

The Nuclear Factor κ B–Activator Gene *PLEKHG5* Is Mutated in a Form of Autosomal Recessive Lower Motor Neuron Disease with Childhood Onset

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Lower motor neuron diseases (LMNDs) include a large spectrum of clinically and genetically heterogeneous disorders. Studying a large inbred African family, we recently described a novel autosomal recessive LMND variant characterized by childhood onset, generalized muscle involvement, and severe outcome, and we mapped the disease gene to a 3.9-cM interval on chromosome 1p36. We identified a homozygous missense mutation (c.1940 T→C [p.647 Phe→Ser]) of the Pleckstrin homology domain–containing, family G member 5 gene, *PLEKHG5*. In transiently transfected HEK293 and MCF10A cell lines, we found that wild-type *PLEKHG5* activated the nuclear factor κ B (NF κ B) signaling pathway and that both the stability and the intracellular location of mutant *PLEKHG5* protein were altered, severely impairing the NF κ B transduction pathway. Moreover, aggregates were observed in transiently transfected NSC34 murine motor neurons overexpressing the mutant *PLEKHG5* protein. Both loss of *PLEKHG5* function and aggregate formation may contribute to neurotoxicity in this novel form of LMND.

Lower motor neuron diseases (LMNDs) are clinically characterized by progressive paralysis with amyotrophy and loss of deep tendon reflexes and fasciculations, because of motor neuron degeneration in the anterior horn of the spinal cord and the brainstem. Diagnosis is confirmed by electrophysiological or histological evidence of muscle denervation, with normal or subnormal motor nerve conduction velocities and normal sensory potentials. The classic form of autosomal recessive proximal spinal muscular atrophy (MIM 253300) is linked to the *SMN1* gene,¹ but numerous LMND variants have been described, differing by localization of motor weakness, mode of inheritance, and age at onset.^{2,3} To date, six other causative genes were reported in pure LMNDs: five with autosomal dominant inheritance (*HSPB8/HSP22* and *HSPB1/HSP27* in distal hereditary motor neuropathy type II [d-HMNII {MIM 158590 and 608634}], *GARS* and *BSCL2* in d-HMNV [MIM 600794], and *DCTN1* in d-HMNVII [MIM 607641]) and one with autosomal recessive inheritance (*IGHMBP2* in d-HMNVII, also known as “spinal muscular atrophy with respiratory distress” [SMARD {MIM 604320}]).^{4–9} All encode proteins that are directly or indirectly involved in two important intracellular pathways: axonal transport and RNA processing.^{10,11}

Elsewhere, we described a novel clinical variant of au-

tosomal recessive LMND with childhood onset in a large inbred family originating from Mali.¹² Affected individuals presented with generalized muscle weakness and atrophy with denervation and normal sensation. Bulbar symptoms and pyramidal signs were absent. In four of the five affected children, the outcome was severe, with loss of walking and the need for permanent respiratory assistance before adulthood. Genetic analyses with the use of a homozygosity mapping strategy assigned this LMND locus to a 3.9-cM (or 1.5-Mb) interval on chromosome 1p36, between loci *DIS508* and *DIS2633* ($Z_{\max} = 3.79$ at $\theta = 0.00$ at locus *DIS253*). Here, we report the identification of the Pleckstrin homology domain–containing, family G member 5 gene, *PLEKHG5* (GenBank accession number NM_020631), located within the candidate region, which is mutated in this pedigree.

Subjects and Methods

Clinical Study

Elsewhere, we reported the linkage data on the African family.¹² Informed consent was obtained from all family members, and the study was approved by the ethics committee of the Catholic University of Louvain.

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Genotyping and Mutation Analysis

We isolated genomic DNA (gDNA) from blood samples, using standard protocols. We performed genotyping for microsatellites as described elsewhere.¹² We used the Primer3 program to design PCR primers that flanked all exons of candidate genes, on the basis of the chromosome 1 draft human sequence. Details on *PLEKHG5* primers are given in table 1. We performed direct sequencing, using the dideoxy chain-termination method (ABI Big Dye 3.1) on a 3100 automated sequencer (ABI Prism [Applied Biosystems]) in accordance with standard procedures and the manufacturer's recommendations. The mutation was verified bidirectionally on gDNA and cDNA. We extracted whole mRNA from fibroblast cell lines of patients (RNeasy [Qiagen]), and we obtained cDNA products by RT-PCR, using oligo(dT) and random hexamers (Transcriptor First Strand cDNA Synthesis Kit [Roche]).

Plasmid Constructions

PLEKHG5 full-ORF expression clones, containing the complete coding cDNA for isoforms BC042606 or BC015231, were sequence verified (Deutsches Ressourcenzentrum für Genomforschung [RZPD]). To introduce the c.1940 T→C amino acid substitution in the *PLEKHG5* plasmids, we amplified cDNA of the patients with primers framing the mutation (table 1). We then restricted the mutated cDNA amplification product and the full-ORF expression clones with two single-cut endonucleases, *BstEII* and *PfIMI* (New England Biolabs). After ligation, we screened for the presence of the mutated insert, and we verified the absence of additional mutation by sequencing analysis. pCMVlacZ plasmid was constructed by insertion of the *Escherichia coli* lacZ coding region into the multiple cloning site of pCMX-PL1.¹³

Cell Culture and Transfection Conditions

HEK293 cells (human embryonic kidney 293 cells) and MCF10A cells (human mammary epithelial cells) were maintained at 37°C in a humidified 5% CO₂ atmosphere, in Dulbecco's modified Eagle medium (DMEM) F12 (Invitrogen) supplemented with 5% (for MCF10A) or 10% (for HEK293) horse serum (Cambrex), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Sigma). Also added to the medium for MCF10A cells was 0.1 µg/ml cholera toxin (Calbiochem), 10 µg/ml insulin (Invitrogen), 0.5 µg/ml hydrocortisone (Sigma), and 20 ng/ml human epidermal growth factor (Invitrogen). Before transfection, exponentially proliferating cells were trypsinized, and 1.6 × 10⁵ MCF10A cells or 2.6 × 10⁵ HEK293 cells were plated in each well of a 6-well plate. Twenty-four hours after plating, cells were transfected using 3 µl of Fugene 6 (Roche) in accordance with the manufacturer's instructions. For luciferase-reporter gene assays and real-time RT-PCR analyses, 0.5 µg of the expression vectors was transfected together with 1 µg of the reporter plasmid (nuclear factor κB [NFκB] *cis*-reporting system [Stratagene]) and with 20 ng of constitutive reporter plasmid (pCMVlacZ) for luciferase activity normalization. Expression vectors were replaced by 50 ng of pFC-MEKK (Stratagene) and 0.5 µg of carrier DNA (pCat), to provide a positive control for NFκB activation. For western-blot analyses and immunofluorescence studies, cells were transfected with 1.5 µg of expression vector DNA.

NSC34 cells (a mouse embryonic spinal cord–neuroblastoma cell line with a motor neuron phenotype, kindly provided by Dr. Neil Cashman, University of Toronto¹⁴) were cultured at 37°C under 5% CO₂ and 95% air in DMEM supplemented with 10%

Table 1. *PLEKHG5* Primers Used for gDNA and cDNA Sequencing Analyses, Plasmid Constructions, and Real-Time RT-PCR

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

fetal calf serum. NSC34 cells in a 24-well plate were transfected with either the wild-type or the mutant *PLEKHG5* plasmids with use of Lipofectamine (Invitrogen). The transfection efficacy was controlled using cotransfection with a green fluorescent protein (GFP)–expressing plasmid.

Enzymatic Assays

Cells were harvested 48 h after transfection. Lysis and enzymatic activity dosages were performed with the β-Gal Reporter Gene Assay (chemiluminescent) kit (Roche) and the Luciferase Reporter Gene assay (high-sensitivity) kit (Roche).

Gene-Expression Analysis by Quantitative RT-PCR

Total RNA was isolated 48 h after transfection and was purified using the Trizol procedure (Invitrogen) in accordance with the manufacturer's instructions. cDNA was transcribed from 3 µg of total RNA by use of random hexamers and M-MLV Reverse Transcriptase (Invitrogen), in the presence of RNase inhibitor (Promega). Quantitative real-time RT-PCR amplification was performed on cDNA by use of the qPCR Master Mix Plus for SYBR Green I (Eurogentec) in the presence of *PLEKHG5*-specific primers (Eurogentec) (table 1). The measurement of the β2 microglobulin gene provided an amplification control that allowed *PLEKHG5* expression to be normalized. Each reaction was performed in triplicate, by use of an MX3000P Real-Time PCR System (Stratagene).

Synthesis of Polyclonal Antibody to *PLEKHG5*

Polyclonal antibodies to *PLEKHG5* were obtained by immunization of two rabbits with two synthesized specific peptides (NH₂-CYLRVKAPAKPGDEG-CONH₂ and NH₂-CKVDIYLDQSNTPSL-CONH₂) and were purified on a sepharose column (Covalab). High reactivity of immunopurified antibodies was confirmed by ELISA.

Western-Blot Analysis

Proteins were harvested 48 h after transfection. Cells were washed three times with ice-cold PBS and then were lysed in 0.32 M sucrose or in lysis buffer (10 mM Hepes [pH 7.8], 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, and 1 mM dithiothreitol) and 10% Nonidet P40 (Sigma), with the addition of the Protease Inhibitors cocktail (Roche). Cell extracts were boiled for 5 min in Laemmli buffer and were submitted to a 7.5% SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes. After electroblotting, membranes were treated with 5% dry milk in TBS buffer (50 mM Tris [pH 8.1] and 150 mM NaCl) containing 0.05% Tween-20, for 1 h at room temperature, and then were hybridized overnight at 4°C with anti-*PLEKHG5* antibodies (1:1,000) diluted in blocking solution. The antigen-antibody complexes were revealed by secondary incubation with horseradish peroxidase–coupled goat anti-rabbit immunoglobulin G antibodies (1:10,000 [Sigma]). Immunoreactive proteins were visualized using enhanced chemiluminescence reagents (Perkin Elmer). Hybridiza-

tion with β -actin antibody (1:1,000 [Sigma]) was performed as a control.

Immunocytochemistry and Microscopy

Transfected HEK293 and MCF10A cells were fixed in 4% formaldehyde 48 h after transfection. We permeabilized cells with 1% Triton X-100 in PBS for 15 min and blocked aspecific fixation sites in 5% nonfat milk for 2 h at room temperature. Cells were

incubated with anti-PLEKHG5 polyclonal antibodies (1:100) for 1 h and then with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:1,000 [Sigma]) for an additional 1 h. Cells were examined using a Zeiss Axioplan 2 imaging microscope equipped with ISIS 3 software (MetaSystem).

Transfected NSC34 murine cells were treated for PLEKHG5 immunofluorescence with the polyclonal anti-PLEKHG5 antibody (1:100) coupled with diamidino-4',6-phenylindole-2 dichlorhydrate staining of the nuclei. Cells were incubated with the pri-

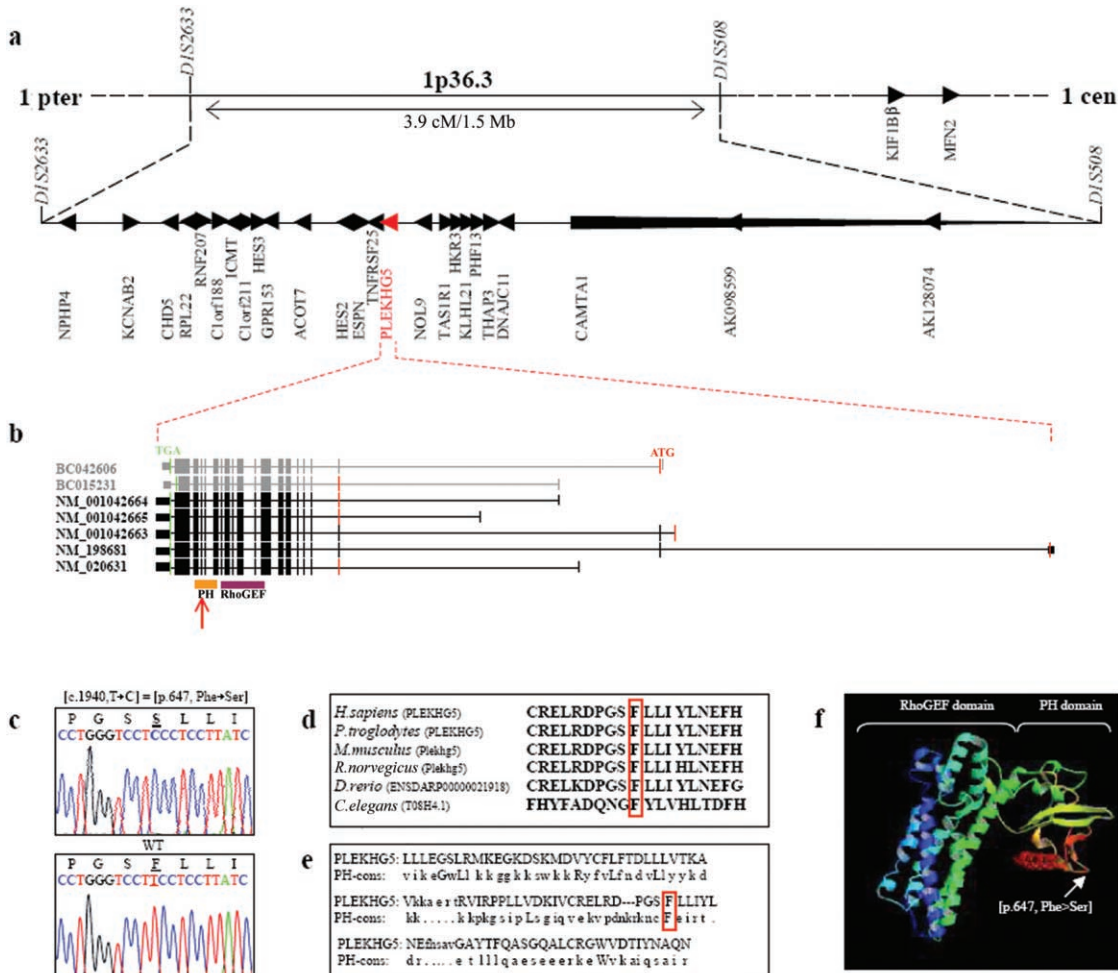


Figure 1. Physical map of the locus associated with generalized LMND, structure of PLEKHG5, and location of the mutation identified in the African family. *a*, Location and transcriptional direction of the 27 sequenced genes (UCSC Genome Browser). *b*, Structure of PLEKHG5 (UCSC Genome Browser). The five isoforms annotated by NCBI genome browser are in black, and the two Mammalian Gene Collection Full ORF mRNAs used for plasmid constructions are in gray. The coding exons are shown as vertical bars, illustrating their approximate size and position. The position of the initiation codon (ATG) at nucleotide +1 is indicated in red, and the position of the stop codon (TGA) is indicated in green. The 5' and 3' UTRs are shown as shorter vertical bars. Locations of the PH and RhoGEF domains are also shown (Pfam). The identified homozygous mutation is located in the PH domain (red arrow). *c*, Electropherogram of affected individuals compared with a control, showing the homozygous c.1940 T→C (p.647 Phe→Ser) mutation (GenBank accession number NM_020631.2). WT = wild type. *d*, Phenylalanine at position 647 (boxed in red) highly conserved across species: *Homo sapiens*, *Pan troglodytes*, *Mus musculus*, *Rattus norvegicus*, *Danio rerio*, and *Caenorhabditis elegans* (UCSC Genome Browser). *e*, Comparison of the PH domain of PLEKHG5 with the PH domain consensus sequence (PH-cons). In this sequence, the eight consensus residues are indicated in capital letters, including the mutant phenylalanine (boxed in red) (Pfam). *f*, Predictive three-dimensional structure of PLEKHG5 (ModBase). The RhoGEF domain has an α -helical structure, and the PH domain consists of seven β -sheets, followed by a C-terminal amphipathic helix. The mutation occurs in the area of the β 5/ β 6 loop of the PH domain, which may be part of the phospholipids binding site.¹⁵

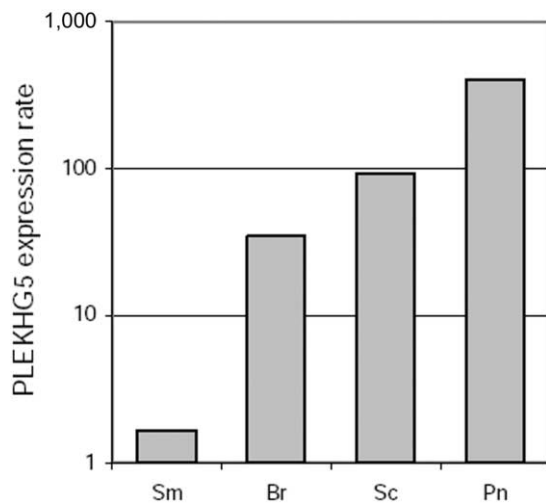


Figure 2. Expression of PLEKHG5 in the human nervous system. PLEKHG5 expression was evaluated in several human nervous tissues by real-time quantitative RT-PCR. All reactions were performed in triplicate, and PLEKHG5 expression was normalized according to $\beta 2$ microglobulin expression. Results were expressed as amplification rates in comparison with PLEKHG5 expression in untransfected HEK293 cell line. PLEKHG5 transcripts were detected in all studied tissues, supporting its ubiquitous expression pattern in the human nervous system, with a predominance in peripheral nerve and spinal cord. Sm = skeletal muscle; Br = brain; Sc = spinal cord; Pn = peripheral nerve.

mary anti-*PLEKHG5* antibody (1:100) overnight, followed by a rhodamine (A-594)-conjugated anti-rabbit secondary antibody (1:800 [Invitrogen]) for an additional 1 h. *PLEKHG5*- and GFP-coexpressing cells were observed using confocal analysis (Leica).

Statistical Analysis

P values were calculated by Welch's *t* test, by use of R software (version 2.3.0.).

Results

We sequenced the 25 candidate and predicted genes located in the 3.9-cM mapped interval on chromosome 1p36 (*NPHP4*, *KCNAB2*, *CHD5*, *RPL22*, *RNF207*, *C1orf188*, *ICMT*, *C1orf211*, *HES3*, *GPR153*, *ACOT7*, *HES2*, *ESPN*, *TNFRSF25*, *PLEKHG5*, *NOL9*, *TAS1R1*, *HKR3*, *KLHL21*, *PHF13*, *THAP3*, *DNAJC11*, *CAMTA1*, *AKO98599*, and *AK128074*), as well as two additional candidate genes known to cause Charcot-Marie-Tooth disease (CMT) and located very close to this chromosomal region (*MFN2* and *KIF1B*) (fig. 1a). In the five affected members of the Malian family, we found a homozygous mutation (c.1940 T→C) resulting in an amino acid substitution (p.647 Phe→Ser) in the pleckstrin homology (PH) domain of the *PLEKHG5* protein (fig. 1c). The mutation was not detected in 300 healthy controls (600 chromosomes), of whom 250 originated from Mali (500 chromosomes). The mutant

phenylalanine is highly conserved across species and is a canonical amino acid residue of the PH consensus sequence (fig. 1d and 1e). We then screened *PLEKHG5* in a sample of four unlinked families and 16 isolated patients with a close phenotype, but we identified no additional mutations.

PLEKHG5 spans 53,917 bp on human chromosome 1 and codes for a member of the Dbl protein family that shares a PH domain and a guanine nucleotide exchange factor for Rho protein (RhoGEF) domain.¹⁶ Interestingly, it has been suggested elsewhere that the *PLEKHG5* protein has an NF κ B-activating function.¹⁷ Five mRNA isoforms annotated by National Center for Biotechnology Information (NCBI) genome browser and two Mammalian Gene Collection Full ORF mRNAs have been reported to code for proteins that mainly differ by their N-terminal end (GenBank accession numbers NM_020631, NM_198681, NM_001042663, NM_001042664, NM_001042665, BC042606, and BC015231) (fig. 1b). According to microarray databases (UniGene, Expression Profile Viewer), *PLEKHG5* is ubiquitously expressed, but predominantly in the peripheral nervous system and brain. Using western blotting and immunofluorescence, we failed to detect endogenous *PLEKHG5* protein in various human cells (fibroblastic cell lines, lymphoblastoid cell lines, cultured amniocytes, and cultured primary hepatocytes) (data not shown). However, using real-time quantitative RT-PCR, we confirmed the ubiquitous expression of *PLEKHG5* in the human nervous system, with a predominance in peripheral nerve and spinal cord (fig. 2).

To further investigate the impact of the c.1940 T→C mutation, two isoforms of *PLEKHG5* (*BC042606* and *BC015231*) (fig. 1b) were transfected in HEK293 and MCF10A cell lines. The wild-type *PLEKHG5* protein was not visualized by western blotting or immunofluorescence before transfection. By contrast, after transfection of expression vectors encoding wild-type *BC015231* and *BC042606* isoforms, we detected protein fragments of 130 and 150 kDa in cell lysates, using polyclonal rabbit anti-*PLEKHG5* antibodies (fig. 3a). In addition, immunofluorescence analysis revealed that wild-type *PLEKHG5* proteins were diffusely localized in cytoplasm (fig. 3b). On the contrary, in cells transfected with the corresponding c.1940 T→C mutant constructs, mutant *PLEKHG5* proteins were consistently undetectable by western blotting, suggesting an instability of the mutant variants (fig. 3a). By immunofluorescence, mutant *PLEKHG5* proteins were not detected with a classic exposure time (0.04 s). However, a light, diffuse cytoplasmic signal could be detected after a longer exposure time (0.34 s) (fig. 3b).

The two isoforms of *PLEKHG5* were then tested for their ability to activate the NF κ B pathway. HEK293 and MCF10A cell lines were transfected with a luciferase-reporter gene responsive to NF κ B, together with expression vectors encoding either wild-type or c.1940 T→C mutated *PLEKHG5* cDNAs. Although the *PLEKHG5* transcript was barely detected in untransfected control cells by use

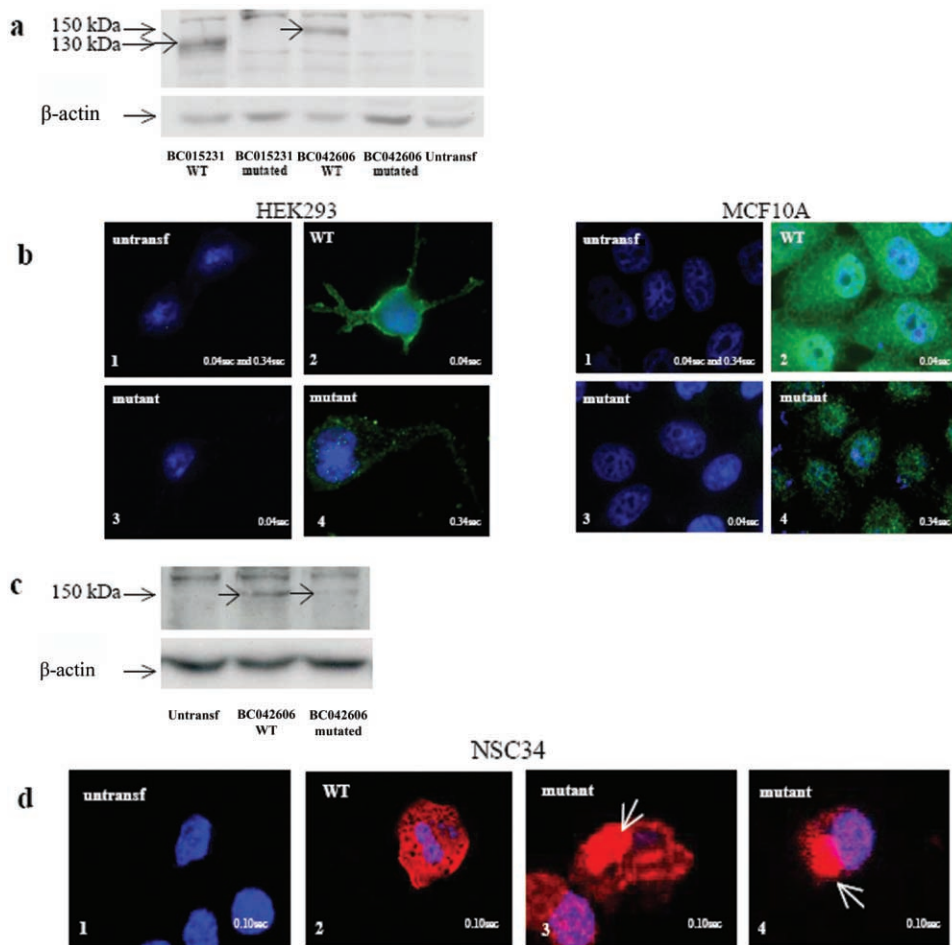


Figure 3. Analysis of wild-type (WT) and mutant PLEKHG5 protein expression in untransfected (untransf) and transiently transfected HEK293, MCF10A, and NSC34 cells. *a*, Analysis by western blotting of PLEKHG5 protein expression in HEK293 cells. PLEKHG5 protein was not detectable in untransfected cells. The 130-kDa and 150-kDa bands were detected in cells overexpressing the BC015231 and BC042606 wild-type isoforms. In contrast, no similar bands were observed in cells transfected with the mutant variants. Similar results were found after distinct protein extraction methods (with 0.32 M sucrose or lysis buffer A; see the “Subjects and Methods” section) and in both HEK293 and MCF10A cells. *b*, Immunofluorescence assays of PLEKHG5 protein expression in HEK293 and MCF10A cells. *b1*, There was an absence of signal in untransfected cells, even with a long exposure time (0.34 s). *b2*, With an exposure time of 0.04 s, cells transfected with wild-type PLEKHG5 showed a diffuse cytoplasmic distribution of PLEKHG5 protein. *b3*, In cells transfected with the mutant constructs, no protein was visualized with an identical exposure time (0.04 s). *b4*, Increased exposure time (0.34 s) revealed a weak signal in the nucleus and the cytoplasm. *c*, Analysis by western blotting of PLEKHG5 protein expression in NSC34 cells. Wild-type PLEKHG5 protein was not detectable in untransfected cells. The 150-kDa band was detected in cells overexpressing the BC042606 wild-type isoform. In contrast, the band intensity was clearly decreased in cells transfected with the BC042606 mutant variant. *d*, Immunofluorescence assays of PLEKHG5 protein expression in NSC34 cells. The transfection efficacy was controlled using cotransfection with a GFP-expressing plasmid. *d1*, There was an absence of signal in GFP-negative nontransfected cells. *d2*, Cells transfected with the wild-type PLEKHG5 plasmid showed a diffuse cytoplasmic distribution of the PLEKHG5 protein. *d3* and *d4*, Presence of large immunoreactive aggregates (white arrows) in the soma of NSC34 motor neurons transfected with the mutant PLEKHG5 plasmids.

of real-time quantitative RT-PCR, its abundance increased up to 1,000-fold after transfection in cells transfected with either wild-type or mutant *PLEKHG5* variants (fig. 4). Consistently, the luciferase activity was more than sixfold higher in the wild-type *PLEKHG5*-transfected cells than in the control cells. By contrast, induction of the luciferase activity was markedly reduced in cells transfected with the mutant isoforms (fig. 5a), therefore demonstrating that

the amino acid substitution severely impaired PLEKHG5 ability to activate the NF κ B pathway. This loss of activity clearly reflects, at least in part, the instability of the mutant protein.

Finally, we transiently transfected murine motor neuronal NSC34 cells with the two wild-type *BC015231* and *BC042606* isoforms and the corresponding mutant constructs. Western-blotting experiments confirmed the in-

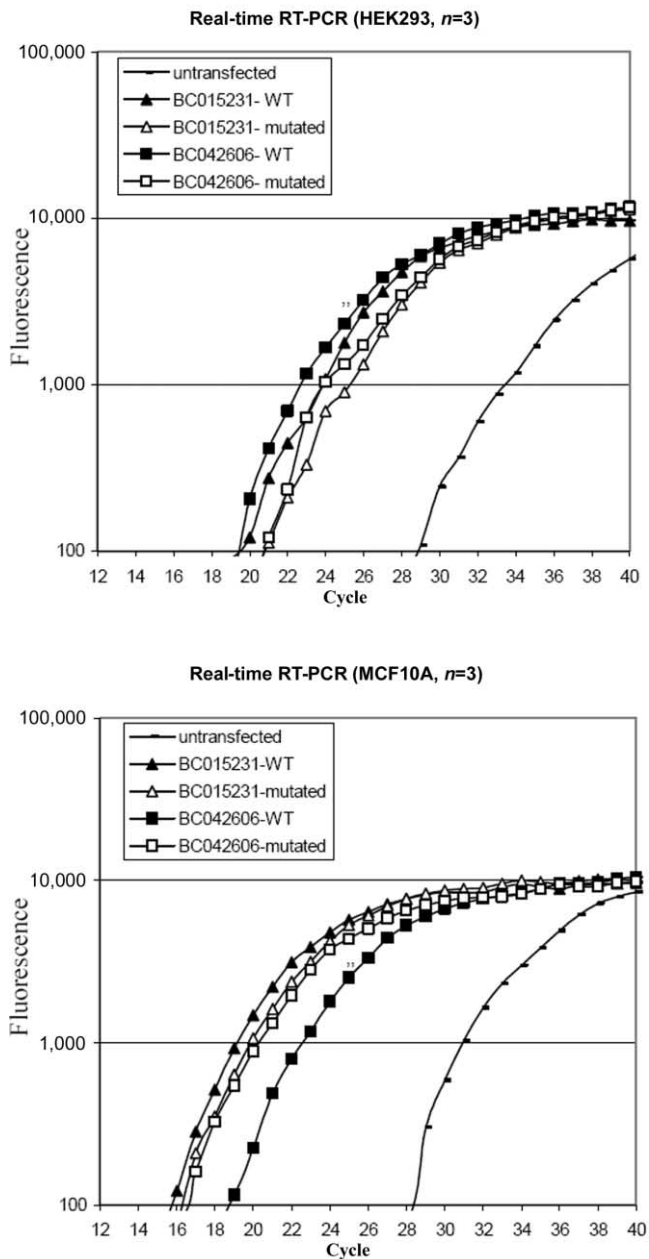


Figure 4. Quantification, by real-time RT-PCR, of *PLEKHG5* expression levels in untransfected and transfected HEK293 and MCF10A cells. At 48 h after transfection, the cells were harvested and were analyzed by real-time RT-PCR for *PLEKHG5* mRNA. The fluorescence was measured in every PCR cycle and was proportional to the accumulation of PCR product. Endogenous expression of *PLEKHG5* was detected in untransfected control cells but was markedly increased in transfected cells (up to 1,000-fold, estimated by $\Delta\Delta C_t$ method). Overexpression of wild-type (WT) and mutated *PLEKHG5* in transfected cells was similar. Triplicate analyses were performed for each sample.

stability of the mutant *PLEKHG5* proteins, which accumulated to much lower level than that of the wild-type proteins (fig. 3c). Detection of the mutant *PLEKHG5* proteins by immunofluorescence revealed formation of aggregates in the motor neuron somas close to the nucleus in the majority (60%–70%) of the transfected cells (fig. 3d). This was observed neither in NSC34 cells transfected with the wild-type counterparts nor in nontransfected cells.

In conclusion, transfection experiments using *PLEKHG5* variants in distinct cellular models supported instability and loss of NF κ B-activating function of the mutant *PLEKHG5* proteins and revealed their involvement in aggregate formation in transfected murine motor neuron cells.

Discussion

The mechanism by which the mutation in the PH domain of *PLEKHG5* leads to an autosomal recessive, generalized LMND is unclear. PH domains are protein modules of ~100 aa, found in a wide range of eukaryotic proteins, many of which are involved in cell signaling and cytoskeletal regulation. They play a membrane-anchoring function because of their ability to bind to phosphoinositides.¹⁸ In Dbl-family proteins, including *PLEKHG5*, PH domains also independently contribute to the allosteric regulation of the RhoGEF domain. This latter domain activates GTPases by stimulating the exchange of GDP to GTP, thereby initiating various signaling mechanisms that regulate neuronal shape and plasticity, dendrite growth, synapse formation, and neuronal survival.^{19–22} Recent experiments show that mutations in the PH domain, impairing phosphoinositide binding, do not systematically affect protein subcellular localization. However, in all cases, these mutations significantly reduce the guanine nucleotide exchange activity of the Dbl proteins.²³

Two proteins sharing a PH domain or a PH/RhoGEF domain have already been shown to account for human neurodegenerative diseases: Dynamin 2 (encoded by *DNM2*) and alsin (encoded by *ALS2*). Mutations of the PH domain of Dynamin 2 have been reported in CMT, dominant intermediate B (DI-CMTB [MIM 606482]), whereas mutations outside the PH domain are responsible for a dominant myopathic phenotype (centronuclear myopathy [MIM 160150]), suggesting that the PH domain could be specifically involved in motor neuron maintenance.^{24,25} Mutations in *ALS2* have been described in three overlapping autosomal recessive diseases: juvenile amyotrophic lateral sclerosis (ALS2 [MIM 205100]), infantile-onset ascending spastic paraplegia (IAHSP [MIM 607225]), and juvenile primary lateral sclerosis (PLS [MIM 606353]).^{26–32} The alsin protein is composed of three guanine nucleotide exchange factor domains, which result in Ran, Rho/Rac1, and Rab5 guanine nucleotide exchange activities, involved in motor neuron maintenance, axonal transport, and neurite outgrowth.^{10,11} The crucial role of the PH/

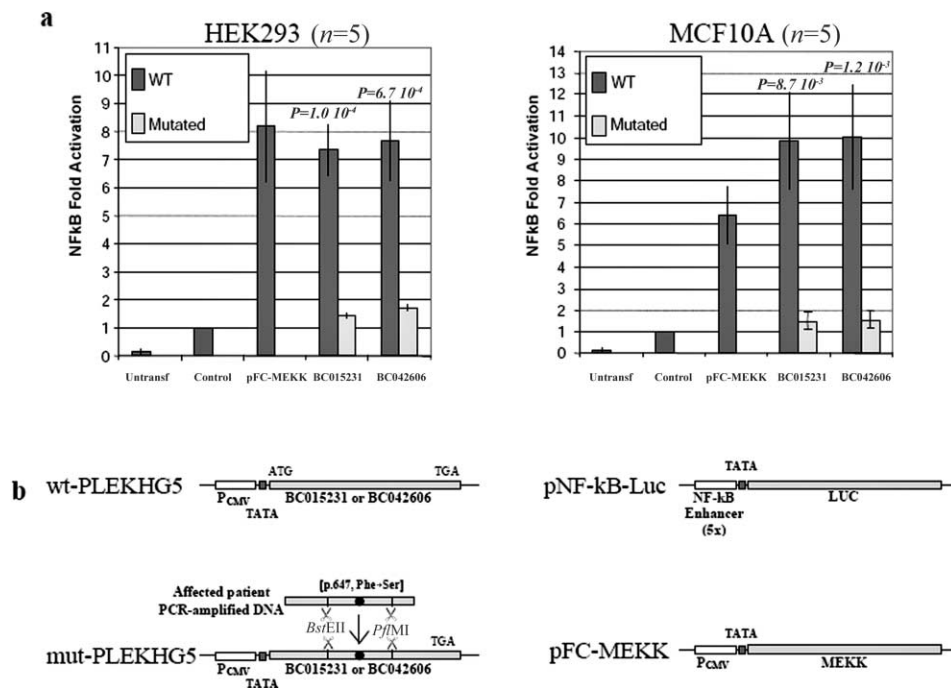


Figure 5. Comparison between wild-type (WT) and mutated PLEKHG5 activity on the NFκB signaling pathway. *a*, Reporter construct consisting of the luciferase gene placed under control of a promoter containing the consensus NFκB binding sites (Stratagene), transfected in HEK293 or MCF10A cells alone (Control), in combination with expression vectors for wild-type and mutated PLEKHG5 proteins (BC015231 and BC042606), or in combination with expression vector for a positive control of NFκB activation (pFC-MEKK). Values are expressed as fold activation over transfection of the reporter plasmid alone. Bars indicate the SD of five independent experiments. The differences between wild-type and mutated PLEKHG5 are statistically significant according to Welch's *t* test. *b*, Schematic representation of the plasmid constructions (see the "Subjects and Methods" section). Untransf. = untransfected. P_{CMV} = CMV promoter; TATA = TATA box.

RhoGEF domain for the alsin-mediated neuroprotection against the toxic effect of the mutant form of SOD1 has recently been demonstrated.^{33,34} Similar to PLEKHG5, endogenous alsin is a low-abundance protein enriched in neural tissues.³⁵ The reported mutations of the *ALS2* gene are rarely located in the PH domain. However, all but one of the causative mutations generate alsin protein truncation, often leading to the loss of the PH domain. Except for the recently reported missense G540E mutation, a loss-of-function mechanism was attributed to the causative homozygous mutations in the *ALS2* gene. Indeed, when expressed in cultured HEK293 cells, most of the disease-associated mutant forms are unstable and are rapidly degraded by the proteasome, as observed with mutant PLEKHG5.³⁵

The Rho family of GTPases are known to activate the NFκB signaling pathway.^{36,37} Here, we confirm the NFκB-activating function of PLEKHG5. The NFκB signaling pathway has not been found to be directly involved in human motor neuronopathies. However, its neuronal antiapoptotic role has been documented in various cellular models, and several studies showed that inhibition of the NFκB pathway promoted apoptosis in neurons.^{38–43} Moreover, down-regulation of NFκB activity has recently been observed in the expanded polyglutamine protein-expressing

neuro-2a cells, suggesting that this pathway could be involved in the pathogenesis of polyglutamine-degenerative disorders, including Kennedy disease (MIM 313200), a spinobulbar muscular atrophy.⁴⁴ Therefore, considering its wide CNS expression pattern and its NFκB-stimulating activity, PLEKHG5 might play a role in neuronal maintenance. Indeed, a mutation in the PH domain leading to the loss of NFκB activation could fail to protect neurons against apoptosis and could compromise the neuronal survival.

Finally, we showed that mutant PLEKHG5 variants formed aggregates in transfected NSC34 murine motor neurons. Interestingly, aggregates were never observed in wild-type-transfected NSC34 cells, and formation of mutant PLEKHG5 aggregates appeared to be specific to the neuronal cell line, because they were not observed in transfected HEK293 and MCF10A cells. This last finding argues against the hypothesis of an artifact of overexpression.⁴⁵ Animal models or human postmortem histological studies would be useful for evaluating whether this observation in vitro is correlated with intracellular inclusions in vivo. Although the neurotoxic or neuroprotective effect of aggregates is still debated, abnormal aggregation is a common feature in several neurodegenerative diseases, including motor neuron diseases.^{45–49} In particular, protein

aggregation has been recognized as a characteristic change in degenerating motor neurons of amyotrophic lateral sclerosis (MIM 105400) with mutant SOD1.⁵⁰ Moreover, transfection experiments revealed abnormal aggregation of various mutated proteins involved in autosomal dominant distal hereditary motor neuropathies, such as the small heat-shock proteins 1 and 8 (HSPB1 and HSPB8), the seipin protein (Bsc12), and the dynactin protein (DCTN1).^{8,9,51–53} In these examples, there is increasing evidence that the misfolded protein and aggregate structures lead to a dominant pathological phenotype by a toxic gain-of-function mechanism or by the combination of both loss of function and toxic gain of function.^{9,51,53–56} Conversely, specific aggregation of mutant proteins involved in autosomal recessive LMNDs has not yet been reported.

Whether PLEKHG5 is directly or indirectly involved in RNA processing or axonal transport in motor neuron, as postulated for the other LMND-causative genes, remains to be further explored. As already described in neurodegenerative diseases with aggregate accumulation, mutant PLEKHG5 could generate toxic interaction with various intracellular partners, including specific components of the axonal cytoskeleton, leading to the disruption of the anterograde transport pathway in motor neurons.^{7,8,11,33,51,56–60}

In conclusion, we have demonstrated that a form of LMND with childhood onset is caused by a homozygous mutation (c.1940 T→C [p.647 Phe→Ser]) in the *PLEKHG5* gene. This mutation caused protein instability, impaired the ability of PLEKHG5 to activate the NFκB pathway in transfected HEK293 and MCF10A cell lines, and eventually led to aggregate formation in a transfected murine NSC34 motor neuron cell line. The observation of a mutation of the *PLEKHG5* gene in a motor neuron degenerative disorder suggests that the RhoGEF-mediated NFκB signaling pathway plays an important function in motor neuron maintenance and could be involved in other human neuronopathies as well.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Expression Profile Viewer, <http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.284232>
 GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for mRNAs [accession numbers NM_020631, NM_198681, NM_001042663, NM_001042664, NM_001042665, BC042606, and BC015231])
 ModBase, <http://modbase.compbio.ucsf.edu/>
 NCBI, <http://www.ncbi.nlm.nih.gov/>
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for spinal muscular atrophy, d-HMNII, d-HMNV, d-HMNVII, SMARD, DI-CMTB, centronuclear myopathy, ALS2, IAHS, PLS, Kennedy disease, and amyotrophic lateral sclerosis)
 Pfam, <http://www.sanger.ac.uk/software/Pfam/> (for protein families and domain)
 Primer3, <http://frodo.wi.mit.edu/>
 RZPD, <http://www.rzpd.de/>
 UCSC Genome Browser, <http://genome.ucsc.edu/>

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