated the clinical and cellular consequences of
422 *KCTD7* mutations. Bi-allelic *KCTD7* mutations cause an early onset (16.8±6 months) progressive
423 myoclonic epilepsy previously ascribed only to older children.^{17, 18} These patients are characterized
424 by movement disorders and developmental delays that may precede onset of intractable myoclonic
425 seizures more often than appreciated based on recorded parental comments. The few ambulatory
426 patients exhibit severe cognitive and psychosocial impairments. All patients exhibit disease
427 progression indicative of an underlying degenerative process despite apparently normal initial
428 developmental milestones. Ultrastructural analysis of a brain biopsy or skin fibroblasts from a total of
429 three different patients revealed shared features, most notably the accumulation of lipid droplets and
430 abnormal phagolysosomes containing undegraded material. Misshapen mitochondrial cristae

431 membranes were also prominent features of skin fibroblasts where sample preparation is more
432 amenable to organelle preservation (**Figs 5 and 6**). The constituents of lipofuscin granules in patient
433 brain appear to be residual bodies derived from lysosomes and could potentially be produced by a
434 partial degradation of unsaturated lipids. Brain biopsies for future patients may provide more
435 valuable information. The ultrastructural features in patient samples together with defective
436 autophagy responses in patient fibroblasts and the corresponding yeast deletion strain are consistent
437 with the possibility that an autophagy-lysosome pathway defect underlies the disease caused by bi-
438 allelic *KCTD7* mutations. Although lysosome pathway defects are implicated in a growing number of
439 neurological disorders, each can manifest differently, presumably owing to the molecular details not
440 yet known. New therapies will be needed that enhance autophagosome-lysosome function without
441 worsening defective bottlenecks downstream in the pathway.

442 Two reported phenotypes for *KCTD7*/*EPM3* patients were not found in the new patient cohort
443 reported here. Clonic eye movements reported for patient-3 diagnosed with opsoclonus-myoclonus
444 syndrome (OMS)¹⁴ were not detected though not formally tested. We also did not detect specific
445 neuronal ceroid lipofuscinosis pathology reported for patient-24, the founder case for *CLN14*
446 designation, although this patient potentially had more severe disease (onset 8 mo, died 17 yr).¹³

447

448 **Causality of *KCTD7* variants**

449 The low frequency in the general population of each of the 30 unique *KCTD7* patient variants,
450 including T64A, and the lack of homozygous patient variants in healthy individuals,³² indicate that
451 these 30 variants are causal for *EPM3*/*CLN14*. This disorder occurs worldwide and the patients in
452 this study have diverse ancestry (e.g. Moroccan, Syrian Sephardi, European, Native American,
453 French Canadian). Genome sequence data from the general population suggest at least 0.05% of
454 healthy unrelated individuals may carry a heterozygous pathogenic *KCTD7* variant.³² The relatively
455 higher than average arginine content of the *KCTD7* protein (7.3% vs. 4-6%) may contribute to the
456 mutation frequency (12 of 30 variants change an Arg, **Table S1**) given that arginine codons have the
457 highest proportion of CpG sequences and CpG mutations (affecting DNA methylation) are by far the
458 most prevalent in the population.³²

459 Additional rare heterozygous *KCTD7* variants were identified in 17 additional patients with
460 related yet clinically distinct disorders (**Table S3**), although any contribution to disease is unknown.
461 However, when considering the genetic complexities of neurobehavioral disorders such as autism,
462 other genetic modifiers with discernable clinical phenotypes may exist. *KCTD7* sibling patients-9/10
463 were diagnosed with autism. The *KCTD7*-related *KCTD13* gene at 16p11.2 is thought to have a role

464 in a small subset of autism cases.¹ Any manifestations of heterozygous *KCTD7* mutations in late
465 onset disorders analogous to heterozygous loss of progranulin in older adults with frontotemporal
466 dementia/FTD versus bi-allelic mutations that cause lipofuscinosis CLN11⁴⁴ are unexplored. Based
467 on yeast studies that first identified the KCTD-like yeast *Whi2*,⁵ *KCTD7* variants could potentially
468 compensate for more deleterious mutations. Spontaneous mutations in the analogous gene *WHI2* can
469 compensate in part for the more detrimental lack of mitochondrial fission factor *Fis1* or several other
470 genes.^{5, 23}

471

472 **Biological and biochemical roles for KCTD7**

473 The biochemical function of *KCTD7* is not known and little is understood about the other 24
474 human family members (*KCTD1-21*, *TNFAIP1*, *KCNRG*, *SHKBP1* and *BTBD10*). Inspired by our
475 finding that yeast *Whi2* is required to suppress cell growth in low amino acid conditions,^{5, 23} it was
476 reasonable to consider that *whi2*-deficient yeast were also defective for autophagy induction.
477 Therefore, we tested for an evolutionarily conserved function between *Whi2* and *KCTD7* in
478 autophagy. We found that both yeast *Whi2* and *KCTD7* are required for normal basal autophagy and
479 low nutrient-induced autophagy (**Figs 7 and 8**). Both yeast *Whi2* and the *KCTD7* homolog, *KCTD11*,
480 were recently shown to suppress *TORC1/mTORC1*, a known inhibitor of autophagy.⁴⁵ The same
481 study failed to detect an effect of *KCTD7* on *TORC1/mTORC1* activity in yeast and primate *COS7*
482 cells. However, a role for *KCTD7* in autophagy-lysosome function is consistent with our
483 ultrastructural studies revealing abnormal autophagosome-lysosome structures, mitochondrial cristae
484 and supernumerary lipid droplets potentially reflecting impaired lipophagy (**Fig 5 and 6**). Related
485 pathologies are observed with aging-related lysosomal dysfunction and progressive decline in
486 chaperone-mediated autophagy rates in late-onset Alzheimer disease,³⁷ perpetuated by oxidation of
487 partially degraded macromolecules derived from mitochondria, glycosphingolipids and other
488 components in autolysosomes resulting in reactive oxygen species that interact with lysosomal iron.⁴⁶

489 Consistent with our finding of abnormal mitochondrial cristae in patient-derived cells, one
490 study investigated the role of yeast *Whi2* in mitophagy (a subtype of autophagy). They reported that
491 the spontaneous *WHI2* mutation in *FIS1* knockout strains, rather than loss of the *FIS1* mitochondrial
492 fission gene, causes a defect in the degradation of yeast mitochondrial organelles.²¹ However, a
493 subsequent study challenged this conclusion, reporting that mitochondrial fission mediated by *FIS1*
494 rather than *WHI2* is required for normal mitophagy.²² Thus, the question remains open.

495 A possible role for *KCTD7* in protein turnover is consistent with having an N-terminal BTB
496 domain, where almost half of the patient mutations identified thus far occur (**Fig 1B**), as BTB

497 proteins can serve as adaptor proteins that retrieve substrates for the CUL3 ubiquitin ligase complex.
498 ⁴⁷ Interesting, several other KCTD family members were identified in screens for cullin-ARIH1
499 complex components,⁴⁸ consistent with serving as an E3 ubiquitin ligase adaptor. CUL3 has many
500 critical roles in cells, and a role for CUL3 in autophagy has gained recent attention. CUL3 and its
501 BTB-Kelch adaptor protein KLHL20 were found to prevent overzealous autophagy by direct
502 ubiquitination and degradation of ULK1, a key upstream positive regulator of autophagy induction.⁴⁹
503 Although KCTD family proteins have not been demonstrated to have a molecular role in autophagy,
504 defective autophagy-lysosome pathways are consistent with their causal roles in neurodegenerative
505 disorders.

506

507

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519

520

521 **Author Contributions**

522 KAM, XT, ALH and JMH contributed to the conception and design of the study; KAM, XT, IC,
523 HML, BW, JAR, XC, YZ, HJK, MEM, TSW, EDH, GWA, ELS, WB, TCM, MP, NM, AG, NRD, PJ,
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525 and JMH contributed to the acquisition and analysis of data; KAM, XT, IC, HML, BW, GHBM,
526 JGM, ALH and JMH contributed to drafting the text and preparing the figures.

527

528 **Potential Conflicts of Interest:** The authors have declared that no conflict of interest exists.

529

530

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532

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- 642
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645 **Figure Legends**

646 **Fig 1. Genetics and clinical features for all KCTD7 patients.**

647 (A) KCTD7 protein changes and clinical features for 37 new and published patients numbered by
648 *KCTD7* variant position and color-coded by protein region as in panel B. Initial DNA sequencing
649 strategies: whole genome (WGS), whole exome (WES), clinical diagnostic sequencing panel (DSP),
650 genome-wide linkage/autozygosity mapping (GWL), Sanger sequencing (Sng), and the heat map of
651 clinical features for all new patients are derived from deidentified clinical data (Table S1) and from
652 published cases as cited by reference number.^{4, 7-11, 13, 14} Note, L108M patient 13 was confirmed by
653 the authors to be distinct from patients-11 and -12. Estimated age of onset (months) for males vs.
654 females is not significant, $p=0.055$ (two-tailed t-test). *Deceased. (B) Map of human KCTD7
655 isoform-1 (289 amino acids) encoded on 4 color-shaded exons (scale units = 10 residues); 30 patient
656 variants are grouped in color-coded clusters as in panel A. BTB domain (transparent box); missense
657 mutations (solid line), nonsense mutations (dashed), frameshift (double line), mutations occurring in
658 >1 family (diamond), different amino acid mutation at the same position in >1 family (square).

659

660 **Fig 2. Earlier disease onset distinguishes KCTD7/EPM3 from other myoclonic epilepsies.**

661 Range (bars) and median (line) age of disease onset for early-onset disorders with myoclonic seizures.
662 TBC1D24 calculated from Supplementary data in Balestrini et al.,²⁹ DNMI,³⁰ CLN1/CLN3,¹⁷
663 KCNC1,²⁸ MERRF,⁵⁰ EPM1A/EPM2/EPM4,²⁷ sialidosis/mucopolipidosis-I
664 <https://emedicine.medscape.com/article/948704-overview>, and for KCTD7/EPM3 from Fig 1A.

665

666 Fig 3. KCTD7 family pedigrees and mutation frequency.

667 (A) Pedigrees of 11 new families from Fig 1A with 18 novel *KCTD7* variants in 15 bi-allelic patients;
668 birth order unknown for family 12. (B) All patient variants listed in the ExAC³² and gnomAD
669 sequence databases <http://gnomad.broadinstitute.org/> for the correct *KCTD7* transcript
670 ENST00000275532 (**Table S2**).

671

672 Fig 4. Patient mutations in the BTB domain affect cullin 3 (CUL3) interactions.

673 (A) Immunofluorescence microscopy of N-terminal HA-tagged KCTD7-BTB (amino acids 1-149) in
674 Kyoto HeLa cells transfected 18 h and stained with 1:1000 anti-HA (Santa Cruz HA Y-11) and
675 1:2000 anti-Myc (Calbiochem Ab-1 OP10L) (similar results without tag and in other cell types
676 tested). Representative of >3 independent experiments. (B) Immunoblots (12% SDS-PAGE, PVDF)
677 of samples described for panels A, C and D probed for anti-HA (1:1000; Santa Cruz Y11), anti-Myc
678 (1:2000; Calbiochem), and anti-actin as loading control (1:10,000; MP Biomedicals 691001),
679 representative of two independent experiments each with both WT and inactive K712R mutant CUL3
680 yielding similar results. (C) Immunofluorescence microscopy of Kyoto HeLa cells transfected with
681 N-terminal 6Myc-tagged CUL3 (K712R) alone and detected with anti-Myc (similar results for wild
682 type 6Myc-CUL3). (D) Parallel samples to panels B and C co-transfected with HA-KCTD7(1-149)
683 and N-terminal 6Myc-cullin3(K712R) and dual-stained with anti-Myc and anti-HA. Individual gray
684 scale and color merged immunofluorescence microscopy images shown. Representative of >3
685 independent experiments per condition. Scale bar = 10 μ m in all panels. (E) Co-immunoprecipitation
686 of WT 6Myc-Cul3 from HEK293 whole cell lysates (WCL) after transient co-transfection with 3His-
687 3Flag-KCTD7 using anti-Flag M2 affinity matrix for immunoprecipitation (IP). The strength of the
688 interaction between CUL3 and KCTD7 variants was quantified as a ratio of the IP cMyc signal to the
689 IP Flag signal and normalized to the WT KCTD7/CUL3 ratio in the total IP. Representative of 3
690 independent experiments is shown. **P<0.000001, *P<0.03

691

692 Fig 5. Abnormal phagolysosomes with lipid droplets in patient brain biopsy.

693 A-D. Electron micrographs of frontal lobe brain biopsy from patient-1 (T64A/R211X) at age 8 years.
694 Electron-dense lipofuscin (lysosome) structures typically contain electron-lucent lipid droplets
695 (arrowheads) that may be encased within the delimiting membrane (arrow) of phagosome-like
696 structures also containing lysosomes, but no curvilinear, fingerprint inclusions, GROD or other

697 characteristics of neuronal ceroid lipofuscinosis (NCL). Images presented are from two independent
698 preparations.

699

700 **Fig 6. Lipid droplets and abnormal phagolysosomes and mitochondria in patient fibroblasts.**

701 Electron microscopy of low (~6) passage cultured skin fibroblasts taken from patients-3 and -4 at
702 ages 4 years and 6 years, respectively. (A) Supernumerary lipid droplets (LD) near mitochondria (m)
703 common to both patients unlike age/passage-matched controls not depicted. LDs engulfed in
704 membrane-bound structures (white arrowheads); normal caveolae pits and caveolae vesicles typical
705 of normal healthy fibroblasts (patient-3, top). (B) Abnormal membrane-bound lysosome structures
706 (black arrows) associated with larger single or partial double membrane phagosome-like
707 compartments sparsely filled with lightly-stained undegraded material (white arrows) and occasional
708 electron-dense lipid whirls (example in left and right panels); lipid droplets (LD) detected in all 3
709 panels; swollen endoplasmic reticulum (ER, right panel). (C) Age- and passage-matched control
710 fibroblasts 5757 (left panel) lack abnormal mitochondria (m) containing double membrane structures
711 (black arrowheads) common to both patient fibroblasts. (D) Quantification of abnormal morphologies
712 presented as the percent of randomly selected cells (n=33, patient-3) and (n=39, patient-4), and ~100
713 fields of control 5757. Cells with supernumerary LDs typically contain over 100 LDs often close to
714 mitochondria.

715

716 **Fig 7. Defective autophagy in *whi2*-deficient yeast.**

717 (A) Immunoblots of wild type and *whi2* knockout strains of *Saccharomyces cerevisiae* (BY4741)
718 expressing autophagy reporter fusion protein GFP-Atg8 expressed by autophagy-responsive yeast
719 *ATG8/LC3* promoter (pr), before and after switching from high to low amino acid medium as
720 described,^{5, 23} probed with 1:1000 anti-GFP (Santa Cruz sc-9996), loading control anti-Pgk
721 (Abcam 113687), and HRP-conjugated secondary antibodies (GE Healthcare, 1:20,000). Induction
722 of the *ATG8* promoter (total GFP in upper+lower bands relative to PGK loading control) and
723 autophagy flux (protease-resistant free GFP liberated by vacuolar protease-dependent digestion of the
724 Atg8 moiety relative to total GFP) are presented in separate graphs as mean +/-SD for 3 independent
725 experiments with/without switching to low amino acids. (B) Experiment and analysis as described for
726 panel A except using the constitutive *PGK* promoter (pr) to express the GFP-Atg8 autophagy reporter.
727 For panels A and B, results are presented as mean +/-SD for 3 independent experiments. Two-tailed t-
728 test: WT vs. $\Delta whi2$, *p<0.05, **p<0.005, ***p<0.001). (C) As described in panel A except using the

729 transcription/translation control GFP reporter (no Atg8) expressed by the constitutive *ADHI*
730 promoter, probed with anti-GFP and loading control anti-Cdc11 (Novus Biologicals NB100-81019).
731

732 **Fig 8. Conservation of autophagy defect in KCTD7 patient fibroblasts.**

733 (A) Immunoblot of control age-/passage-matched human fibroblasts and fibroblasts derived from
734 patient-3 and -4 (passage 6-8) for endogenous autophagy protein LC3 (1:1000 anti-LC3, Cell
735 Signaling 2775) and HSP90 loading control (BD Biosciences 610419) detected with HRP-conjugated
736 secondary antibodies (GE Healthcare, 1:20,000), developed using ECL-Prime (GE Healthcare).
737 Compare LC3-II levels before versus after 1 h with 15 μ M chloroquine (Sigma C6628) or carrier
738 control to assess basal autophagy (arrows). (B) Quantified data for panel A, pooling 5757 and 498
739 control cells versus patient cells from 3 independent experiments, each performed in duplicate or
740 triplicate for each of the 4 cell lines (n=11 per condition) and presented as the ratio of LC3-II levels
741 +/-15 μ M chloroquine after correcting for loading; mean+/-SE, 2-tailed t-test, p = 0.00065. (C)
742 Autophagy assay as in panel A except cells were treated 3 h +/-glucose, +/-amino acids, +/-100 nM
743 Bafilomycin A1 (Enzo). (D) Quantification for panel C presented as mean+/-SE of LC3-II levels
744 adjusted to relative loading controls and calculated as the fold change over control 5757 in full
745 medium for 6-8 replicates in 4 independent experiments. Two-tailed t-test, *p<0.05. (E) Autophagy
746 assay as in panel A except cells were incubated in amino acid-free/serum-free Earle's balanced salt
747 solution (Thermo Fisher). The change in LC3-II/LC3-I ratios relative to time 0 is presented as
748 mean+/-SE for 3 independent experiments; 2-tailed t-test for control-5757 vs. patient-3 at 2 h and 3 h
749 (p=0.002 and p=0.018), and patient-4 (p=0.023 and p=0.020, respectively). (F) Light microscopy
750 (Nikon TE200) of control and mouse *Kctd7*-specific shK7.1 shRNA knockdown (TRCN0000069304
751 transfected with FuGENE or Lipofectamine2000) in N2a mouse neuroblastoma cells refed +/-serum
752 48 h to monitor neurite outgrowths (arrows). (G) Immunoblot confirmation of endogenous *Kctd7*
753 knockdown by shK7.1 with 1:1000 anti-KCTD7 (Abcam ab83237) and anti-HSP90 (BD Biosciences
754 610419) loading control. Lysates and immunoblots were prepared as described for panel A, except
755 visualized using Amersham Hyperfilm ECL (GE Healthcare).

756

757 **Supporting Materials**

758 Table S1. Supporting clinical data

759 Table S2. *KCTD7*/EPM3 genetic data

760 Table S3. *KCTD7* heterozygous variant data

A

Patient	Family	Mutations		Sequencing strategy	Gender	Onset (months)	Initial symptom		Seizure type					Movement disorder			Progression	Retinal abnor.	Source
		allele 1	allele 2				Seizures	Mov. disor.	Dev. delay	Myoclonus	Tonic conic	Atonic	Hypomotor	Ataxia	Tremors	Dyskinesia			
1	1	T64A	R211X	WES	M	23												New	
2	2	R70W	R84Q	WES	F	20												New	
3	3	R84W	D106fs	WES	M	5												14	
4	4	R94W	R94W	GWL	M	10												8	
5	5	R94W	R94W	WGS	M	18												7	
6	6	R99X	R99X	WGS	F	18												4	
7	6	R99X	R99X	WGS	F	24								No data				4	
8*	6	R99X	R99X	WGS	F	16												4	
9	7	G105E	G114E	WES	M	16												New	
10	7	G105E	G114E	WES	M	16												New	
11	8	L108M	L108M	DSP	F	18												7,9	
12	8	L108M	L108M	DSP	M	18												7,9	
13	9	L108M	L108M	WES	M	13												12	
14	10	R112C	R112C	WES	M	22												New	
15	11	D115Y	N273I	WGS	M	18												7	
16*	12	R121L	W235S	WES	M	18												New	
17	12	R121L	W235S	WES	F	18												New	
18	13	R153H	R153H	WES	F	15												New	
19	14	R177H	A178V	DSP	F	24												New	
20	15	V179fs	V179fs	WES	M	10												New	
21	15	V179fs	V179fs	Sngr	F	19												New	
22	16	R181W	D229Y	WES	M	24												New	
23*	17	R184C	R184C	WES	M	9								NA				13	
24*	17	R184C	R184C	WES	F	8								NA				13	
25	18	I199fs	I199fs	WGS	F	9												7	
26	19	I199fs	I199fs	WGS	M	18												7	
27	20	P205L	V259E	DSP	M	16												New	
28*	21	D229Y	L271H	WES	F	15												New	
29	21	D229Y	L271H	WES	M	15												New	
30*	22	F232fs	F232fs	WES	M	9												11	
31	22	F232fs	F232fs	WES	M	6												11	
32	23	N273I	N273I	WGS	M	18								NA				7	
33	24	Y276C	Y276C	WES	F	24												10	
34	24	Y276C	Y276C	WES	F	24												10	
35	24	Y276C	Y276C	WES	F	15								No data				10	
36	25	W289X	W289X	WGS	M	18												7	
37	25	W289X	W289X	WGS	F	36												7	

Fig 1
Metz et al.

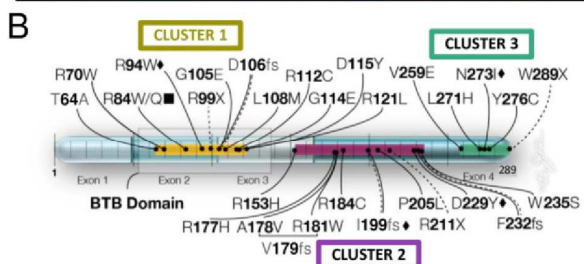


Fig1 Patients

101x159mm (300 x 300 DPI)

Fig 2
Metz et al.

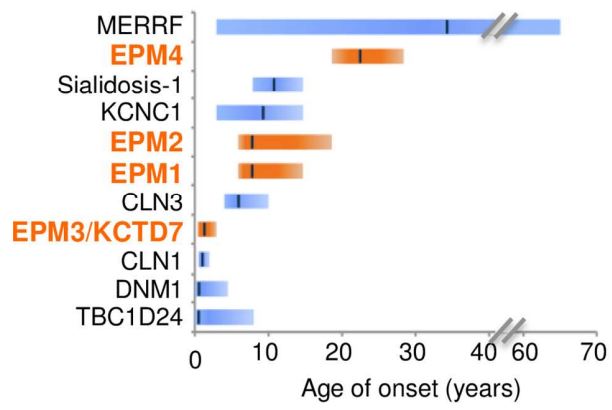


Fig 2 Age of onset

125x121mm (300 x 300 DPI)

Fig 3
Metz et al.

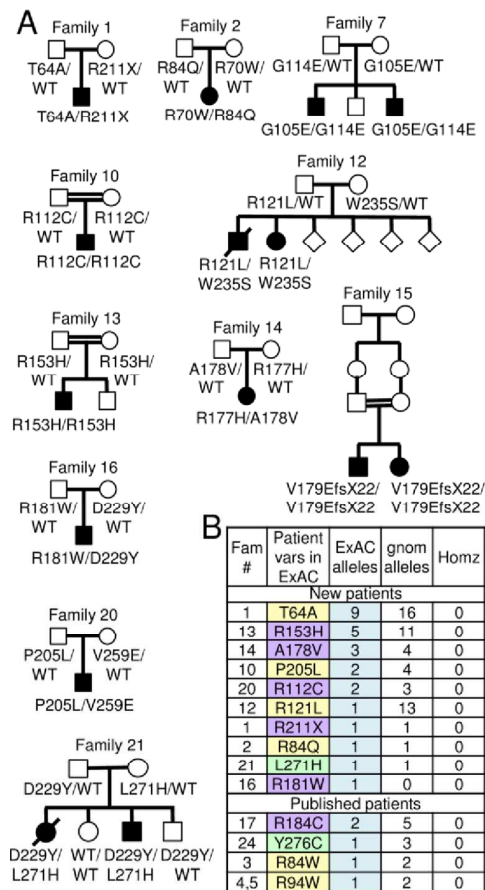


Fig 3 Genetics

107x168mm (300 x 300 DPI)

Fig 4
Metz et al.

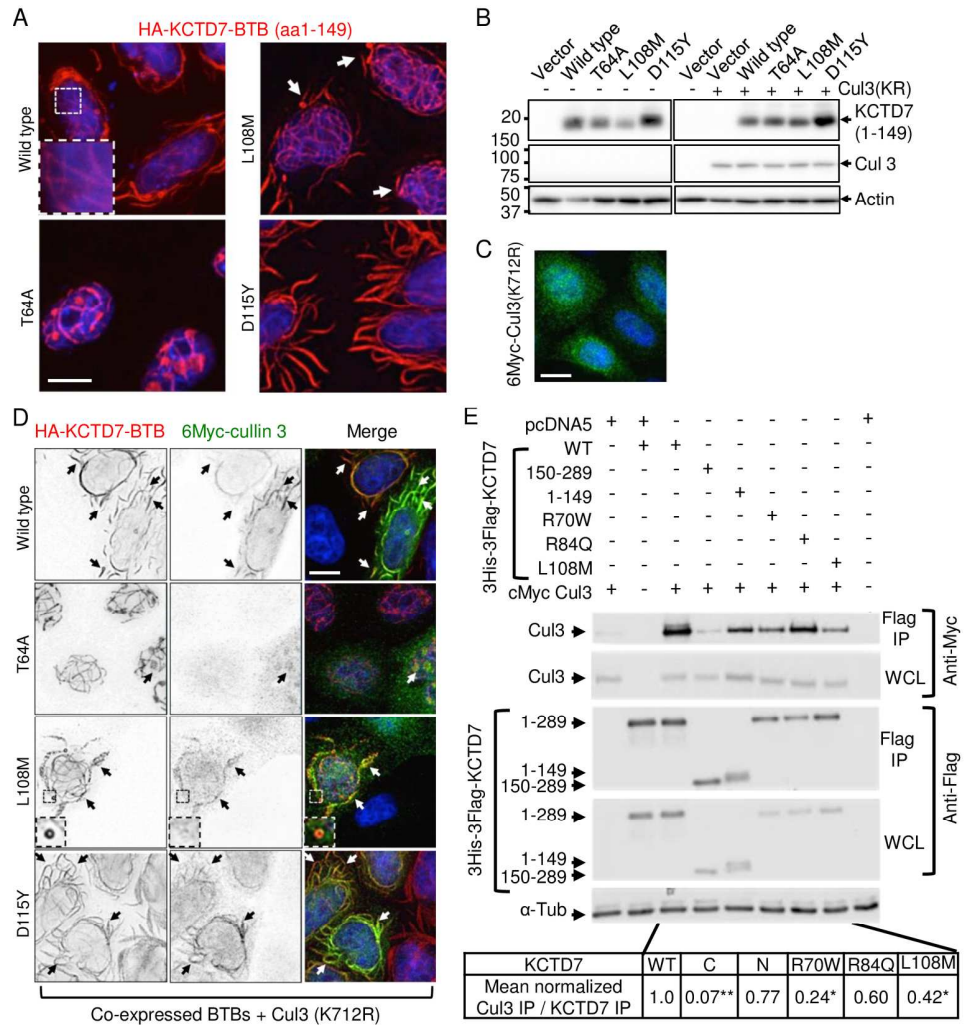


Fig 4 KCTD7 function

171x200mm (300 x 300 DPI)

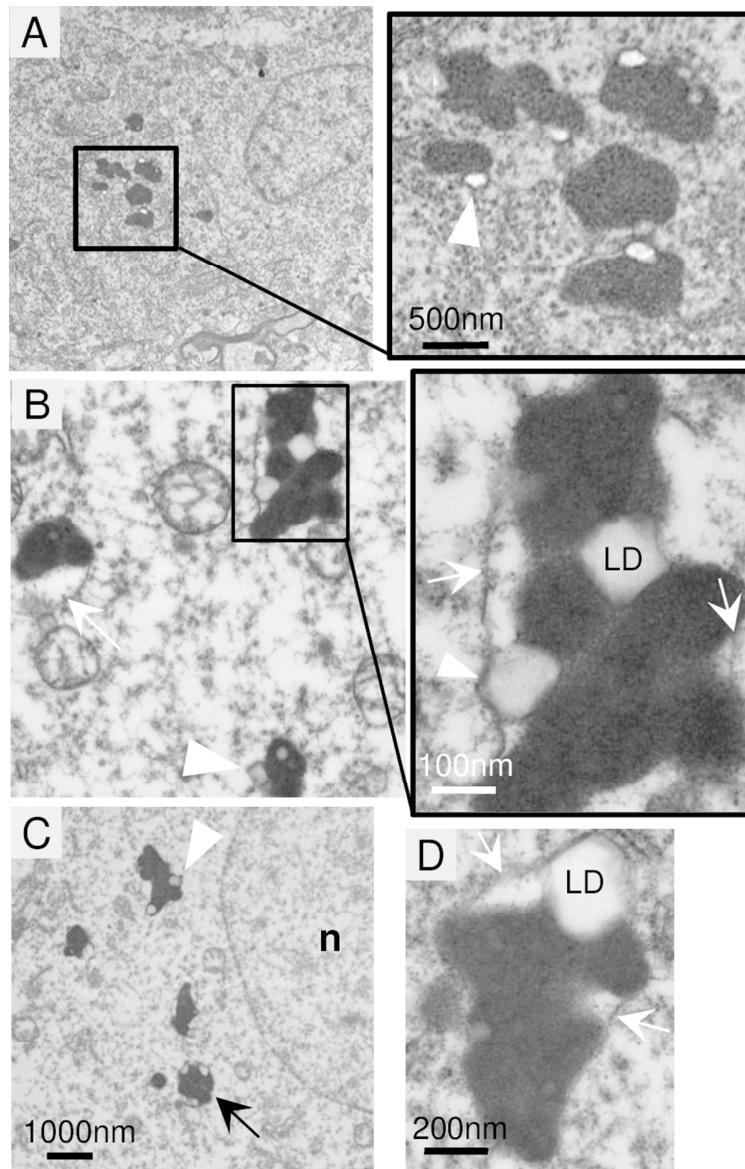


Fig 5 Brain biopsy

75x117mm (300 x 300 DPI)

Fig 6 Metz et al.

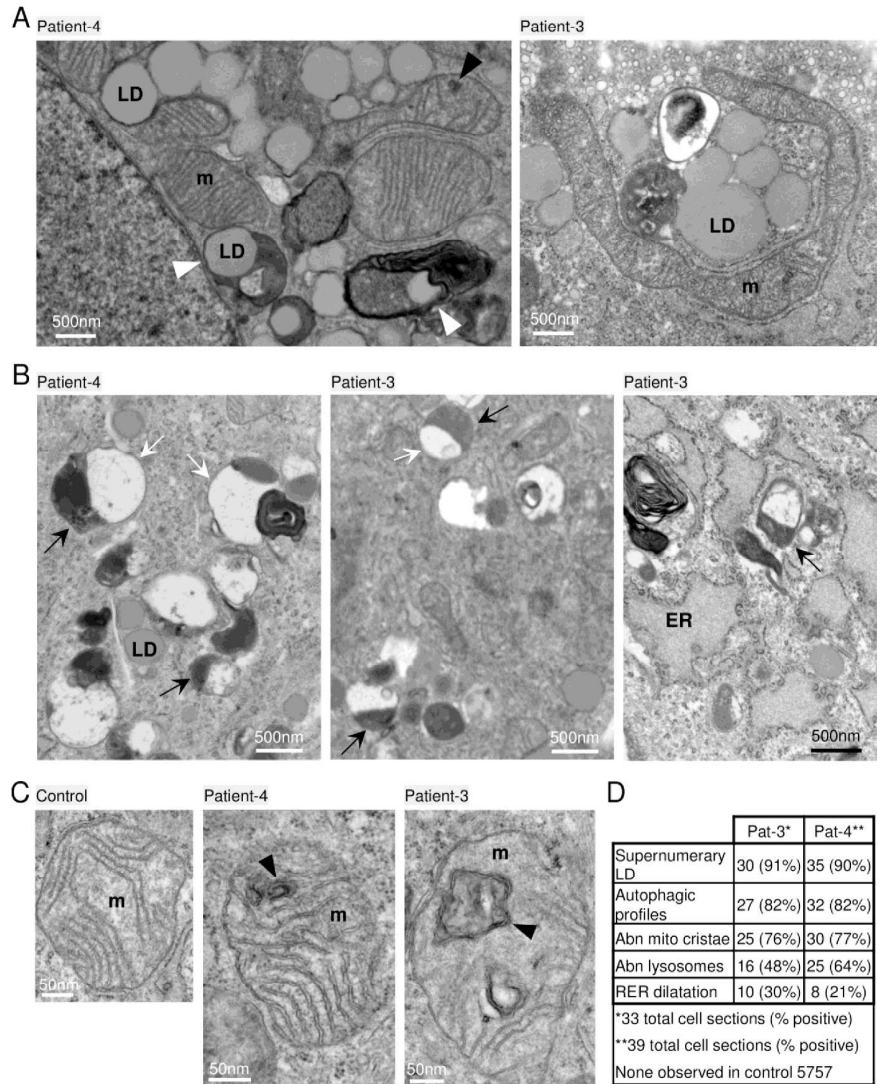


Fig 6 Fibroblast ultrastructure

173x220mm (300 x 300 DPI)

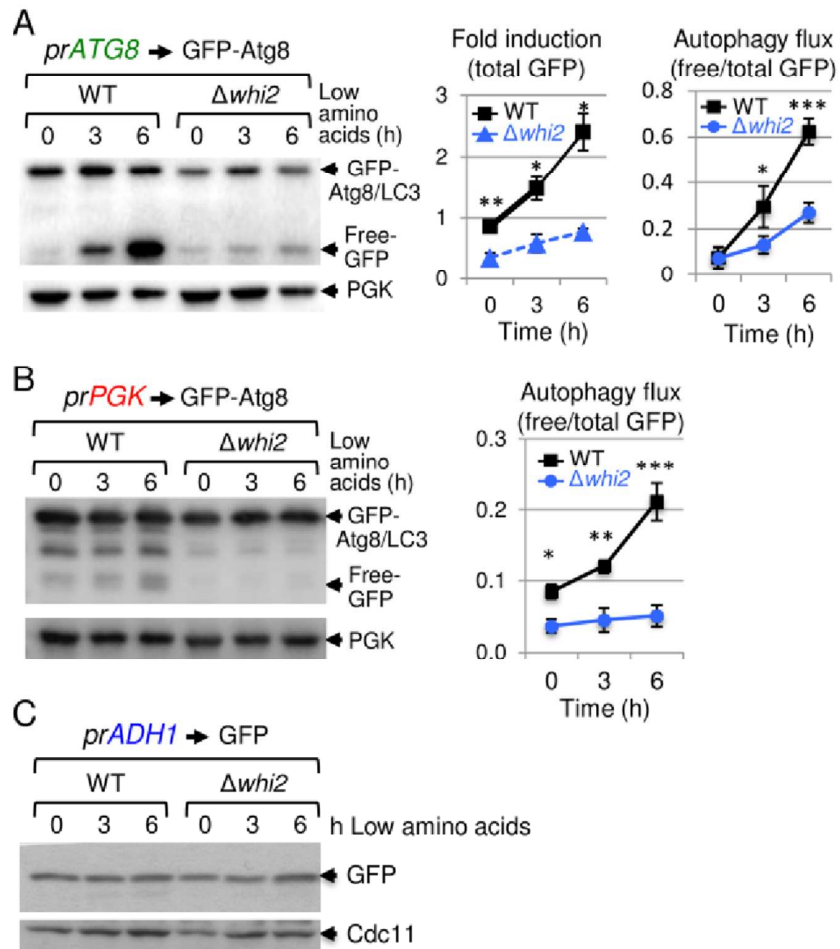
Fig 7
Metz et al.

Fig 7 Yeast autophagy assays

92x112mm (300 x 300 DPI)

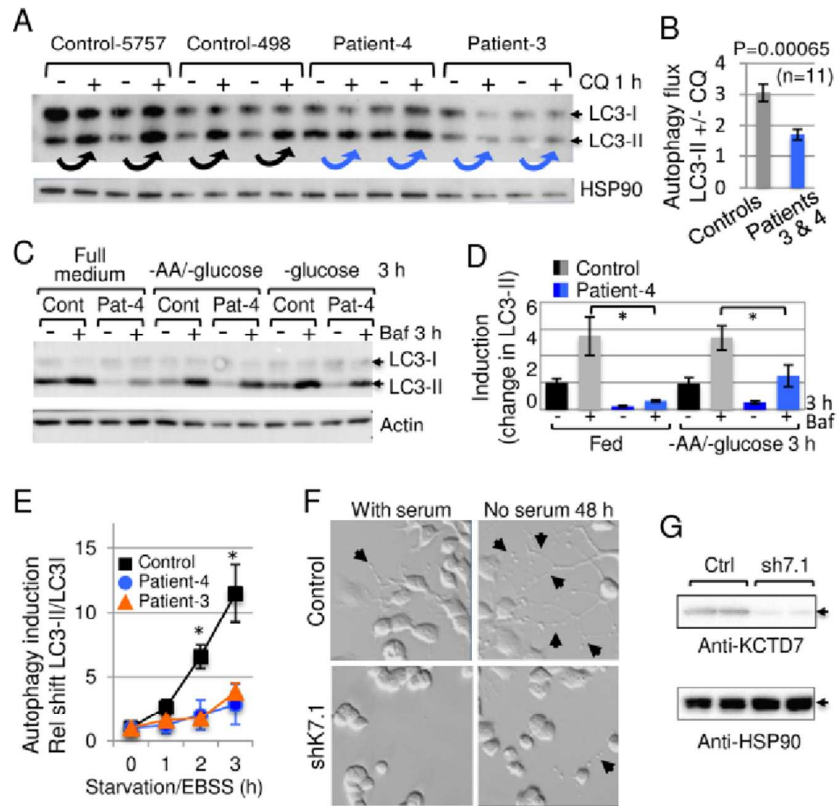
Fig 8
Metz et al.

Fig 8 Patient fibroblast autophagy assays

93x91mm (300 x 300 DPI)