

A Novel Missense Mutation of *CMT2P* Alters Transcription Machinery

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Objective: Charcot–Marie–Tooth type 2P (CMT2P) has been associated with frameshift mutations in the RING domain of *LRSAM1* (an E3 ligase). This study describes families with a novel missense mutation of *LRSAM1* gene and explores pathogenic mechanisms of CMT2P.

Methods: Patients with CMT2P were characterized clinically, electrophysiologically, and genetically. A neuronal model with the *LRSAM1* mutation was created using CRISPR/Cas9 technology. The neuronal cell line along with fibroblasts isolated from the patients was used to study RNA-binding proteins.

Results: This American family with dominantly inherited axonal polyneuropathy reveals a phenotype similar to those in previously reported non-US families. The affected members in our family cosegregated with a novel missense mutation Cys694Arg that alters a highly conserved cysteine in the RING domain. This mutation leads to axonal degeneration in the in vitro neuronal cell line. Moreover, using protein mass spectrometry, we identified a group of RNA-binding proteins (including FUS, a protein critically involved in motor neuron degeneration) that interacted with *LRSAM1*. The interactions were disrupted by the Cys694Arg mutation, which resulted in reduction of intranuclear RNA-binding proteins.

Interpretation: Our findings suggest that the mutant *LRSAM1* may aberrantly affect the formation of transcription machinery. Given that a similar mechanism has been reported in motor neuron degeneration of amyotrophic lateral sclerosis, abnormalities of RNA/RNA-binding protein complex may play a role in the neuronal degeneration of CMT2P.

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Charcot–Marie–Tooth disease (CMT) affects 1 of 2,500 people.¹ Patients with dominantly inherited CMT are separable into 2 groups: CMT1 and CMT2. CMT1 is characterized by slowed nerve conduction velocities and abnormally developed myelin (dysmyelination), whereas CMT2 shows axonal loss and reduced amplitude of nerve responses with normal or minimally decreased conduction velocities.^{2,3} Most patients with CMTs share many phenotypic features, including chronic sensory loss, muscle weakness, and atrophy in distal limbs.

Mutations in leucine rich repeat and sterile alpha motif 1 (*LRSAM1*, also called Tal or RIFLE) gene on human chromosome 9 have been associated with CMT2P, a dominantly inherited axonal type of CMT. *LRSAM1* is

an E3 ubiquitin ligase that consists of an N-terminal leucine-rich repeat domain (LRR), an ezrin-radixin-moesin domain (SAM), a coil–coil region, a sterile alpha motif domain (PTAP), and a C-terminal RING domain (Fig 1A). CMT2P was initially found in a non-US consanguineous family with an autosomal recessive mutation of p.Glu638AlafsX7.⁴ Subsequently, 3 additional non-US families with dominantly inherited axonal CMT have been linked to different mutations in *LRSAM1*.^{5–7} All these mutations alter a major portion of RING-domain amino acid sequence of *LRSAM1* by either a frame shift or insertion of additional amino acids.

RING-based E3s are encoded by >600 human genes and involve diverse cellular functions. The RING domain is usually typified by an amino acid sequence

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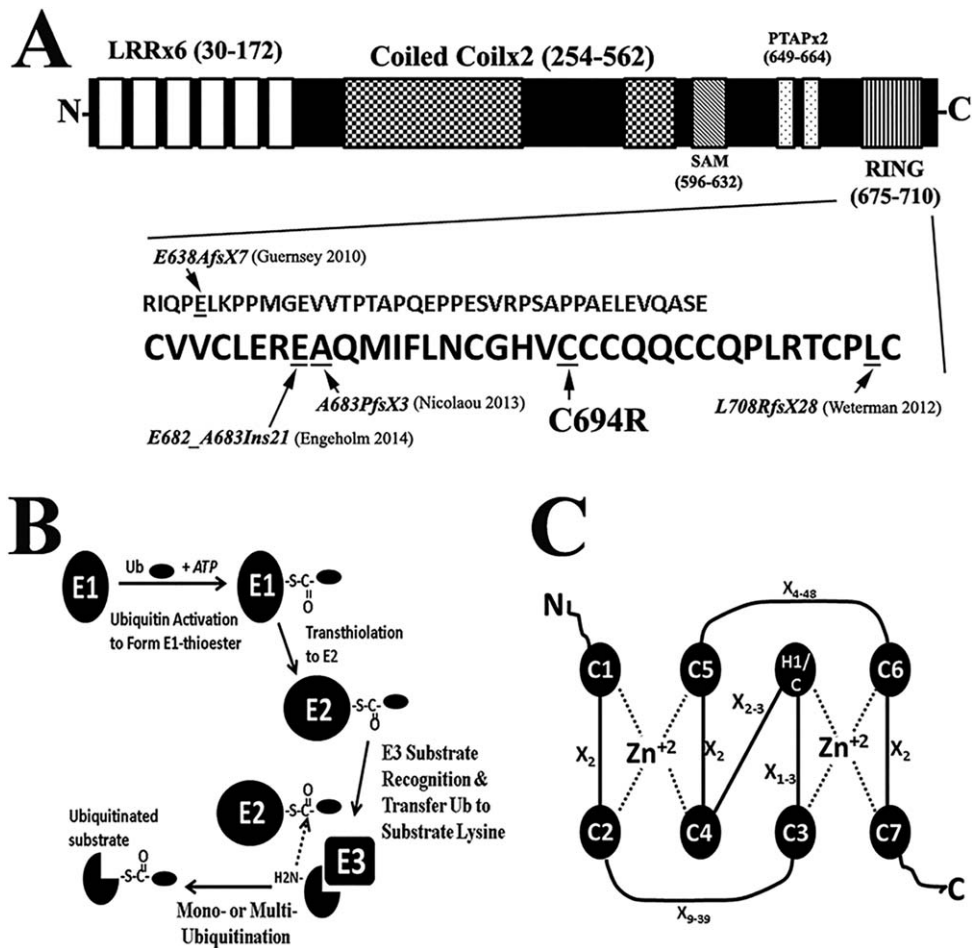


FIGURE 1: LRSAM1 is an E3 ubiquitin ligase. (A) The diagram in the upper panel shows different domains in LRSAM1. This is based on information provided in UniProt (<http://www.uniprot.org/>). Domain name followed by “x + number” indicates how many times that this domain repeats. The lower panel shows a segment of amino acid sequence adjacent to the N-terminal of RING domain (small font), followed by amino acid sequence of RING domain (large font). All 4 previously published mutations were marked at their starting residues. Cys694Arg (=C694R) indicates the residue that was mutated in the family of the present study. (B) This diagram shows steps of ubiquitination reaction (modified from Metzger et al⁸). (C) This diagram depicts a typical structural organization of E3 RING domain (modified from Deshaies and Joazeiro²⁹). Notice that highly conserved cysteines and histidine coordinate 2 zinc ions that are critical for the RING domain structure stabilization and interaction with E2.

C-X₂-C-X₍₉₋₃₉₎-C-X₍₁₋₃₎-H-X₍₂₋₃₎-C-X₂-C-X₍₄₋₄₈₎-C-X₂-C, where C is cysteine, H is histidine, and X is any amino acid.⁸ Cysteines and histidines are highly conserved and critical in maintaining the structure of E3 proteins by binding two atoms of zinc (see Fig 1C). RING-based E3s often function via ubiquitination of their targeted proteins (see Fig 1B).

Here, we report a family with CMT2P. The affected members did cosegregate with a novel missense mutation that changed a highly conserved cysteine to arginine in the RING domain of *LRSAM1*. Moreover, we found that this mutation may impair the formation of transcription machinery.

Subjects and Methods

Patients

Five patients and 3 nonaffected members from the proband's family (Fig 2) were evaluated (by J.L.) at Vanderbilt Medical

Center. A sporadic case was from the University of Miami. This study was approved by the institutional review board at both institutions. Written consent was obtained from all participants.

In addition to medical history and neurological examination, the CMT neuropathy score (CMTES)⁹ was obtained from most patients (listed in Table 1). The score is comprised of sensory and motor symptoms in limbs, and physical findings in limb sensation and muscle strength. The electrophysiological portion of the score was omitted, which will be described separately below. CMTES ranges from 0 to 28, with higher scores indicating an increase of disease severity.

Nerve Conduction Studies

Nerve conduction studies (NCS) data were acquired using conventional methods.¹⁰ For motor nerves, the distal stimulation distances for motor conduction studies were 7cm in the arms and 9cm in the legs. For the sensory nerves, the stimulation

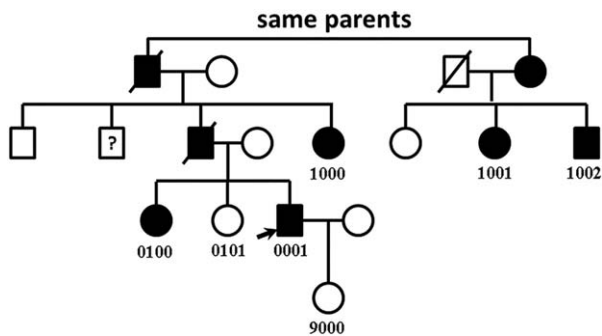


FIGURE 2: Pedigree of the studied family. Arrow indicates the proband. Subjects marked with numerical codes were examined clinically, electrophysiologically, and genetically. Members without a numerical code provided information on history and physical examination but no DNA samples. Medical history and physical examination were not available for the member labeled with a question mark. Note that there is a male-to-male transmission in the pedigree, supporting an autosomal dominant inheritance.

distance was 14cm for median, ulnar, and sural nerves but 10cm for radial nerve.

DNA Sequencing

NEXT GENERATION SEQUENCING. The proband's DNA was initially evaluated by targeted gene panel next generation sequencing, a service provided by Medical Neurogenetics (Atlanta, GA). The test was performed on the Illumina (San

Diego, CA) HiSeq 2500 platform. Analysis used BWA and GATK software packages to align short reads and call variants. The sequencing covered > 98% of nucleotides within exons that were interrogated at >×20 depth. Average depth of coverage of all nucleotides within exons was approximately ×350. The sequencing targeted the following 42 genes (Mendelian Inheritance in Man #) that were known to relate to CMTs: *AARS* (601065), *AIFM1* (300169), *ARHGEF10* (608236), *BAG3* (603883), *BSCL2* (606158), *CTDP1* (604927), *DHTKD1* (614984), *DNM2* (602378), *EGR2* (129010), *FGD4* (611104), *FIG4* (609390), *GAN* (605379), *GARS* (600287), *GDAP1* (606598), *GJB1* (304040), *GNB4* (610863), *HINT1* (601314), *HK1* (142600), *HSPB1* (602195), *HSPB8* (608014), *INF2* (610982), *KARS* (601421), *LITAF* (603795), *LMNA* (150330), *LRSAM1* (610933), *MED25* (610197), *MFN2* (608507), *MPZ* (159440), *MTMR2* (603557), *NDRG1* (605262), *NEFL* (162280), *PDK3* (602526), *PMP22* (601097), *PRPS1* (311850), *PRX* (605725), *RAB7A* (602298), *SBF2* (607697), *SH3TC2* (608206), *SLC12A6* (604878), *TFG* (602498), *TRPV4* (605427), *YARS* (603623). In addition, whole mitochondrial DNA was sequenced.

SANGER SEQUENCING. The specific missense mutations in *LRSAM1* and *MTMR2* were verified by Sanger sequencing in all participants. DNA of blood cells was extracted from all participants using a commercial kit (#A1620; Promega, Madison, WI). The test was performed at Vanderbilt Genome core

TABLE 1. Clinical Features in Patients with Charcot–Marie–Tooth Type 2P

Code	0001	1000	0100	1001	1002
Sex/age, yr	M/51	F/71	F/56	F/66	M/61
Onset age, yr	30s	43	20s	40s	30s
Weakness	HI 4; ADF 4	ADF 4	ADF 4	None	ADF 4
Vibration ^a	Knee	Knee	Knee	Knee	Knee
Pinprick	Knee	Midcalf	Midcalf	Distal leg	Ankle
DTR	Ankle	All joints	All joints	All joints	All joints
Foot deformities ^b	None	None	None	None	None
PSw	No	Yes	Yes	Yes	No
CMTES	18	7	12	8	6
LRSAM1 ^c	C694R	C694R	C694R	C694R	C694R
MTMR2 ^d	E430Q	No	E430Q	No	No

^aLevel of abnormal vibration.

^bHigh arch feet or hammer toes.

^cC694R = Cys694Arg. Note that the C694R mutation is absent in all tested unaffected members (0101 and 9000).

^dE430Q = Glu430Gln.

ADF = ankle dorsal flexor; CMTES = an examination subscore from Charcot–Marie–Tooth neuropathy score, which omitted nerve conduction studies; DTR = joints with absent deep tendon reflex; F = female; HI = hand intrinsic muscles; M = male; MTMR2 = mutation in myotubularin related protein 2 gene; PSw = pelvic swing.

(VANTAGE). Sequences were analyzed using Sequence Scanner (V1.0; Applied Biosystems, Foster City, CA).

NSC34 Neuronal Cell Line Culture and Primary Human Fibroblast Culture

NSC34 cell lines (murine motoneuron–neuroblastoma hybrid; CELLutions Biosystems, Duluth, GA) were maintained in Dulbecco modified Eagle medium (DMEM) high-glucose medium (Cat# 11995; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Cat# 10082-147, Thermo Fisher Scientific). The cells were cultured on coverslips coated with 100 μ g/ml laminin (Cat# 23017-015, Thermo Fisher Scientific) in 24-well plates. The spinal motor neuron features of this cell line have been extensively documented.¹¹

Skin biopsies of the proband and sex/age-matched control were obtained in the Neurology Clinic of Vanderbilt University Medical Center. Tissues were washed in phosphate-buffered saline (PBS) supplemented with penicillin and streptomycin, cut into small pieces, digested overnight in DMEM high-glucose medium supplemented with 20% FBS and 0.6mg/ml collagenase II (Cat# LS004205; Worthington Biochemical Corporation, Lakewood, NJ), and cultured in DMEM/20% FBS. Fibroblast outgrowth started at day 3 to 5. The remaining skin pieces were removed after 1 week. Culture fibroblasts were allowed to grow into 90% confluence.

CRISPR/Cas9 Knockout of LRSAM1 in NSC34 Cell Line

CRISPR/Cas9 constructs were purchased from GeneCopoeia (Rockville, MD). Their designs will be detailed below. The NSC34 cells were transfected with CRISPR/Cas9-mCherry-*LRSAM1* vector by using Effectene (Cat#301425; Qiagen, Valencia, CA). After 3 days, cells were seeded as single colonies (1 cell/well) in 96-well plates. After 2 to 3 weeks, clones were selected based on immunoblot.

Plasmids and Transfection

Human *LRSAM1* plasmid (Genebank ID: BC009239; plasmid 1XHA-*LRSAM1*) was purchased from ABM (Richmond, BC, Canada; Cat# PV024451). Mutations were created using site-directed mutagenesis (primers for the reaction: forward, 5'-TGTGGCCACGTCCGCTGCTGCCAGC-3'; reverse, 5'-GCTGGCAGCAGCGGACGTGGCCACA-3'). pcDNA3-EGFP was from Addgene (Cambridge, MA). Primers for *LRSAM1* insertion were: forward, 5'-CGGGGTACCATGCCGCTCTTCTTCCG-3'; reverse, 5'-CATCTACCACAGCAGCacGCGGCCGCTAAACTAT-3'. KpnI site was added. The stop codon was deleted. Two extra bases were added to avoid frame shift. Following ligation and transformation to DH5a cells, the accuracy of the plasmids was verified by DNA sequencing. The plasmids were transfected into NSC34 cells by using Effectene (Cat#301425, Qiagen).

Protein Pulldown Assay with Protein Mass Spectrometry

Protein lysates were collected from NSC34 cells transfected with *LRSAM1* plasmids. Ten microliters of anti-green

fluorescence protein (GFP) magnetic beads (from Vanderbilt Antibody and Protein Resource core) were added to each sample. These were rotated overnight at 4°C. Beads were washed extensively in radioimmunoprecipitation assay (RIPA) buffer. After washing, beads were collected and resuspended in 20 μ l of protein sample buffer for the study of mass spectrometry described below.

Shotgun proteomic analysis of eluate was performed by first partially resolving eluted proteins at about 1.5cm using a 10% Novex (Thermo Fisher Scientific) precast gel, excising the protein region, and then performing in-gel tryptic digestion to recover peptides. These peptides were analyzed via MudPIT (Multidimensional Protein Identification Technology) as described.¹² Briefly, digested peptides were loaded onto a biphasic precolumn consisting of 4cm of reverse phase (RP) material followed by 4cm of strong cation exchange (SCX) material. Once loaded, this column was placed in line with a 20cm RP analytical column packed into a nanospray emitter tip directly coupled to a linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific). A subset of peptides were eluted from the SCX material onto the RP analytical material via a pulse of volatile salt; those peptides were separated by an RP gradient and then ionized directly into the mass spectrometry. This proceeded for a total of 8 salt elution steps over the course of approximately 16 hours of data acquisition. Both the intact masses (mass spectrometry) and fragmentation patterns (tandem mass spectrometry [MS/MS]) of the peptides were collected, and the peptide MS/MS spectral data were searched against the mouse protein database to which the GFP fusion protein and common contaminants had been appended using Sequest.¹³ Resulting identifications were then collected and filtered using Scaffold (<http://www.proteomesoftware.com>).

Antibodies

Mouse monoclonal anti-FUS (Cat# 60160-1-Ig) antibody was purchased from Proteintech (Rosemont, IL). Rabbit polyclonal anti-G3BP1 (Cat# A302-033A), anti-BCLF1 (Cat# A300-608A), and anti-KHDR1 (Cat# A302-110A) antibodies were from Bethyl Laboratories (Montgomery, TX). Mouse monoclonal anti-LRSAM1 (Cat# ab73113) was from Abcam (Cambridge, MA). Mouse monoclonal anti-HDAC3 (Cat# 3949), rabbit polyclonal anti-TDP43 (Cat# 3448), and rabbit polyclonal anti- β -Actin (Cat# 8457) were from Cell Signaling Technology (Danvers, MA).

Coimmunoprecipitation and Western Blot

Nuclear or cytoplasmic fractionation of human fibroblasts or NSC34 was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Cat#78833, Thermo Fisher Scientific). Cells were incubated with primary antibodies overnight at 4°C with rotation (70rpm). Protein G agarose beads (Cat# 15920-010; Life Technologies, Carlsbad, CA) were added for another 2-hour incubation at 4°C. Samples were eluted with Laemmli sample buffer (Cat# 161-0737; Bio-Rad, Hercules, CA), resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for immunoblotting.

TABLE 2. Electrophysiological Findings

Code	Sensory Nerve Conduction ^a			Motor Nerve Conduction ^a		
	Median	Ulnar	Sural	Median	Ulnar	Peroneal
Norm ^b	3.5/22.0/50	3.5/10.0/50	4.4/6.0/40	4.4/4.0/49	3.3/6.0/49	6.5/2.0/44
0001	3.6/10.0/46	2.5/7.0/55	NR	4.3/10.2/52	2.7/8.2/61	5.6/1.5/33
1000	NR	NR	NR	6.3/2.7/61	4.4/3.7/82	6.3/1.3/32
0100	NR	NR	NR	3.9/8.8/58	3.2/2.5/72	NR
1001	ND	—	—	—	—	—
1002	NR	NR	NR	4.6/7.6/52	3.4/13.2/54	7.6/2.4/35
F2088 ^c	3.2/5.0/50	ND	NR	3.4/8.6/57	ND	6.1/0.3/42

^aDistal latency (ms)/amplitude (mv)/conduction velocity (m/s).
^bNormative values. For distal latency, the numbers listed are the upper limit of normal. For amplitude and conduction velocity, the numbers listed are the lower limit of normal.
^cThis was the code for the patient from the University of Miami.
 ND = not done; NR = no response.

Whole-cell proteins were extracted using RIPA buffer (Cat# R0278; Sigma, St Louis, MO) with proteinase/phosphatase inhibitor cocktail (Cat# 5872, Cell Signaling Technology). Samples were loaded into SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane. The membranes were blotted with 5% nonfat milk and incubated overnight at 4°C with primary antibodies followed by horseradish peroxidase-conjugated antirabbit or antimouse secondary antibodies (Cat# AP307P or AP308P; Millipore, Billerica, MA). The immune complexes were detected by enhanced chemiluminescence (Cat# NEL103001; PerkinElmer, Waltham, MA).

Immunofluorescence Staining

Immunostaining was performed on cells cultured on coverslips. Cells were fixed in 4% paraformaldehyde for 1 hour and then permeabilized with PBS containing 5% normal serum and 0.5% Triton X-100. Cells were incubated overnight with primary antibodies at 4°C. After washing, cells were stained for 1 hour with secondary antibodies. The stained slides were observed under a fluorescence microscope (DM6000B; Leica Microsystems, Wetzlar, Germany).

Statistics

We compared continuous variables between 2 groups using Student *t* test. All statistical analysis was performed using Prism software version 6.0 (GraphPad Software, San Diego, CA) or SAS version 9.4 (SAS Institute, Cary, NC) and a probability value < 0.05 was used to determine significance. Data are presented as the mean ± standard deviation.

Results

Phenotypic and genotypic presentation is consistent with a dominantly inherited axonal polyneuropathy—CMT2.

Clinical Phenotypes

PATIENT 0001. The proband is a 51-year-old man who first developed ankle weakness in his early 30s. He tripped himself frequently and had to wear a pair of tall boots to protect his ankles. His hands and arms were less affected. His NCS revealed a sensory motor axonal polyneuropathy. Neurological examination revealed normal mental status and cranial nerve functions. Muscle strength was 4 on the Medical Research Council (MRC) scale in hand intrinsic muscles and ankle dorsal flexors but 5 in other muscles. Sensation was decreased to pinprick in hands and legs up to the knees and to vibration at ankles and knees. Deep tendon reflexes (DTRs) were absent at ankle joints but normal in other joints.

PATIENT 1000. This is a 71-year-old woman who became symptomatic at 43 years of age with balance issues and multiple falls. She walked with a cane. She developed numbness in feet and distal legs but was asymptomatic in hands. Neurological examination detected muscle weakness in ankle dorsal flexors (4 on MRC) but 5 in other muscles. Vibration was reduced up to knee joints. DTRs were absent in all joints.

We evaluated 3 additional affected members (0100, 1001, and 1002) who presented with phenotypes similar to the 2 patients above. Details are listed in Table 1. Taken together, there were several notable clinical features: (1) none had significantly foot deformities (high arch feet or hammer toes); (2) loss of vibration sense was severe in all cases, and often up to the knee level; this appeared to be disproportional to muscle weakness; and

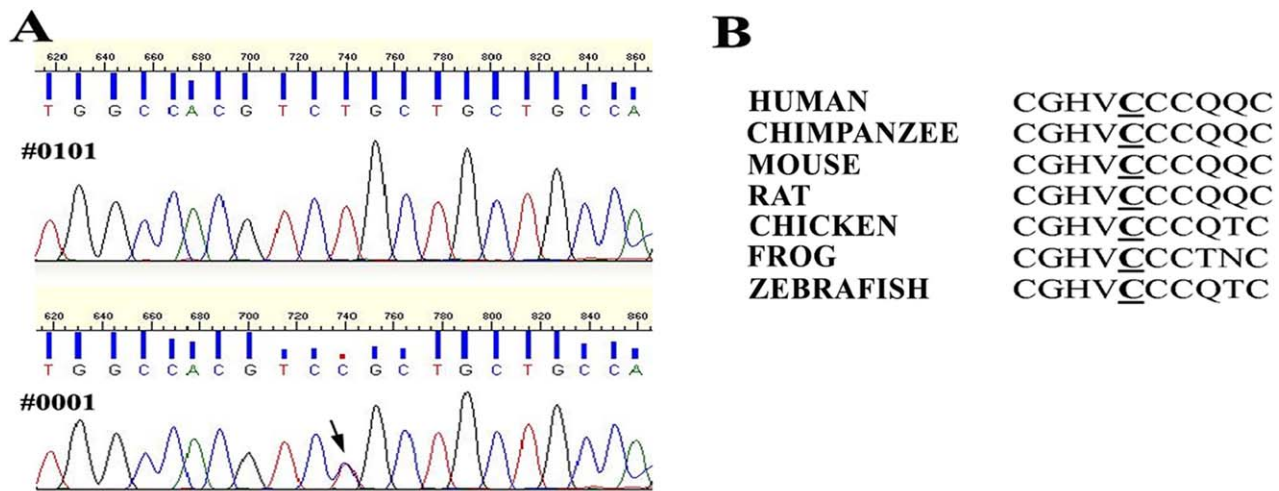


FIGURE 3: Sanger sequencing. (A) Original traces by Sanger sequencing were displayed for Subjects 0101 (unaffected) and 0001 (the proband). The mutated nucleotide is indicated by an arrow. (B) Sequence alignment shows that cysteine 694 (underlined) is conserved in all species listed. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

(3) excessive pelvic swings were noticed in 3 affected members; this was presumably an indication of denervation in pelvic muscles.

Electrophysiological Findings

Six affected patients were evaluated by NCS (Table 2). The findings were consistent with a sensory motor axonal polyneuropathy due to the following reasons:

1. Sensory nerve action potentials (SNAPs) in legs were absent in all cases. SNAP amplitudes in arms were decreased in all cases. In contrast, conduction velocities were normal except for Case #0001 showing a mildly reduced conduction velocity in the median sensory nerve due to carpal tunnel syndrome.
2. A similar pattern of abnormalities was also observed in motor nerve conduction studies. This was particularly evident in Cases 1000 and 0100, which had severely decreased amplitudes of compound muscle action potentials but showed completely normal conduction velocities in the ulnar nerves.
3. Needle electromyography was performed in Cases 0001 and F2088 and showed denervation in distal leg muscles.

DNA analysis showed a missense mutation that changed a highly conserved cysteine to arginine in the RING domain of *LRSAM1*.

The proband DNA was initially sequenced by targeted gene panel next generation sequencing, a commercial diagnostic service provided by Medical Neurogenetics. This technique was briefly described in the Subjects and Methods section. This test identified 2 heterozygous missense mutations, c.(2080T>C) in *LRSAM1* on

chromosome 9 and c.(1288G>C) in *MTMR2* on chromosome 11. The mutations changed cysteine to arginine (Cys694Arg) in *LRSAM1*, and glutamate to glutamine (E430Q) in *MTMR2*. In addition, whole mitochondrial DNA was sequenced and revealed no mutation.

We verified these 2 missense mutations using a Sanger sequencer (Fig 3). Cys694Arg was only found in affected family members, being absent in nonaffected family members (see Fig 2 and Table 1). This cosegregation between Cys694Arg in *LRSAM1* and affected individuals supported its causal role of this disease. In contrast, the heterozygous Glu430Gln (E430Q) mutation in *MTMR2* was detected in 2 affected members and 1 nonaffected member but was absent in 3 affected members. This random distribution strongly rejected its causal role for this disease.

Cysteine at the 694 residue of *LRSAM1* is highly conserved (see Fig 3). The mutation was evaluated by 2 servers: PolyPhen-2¹⁴ and SIFT.¹⁵ In a study, 80% and 82% of a group of disease-causing mutations were correctly predicted to be deleterious by the 2 online tools.¹⁶ Both PolyPhen-2 and SIFT predicted that Cys694Arg was deleterious. In addition, this mutation was evaluated by MutationTaster (“disease causing”) and the likelihood ratio test score (“deleterious”).

We searched Cys694Arg mutation in the GEM.app/GENESIS database. This National Institutes of Health–supported online database stores genetic variants identified from hundreds of patients with CMT and related disorders.¹⁷ The Cys694Arg mutation of *LRSAM1* was found in a 48-year-old man with a diagnosis of idiopathic sensory motor axonal polyneuropathy (see NCS results for F2088 in Table 2). He reported no family history.

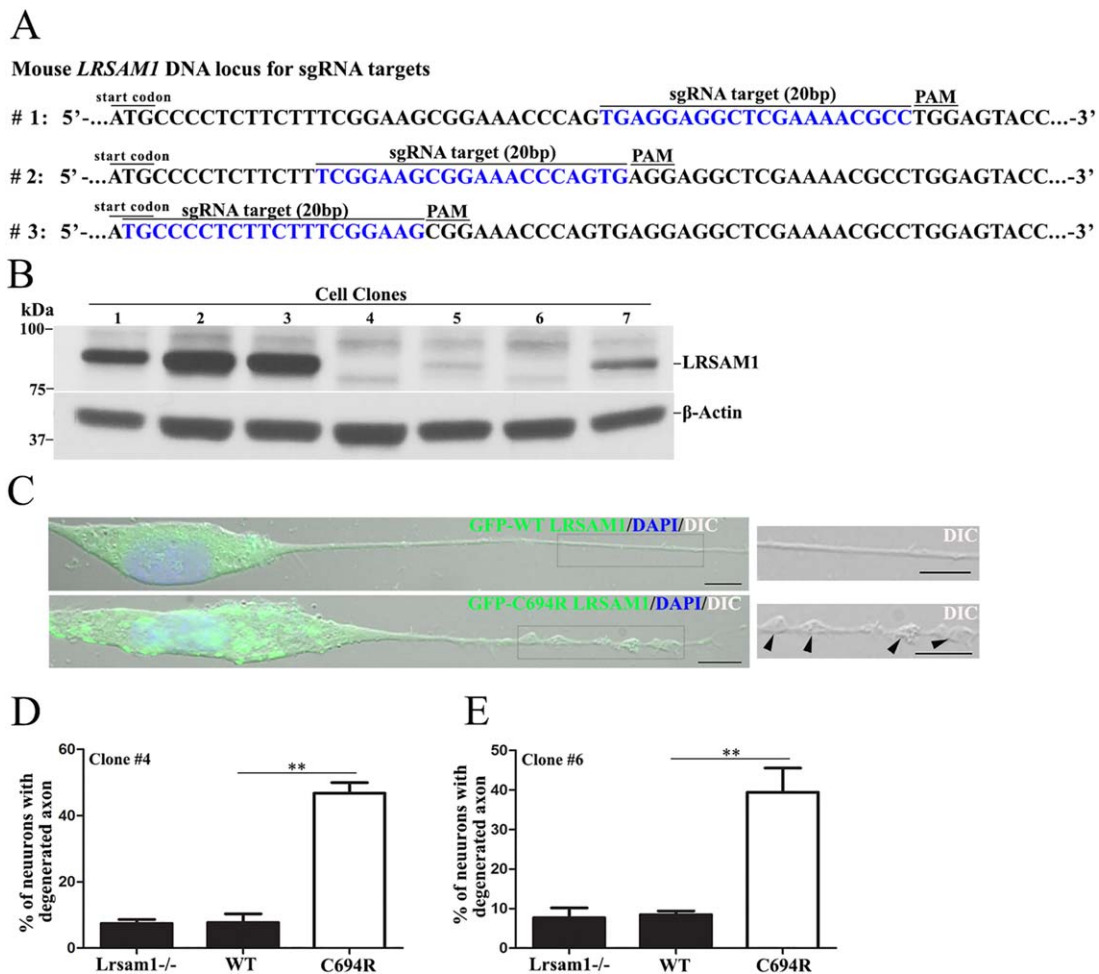


FIGURE 4: Cys694Arg LRSAM1 in *Lrsam1*^{-/-} neuronal cell line causes axonal degeneration. (A) This diagram illustrates the mouse *Lrsam1* loci. Two 20nt-targeting sequences of single-guide RNA (sgRNA) are indicated with lighter type. Protospacer-adjacent motifs (PAM) are lined on the top. (B) Western blot of LRSAM1 in NSC34 clonal cell lines after *Cas9*/*sg-Lrsam1* transfection showed loss of LRSAM1 expression in clones 4 and 6, which were knocked down by 2 different sgRNA sequences. β -Actin was used as a loading control. (C) *Lrsam1*^{-/-} NSC34 cells were transfected with constructs expressing green fluorescence protein (GFP)-wild-type (WT) human LRSAM1 or GFP-Cys694Arg human LRSAM1. Three days after transfection, cells were fixed in 4% paraformaldehyde and imaged to observe neurites with beads (arrowheads). Scale bars = 10 μ m. (D) The percentage of cells with beads were manually counted. Degenerative cells were defined as cells that had neurites with ≥ 2 beads. There was a significant increase of neurites with beads in Cys694Arg LRSAM1 cells ($46.8 \pm 5.6\%$, $n = 119$ cells from 3 wells of *Lrsam1*^{-/-} cell clone 4), compared with that in WT cells ($7.8 \pm 4.4\%$, $n = 133$ cells from 3 wells) and nontransfected *Lrsam1*^{-/-} cells ($7.5 \pm 2.0\%$, $n = 168$ cells from 3 wells). (E) The same quantification was performed in cells of *Lrsam1*^{-/-} cell clone 6. Again, there was a significant difference between WT LRSAM1 ($8.5 \pm 1.6\%$) or nontransfected *Lrsam1*^{-/-} cells ($7.7 \pm 4.3\%$) and Cys694Arg LRSAM1 ($39.5 \pm 10.6\%$) cells (116 cells from 3 wells for WT LRSAM1, 124 cells from 3 wells for nontransfected *Lrsam1*^{-/-}, and 115 cells from 3 wells for Cys694Arg LRSAM1). ** $p < 0.01$. DAPI = 4,6-diamidino-2-phenylindole; DIC = differential interference contrast. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

Finally, the following analysis also supports the pathogenicity of the Cys694Arg mutation. The Cys694Arg allele was present in 1 allele of 107,784 chromosomes in the ExAC database. The Exome Variant Server database does not list this allele in 13,000 chromosomes. The GENESIS database contains 5,400 exomes. The vast majority of enrolled subjects were associated with a neurodegenerative disease, including 587 cases with CMT2. None, except F2088, had the Cys694Arg allele.

Expression of Human Cys694Arg Mutant LRSAM1, but Not Wild-Type LRSAM1, in LRSAM1 Null (*Lrsam1*^{-/-}) Neuronal Cell Line Results in Axonal Degeneration

To further test the causal role of the Cys694Arg mutation, we used CRISPR/Cas9 to disrupt the *Lrsam1* gene in NSC34 mouse neuronal cell line (Fig 4). Because the transfection only took place in about 20 to 30% of NSC34 cells, we selected multiple single cell clones. A complete knockdown of *Lrsam1* (*Lrsam1*^{-/-} cells) was

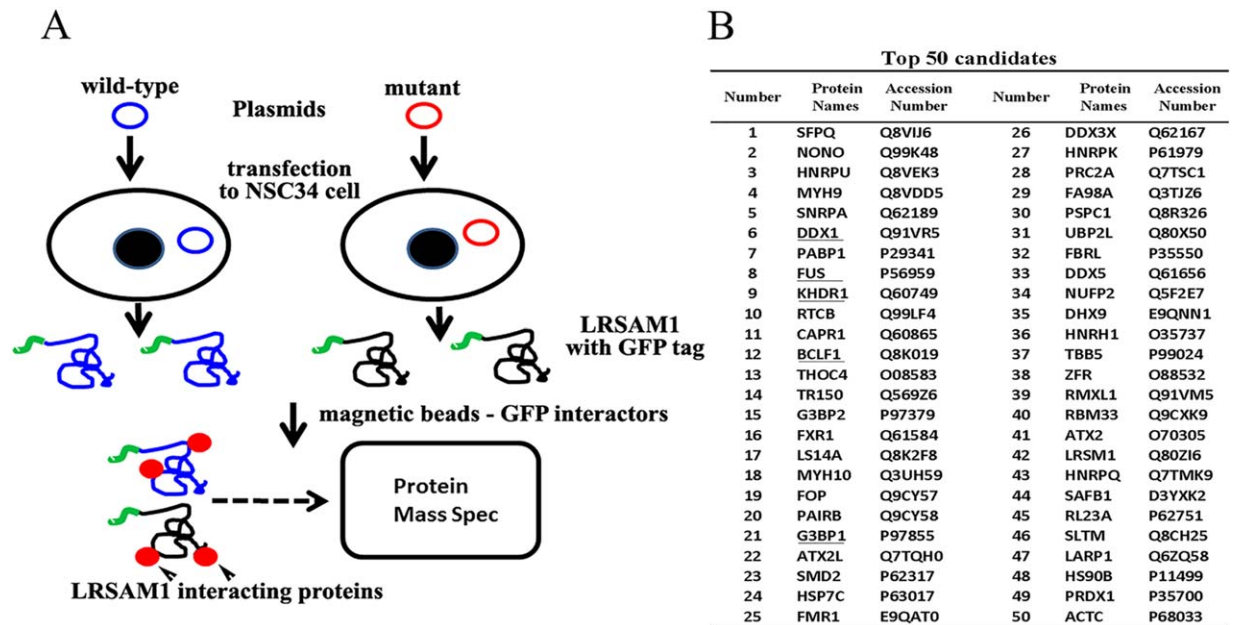


FIGURE 5: Pull-down assay and protein identification with mass spectrometry. (A) Diagram to illustrate the procedure of the pull-down assay. *Lrsam1*^{-/-} NSC34 cells were transfected with plasmids expressing wild-type human LRSAM1 (left) or mutant human LRSAM1 (right). The LRSAM1 was tagged by green fluorescence protein (GFP). The protein lysates were eluted through a column containing magnetic beads conjugated with GFP antibodies. This extracted LRSAM1 and its interacting proteins. The LRSAM1 interacting proteins were then identified by protein mass spectrometry. (B) A list of the top 50 candidate proteins is displayed. Because the mass spectrometry was only semiquantitative, it cannot provide sufficient accuracy to ascertain the difference of levels of candidate proteins between wild-type cells and mutant cells. We thus identify the top 50 proteins only based on their relative abundance, regardless of whether the candidate proteins were from the wild-type cells or mutant cells. The 5 underlined proteins were selected for further verification by coimmunoprecipitation (co-IP). This selection was based on their relative abundance, known biological functions, and availability of antibodies suitable for co-IP experiments. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

verified by Western blot in several clones, which were devoid of LRSAM1 expression (lanes 4 and 6 in Fig 4B). Depletion of LRSAM1 in NSC34 resulted in no phenotype in culture, which was consistent with the negligible phenotype observed in *Lrsam1*^{-/-} mice.¹⁸

NSC34 cells from 2 *Lrsam1*^{-/-} clones were transfected with plasmids expressing either wild-type human LRSAM1 or Cys694Arg LRSAM1. Transfected cells were examined under phase contrast microscopy. Axons with multiple beads, a sign of axonal degeneration,¹⁹ were manually counted. Percentage of abnormal axons was drastically increased in Cys694Arg cells, compared with that in wild-type cells (see Fig 4C–E). This finding supports the causal role of Cys694Arg mutation in CMT2P.

Cys694Arg Mutation Alters Formation of Nuclear Transcription Machinery

To understand how the Cys694Arg mutation causes the disease, we combined a pull-down assay and protein mass spectrometry to identify proteins that may interact with LRSAM1. The procedure is illustrated in Figure 5A. Plasmids expressing wild-type or Cys694Arg human LRSAM1 tagged with GFP (LRSAM1-GFP) were transfected into the *Lrsam1*^{-/-} NSC34 cells. LRSAM1-GFP

along with its interacting proteins was pulled down by magnetic beads conjugated with a specific GFP antibody or GFP interactor.

The pulled-down proteins listed in Figure 5B, including LRSAM1 itself, revealed a cluster of RNA-binding proteins among the top candidates. One top candidate, FUS, has been critically involved in the pathogenesis of motor neuron degeneration in amyotrophic lateral sclerosis (ALS).²⁰

We chose 5 RNA-binding proteins (FUS, G3BP1, DDX1, BCLF1, and KHDR1; underlined in Fig 5B) based on their relative levels, known biological functions, and availability of antibodies suitable for the co-IP experiments. Their interactions with LRSAM1 were verified in human fibroblasts or NSC34 cells by co-IP experiments. Two (FUS and G3BP1) of the 5 proteins were further analyzed and are shown in Figure 6. FUS levels in cytoplasm were low and only pulled down a negligible amount of LRSAM1. As expected, FUS was abundant in nuclei. FUS antibodies pulled down LRSAM1 from nuclear proteins of normal fibroblasts but failed to pull down Cys694Arg LRSAM1 from fibroblasts of patients with CMT2P. A disruption of protein–protein interaction was also observed between Cys694Arg

LRSAM1 and G3BP1. These changes were associated with a decrease of nuclear FUS and G3BP1 levels in CMT2P fibroblasts. TDP43, another RNA-binding protein known to interact with FUS in transcription protein complex (confirmed in Fig 6A), was also decreased in the

nuclei. The decrease of nuclear FUS and TDP43 was further substantiated by immunostaining the human fibroblasts. The levels of nuclear FUS fluorescence intensity were significantly decreased compared with those in normal fibroblasts. Unlike a previous study,²¹ we could

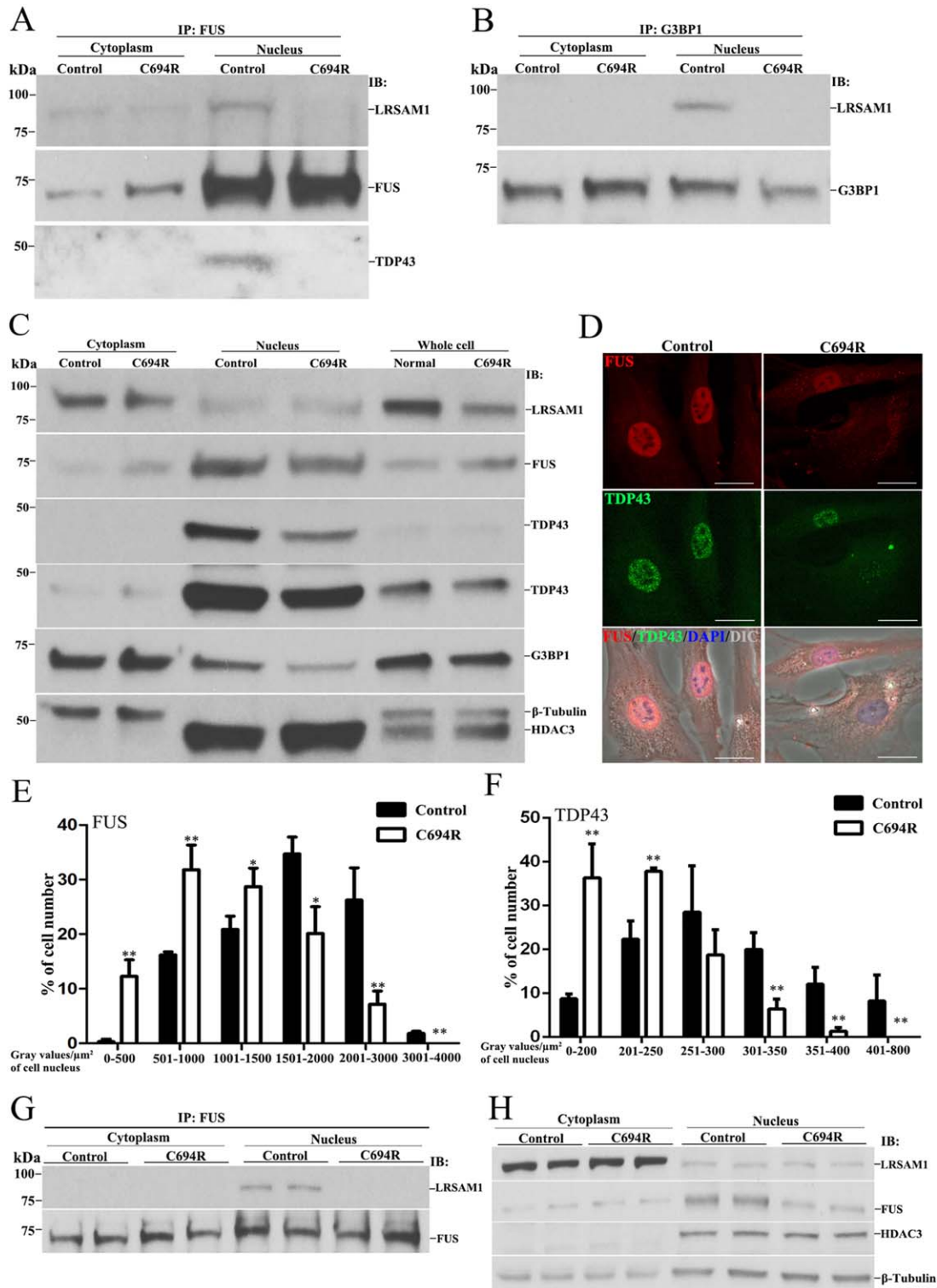


FIGURE 6.

not confirm the interaction between Tsg101 and LRSAM1 in human fibroblasts and our mass spectrometry assay even while Tsg101 was present.

Furthermore, we expressed wild-type and Cys694Arg human LRSAM1 in *Lrsam1*^{-/-} NSC34 neuronal cell line. co-IP was performed. Again, we found the disruption of interaction between mutant LRSAM1 and FUS and the decrease of nuclear FUS levels (see Fig 6G, H).

Taken together, the Cys694Arg mutation of LRSAM1 alters interactions between LRSAM1 and some RNA-binding proteins that are required for nuclear transcription machinery. It should be clarified that an increase of interaction between mutant LRSAM1 and other RNA-binding proteins is possible. These abnormalities are expected to change the protein stoichiometry of transcription machinery, leading to the alterations of RNA metabolism.²⁰

Discussion

Our study has identified a family and a sporadic case who are afflicted by an inherited sensory motor axonal polyneuropathy. This disease appears to be caused by a missense mutation (Cys694Arg) in *LRSAM1*. This conclusion is supported by the following evidence: (1) the affected individuals are cosegregated with the mutation; (2) although CMT2P has not been reported in the American population, 4 previously reported non-US families from Canada, England, Germany, and Cyprus displayed a phenotype similar to the American family⁴⁻⁷; (3) a sporadic case with the same mutation shares a phenotype similar to our family; (4) the Cys694Arg allele changes a highly conserved cysteine that resides in the

RING domain of LRSAM1; studies have shown that these conserved cysteines are critical for the function of any E3 ubiquitin ligase in general⁸; and (5) mutant LRSAM1, but not wild-type LRSAM1, caused axonal degeneration in a culture neuronal cell line with endogenous LRSAM1 depleted by CRISPR/Cas9.

Other than family history, our patients are clinically indistinguishable from patients with idiopathic axonal polyneuropathy (IAP). Case F2088 with a sporadic axonal polyneuropathy further highlights this point. Given the high prevalence of IAP,²² extensive laboratory workup would result in high costs to search for an etiology. With the remarkable advance of DNA sequencing technology and the declining price for DNA testing, an early diagnosis using genetic testing may eliminate considerable costs in these patients. This advantage will become even more evident along with the discovery of new genetic causes for neuropathies.

To understand how Cys694Arg causes axonal degeneration, we employed protein mass spectrometry and identified a cohort of proteins that may interact with LRSAM1. RNA-binding proteins were disproportionately abundant among these candidates. Two proteins from the cohort (FUS and G3BP1) were further studied in detail for their interactions with LRSAM1 by co-IP. Interestingly, the interactions were disrupted by the Cys694Arg mutation. Their levels in nuclei were decreased in mutant cells, leading to a decreased availability of RNA-binding proteins for the formation of RNA/protein complex. Although TDP43 does not interact with LRSAM1 directly, it does complex with FUS. TDP43 levels were also reduced in the nuclei (see Fig 6C, D, F).

FIGURE 6: Cys694Arg mutation disrupts interactions between LRSAM1 and RNA-binding proteins. (A) Cytoplasmic and nuclear fractions were extracted from normal and Cys694Arg human fibroblasts. Proteins were immunoprecipitated (IP) with anti-FUS antibody, and the precipitated proteins were immunoblotted (IB) with anti-LRSAM1 and anti-FUS antibody. FUS antibody was able to pull down LRSAM1 in nuclear fractions of normal fibroblasts but failed to pull down LRSAM1 in the nuclear fractions of Cys694Arg fibroblasts. (B) IP using anti-G3BP1 antibody was carried out in cytoplasmic and nuclear fractions from the fibroblasts. The G3BP1 antibody was able to pull down LRSAM1 in cytoplasm and nuclear fractions of control fibroblast cells but failed to do so in Cys694Arg fibroblasts. (C) Although TDP43 does not directly interact with LRSAM1, it is well known to form a protein complex with FUS. We thus tested whether TDP43 is also decreased in the nuclei. Whole cell, cytoplasmic, and nuclear proteins were extracted from the fibroblasts and examined by Western blot for levels of LRSAM1, FUS, and G3BP1. β -Tubulin was included as a loading control of the whole cell and cytoplasmic extracts. HDAC3 served as a loading control of the nuclear extracts. Note that row 4 was an overexposure. To clearly show the difference between normal and mutant cells, we also displayed the image with a low exposure in row 3. (D) The fibroblasts were stained with antibodies against FUS. There was a decrease of FUS immunoreactivity in the nuclei of Cys694Arg fibroblasts, compared with those in normal fibroblasts. Nuclei were also stained by 4,6-diamidino-2-phenylindole (DAPI). Scale bars = 10 μ m. (E, F) Fluorescence intensity of FUS and TDP43 were quantified from nuclei of fibroblasts. The FUS and TDP43 intensity in Cys694Arg fibroblast cells had a left shift toward higher levels ($n = 390$ from 3 wells), compared to those in normal fibroblasts ($n = 417$ from 3 wells). * $p < 0.05$, ** $p < 0.01$. (G) *Lrsam1*^{-/-} NSC34 cells of clone 4 was transfected with plasmids expressing wild-type (WT) human LRSAM1 or Cys694Arg LRSAM1. Three days after transfection, cell colonies were selected to ensure the uniformity of LRSAM1 expression in all cells. Protein lysates from cytoplasmic or nuclear fractions were used for IP. FUS antibody was able to pull down LRSAM1 in the nuclear fractions of WT LRSAM1 cells but failed to pull down LRSAM1 in the nuclear fractions of Cys694Arg cells. (H) Western blot of LRSAM1 and FUS was performed in the cytoplasmic or nuclear fractions extracted from WT LRSAM1 or Cys694Arg cells. The levels of nuclear FUS were decreased in the Cys694Arg cells compared with that in WT LRSAM1 cells.

This finding is pathogenically relevant. Both FUS and TDP43 as RNA-binding proteins are known to regulate the stabilization of RNA. The protein complex may also directly regulate transcription by associating with gene regulatory elements.²⁰ These nuclear proteins are excluded from nuclei into the cytoplasm of motor neurons in ALS, leading to dysregulation of RNA metabolism. This has emerged as one of the most promising mechanisms in the pathogenesis of motor neuron degeneration in ALS. This mechanism is further highlighted by multiple recent observations. For instance, the most prevalent ALS mutation in *C9Orf* gene impairs nuclear transport of TDP43 through RanGAP1 complex. Correction of this transport defect rescues neuronal degeneration in ALS models in vitro and in vivo.²³ Our study indicates that mutations in CMT2P may also be involved in this mechanism by altering stoichiometry of RNA-binding protein complex.

It is still unclear how mutations in the RING domain of LRSAM1 lead to the mislocalization of RNA-binding proteins from the nuclei to the cytoplasm. Ubiquitination is required for targeting certain proteins into the nuclear transport protein complex.²⁴ It remains to be determined whether the E3 ligase activity of LRSAM1 is required to ubiquitinate the RNA-binding proteins, such as FUS or G3BP1, and whether the ubiquitination is necessary for the nuclear transport of these RNA-binding proteins.

We have noticed that a study has shown interactions between Tsg101 and LRSAM1.²¹ We could not confirm the interaction in our mouse and human cells. In addition, a recent publication shows that LRSAM1 was hardly identifiable in endosomes where Tsg101 resides.¹⁸ Thus, this finding also does not support the interaction between LRSAM1 and Tsg101. SIMPLE (also called LITAF) was reported to interact with Tsg101.²⁵ However, mutations in *SIMPLE/LITAF* cause CMT1C, de-/dysmyelinating polyneuropathy with slowed conduction velocity.²⁶ This phenotype is conspicuously different from that in CMT2P. Nevertheless, this discrepancy could be due to different types of cells used between the studies. It also makes no difference for our key findings on RNA-binding proteins.

CMT2P families reported previously were associated with frameshift mutations or amino acid insertion.⁵ Although mutant LRSAM1 was expected to be produced in the 3 families with autosomal dominant inheritance, the phenotype in the 3 families was highly similar to that in the Canadian family with a recessive frameshift mutation. The recessive mutation was claimed to eliminate the expression of LRSAM1.⁴ Therefore, the disease in the 3 families was interpreted as a result of dominant

negative effect by the mutant LRSAM1. However, because the mutation in the Canadian family resides in the terminal portion of *LRSAM1* gene, antibody against the terminal portion of LRSAM1 may not detect the mutant LRSAM1 proteins. Therefore, it is still questionable whether the disease is related to any loss of function of LRSAM1. In line with this notion, deletion of *Lrsam2* in mice results in negligible phenotype.¹⁸ Nevertheless, regardless of gain or loss of function of LRSAM1 in these families, these two mechanisms are not mutually exclusive. The mutant LRSAM1 may either decrease its interaction with RNA-binding proteins or gain new interacting partners.

Interestingly, a similar situation has also been observed in a familial Parkinson disease case with mutations in *Parkin* gene. Parkin is also an E3 ubiquitin ligase with a RING domain at its c-terminal. Missense mutations affecting conserved cysteine in the RING domain of Parkin have been found in patients with inherited Parkinson disease.²⁷ Remarkably, a subgroup of patients with mutations in *LRSAM1* have developed phenotypes of Parkinson disease.²⁸

In summary, we have identified a novel missense mutation that alters cysteine to arginine in the RING domain of LRSAM1. We have shown multiple lines of evidence suggesting that this mutation is causal for CMT2P. Our study also shows that this mutation may affect the formation of RNA-binding protein complex, a potential mechanism of neuronal degeneration in CMT2P.

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Author Contributions

B.H. and J.L. contributed to study concept and design. All authors contributed to data acquisition and analysis. B.H. and J.L. contributed to drafting the manuscript and figures.

Potential Conflicts of Interest

Nothing to report.

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