

**REPORT****A novel mutation in VCP causes Charcot–Marie–Tooth Type 2 disease**

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Mutations in VCP have been reported to account for a spectrum of phenotypes that include inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia, hereditary spastic paraplegia, and 1–2% of familial amyotrophic lateral sclerosis. We identified a novel VCP mutation (p.Glu185Lys) segregating in an autosomal dominant Charcot–Marie–Tooth disease type 2 family. Functional studies showed that the Glu185Lys variant impaired autophagic function leading to the accumulation of immature autophagosomes. VCP mutations should thus be considered for genetically undefined Charcot–Marie–Tooth disease type 2.

**Keywords:** neuropathy; whole-exome sequencing; autophagy; hereditary motor and sensory neuropathies; neurodegeneration

**Abbreviations:** ALS = amyotrophic lateral sclerosis; CMT = Charcot–Marie–Tooth; IBMPFD = inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia

**Introduction**

Charcot–Marie–Tooth disease type 2 (CMT2) is a clinically and genetically heterogeneous disorder that leads to axonal degeneration of the peripheral nerve. At the time of writing, it is estimated that up to 70% of patients with CMT2 do not obtain

a genetic diagnosis (Saporta *et al.*, 2011; Murphy *et al.*, 2012). However, whole exome sequencing has led to astounding progress in gene identification in CMT2, which will ultimately improve genetic diagnostics (Timmerman *et al.*, 2014). In addition, the identification of >50 genes for CMT2 has improved our understanding of the phenotypic and genotypic intersection of peripheral

neuropathies with other motor neuron and axonal degenerative disorders. The gene encoding valosin-containing protein (VCP) is an example of a gene associated with a spectrum of related disorders due to reports that have linked missense mutations in VCP to cause inclusion body myopathy, Paget's disease of the bone, frontotemporal dementia (IBMPFD), hereditary spastic paraplegia, and 1–2% of familial amyotrophic lateral sclerosis (ALS) (Kimonis *et al.*, 2000; Watts *et al.*, 2004; Johnson *et al.*, 2010; de Bot *et al.*, 2012). VCP, in association with ubiquitin binding proteins, facilitates the degradation of proteins through both the proteasomal and autophagic pathways (Meyer *et al.*, 2012). Recent evidence suggests that pathogenic VCP mutations preferentially disrupt autophagy leading to cellular degeneration (Ju *et al.*, 2009).

Using whole exome sequencing in a family with autosomal dominant CMT2, we identified a novel missense mutation (p.Glu185Lys) in VCP that is located in the same L1 domain as other pathogenic mutations. Our results suggest that the phenotypic spectrum associated with VCP should be expanded to include CMT2.

## Materials and methods

### Consent and exome sequencing

A CMT family with eight individuals was ascertained. Informed consent was obtained from all individuals and the Institutional Review Boards at the participating medical centres approved the study. Neurological exam and neurophysiological studies were performed. For detailed methods and functional studies see the online Supplementary material.

## Results

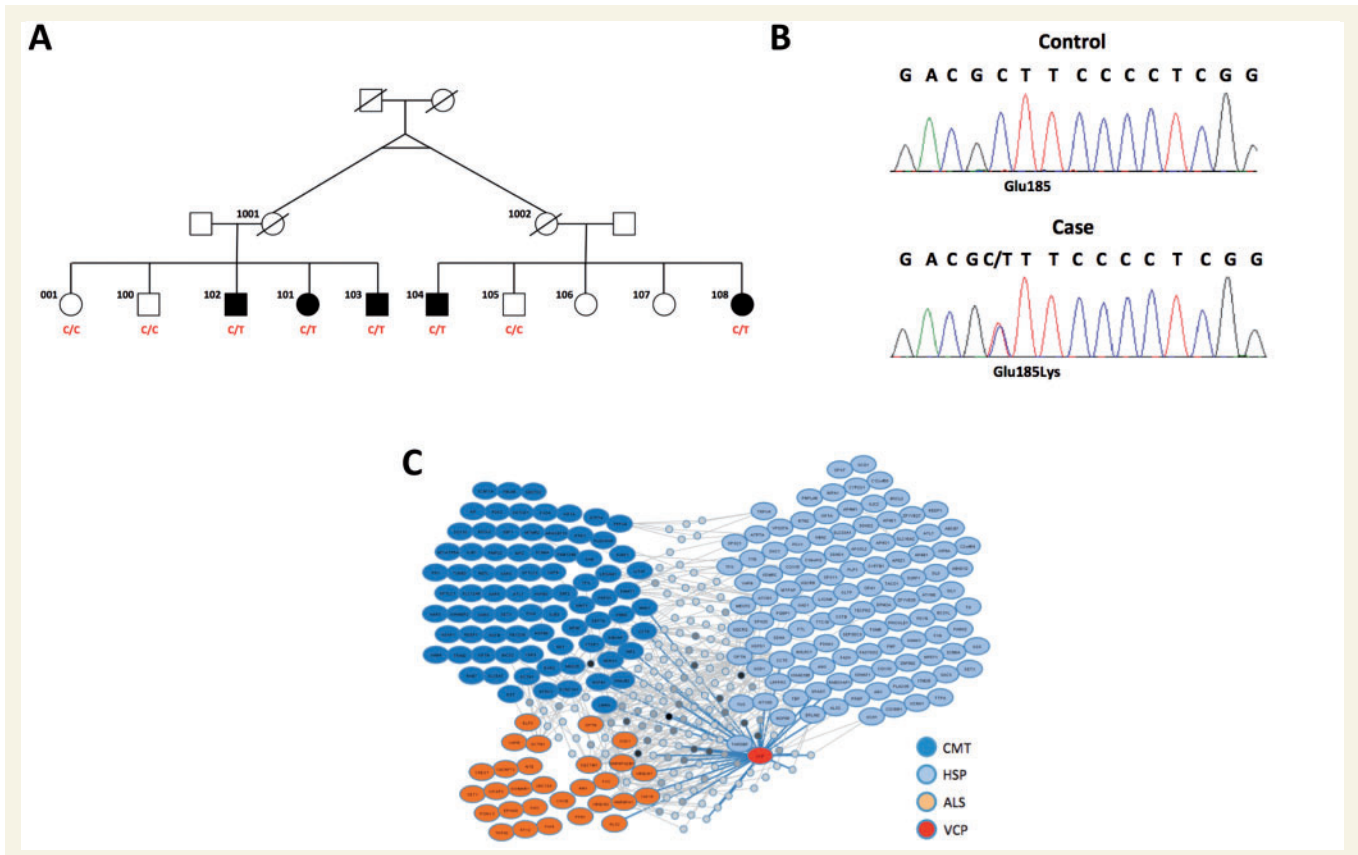
### Clinical findings

We evaluated eight family members from a single generation of a family thought to have a dominantly inherited peripheral neuropathy (Fig. 1). Dominant inheritance is presumed based on family history describing that the identical twins Subjects 1001 and 1002 were reportedly to have always had long, thin legs. They had high arches and hammer toes and developed weakness and sensory loss in their fourth and fifth decade of life. Subject 1001 passed away at 87 years of age and Subject 1002 passed away at 84 years, both of natural causes. Five individuals had abnormalities on their neurological examinations and nerve conduction studies. The onset of symptoms began in early childhood for Subject 0101, early adulthood in Subject 0108, and after age 50 in Subjects 0102, 0103 and 0104. The earliest affected individual required shoes connected by a bar to aid in ambulation in early childhood. She was a slow runner and could not keep up with her peers as a girl. She underwent triple arthrodesis in 1998 and 2000. She continued to have difficulty with balance and noticed a progressive inability to feel touch below her knees. She began wearing bilateral ankle foot orthoses in 2009, and in 2010 she used these along with forearm crutches for mobility. She

subsequently developed difficulty using her hands for fine movements such as manipulating buttons or fastening jewellery. In 2009, neuropsychiatric testing and evaluation resulted in the diagnosis of adjustment disorder with changes in mood and behaviour requiring anxiolytic antidepressant or other psychotropic medication. She has long-standing dysarthria, dyspnoea, and persistent cough. Subject 0108 first noted weakness in her early 20s when she needed to wear high heels at work. She obtained orthotics in her late 20s and has gradually developed problems with balance. The three remaining subjects all developed problems with balance, loss of sensation, and difficulties with fine movements of their hands after the age of 50. The adult CMT Neuropathy Score (CMTNSv2) was used to measure impairment in all five subjects (Murphy *et al.*, 2011). Impairment was considered to be mild in two, moderate in two and severe in one individual. All affected subjects had absent Achilles deep tendon reflexes. Four of the five had clear pes cavus; the fifth had high arches but not hammer toes. The clinical features of the affected five individuals are summarized in Table 1. Four of the five affected patients gradually developed symmetrical, length-dependent weakness resulting in an inability to walk on their heels and frequent ankle pains. The same four patients subsequently developed weakness in intrinsic hand muscles (Table 1). Additionally, weakness in the proximal leg or arm muscles was not observed. No scapular winging was present, creatine kinase values were normal, and no fasciculations were observed. Atrophy was detected in weak calf and intrinsic hand muscles. All five patients had length-dependent small (pin-prick) and large (vibration) fibre sensory loss (Table 1). All five patients had difficulty with balance that was exacerbated by situations in which vision was impaired as by darkness or walking in a crowd. Nerve conduction studies were abnormal in all five affected individuals. The abnormalities were characterized as intermediate slowing of motor conduction velocities and axonal features of both motor and sensory nerves (Supplementary Tables 1 and 2). The EMG did not demonstrate any spontaneous activity as positive sharp waves or fibrillations. The proband (Subject 0101) had previously undergone commercial testing for CMT that did not reveal mutations in *GJB1* (Cx32), *MPZ*, *NEFL*, *GDAP1*, *MFN2*, *LMNA*, *RAB7A*, *GARS* and *HSPB1*. Alkaline phosphatase and creatine kinase levels were normal.

### Genomic studies

We used the GEM.app software (Gonzalez *et al.*, 2013) to focus our genomic analysis on rare and conserved variants that followed an autosomal dominant mode of inheritance in whole exome sequencing data. Only variants were considered that meet the following criteria: (i) non-synonymous change; (ii) not present in NHLBI EVS 6500; (iii) not present in 2700 families within the GEM.app database; (iv) Genomic Evolutionary Rate Profiling (GERP) > 3 OR PhastCons > 0.6; and (v) Genotype Quality > 75 (as previously described in Montenegro *et al.*, 2011). This resulted in the identification of three candidate variants of which only a variant in VCP co-segregated with disease. This missense change, c.553C > T (p.Glu185Lys) in VCP (NM\_007126.3) was not observed in 9200 exomes, had a very high GERP conservation score of 6.07, PhastCons score of 1, and was predicted to be

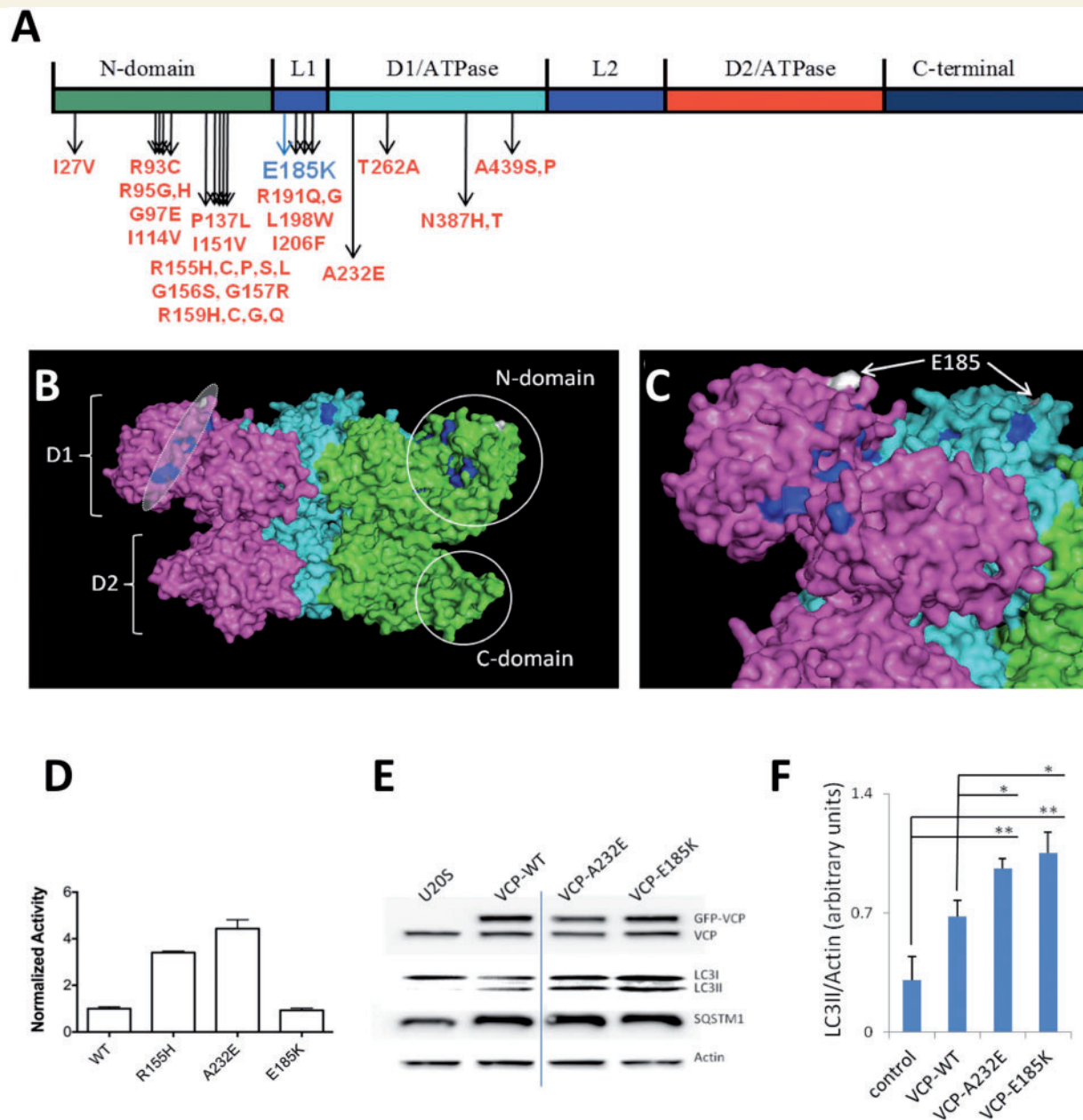


**Figure 1** Genomic studies of a large CMT2 family. (A) Pedigree of the studied family with genotypes of the VCP mutation in all available individuals. Black symbols – affecteds. (B) Sanger sequencing traces confirmed the presence of the mutation. (C) Visualization of VCP yeast 2-hybrid interactions with known CMT, hereditary spastic paraplegia and ALS proteins, allowing for one intermediate interacting node (Gonzalez *et al.*, 2013; Sreedharan and Brown, 2013; Timmerman *et al.*, 2013). Direct VCP interactions are highlighted. The figure was generated using interaction data from BioGRID and the GEM.app application (Stark *et al.*, 2006; Gonzalez *et al.*, 2013).

**Table 1** Clinical features of family with E187K VCP mutation

Patient ID	Patient age (years)	CMTNSv2	Distal weakness LL	Proximal weakness LL	Distal weakness UL	Proximal weakness UL	Vibration LL	Vibration UL	Cutaneous LL	Cutaneous UL
0101	59	22 (severe)	+ (4–,5)	– (5,5)	+ (4–,4,4–)	– (5,5,5)	Red Toes, ankles and knees	Red fingers, wrist	Abs toes, ankles, knees	Normal
0102	64	12 (moderate)	+ (4–,5)	– (5,5)	+ (4,5,4)	– (5,5,5)	Abs toes, Red ankles	Normal	Abs toes, Red ankles	Normal
0103	66	16 (moderate)	– (5,5)	– (5,5)	– (5,5,5)	– (5,5,5)	Abs toes, Red ankles, knees	Red fingers	Abs toes, ankles, knees	Normal
0108	48	5 (mild)	+ (4,5)	– (5,5)	+ (4+,4+,4+)	– (5,5,5)	Abs toes	Normal	Absent toes	Normal
0104	66	7 (mild)	+ (0,3)	+ (4,4–)	+ (4+,5,4)	– (5,5,5)	Abs toes, Red ankles	Normal	Red toes	Normal

Motor weakness based on MRC scale (0–5): ‘+’ = weakness present, ‘–’ = no weakness detected. Lower limb distal weakness assessed by anterior tibialis and gastrocnemius; lower limb proximal weakness assessed by iliopsoas and quadriceps; upper limb distal weakness assessed by first dorsal interosseous, abductor pollicis brevis, and adductor digiti minimi; upper limb proximal weakness assessed by deltoids, biceps brachii, and triceps. Vibration based on Rydell tuning fork with ‘5’ on scale of ‘8’ being considered normal and cutaneous based on Pinprick sensation: Normal is no decrease compared to the examiner. Red = reduced; abs = absent up to level indicated. Both motor and sensory evaluations were based on worst score observed of the two limbs. CMTNSv2 scores are separable into < 10 (mild), 11–20 (moderate) or > 20 (severe) impairment (Murphy *et al.*, 2011). LL = lower limb; UL = upper limb.



**Figure 2** Functional evaluation of the VCP mutation. (A) Linear diagram of VCP with the location of 29 identified missense mutations at 18 different residues (in red). The location of the E185K mutation is shown in blue. (B–C) Renderings of the crystallographic structure of three subunits from a VCP hexamer (each individual monomer is a unique colour). (B) Hexameric VCP with the positions of the D1 domain (top barrel) and D2 domain (bottom barrel) with the N and C domains protruding to the sides. The dotted oval denotes where all 18 mutant residues (blue) reside. (C) Close-up view of the N-D1 interface where all known mutations reside (in blue) and the position of E185 (white). (D) Recombinant human VCP protein was purified from *E. coli* and its basal rate of ATPase was assayed. The ATPase activity of VCP-WT was arbitrarily designated as 1. (E) Representative immunoblot for VCP, LC3, SQSTM1 and actin of U20S cells transiently expressing VCP-WT-GFP, VCP-A232E-GFP or VCP-E185K-GFP for 2 days. (F) Quantitation of LC3II/actin levels from three independent experiments. \* $P < 0.05$  and \*\* $P$ -value  $< 0.005$  by student  $t$ -test.

'damaging' by four of five protein prediction programs (PolyPhen-2, MutationAssessor, LRT, MutationTaster, SIFT) (Table 2). VCP had previously been shown to cause a spectrum of phenotypes from ALS, frontotemporal dementia, inclusion body myopathy, Paget's disease of bone, and hereditary spastic paraplegia, but not CMT2.

Parametric linkage analysis was performed with the following assumptions: (i) 100% penetrance; (ii) autosomal dominant mode of inheritance; (iii) disease frequency of  $1 \times 10^{-6}$ ; and (iv) minor allele frequency of  $1 \times 10^{-6}$ . This analysis resulted in a log of odds (LOD) score of 2.107 for the c.553C > T VCP mutation in this family.

**Table 2** *In silico* scores

Severity prediction	Value	Range
GERP score	6.07	–12.3 to 6.17
PhastCons	1	0 to 1
PolyPhen-2	Probably damaging	Benign, possibly damaging, probably damaging
MutationTaster	Disease causing	Polymorphism to disease causing
MutationAssessor	High	Neutral, low, medium, high
LRT	Deleterious	Unknown, neutral, deleterious
SIFT	Tolerated	Tolerated to damaging

Nearly all *in silico* scores predicted a significantly damaging mutation.  
GERP = Genomic Evolutionary Rate Profiling.

To understand the role of VCP in a gene network composed of known Mendelian genes for ALS, hereditary spastic paraplegia and CMT, we analysed unbiased, experimentally derived protein–protein interactions based on data from BioGRID (Stark *et al.*, 2006). VCP directly interacts with 14 known CMT, hereditary spastic paraplegia, and ALS proteins, and further interacts with 19 ‘intermediate’ proteins that directly connect to proteins in the known disease gene clusters (Fig. 1C). Thus, VCP appears to represent an important node negotiating between these different disease gene clusters, which may explain its involvement in a number of phenotypes.

## Functional evaluation

The E185K variant resides within the linker domain between the N-domain and the D1 ATPase domain. Several other known pathogenic mutations are within this amino acid stretch and include R191Q, R191G, and L198W (Fig. 2A). More importantly, the E185 mutant residue is adjacent to other pathogenic mutations when superimposed onto the crystal structure of VCP (Fig. 2B and C).

To further assess the potential pathogenicity of the E185K mutation, we compared the intrinsic ATPase activity of VCP-WT, the previously reported IBMPFD mutations VCP-R155H and VCP-A232E with VCP-E185K. As demonstrated previously, the intrinsic ATPase activity of recombinant VCP-R155H and VCP-A232E was elevated by 3–4-fold (Fig. 2D) (Niwa *et al.*, 2012). In contrast, VCP-E185K’s ATPase activity was unchanged as compared to VCP-WT (Fig. 2D). To further assess the pathogenicity, we transiently transfected VCP-WT, VCP-A232E and VCP-E185K fused to a C-terminal green fluorescent protein tag into U20S cells and evaluated the levels of VCP, the autophagosome protein LC3, SQSTM1 and a loading control actin via immunoblot (Fig. 2E). As previously described, VCP-A232E expression results in an increase in LC3II protein levels (Fig. 2E) (Ju *et al.*, 2009). Similarly, VCP-E185K expression caused an increase in LC3II levels consistent with the previously defined disruption in autophagosome maturation by VCP disease mutations (Fig. 2E and F).

## Discussion

By applying a comprehensive genomic approach and linkage analysis we have identified a CMT2 family with a significant mutation

in VCP. The combination of length-dependent sensory loss clinically and by electrophysiology, intermediate slowing of motor nerve conduction suggestive of myelin abnormalities and length-dependent weakness represents a novel phenotype for patients with VCP mutations. The phenotype is distinct from that of ALS or inclusion body myopathy. Because of the intermediate slowing on motor nerve conduction, this family could also be considered to have dominant-intermediate CMT rather than CMT2. The expansion of the phenotypic spectrum of VCP to include CMT is part of a broader trend, especially in neurological diseases, where genotypic and phenotypic overlap is increasingly recognized. This is mainly owed to the wider application of next-generation sequencing and other comprehensive forms of genome studies.

The E185K mutation in VCP is unique in that it causes distinctly one phenotype, CMT2, within a family. Most other previously reported VCP mutations have caused a spectrum of disease phenotypes ranging from inclusion body myopathy, Paget’s disease, and FTD to ALS. The exception being patients identified with sporadic ALS and unique VCP variants (DeJesus-Hernandez *et al.*, 2011; Abramzon *et al.*, 2012; Koppers *et al.*, 2013). A CMT2 phenotype may be considered a milder form of IBMPFD/ALS as some patients with IBMPFD/ALS have been reported to have sensory/motor neuropathies in addition to the other phenotypes (Kimonis *et al.*, 2008). This milder phenotypic expression may explain the normal ATPase activity of the E185K mutation. An elevated basal ATPase activity is characteristic for most but not all IBMPFD/ALS (Niwa *et al.*, 2012).

Studies suggest that IBMPFD mutations in VCP lead to an impairment in protein degradation and specifically autophagy (Ju *et al.*, 2009). Expression of IBMPFD mutations in tissue culture or animal models leads to the accumulation of non-degradative autophagosomes (Ju *et al.*, 2009). More recently it has been demonstrated that VCP is instrumental in the autophagic degradation of mitochondria or mitophagy. VCP facilitates the extraction of ubiquitinated MFN1 and MFN2 from the outer mitochondrial membrane thus initiating mitophagy (Tanaka *et al.*, 2010). Interestingly, mutations in MFN2 lead to CMT2 suggesting a functional connection in the pathogenesis of these two hereditary neuropathies (Zuchner *et al.*, 2004). Our protein network analysis further underlines the role of VCP as a hub with surprisingly many connections into CMT, ALS and hereditary spastic paraplegia gene/protein clusters. Future studies may determine that

mutations at E185K specifically disrupt mitophagy as opposed to global macroautophagy.

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## Supplementary material

Supplementary material is available at *Brain* online.

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