

## Sensory neuropathy with bone destruction due to a mutation in the membrane-shaping atlastin GTPase 3

Uwe Kornak,<sup>1,2,\*</sup> Inès Mademan,<sup>3,4,\*</sup> Marte Schinke,<sup>5,\*</sup> Martin Voigt,<sup>6</sup> Peter Krawitz,<sup>1</sup> Jochen Hecht,<sup>1</sup> Florian Barvencik,<sup>5</sup> Thorsten Schinke,<sup>5</sup> Sebastian Gießelmann,<sup>6</sup> F. Timo Beil,<sup>7</sup> Adolf Pou-Serradell,<sup>8</sup> Juan J. Vilchez,<sup>9,10,11</sup> Christian Beetz,<sup>12</sup> Tine Deconinck,<sup>3,4</sup> Vincent Timmerman,<sup>3,13</sup> Christoph Kaether,<sup>14</sup> Peter De Jonghe,<sup>3,4,15</sup> Christian A. Hübner,<sup>6</sup> Andreas Gal,<sup>16</sup> Michael Amling,<sup>5</sup> Stefan Mundlos,<sup>1,2,17</sup> Jonathan Baets<sup>3,4,15,†</sup> and Ingo Kurth<sup>6,†</sup>

1 Institute of Medical Genetics and Human Genetics, Charité-Universitätsmedizin Berlin, 13353 Berlin, Germany

2 Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany

3 Neurogenetics Laboratory, Institute Born-Bunge, University of Antwerp, 2610 Antwerp, Belgium

4 Neurogenetics Group, VIB Department of Molecular Genetics, University of Antwerp, 2610 Antwerp, Belgium

5 Department of Osteology and Biomechanics, University Medical Centre Hamburg-Eppendorf, 20246 Hamburg, Germany

6 Institute of Human Genetics, Jena University Hospital, 07743 Jena, Germany

7 Department of Orthopaedics, University Medical Centre Hamburg Eppendorf, 20246 Hamburg, Germany

8 Department of Neurology, Hospital del Mar, University of Barcelona, 08003 Barcelona, Spain

9 Department of Neurology, Hospital Universitari i Politècnic La Fe, 46026 Valencia, Spain

10 Department of Medicine, University of Valencia, 46026 Valencia, Spain

11 Biomedical Network Research Centre on Neurodegenerative Disorders (CIBERNED), Instituto de Salud Carlos III, 28029 Madrid, Spain

12 Department of Clinical Chemistry, Jena University Hospital, 07747 Jena, Germany

13 Peripheral Neuropathy Group, VIB Department of Molecular Genetics, University of Antwerp, 2610 Antwerp, Belgium

14 Leibniz Institut für Altersforschung-Fritz Lipmann Institut, 07745 Jena, Germany

15 Department of Neurology, Antwerp University Hospital, 2650 Antwerp, Belgium

16 Institute of Human Genetics, University Medical Centre Hamburg Eppendorf, Hamburg 20246, Germany

17 Berlin-Brandenburg Centre for Regenerative Therapies (BCRT), Charité Universitätsmedizin Berlin, 13353 Berlin, Germany

\*.†These authors contributed equally to this work.

Correspondence to: Ingo Kurth  
Jena University Hospital,  
Institute of Human Genetics,  
Kollegiengasse 10,  
07743 Jena,  
Germany  
E-mail: ingo.kurth@med.uni-jena.de

Many neurodegenerative disorders present with sensory loss. In the group of hereditary sensory and autonomic neuropathies loss of nociception is one of the disease hallmarks. To determine underlying factors of sensory neurodegeneration we performed whole-exome sequencing in affected individuals with the disorder. In a family with sensory neuropathy with loss of pain perception and destruction of the pedal skeleton we report a missense mutation in a highly conserved amino acid residue of atlastin GTPase 3 (ATL3), an endoplasmic reticulum-shaping GTPase. The same mutation (p.Tyr192Cys) was identified in a second family with similar clinical outcome by screening a large cohort of 115 patients with hereditary sensory and autonomic

neuropathies. Both families show an autosomal dominant pattern of inheritance and the mutation segregates with complete penetrance. *ATL3* is a paralogue of *ATL1*, a membrane curvature-generating molecule that is involved in spastic paraplegia and hereditary sensory neuropathy. *ATL3* proteins are enriched in three-way junctions, branch points of the endoplasmic reticulum that connect membranous tubules to a continuous network. Mutant *ATL3* p.Tyr192Cys fails to localize to branch points, but instead disrupts the structure of the tubular endoplasmic reticulum, suggesting that the mutation exerts a dominant-negative effect. Identification of *ATL3* as novel disease-associated gene exemplifies that long-term sensory neuronal maintenance critically depends on the structural organisation of the endoplasmic reticulum. It emphasizes that alterations in membrane shaping-proteins are one of the major emerging pathways in axonal degeneration and suggests that this group of molecules should be considered in neuroprotective strategies.

**Keywords:** axon; HSAN; sensory neurons; neurodegeneration; nociception

**Abbreviations:** HSAN = hereditary sensory and autonomic neuropathies; PDI = protein disulphide isomerase; SPG = spastic paraplegia

## Introduction

Axonopathies are neurodegenerative disorders that mainly affect the long processes of neurons (Suter and Scherer, 2003; Züchner and Vance, 2005). The longest axons can measure up to 1 m, posing a major challenge for crucial mechanisms such as trafficking, energy use, signalling and cytoskeletal organization. Axonopathies of cortical motor neurons comprise the group of hereditary spastic paraplegias (SPGs), whereas lower motor neurons are typically affected in distal hereditary motor neuropathies. Both diseases are characterized by a length-dependent axonal degeneration and are distinct from motor neuron diseases where a combined degeneration of both upper and lower motor neuron cell bodies occurs.

Hereditary neuropathies can affect sensory axons and include hereditary motor and sensory neuropathies and hereditary sensory and autonomic neuropathies (HSAN). In HSAN, sensory loss ranges from local numbness over loss of proprioception to the complete inability to experience pain. As a consequence, affected individuals are highly exposed to injuries and mutilations. Loss of pain perception also causes abnormal mechanical loading in the distal weight-bearing parts of the skeleton (Leipold *et al.*, 2013). This can finally result in neuropathic arthropathy and spontaneous fractures. Fracture healing can be severely impaired leading to resorption of bones. The disorder is often complicated by osteomyelitis (Marik *et al.*, 2009). In spite of the heavy burden of disease, only supportive care is currently available for patients affected by HSAN. Autosomal dominant and autosomal recessive forms of the disorder exist and known culprit genes encode proteins crucial for sphingolipid metabolism, neurotrophin action, axonal transport, DNA methylation, and membrane shaping of organelles (Rotthier *et al.*, 2012). Nonetheless, the majority of HSAN cases remain genetically unresolved (Rotthier *et al.*, 2012).

Further identification of causal HSAN genes is essential to improve genetic diagnosis and the development of therapeutic strategies. Unravelling the genetic architecture of HSAN will enlighten the mechanistic understanding of axonal degeneration and provide potential insights in other more common acquired forms of PNS neurodegeneration such as diabetic neuropathy or toxic neuropathies.

## Materials and methods

### Patient data

Written informed consent was obtained from the study participants after approval from the Institutional Review Boards at the participating institutions.

### Exome sequencing

Libraries for whole exome sequencing were prepared using NEBNext<sup>®</sup> DNA Library Prep Master Mix Set for Illumina (New England Biolabs) according to the manufacturer's protocols. Exonic and adjacent intronic sequences were enriched from genomic DNA with the use of the SureSelect Human Exome Kit V4 (Agilent Technologies) and run on a HiSeq 2000 Sequencer (Illumina). The average sequencing depth was  $>40\times$  and  $>90\%$  of the exome target region was covered by  $>10$  reads. Variants were detected with GATK toolkit version 2.6 (McKenna *et al.*, 2010) and the exome genotyping accuracy was estimated to be  $>0.9999$  based on the variant calls (Heinrich *et al.*, 2013). Variants were detected with SAMtools, annotated with ANNOVAR, and analysed using the Gene-Talk platform and Exomiser (Wang *et al.*, 2010; Kamphans and Krawitz, 2012). The pathogenic potential of individual candidate variants was evaluated by MutationTaster (Schwarz *et al.*, 2010).

### Mutation screening

According to standard protocols, total genomic DNA was isolated from blood samples of patients and family members and was used as a template in PCRs. All coding regions and exon–intron boundaries of *ATL3* (NM\_015459) were amplified by oligonucleotide primers designed with Primer3 (sequences available upon request) and sequenced on an ABI3730xl DNA Analyzer (Applied Biosystems) or the Illumina MiSeq system (Illumina).

### Haplotype analysis

Genotyping was performed to investigate the haplotypes in the families. Six highly informative short tandem repeat markers at the 5' and 3' end of *ATL3* (D11S1765, D11S4076, D11S1883, D11S1889, D11S987, D11S1296) were used. PCR amplification of short tandem repeats was done with fluorescently-labelled primer

pairs. The fragments were added to a mixture of formamide and GeneScan™ 500 Liz® Size Standard (Applied Biosystems) and size-separated on an ABI3730xl DNA Analyzer. For analysing the genotyping results the Local Genotype Viewer, an in-house developed software program was applied (<http://www.vibgeneticservicefacility.be/>).

## Cloning

Human *ATL3* (NM\_015459) and human Lunapark (*KIAA1715*, NM\_030650) were cloned from complementary DNA derived from human prostate adenocarcinoma cells (LNCaP-cells). The amplicon for *ATL3* lacking the stop codon was subcloned into a modified pCIneo vector (Promega) that contains the coding sequence for c-myc-tag at the 3' end. Mutation c.575A>G was inserted by PCR-based mutagenesis. Lunapark complementary DNA was subcloned into pEGFP-N2 (Clontech). All inserts were sequence verified using Sanger sequencing. Primer sequences are available upon request.

## Immunohistochemistry

COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with foetal calf serum and 1% penicillin/streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells were transfected with the respective expression constructs using Lipofectamine® 2000 (Invitrogen) and fixed after 24 h with 4% paraformaldehyde. Cells were blocked with 3% normal goat serum and 0.3% Triton™ X-100 in PBS for 30 min, incubated with primary antibody in blocking solution at 4°C overnight and incubated with Alexa Fluor® 488 or 555 secondary antibodies (1:2000; Molecular Probes). DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) was used for nucleic acid staining. Images were taken with a Leica TCS SL confocal microscope. The following antibodies were used: mouse monoclonal anti-myc (clone 9E10, Sigma Aldrich); rabbit-polyclonal anti-myc [c-Myc (A14) sc-789, Santa Cruz], mouse monoclonal anti-giantin (G1/133, Alexis), mouse-monoclonal anti-PDI (SPA-891, Stressgen), mouse monoclonal anti-tubulin (clone DM1A, Sigma-Aldrich).

## Results

### Family 1

#### Clinical features

We studied a German family with autosomal dominant HSAN1 (Fig. 1). Typical first manifestations were abnormal calluses of the feet and valgus deformity of the great toe, which became apparent between 14 and 30 years of age, initially without any noticeable numbness. In the following years painless chronic ulcerations and fractures of the metatarsals developed, which showed delayed healing and occasionally resulted in bone destruction. Increased radiotranslucency of distal bones of the lower extremities, often interpreted as osteomyelitis, and acroosteolysis without prior fracture were also observed. In the most severely affected individual (Patient III<sub>1</sub>) amputation below the knees was the only remaining option after repeated attempts to surgically prevent further progression. Motor nerve conduction velocities were in the normal range, whereas sensory nerve conduction studies showed an axonal sensory neuropathy. Accordingly, there was



**Figure 1** Clinical findings in HSAN1. Typical radiographic presentation of the phenotype in the German family (Family 1). In the severely affected Patient III<sub>1</sub> multiple fractures, destruction and secondary synostoses of metatarsal and phalangeal bones are seen at the age of 33 years. Also in the distal tibia a demineralized area is visible (asterisk). In the more mildly affected Patient IV<sub>1</sub> a healed fracture of metatarsal V, a persisting fracture of metatarsal III and a valgus deformity of the great toe are seen at the age of 32.

no evidence for muscle atrophy or pes cavus. Upper extremities were clinically not affected and autonomic function was normal. Furthermore, no delayed psychomotor development, spasticity or any other signs for CNS involvement were present. Clinical findings are summarized in Table 1.

#### A mutation in *ATL3* causes sensory neuropathy with bone destruction

We performed whole-exome sequencing in two distant affected members of Family 1 (Fig. 2A). After quality filtering and exclusion of known polymorphisms we obtained 10 heterozygous variants of which seven were considered potentially disease-causing (Supplementary Table 1). Highest pathogenicity scores were obtained for missense variants in *NVL* and *ATL3*. Whereas the variant in *NVL* was present in unaffected family members, the mutation c.575A>G (p.Tyr192Cys) (NM\_015459, NP\_056274) in *ATL3* segregated with the disease phenotype assuming complete penetrance (Fig. 2A). The *ATL3* variant was absent from dbSNP, the 1000 Genomes project, and the Exome Variant Server. Moreover, the relevant tyrosine residue in *ATL3* is located in the GTPase-domain of the protein and is evolutionarily conserved throughout species and among all three human *ATL* homologues, suggesting that Tyr192 is important for the proper function of the protein (Fig. 2B).

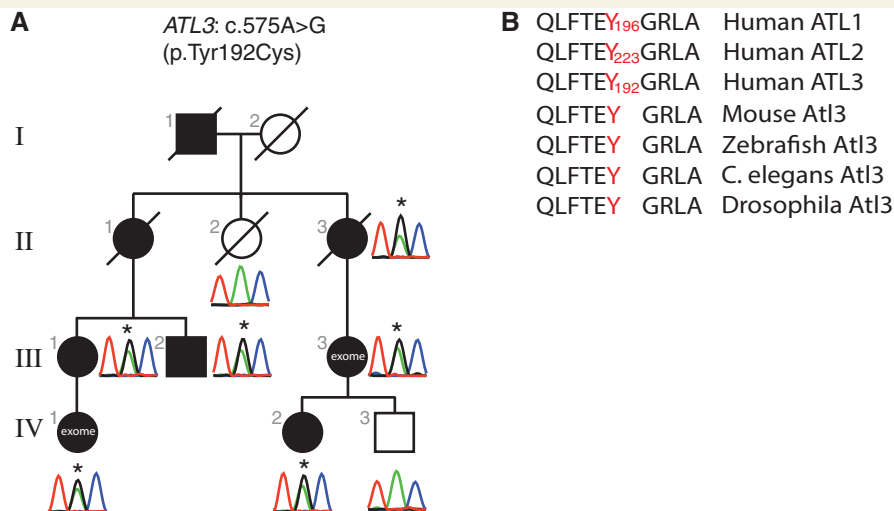
#### Analysis of a large patient cohort for *ATL3* mutations

To corroborate a role for *ATL3* in sensory neuropathies, 115 individuals were screened for mutations in the gene: 76 were diagnosed with HSAN, in 35 a diagnosis of hereditary sensory neuropathy was made due to the absence of overt autonomic features; the remaining four individuals were diagnosed with hereditary motor and sensory

**Table 1** Clinical findings in the individuals harbouring the p.Tyr192Cys mutation in ATL3

Patient ID	A-III-1	A-IV-1	A-III-3	A-IV-2	B-II-1	B-III-1	B-III-2
Origin	Germany	Germany	Germany	Germany	Spain	Spain	Spain
Age at onset (years)	14	30	not known	33	35	14	16
Foot ulcers	+++	+	-	-	-	+	+
Hallux valgus	++	++	+	+	-	+	+
Foot arthropathy	+	+	+	+	+	-	-
Fractures Metatarsals	+++	++	-	+	-	+	+
Foot bone destruction	+++	++	++	+	-	+	+
Amputations	Lower legs	-	-	-	-	Second toe and metatarsal	Second toe and metatarsal
Disturbed touch sensitivity	++	+	n.d.	n.d.	-	+	+
Disturbed pain sensitivity	+	+	+	+	-	+	+
Autonomic dysfunction	-	-	-	-	-	-	-
Spasticity/brisk reflexes	-	-	-	-	-	-	-
NCS	Axonal sensory neuropathy	Sensory neuropathy	n.d.	n.d.	n.d.	Axonal sensory neuropathy	Axonal sensory neuropathy
Others	Nail necrosis	DXA T-score -1	Prominent entheses	Prominent entheses	-	Absent ankle jerks	Absent ankle jerks
Initial diagnosis	Familial acroosteolysis	Familial acroosteolysis	Familial acroosteolysis	Familial acroosteolysis	HSAN type I	HSAN type I	HSAN type I
State	Het	Het	Het	Het	Het	Het	Het
Mutation	c.575A>G	c.575A>G	c.575A>G	c.575A>G	c.575A>G	c.575A>G	c.575A>G
Consequence	p.Tyr192Cys	p.Tyr192Cys	p.Tyr192Cys	p.Tyr192Cys	p.Tyr192Cys	p.Tyr192Cys	p.Tyr192Cys

Het = heterozygous; n.d. = not done; - = not present; + = present; ++ = prominent; +++ = severe.

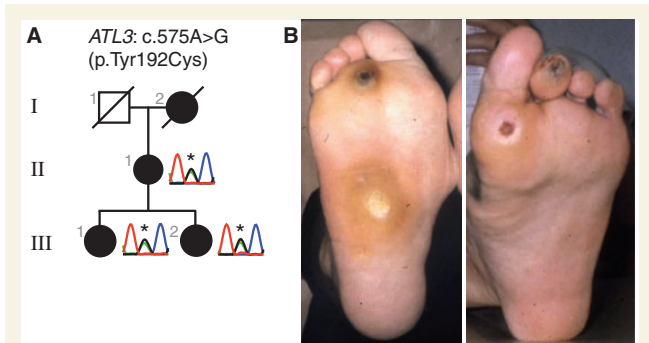


**Figure 2** An *ATL3*-mutation causes HSAN type 1. (A) Pedigree of Family 1 with autosomal-dominant sensory neuropathy. Individuals in which exome-sequencing was performed are indicated. A heterozygous missense mutation in *ATL3* (c.575A>G, p.Tyr192Cys) segregated with the disease in individuals available for testing. (B) Multiple-sequence alignment showing evolutionary conservation of amino acid residue p.Tyr192 in humans, mice, zebrafish, worms and flies. The amino acid is also conserved in human *ATL1* (p.Tyr196) and human *ATL2* (p.Tyr223).

neuropathy type 2 with prominent ulcero-mutilations. Twenty-five individuals had a familial history compatible with an autosomal dominant inheritance; the remaining individuals were isolated cases. A broad range of ages at onset was observed in the study cohort.

In seven individuals onset was before the age of 1, in 14 individuals first symptoms occurred in childhood, in 15 cases the disease started in adolescence and 32 individuals had an adult onset of disease. No exact age at onset was known for the remaining cases.





**Figure 3** p.Tyr192Cys mutation in ATL3 in a second family with HSAN1. (A) The same mutation in ATL3, c.575A>G, p.Tyr192Cys, was identified in a second family of Spanish origin with HSAN1 (Family 2). (B) Valgus deformity and ulcerations in two affected individuals from the family.

Within this cohort we identified the same heterozygous missense-mutation (p.Tyr192Cys) in ATL3 segregating with autosomal dominant HSAN type 1 in a Spanish family (Fig. 3A). Haplotype analysis at the *ATL3* locus of both families suggested that they share the same alleles compatible with identity by descent (Supplementary Fig. 1).

## Family 2

### Clinical features

The disease phenotype in Family 2 was very similar to that of Family 1. Detailed clinical and electrophysiological studies were performed in Patients B-III-1 and B-III-2 who both displayed bilateral hyperkeratosis and plantar ulcers in the second decade of life, at 14 years and 16 years of age, respectively (Fig. 3B). There was no history of spontaneous lancinating pain nor were there symptoms suggesting autonomic disturbance. Distal impairment of sensory function affected superficial touch, pain and temperature. Muscle strength was normal whereas tendon reflexes were diminished and absent at the ankles. Electrophysiological studies were performed at the age of 22 and 28, respectively, and showed a sensory axonal neuropathy in the lower extremities. X-ray imaging in both patients revealed bone destruction eventually necessitating the amputation of the second toe and metatarsal bilaterally in Patient B-III-1 and on the left side in Patient B-III-2. The mother of both patients (Patient B-II-1) refused formal examination but had a history of left foot arthropathy necessitating surgery at the age of 35. Her mother who died at the age of 76 also had trophic abnormalities of the feet.

## ATL3 but not ATL3-Tyr192Cys is enriched in three-way junctions of the endoplasmic reticulum

ATL3 is a member of the atlastin family of endoplasmic reticulum-shaping membrane-bound GTPases. Whereas ATL1 is enriched in the CNS, ATL2 and ATL3 are more broadly expressed (Rismanchi *et al.*, 2008). Beside expression in the CNS, ATL3 is present in

dorsal root ganglia neurons (Hawrylycz *et al.*, 2012) (Supplementary Figs 2 and 3).

ATL3 localized to endoplasmic reticulum tubules upon heterologous expression in COS-7 cells and accumulated in punctuate structures corresponding to three-way junctions (Fig. 4A and B). These crossing-points of the endoplasmic reticulum connect tubules to build a polygonal network (Chen *et al.*, 2012, 2013). The accumulation of ATL3 in three-way junctions was more prominent than that of heterologously expressed ATL1 (not shown). In contrast, ATL3-Tyr192Cys entirely lost its association to three-way junctions, but revealed a condensed immunoreactive structure in proximity to the nucleus and localized to few unbranched tubules throughout the cell (Fig. 4C). This finding demonstrates that the missense mutation in ATL3 results in mislocalization of the mutant protein.

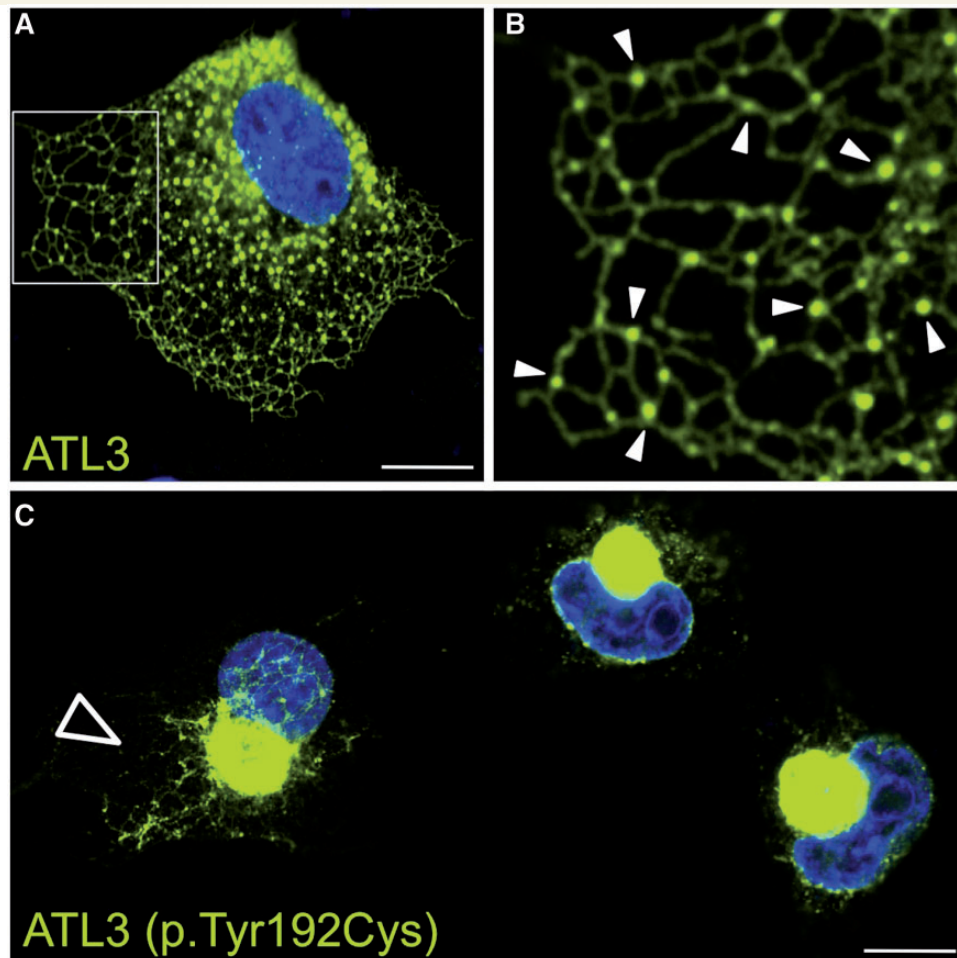
## ATL3-Tyr192Cys disrupts the endoplasmic reticulum network

The impact of the mutant on the overall architecture of the endoplasmic reticulum and Golgi apparatus was analysed. ATL3 co-localized with protein disulphide isomerase (PDI), a marker of the reticular endoplasmic reticulum network including peripheral tubules (Fig. 5A). However, in cells overexpressing ATL3-Tyr192Cys, distribution of PDI was drastically altered and restricted to the condensed area marked by mutant ATL3 (Fig. 5B). This indicates that the endoplasmic reticulum structure collapsed upon expression of the mutant protein and suggests a dominant-negative effect underlying *ATL3*-related HSAN. In line with this, three-way junctions visualized by overexpression of the Lunapark protein were also redistributed to perinuclear aggregates in ATL3-Tyr192Cys expressing cells (Fig. 5C–H). This dominant negative effect is further underlined by the fact that heterozygous microdeletions containing *ATL3* have been described in healthy individuals. In contrast with the strong effect on the endoplasmic reticulum, gross morphology of the *cis*-Golgi compartment and the microtubules was unchanged on ATL3-Tyr192Cys expression (Fig. 5I–T). However, more subtle changes of these structures cannot be detected by this analysis. No rescue or partial restoration of the endoplasmic reticulum structure or the localization of mutant ATL3 protein was observed upon co-expression with wild-type ATL3 or other membrane-shaping proteins, i.e. FAM134B, RTN4, REEP1, SPAST or ATL1 (data not shown). In summary, the mutation p.Tyr192Cys in ATL3 severely disrupts the regular structure of the endoplasmic reticulum in line with its pathogenicity.

## Discussion

Our study further broadens the genetic understanding of PNS neurodegeneration by the identification of a novel causal gene for HSAN type 1, a genetically heterogeneous subgroup of sensory neuropathies.

The most striking clinical characteristics of the disorder were adult onset foot deformities followed by painless ulcerations and destruction of distal bones of the lower extremities, features that

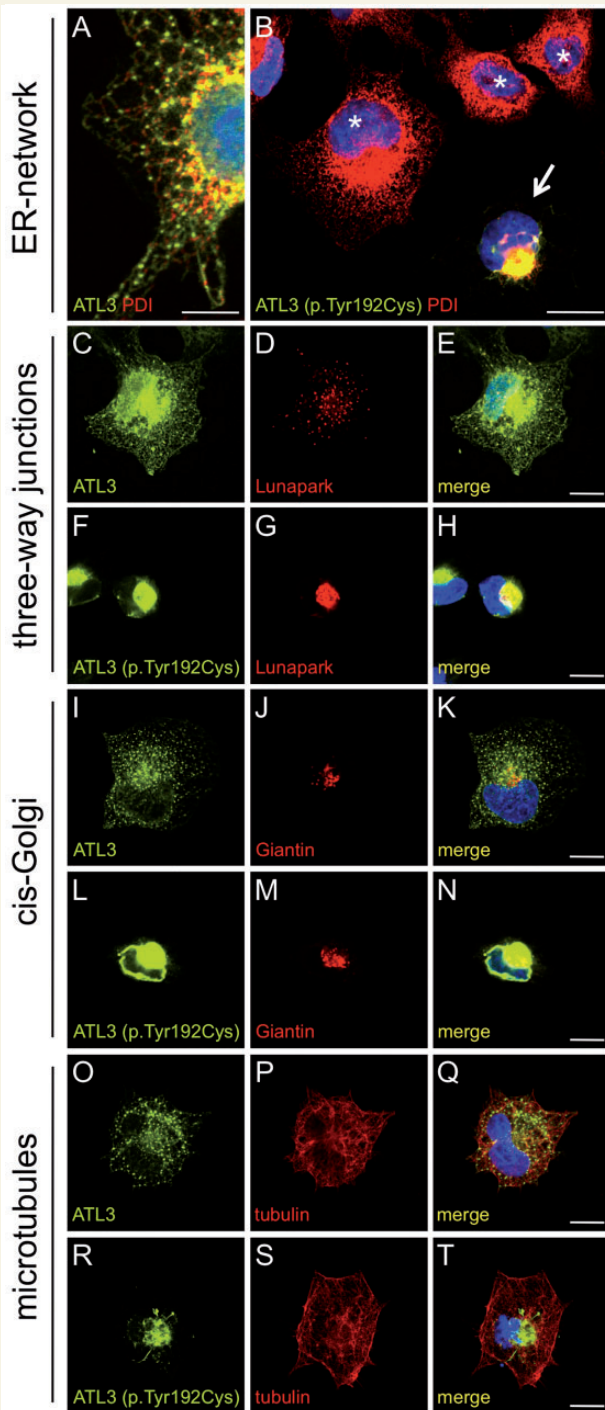


**Figure 4** Association with the endoplasmic reticulum and three-way junctions is lost in cells expressing the p.Tyr192Cys mutation in ATL3. (A) C-terminally myc-tagged human ATL3 localizes to endoplasmic reticulum-tubules and is enriched in three-way junctions upon heterologous expression in COS-7 cells. Scale bar = 20  $\mu$ m. (B) Magnification of the peripheral endoplasmic reticulum (white rectangle in A). Crossing points of the tubules, i.e. three-way junctions, are exemplarily marked by arrowheads. (C) Myc-tagged ATL3 harbouring the p.Tyr192Cys mutation showed a condensed perinuclear localization. Some cells revealed few unbranched tubular structures that stained positive with anti-myc antibodies (white arrowhead). Scale bar = 20  $\mu$ m.

are frequent in HSAN1 and Charcot neuroarthropathy (Davidson *et al.*, 2012; Roththier *et al.*, 2012). Bone destruction in the patients reported here was rapidly progressive. It is currently unclear whether bone resorption is caused by continued loading of the fractured bone, or, if other factors such as autonomic dysfunction or lack of anti-inflammatory neurotransmitters might also play a role in this process (Mabilleau and Edmonds, 2010). Osteomyelitis as a result of neglected ulcerations further worsens the clinical outcome. Notably, bone resorption was reported to be inhibited by bisphosphonate therapy in a patient with HSAN1 and might be a conceivable therapeutic option (Marik *et al.*, 2009). In the individuals with *ATL3* mutations upper extremity sensory involvement was not observed and muscle weakness and atrophy were absent. This is in contrast to other subtypes of the disorder, including those caused by mutations in the related *ATL1* gene that result in HSAN1 with a sensorimotor neuropathy and distal muscular atrophy (Guelly *et al.*, 2011). Furthermore, *ATL1* mutations causing HSAN1 can result in some degree of upper motor

neuron involvement whereas no such feature was found in the present study. Although *ATL1* and *-3* seem largely co-expressed in CNS neurons the reason for the missing propensity of *ATL3* mutations to cause upper motor neuron damage is unclear, but might be partially explained by declining *ATL3* expression with advancing CNS development and lower expression levels compared to *ATL1*. Notably, *ATL3* is highly expressed in sensory neurons in line with the major side of pathology.

What is the cellular pathology of the *ATL3*-associated disorder? The architecture and curvature of organelle membranes depend on different mechanisms including the insertion of integral membrane proteins into lipid bilayers (Shibata *et al.*, 2009; Park and Blackstone, 2010; Pendin *et al.*, 2011). Proteins of the reticulon family are prototypes in the bending and stabilization of the membrane curvature of the endoplasmic reticulum (Voeltz *et al.*, 2006). This class of molecules inserts a wedge-shaped reticulon-domain composed of hydrophobic hairpins into the outer leaflet of the lipid-bilayers to induce membrane bending.



**Figure 5** Disruption of the endoplasmic reticulum network by the p.Tyr192Cys mutation in ATL3. (A) Myc-tagged ATL3 colocalizes with the endoplasmic reticulum-marker PDI. (B) The reticular staining pattern of PDI in untransfected cells (asterisks) is disrupted in p.Tyr192Cys-overexpressing cells (arrow). (C–E) Myc-tagged ATL3 colocalizes with C-terminally GFP-tagged human Lunapark in three-way junctions of the endoplasmic reticulum. For unification Lunapark\_GFP was given red pseudo-colour. (F–H) Lunapark\_GFP is re-distributed to the perinuclear condensation of overexpressed ATL3-p.Tyr192Cys. For unification Lunapark\_GFP was given red pseudo-colour. (I–N) No morphological changes comparable to the severe

ATL3 contains a reticulon-domain in addition to its GTPase domain. Upon GTP binding the cytosolic GTPase domains of atlastin GTPases dimerize. Subsequent GTP hydrolysis causes a major conformational change that pulls atlastin GTPases in opposing membranes together to facilitate membrane fusion (Bian *et al.*, 2011; Byrnes and Sonderrmann, 2011; Liu *et al.*, 2012). By this mechanism called homotypic fusion, atlastin GTPase proteins connect tubules of the endoplasmic reticulum to networks (Hu *et al.*, 2009; Muriel *et al.*, 2009; Orso *et al.*, 2009). According to our data ATL3 is particularly enriched in three-way junctions of the endoplasmic reticulum where it might facilitate the composition of the polygonal endoplasmic reticulum network. The p.Tyr192Cys mutation in ATL3 affects a residue in the GTPase-domain that is evolutionarily conserved throughout species and among all three human atlastin GTPase homologues, suggesting that Tyr192 is important for the proper function of the protein. Remarkably, the mutation p.Tyr196Cys in ATL1 has been identified as cause of autosomal-dominant SPG3A with white matter changes and seizures (for alignment see Fig. 2B) (McCorquodale *et al.*, 2011). Tyr196 in ATL1, which is homologous to p.Tyr192 in ATL3, is located at the interface of the GTPase domains and its replacement by cysteine impaired dimerization *in vitro* (Byrnes and Sonderrmann, 2011). Our data indicate that the mutation in Tyr192 of the ATL3 protein results in a disruption of the cellular endoplasmic reticulum network.

The endoplasmic reticulum plays a major role in various cellular processes, such as  $\text{Ca}^{2+}$  storage, lipid and glycan synthesis, and biosynthesis of secretory membrane proteins (Hu *et al.*, 2011). Alterations in the architecture of the endoplasmic reticulum thus likely interfere with crucial cellular processes and might also induce endoplasmic reticulum stress. The central position of this cellular compartment is reflected by its tight connections to other organelles, which might be particularly disturbed by an altered endoplasmic reticulum morphology. Membranes of the endoplasmic reticulum, for example, provide extensive contact sites to microtubules and endosomes, indicating functional coupling (Friedman *et al.*, 2013). Endosomes that acquire RAB7, a marker of late endosomes, are almost completely and constitutively bound to the endoplasmic reticulum (Friedman *et al.*, 2013). RAB7 plays a crucial role in the signalling complex formed by TRKA and nerve growth factor (NGF) (Saxena *et al.*, 2005; Zhang *et al.*, 2013) and mutations in the latter proteins cause HSN4 (MIM608600) and HSN5 (MIM608654), respectively (Indo *et al.*, 1996; Einarsdottir *et al.*, 2004). Mutations in RAB7 itself cause ulcero-mutilating Charcot–Marie–Tooth type 2B that is also part of the HSN disease spectrum (MIM600882)

#### Figure 5 Continued

alterations in the endoplasmic reticulum were observed for the cis-Golgi compartment in ATL3-p.Tyr192Cys expressing cells. The cis-Golgi was stained with an anti-Giantin antibody. (O–T) Microtubules are preserved in ATL3-p.Tyr192Cys expressing cells as indicated by co-staining of tubulin. Scale bars = 10  $\mu\text{m}$  in A; 20  $\mu\text{m}$  in B–T.



(Verhoeven *et al.*, 2003). Major contact sites of the endoplasmic reticulum also exist with mitochondria to facilitate lipid and  $\text{Ca}^{2+}$  exchange (Kornmann, 2013). Mutations in MFN2, a protein that tethers endoplasmic reticulum tubules to mitochondria (de Brito and Scorrano, 2008) likewise cause an axonopathy, Charcot–Marie–Tooth disease 2A2 (MIM609260) (Züchner *et al.*, 2004). Finally, the architecture of the endoplasmic reticulum might also influence autophagy, a mechanism of intracellular bulk recycling that plays a fundamental role in neuronal homeostasis (Wong and Cuervo, 2010; Mizushima *et al.*, 2011). A large pool of autophagosomes has recently been shown to originate from contact sites between the endoplasmic reticulum and mitochondria (Hamasaki *et al.*, 2013). However, ATL3 function might not be restricted to the endoplasmic reticulum. Functional studies indicate that atlastin GTPases also influence the morphology of the Golgi apparatus and regulate the size of lipid droplets, endoplasmic reticulum-derived lipid storage pools that play a role in metabolism (Zhu *et al.*, 2003, 2006; Rismanchi *et al.*, 2008; Klemm *et al.*, 2013). Atlastin also regulates the bone morphogenetic protein (BMP) pathway in zebrafish (Fassier *et al.*, 2010). This crosstalk might also be relevant for bone regeneration, a process that is clearly impaired in patients with HSN1.

Despite remarkable non-allelic genetic heterogeneity of axonopathies, one of the common emerging disease pathways turns out to be an impairment of the structural organization of organelles. Mutations in *RTN2* (reticulon 2) have been shown to cause SPG12 (MIM604805) supporting a role for shaping proteins in axonal maintenance (Montenegro *et al.*, 2012). Moreover, nearly half of the patients with hereditary SPGs carry mutations in *SPAST* (SPG4, MIM182601), *REEP1* (SPG31, MIM610250), and *ATL1* (SPG3A, MIM182600), likewise encoding reticulon-domain containing endoplasmic reticulum-associated proteins (Blackstone *et al.*, 2011; Blackstone, 2012). Close functional connections exist among these molecules as ATL1 interacts directly with reticulons, REEP1, and the microtubule-severing SPAST protein (Evans *et al.*, 2006; Sanderson *et al.*, 2006; Park *et al.*, 2010). REEP1-deficient mice also showed a reduced complexity of the peripheral endoplasmic reticulum in cortical motor neurons (Beetz *et al.*, 2013). Finally, mutations in *FAM134B*, encoding another reticulon-domain containing protein of the endoplasmic reticulum and Golgi-compartment, cause autosomal recessive HSN2 (MIM613115) (Kurth *et al.*, 2009).

It has recently become evident that several axonopathies are allelic disorders (Timmerman *et al.*, 2013). Although mutations in *REEP1* were initially associated with SPG31 (Züchner *et al.*, 2006), a mutation in the same gene was also found in hereditary motor neuropathy 5B (MIM614751) (Beetz *et al.*, 2012). *ATL1* mutations can cause either SPG3A (Zhao *et al.*, 2001) or HSN1D (MIM613708) (Guelly *et al.*, 2011). In this respect, it may turn out that mutations in *ATL3* are not unique to HSN1 either, but could be implicated in other forms of the continuous spectrum of axonopathies.

In summary, our findings strengthen the concept of membrane-shaping defects as a common molecular pathway in axonal degeneration and identify ATL3 as important molecule for the long-term maintenance of sensory neurons.

## Acknowledgements

We are grateful to the families participating in the study. We thank Dr. Eva-Maria Wicklein for providing clinical reports. The authors declare no conflict of interest.

## Funding

This work was supported by grants of the DFG to I.K. (KU 1587/2-1), C.A.H. (HU 800/5-1, RTG 1715, HU 800/6-1, HU 800/7-1), S.M. (SFB665) and by the German Federal Ministry of Education and Research to U.K. and S.M. (01EC1006A, OsteoPath). Funding to J.B., P.D.J. and V.T. was provided by the University of Antwerp, Medical Foundation Queen Elisabeth (GSKE) and the 'Association Belge contre les Maladies Neuromusculaires' (ABMM). I.M. is supported by a Ph.D. fellowship of the agency for Innovation by Science and Technology (IWT). J.J.V. received research support from the CIBNED, IRDiRC (IR11/TREAT-CMT) and Fundación Gemio.

## Supplementary material

Supplementary material is available at *Brain* online.

## Web resources

NCBI, <http://www.ncbi.nlm.nih.gov/>  
 1000 Genomes, <http://www.1000genomes.org>  
 MutationTaster, <http://www.mutationtaster.org>  
 NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>  
 Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>  
 PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>  
 Primer3, <http://primer3.wi.mit.edu/>  
 UCSC Genome Browser, <http://genome.ucsc.edu/>  
 UniGene, <http://www.ncbi.nlm.nih.gov/uniGene>  
 UniProt, <http://www.uniprot.org/>  
 GeneTalk, <http://www.gene-talk.de/>  
 Exomizer, <https://www.sanger.ac.uk/resources/databases/exomizer/>  
 Allen Spinal Cord Atlas, <http://mousespinal.brain-map.org/>

## References

- Beetz C, Koch N, Khundadze M, Zimmer G, Nietzsche S, Hertel N, *et al.* A spastic paraplegia mouse model reveals REEP1-dependent ER shaping. *J Clin Invest* 2013; 123: 4273–82.
- Beetz C, Pieber T, Hertel N, Schabhüttl M, Fischer C, Trajanoski S, *et al.* Exome sequencing identifies a REEP1 mutation involved in distal hereditary motor neuropathy type V. *Am J Hum Genet* 2012; 91: 139–45.
- Bian X, Klemm R, Liu T, Zhang M, Sun S, Sui X, *et al.* Structures of the atlastin GTPase provide insight into homotypic fusion of endoplasmic reticulum membranes. *Proc Natl Acad Sci USA* 2011; 108: 3976–81.



- Blackstone C. Cellular pathways of hereditary spastic paraplegia. *Annu Rev Neurosci* 2012; 35: 25–47.
- Blackstone C, O’Kane CJ, Reid E. Hereditary spastic paraplegias: membrane traffic and the motor pathway. *Nat Rev Neurosci* 2011; 12: 31–42.
- Byrnes L, Sondermann H. Structural basis for the nucleotide-dependent dimerization of the large G protein atlastin-1/SPG3A. *Proc Natl Acad Sci USA* 2011; 108: 2216–21.
- Chen S, Novick P, Ferro-Novick S. ER network formation requires a balance of the dynamin-like GTPase Sey1p and the Lunapark family member Lnp1p. *Nat Cell Biol* 2012; 14: 707–16.
- Chen S, Novick P, Ferro-Novick S. ER structure and function. *Curr Opin Cell Biol* 2013; 25: 428–33.
- Davidson G, Murphy S, Polke J, Laura M, Salih M, Muntoni F, et al. Frequency of mutations in the genes associated with hereditary sensory and autonomic neuropathy in a UK cohort. *J Neurol* 2012; 259: 1673–85.
- de Brito OM, Scorrano L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* 2008; 456: 605–10.
- Einarsdóttir E, Carlsson A, Minde J, Toolanen G, Svensson O, Solders G, et al. A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. *Hum Mol Genet* 2004; 13: 799–805.
- Evans K, Keller C, Pavur K, Glasgow K, Conn B, Luring B. Interaction of two hereditary spastic paraplegia gene products, spastin and atlastin, suggests a common pathway for axonal maintenance. *Proc Natl Acad Sci USA* 2006; 103: 10666–71.
- Fassier C, Hutt JA, Scholpp S, Lumsden A, Giros B, Nothias F, et al. Zebrafish atlastin controls motility and spinal motor axon architecture via inhibition of the BMP pathway. *Nat Neurosci* 2010; 13: 1380–7.
- Friedman JR, Dibenedetto JR, West M, Rowland AA, Voeltz GK. Endoplasmic reticulum-endosome contact increases as endosomes traffic and mature. *Mol Biol Cell* 2013; 24: 1030–40.
- Guelly C, Zhu P-P, Leonardis L, Papić L, Zidar J, Schabhüttl M, et al. Targeted high-throughput sequencing identifies mutations in atlastin-1 as a cause of hereditary sensory neuropathy type I. *Am J Hum Genet* 2011; 88: 99–105.
- Hamasaki M, Furuta N, Matsuda A, Nezu A, Yamamoto A, Fujita N, et al. Autophagosomes form at ER-mitochondria contact sites. *Nature* 2013; 495: 389–93.
- Hawrylycz MJ, Lein ES, Guillozet-Bongaarts AL, Shen EH, Ng L, Miller JA, et al. An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature* 2012; 489: 391–9.
- Heinrich V, Kamphans T, Stange J, Parkhomchuk D, Dickhaus T, Hecht J, et al. Estimating exome genotyping accuracy by comparing to data from large scale sequencing projects. *Genome Med* 2013; 5: 69.
- Hu J, Prinz WA, Rapoport TA. Weaving the web of ER tubules. *Cell* 2011; 147: 1226–31.
- Hu J, Shibata Y, Zhu P-P, Voss C, Rismanchi N, Prinz W, et al. A class of dynamin-like GTPases involved in the generation of the tubular ER network. *Cell* 2009; 138: 549–61.
- Indo Y, Tsuruta M, Hayashida Y, Karim MA, Ohta K, Kawano T, et al. Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat Genet* 1996; 13: 485–8.
- Kamphans T, Krawitz PM. GeneTalk: an expert exchange platform for assessing rare sequence variants in personal genomes. *Bioinformatics* 2012; 28: 2515–6.
- Klemm RW, Norton JP, Cole RA, Li CS, Park SH, Crane MM, et al. A conserved role for atlastin GTPases in regulating lipid droplet size. *Cell Rep* 2013; 3: 1465–75.
- Kornmann B. The molecular hug between the ER and the mitochondria. *Curr Opin Cell Biol* 2013; 25: 443–8.
- Kurth I, Pamminer T, Hennings J, Soehendra D, Huebner A, Rotthier A, et al. Mutations in FAM134B, encoding a newly identified Golgi protein, cause severe sensory and autonomic neuropathy. *Nat Genet* 2009; 41: 1179–81.
- Leipold E, Liebmann L, Korenke GC, Heinrich T, Giesselmann S, Baets J, et al. A de novo gain-of-function mutation in SCN11A causes loss of pain perception. *Nat Genet* 2013; 45: 1399–404.
- Liu T, Bian X, Sun S, Hu X, Klemm R, Prinz W, et al. Lipid interaction of the C terminus and association of the transmembrane segments facilitate atlastin-mediated homotypic endoplasmic reticulum fusion. *Proc Natl Acad Sci USA* 2012; 109: E2146–54.
- Mabilleau G, Edmonds ME. Role of neuropathy on fracture healing in Charcot neuro-osteoarthropathy. *J Musculoskelet Neuronal Interact* 2010; 10: 84–91.
- Marik I, Kuklik M, Kuklikova D, Kozłowski K. Hereditary sensory and autonomic neuropathy type IV orthopaedic complications. *J Pediatr Orthop B* 2009; 18: 138–40.
- McCorquodale D, Ozomaro U, Huang J, Montenegro G, Kushman A, Citrigno L, et al. Mutation screening of spastin, atlastin, and REEP1 in hereditary spastic paraplegia. *Clin Genet* 2011; 79: 523–30.
- McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernysky A, et al. The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* 2010; 20: 1297–303.
- Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol* 2011; 27: 107–32.
- Montenegro G, Rebelo AP, Connell J, Allison R, Babalini C, D’Aloia M, et al. Mutations in the ER-shaping protein reticulon 2 cause the axon-degenerative disorder hereditary spastic paraplegia type 12. *J Clin Invest* 2012; 122: 538–44.
- Muriel M-P, Dauphin AI, Namekawa M, Gervais A, Brice A, Ruberg M. Atlastin-1, the dynamin-like GTPase responsible for spastic paraplegia SPG3A, remodels lipid membranes and may form tubules and vesicles in the endoplasmic reticulum. *J Neurochem* 2009; 110: 1607–16.
- Orso G, Pendin D, Liu S, Tosetto J, Moss T, Faust J, et al. Homotypic fusion of ER membranes requires the dynamin-like GTPase atlastin. *Nature* 2009; 460: 978–83.
- Park SH, Blackstone C. Further assembly required: construction and dynamics of the endoplasmic reticulum network. *EMBO Rep* 2010; 11: 515–21.
- Park SH, Zhu P-P, Parker RL, Blackstone C. Hereditary spastic paraplegia proteins REEP1, spastin, and atlastin-1 coordinate microtubule interactions with the tubular ER network. *J Clin Invest* 2010; 120: 1097–110.
- Pendin D, McNew J, Daga A. Balancing ER dynamics: shaping, bending, severing, and mending membranes. *Curr Opin Cell Biol* 2011; 23: 435–42.
- Rismanchi N, Soderblom C, Stadler J, Zhu P-P, Blackstone C. Atlastin GTPases are required for Golgi apparatus and ER morphogenesis. *Hum Mol Genet* 2008; 17: 1591–604.
- Rotthier A, Baets J, Timmerman V, Janssens K. Mechanisms of disease in hereditary sensory and autonomic neuropathies. *Nat Rev Neurol* 2012; 8: 73–85.
- Sanderson CM, Connell JW, Edwards TL, Bright NA, Duley S, Thompson A, et al. Spastin and atlastin, two proteins mutated in autosomal-dominant hereditary spastic paraplegia, are binding partners. *Hum Mol Genet* 2006; 15: 307–18.
- Saxena S, Bucci C, Weis J, Kruttgen A. The small GTPase Rab7 controls the endosomal trafficking and neurotogenic signaling of the nerve growth factor receptor TrkA. *J Neurosci* 2005; 25: 10930–40.
- Schwarz JM, Rodelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nat Methods* 2010; 7: 575–6.
- Shibata Y, Hu J, Kozlov M, Rapoport T. Mechanisms shaping the membranes of cellular organelles. *Annu Rev Cell Dev Biol* 2009; 25: 329–54.
- Suter U, Scherer SS. Disease mechanisms in inherited neuropathies. *Nat Rev Neurosci* 2003; 4: 714–26.
- Timmerman V, Clowes VE, Reid E. Overlapping molecular pathological themes link Charcot-Marie-Tooth neuropathies and hereditary spastic paraplegias. *Exp Neurol* 2013; 246: 14–25.
- Verhoeven K, De Jonghe P, Coen K, Verpoorten N, Auer-Grumbach M, Kwon JM, et al. Mutations in the small GTP-ase late endosomal protein RAB7 cause Charcot-Marie-Tooth type 2B neuropathy. *Am J Hum Genet* 2003; 72: 722–7.

- Voeltz G, Prinz W, Shibata Y, Rist J, Rapoport T. A class of membrane proteins shaping the tubular endoplasmic reticulum. *Cell* 2006; 124: 573–86.
- Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res* 2010; 38: e164.
- Wong E, Cuervo AM. Autophagy gone awry in neurodegenerative diseases. *Nat Neurosci* 2010; 13: 805–11.
- Zhang K, Fishel Ben Kenan R, Osakada Y, Xu W, Sinit RS, Chen L, et al. Defective axonal transport of Rab7 GTPase results in dysregulated trophic signaling. *J Neurosci* 2013; 33: 7451–62.
- Zhao X, Alvarado D, Rainier S, Lemons R, Hedera P, Weber C, et al. Mutations in a newly identified GTPase gene cause autosomal dominant hereditary spastic paraplegia. *Nat Genet* 2001; 29: 326–31.
- Zhu P-P, Patterson A, Lavoie B, Stadler J, Shoeb M, Patel R, et al. Cellular localization, oligomerization, and membrane association of the hereditary spastic paraplegia 3A (SPG3A) protein atlastin. *J Biol Chem* 2003; 278: 49063–71.
- Zhu P-P, Soderblom C, Tao-Cheng J-H, Stadler J, Blackstone C. SPG3A protein atlastin-1 is enriched in growth cones and promotes axon elongation during neuronal development. *Hum Mol Genet* 2006; 15: 1343–53.
- Züchner S, Mersiyanova IV, Muglia M, Bissar-Tadmouri N, Rochelle J, Dadali EL, et al. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. *Nat Genet* 2004; 36: 449–51.
- Züchner S, Vance JM. Emerging pathways for hereditary axonopathies. *J Mol Med (Berl)* 2005; 83: 935–43.
- Züchner S, Wang G, Tran-Viet KN, Nance MA, Gaskell PC, Vance JM, et al. Mutations in the novel mitochondrial protein REEP1 cause hereditary spastic paraplegia type 31. *Am J Hum Genet* 2006; 79: 365–9.