

ZFYVE27 (SPG33), a Novel Spastin-Binding Protein, Is Mutated in Hereditary Spastic Paraplegia

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Spastin, the most commonly mutated protein in the autosomal dominant form of hereditary spastic paraplegia (AD-HSP) has been suggested to be involved in vesicular cargo trafficking; however, a comprehensive function of spastin has not yet been elucidated. To characterize the molecular function of spastin, we used the yeast two-hybrid approach to identify new interacting partners of spastin. Here, we report ZFYVE27, a novel member of the FYVE-finger family of proteins, as a specific spastin-binding protein, and we validate the interaction by both in vivo coimmunoprecipitation and colocalization experiments in mammalian cells. More importantly, we report a German family with AD-HSP in which ZFYVE27 (SPG33) is mutated; furthermore, we demonstrate that the mutated ZFYVE27 protein shows an aberrant intracellular pattern in its tubular structure and that its interaction with spastin is severely affected. We postulate that this specific mutation in ZFYVE27 affects neuronal intracellular trafficking in the corticospinal tract, which is consistent with the pathology of HSP.

Impaired axonal transport has been implicated in many neurodegenerative disorders. One such group of neurodegenerative disorders that primarily affects lower limbs is hereditary spastic paraplegia (HSP). HSPs are characterized by progressive spastic paralysis of the legs, usually caused by length-dependent distal degeneration of the corticospinal tract axons.¹ Clinically, HSPs are classified as either the pure or the complex form.² In the pure form of HSP, spastic paraplegia occurs in relative isolation and is often accompanied by urinary urgency. The complex form of HSP is associated with additional neurological or nonneurological features. HSPs are genetically complex: autosomal dominant, recessive, and X-linked modes of inheritance are reported for both pure and complex HSP.² Within each inheritance pattern group, there is further locus heterogeneity. For autosomal dominant HSP (AD-HSP), 13 different loci are reported; also, 13 loci for the autosomal recessive form and 3 loci for the X-linked disease are known. It is likely that more loci will be identified in the near future. Eleven of the HSP genes are now identified. However, mutation in spastin (MIM 604277) is the most common cause of HSP and accounts for up to 40% of AD-HSP cases.³⁻⁶

Spastin is a member of the AAA family of proteins and is ubiquitously expressed in all tissues. The AAA proteins are proven or putative ATPases, and they are characterized by a conserved C-terminal domain containing one or two AAA cassettes.⁷ The N-terminal domain of the protein, which contains recognition sites for substrates or adaptors, is highly divergent and, therefore, confers specificity to its cellular function.⁸

A comprehensive function of spastin is not yet elucidated; previous reports suggested that spastin is principally involved in microtubule dynamics and severing.⁹⁻¹¹ However, the expression of spastin in punctate vesicles in cultured cells also suggests a role in vesicular trafficking, which is now supported by emerging reports.^{12,13} Furthermore, the N-terminal portion of spastin contains a microtubule-interacting and trafficking (MIT) domain, which is predicted to play a role in the endosomal trafficking process.¹⁴ The MIT domain corresponds to a slightly extended version of the End13-SNX15-Pa1B (ESP) homology domain, which is also present in VPS4, SKD1, RPK118, and SNX15, all of which have a well-established role in endosomal trafficking.¹⁵⁻¹⁸

Spastin is a multifaceted protein, and, to elucidate its function, we performed a yeast two-hybrid assay. In our screen, we used spastin-lacking exon 4 (GenBank accession number NM_199436) (referred to as "spastin") as bait against a human brain cDNA library and tested 2.2×10^6 clones. This spastin splice variant is the major transcript in adult spinal cord and could be pathologically relevant.¹⁹ Stringent screening revealed that 31 clones were able to grow in selective media and were also positive for α -galactosidase activity (data not shown). Sequencing of these clones led to the identification of seven ORFs of five unique genes (table 1). Clone 18, which was identified as a potential spastin-interacting protein, encodes for ZFYVE27 and contains the C-terminal fragment of ZFYVE27 (amino acids 209-318) and the 3' UTR, which corresponds to nucleotides 822-2746 of the ZFYVE27 cDNA (ZFYVE27-c isoform

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Table 1. Genes Identified as Putative Spastin-Interacting Proteins in our Yeast Two-Hybrid Assay

Clone ^a	Gene ^b	Chromosomal Location ^c	Amino Acids ^d
3	<i>Reticulon3</i>	11q13	57–236
5	<i>CRELD5</i>	3p25.2	201–421
6	<i>COPS5</i>	8q13.2	67–335
8	<i>Reticulon1</i>	14q23.1	632–776
10	<i>Reticulon1</i>	14q23.1	632–776
18	<i>ZFYVE27</i>	10q24.2	209–319
22	<i>Reticulon3</i>	11q13	85–236

^a Identity of the clone designated in our yeast two-hybrid experiment.

^b Name of the gene corresponding to the clone.

^c Chromosomal location of the gene.

^d Encoding amino acid sequence present in the clone.

[GenBank accession number AK097945]). ZFYVE27 is a novel protein that belongs to the FYVE-finger family of proteins. The FYVE-finger domain is suggested to be responsible for endosomal localization of these proteins, and the majority of the FYVE-finger proteins serve as regulators of endocytic membrane trafficking.^{20,21} The fact that ZFYVE27 is a putative endosomal protein, combined with the recent finding of spastin interaction with endosomal protein CHMP1B,¹² prompted us to investigate the interaction of ZFYVE27 with spastin and its physiological relevance.

In the Ensembl database, four different splice variants of *ZFYVE27* are reported, as shown in figure 1A. To amplify the complete ORF of *ZFYVE27*, we performed RT-PCR on human brain RNA with a primer pair flanking the complete ORF of the transcripts, and we were able to amplify a single ~1-kb product (fig. 1B). Sequencing revealed that this product corresponds to the variant form, *ZFYVE27-c* (fig. 1A). We used this *ZFYVE27-c* product in our further investigations and referred to it as “ZFYVE27.”

To determine the intracellular distribution of ZFYVE27, we generated epitope-tagged ZFYVE27 constructs. Analysis of cells, which were transfected with E2-ZFYVE27 or green fluorescent protein (GFP)-ZFYVE27 construct, showed that ZFYVE27 is predominantly expressed in punctate vesicles (fig. 2A and 2B). However, in a small proportion of cells, a tubular pattern of expression was also observed (fig. 2C). Overall, the intracellular distribution of ZFYVE27 in punctate vesicles was comparable to the expression pattern reported for other well-characterized members of the FYVE-finger family of proteins.^{22–24} Colocalization studies with subcellular markers revealed an overlapping expression with the endosomal marker, *EEA1*^{22,23} (fig. 2D–2F), and the endoplasmic reticulum (ER) marker, *RTNI*^{25,26} (fig. 2G–2I). ZFYVE27 expression in the vesicles originating from both ER and endosome could be dependent on the metabolic stage of the cell after the transient transfection. Presumably, the biogenesis of ZFYVE27 occurs in ER, and then it could be sorted to the endosome through its FYVE-finger

domain interaction with the endosomal membrane. Examination of HeLa cells coexpressing GFP-ZFYVE27 and c-Myc-spastin by immunofluorescence microscopy revealed an obvious colocalization in vesicular-like structures in the cytoplasm (fig. 2J–2L). The colocalization of ZFYVE27 and spastin in discrete vesicles in the cytosol also highlights the physiological interaction between spastin and ZFYVE27.

To investigate the interaction of ZFYVE27 with spastin, we performed coimmunoprecipitation studies by cotransfecting NIH 3T3 cells with E2-ZFYVE27 and c-Myc-spastin constructs. When E2-ZFYVE27 was immunoprecipitated from total protein lysate of the transfected cells, c-Myc-spastin (64 kDa) could also be detected in the precipitated fraction (fig. 3A). Similarly, E2-ZFYVE27 (42 kDa) could be coimmunoprecipitated with c-Myc-spastin (fig. 3B). To determine the domain of spastin, which is responsible for the interaction, we generated N-terminal (c-Myc-spastin Δ NT) and C-terminal (c-Myc-spastin Δ AAA) deletion constructs of spastin for immunoprecipitation experiments. The c-Myc-spastin Δ NT lacks the first 300 amino acids, including the MIT domain, whereas, in the c-Myc-spastin Δ AAA construct, amino acids 398–583 were deleted.

Our results revealed that E2-ZFYVE27 was able to interact with c-Myc-spastin Δ AAA (fig. 3C) but not with c-Myc-spastin Δ NT (data not shown), suggesting that spastin mediates interaction with ZFYVE27 through its N-terminal domain, which consists of a MIT motif. Interestingly, the N-terminal spastin domain is also responsible for the interaction with other proteins—for example, the endosomal protein CHMP1B,¹² microtubules,⁹ and the centrosomal protein NA14.²⁷ To validate the interaction between spastin and ZFYVE27 at the endogenous level, we performed an immunoprecipitation assay, using antispastin antibodies with protein extract derived from HeLa cells transfected with E2-ZFYVE27. Our analysis confirmed that endogenous spastin interacts with E2-ZFYVE27 (fig. 3D).

The fact that ZFYVE27 is a spastin-interacting protein and its expression in brain led us to consider *ZFYVE27* as a candidate gene for HSP. The *ZFYVE27* gene is located on chromosome 10q24.2 and consists of 13 exons. We investigated genomic DNA of 43 patients with AD-HSP for mutation in *ZFYVE27* (for primer sequences, see table 2). Our mutational analysis detected a single sequence variant in the exon 6 of *ZFYVE27* in the genomic DNA of one patient with AD-HSP (III.2 in fig. 4B). We identified a transversion, G→T, at position 572 of *ZFYVE27* cDNA (*ZFYVE27-a* isoform [GenBank accession number NM_001002261]),

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

Figure 1. Spliced isoforms of ZFYVE27. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

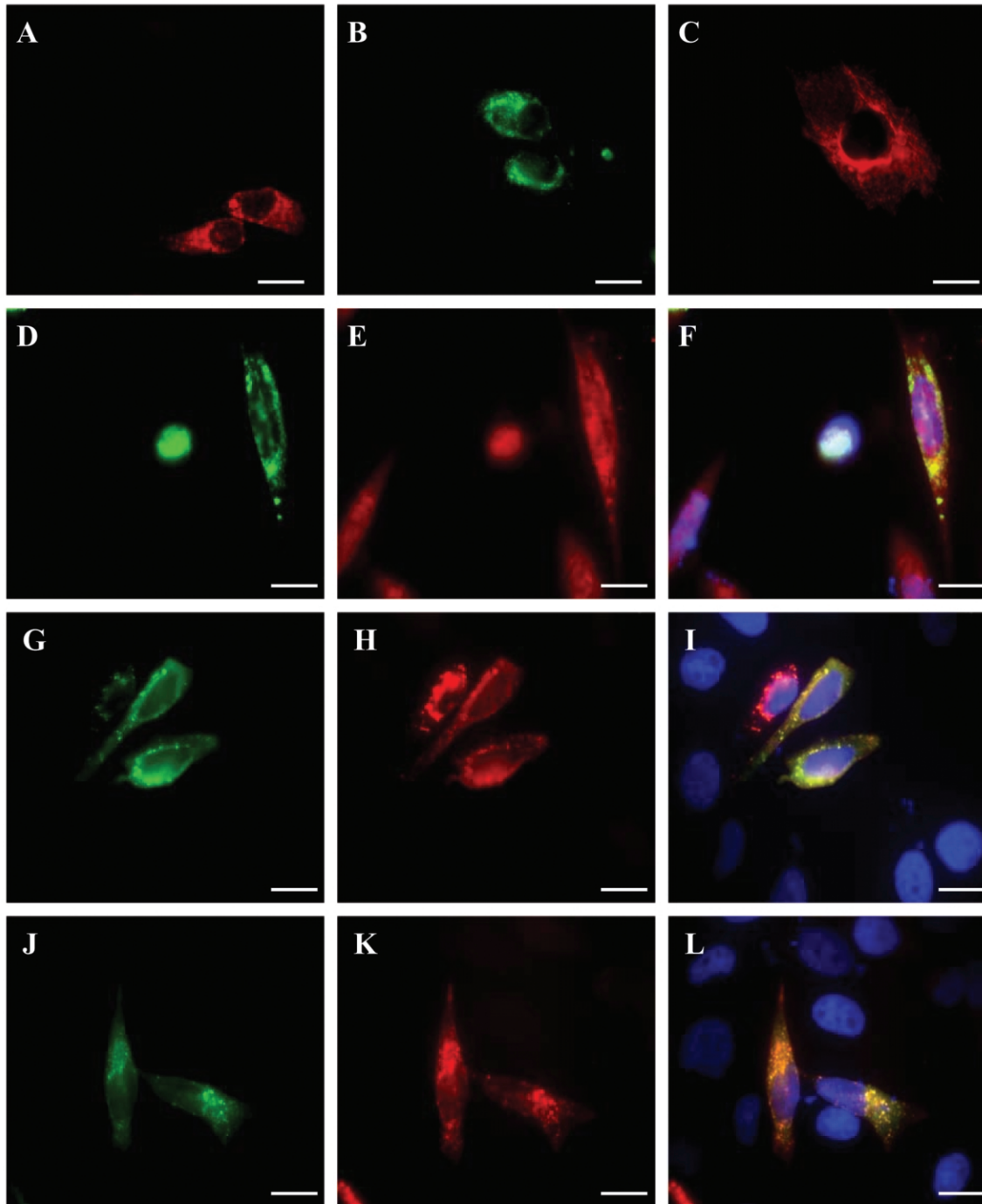


Figure 2. Intracellular distribution of ZFYVE27 and colocalization with endosomal marker (*EEA1*), ER marker (*RTN1*), and spastin in mammalian cells. HeLa cells were transiently transfected with either E2-tagged ZFYVE27 or GFP-ZFYVE27 constructs. *A*, E2-tagged ZFYVE27 expressed in the punctate vesicles distributed in the cytoplasm. *B*, GFP-ZFYVE27 also showing vesicular expression in the cytosol. *C*, A small proportion of cells revealing a tubular pattern of expression, which seems to arise from the perinuclear region and is then distributed in the peripheral region of the cytoplasm. *D–F*, HeLa cells transiently transfected with GFP-ZFYVE27 and immunostained with *EEA1*. *D*, HeLa cell expressing GFP-ZFYVE27 in cytoplasmic vesicles. *E*, Staining of the same cell, with *EEA1* antibody showing expression in punctate vesicles in the cytoplasm. *F*, Subset of cytoplasmic vesicles showing coexpression of GFP-ZFYVE27 and *EEA1*, as observed in the superimposed image. The nuclei of the cells were stained with 4',6-diamidino-2-phenylindole (DAPI). *G–I*, Colocalization of GFP-ZFYVE27 and ER marker, *E2-RTN1*, in the HeLa cells. *G*, GFP-ZFYVE27; *H*, *E2-RTN1*; *I*, overlay image. An overlapping expression pattern in vesicular structure around the perinuclear region can be seen between GFP-ZFYVE27 and *E2-RTN1* in the cytoplasm. *J–L*, Expression of GFP-ZFYVE27 and c-Myc-spastin. *J*, GFP-ZFYVE27; *K*, c-Myc-spastin; *L*, overlay image. A striking level of colocalization between GFP-ZFYVE27 and c-Myc-spastin can be observed in cytoplasmic vesicles. Scale bar is 20 μm .

which leads to a missense mutation, G191V, in the protein (fig. 4A). Sequencing of the *ZFYVE27* gene in the genomic DNA of other family members (IV.2, IV.3, and IV.4) revealed that the mutation c.572G→T (p.G191V) segregated with the clinical phenotype in the affected individuals (IV.2 and IV.3) and was not detected in the clinically unaffected individual (IV.4) (fig. 4B). An extensive GenBank search of *ZFYVE27* EST clones for the presence of this base exchange failed to detect any EST containing the identified sequence variant. Furthermore, this mutation was not found in 210 control chromosomes (data not shown). Thus, the detected base exchange is, most probably, causative for the HSP in this family and is not a rare polymorphism.

The clinical features of the affected members suggest that theirs is a pure form of HSP. The index patient, III.2, had had pes equinus for many years when she noticed a gait disorder at age 50 years. She then manifested hyperreflexia and spasticity of the lower limbs, a positive Babinski sign for an ankle clonus. Sensitivity was normal. Six years ago, she needed walking sticks, some years later a wheelchair was necessary, and now she is bedridden. Her daughter (IV.2), aged 47 years, also had pes equinus for many years. Five years ago, she developed a gait dis-

Table 2. Primer Sequences for Amplifying and Sequencing of *ZFYVE27*

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order. She also has hyperreflexia and spasticity of the legs, as well as an ankle clonus. The index patient's son (IV.3) is reported to have pes equinus, spasticity of the legs, and a gait disorder. However, the definite age of onset of the symptoms could not be determined from the family history. Cognitive deficits or additional neurological symptoms are not present in this family. Thus, this SPG form seems to be a pure form of HSP.

ZFYVE27 is located at 10q24.2. In the vicinity of this locus, two SPG loci were previously mapped—namely, *SPG9* (MIM 601162), which is located between 10q23.3 and 10q24.2,²⁸ and *SPG27* (MIM 609041), which is mapped within 10q22.1-q24.1.²⁹ *ZFYVE27* could be excluded as a candidate gene for *SPG27* subtype, since *ZFYVE27* is located distal to the critical region of the locus. Recent updated mapping of the *SPG9* locus (M. Seri, personal communication) also excluded *ZFYVE27* from the refined region.

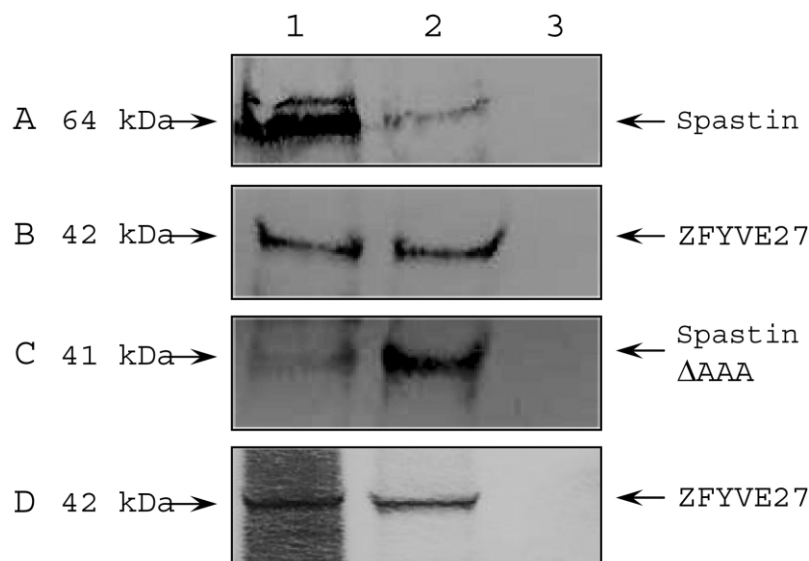


Figure 3. In vivo interaction between spastin and *ZFYVE27* in mammalian cells. NIH 3T3 cells were cotransfected with c-Myc-spastin and E2-*ZFYVE27* constructs. Soluble protein lysate was prepared after 24–48 h of transfection. *A*, Immunoprecipitation performed with E2-tag antibody, and, subsequently, Western blot performed with c-Myc antibody. *Lane 1*, standard protein lysate, which serves as a control for transfection efficiency; *lane 2*, immunoprecipitated fraction; *lane 3*, mock precipitation done without E2-tag antibody. A 64-kDa band corresponding to c-Myc-spastin can be seen in lane 1 and lane 2 but not in lane 3. *B*, Precipitation done with c-Myc antibody and the immunoblot done with E2 antibody. A 42-kDa band corresponding to E2-*ZFYVE27* can be detected in protein standard (*lane 1*) and precipitated fraction (*lane 2*) but not in mock experiment (*lane 3*). *C*, Precipitation performed with cells cotransfected with c-Myc-spastin Δ AAA, E2-*ZFYVE27* with E2 antibody, and the Western blot with c-Myc antibody. A c-Myc-spastin Δ AAA protein can be detected as a 41-kDa band in the precipitated fraction (*lane 1*) as well as in the standard protein fraction (*lane 2*) but not in the mock precipitate (*lane 3*). *D*, Immunoprecipitation assay between endogenous spastin and E2-*ZFYVE27*, with the use of antispastin antibody. *Lane 1*, immunoprecipitated fraction; *lane 2*, protein standard; *lane 3*, mock precipitation. The endogenous spastin was able to coprecipitate E2-*ZFYVE27*, since, in the immunoblot, a 42-kDa band corresponding to E2-*ZFYVE27* could be detected in both lanes 1 and 2 with the use of E2 antibody.

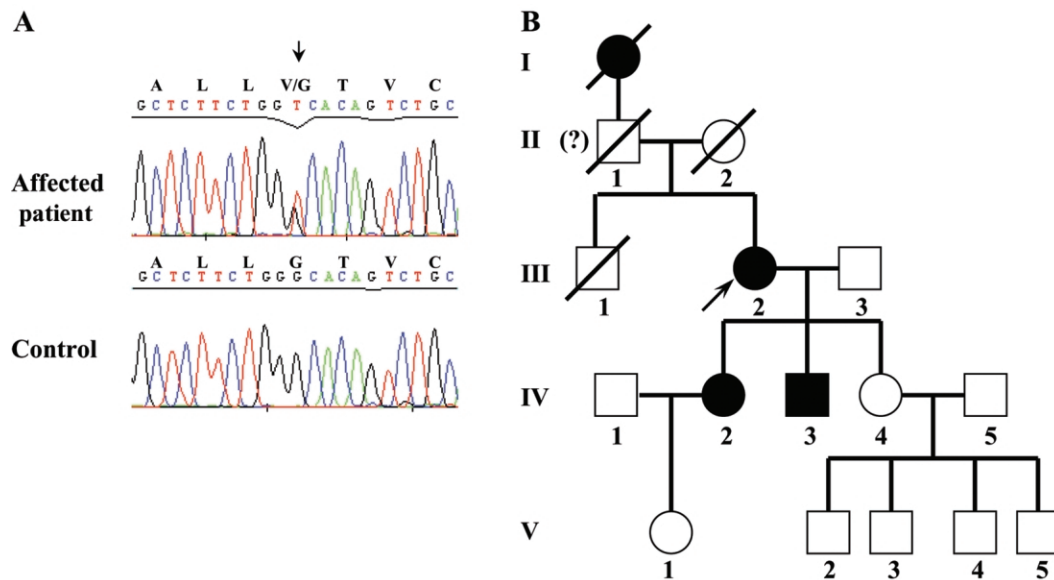


Figure 4. Identification of a mutation in the *ZFYVE27* gene in a family with AD-HSP. *A*, Forward sequence of a PCR product of exon 6 of the *ZFYVE27* gene in genomic DNA from an affected patient and a control. The mutated nucleotide is indicated by a black arrow; the identified mutation G→T leads to the amino acid substitution G191V in *ZFYVE27*. *B*, Pedigree of a five-generation German family with AD-HSP, showing segregation of G191V in affected family members. We could not assert whether individual II.1 was symptomatic, because of limited family history.

Therefore, the *ZFYVE27* locus should be considered a new subtype of HSP, which we termed “SPG33.”

The identified mutation G191V of *ZFYVE27* isoform-a (position G105V of isoform-c) is located near the vicinity of the third transmembrane (TM) motif. *In silico* topology prediction, with the use of SOSUI software, revealed that the G105V mutation shifts the TM motif forward by three amino acids (fig. 5A), which could have a conformational effect in the three-dimensional structure of *ZFYVE27*, particularly on the FYVE-finger domain. Phylogenetic analysis revealed that the G105 residue of *ZFYVE27*-c is conserved in most vertebrates (fig. 5B). To study the functional implication of the G105V exchange mutation on the *ZFYVE27* protein, we introduced this mutation in the GFP-*ZFYVE27* construct and performed *in vivo* immunoprecipitation assay. We used c-Myc antibody to precipitate spastin from the protein lysate derived from cells cotransfected with c-Myc-spastin and GFP-*ZFYVE27*(G105V). Immunoblot analysis with GFP antibody detected either no band (fig. 6A, panel *i*) or, by sample overloading, a very weak band (fig. 6A, panel *ii*) equivalent to protein size of GFP-*ZFYVE27*(G105V). The *ZFYVE27* interacts with spastin through its C-terminal domain, and the G105V mutation could lead to misfolding of the C-termini of the FYVE-finger domain, which could be consequential to this interaction.

Next, we investigated the effect of this mutation on the intracellular distribution of *ZFYVE27* protein. Analysis of the transfected cells by fluorescence microscopy indicated that, in addition to diffuse vesicular expression (fig. 6B),

a significant proportion of cells showed expression in tubular structures or in plasma membrane (fig. 6C–6D). Examination of cells coexpressing GFP-*ZFYVE27*(G105V) and c-Myc-spastin by immunofluorescence microscopy revealed weak and diffuse colocalization in a subset of vesicles in the cytoplasm (fig. 6E–6G). The observation that a significant proportion of cells showed a tubular expression, presumably in the ER network, suggests that the G105V mutation might hinder or delay the biogenesis process of *ZFYVE27*.

Our genetic and biochemical studies strongly suggest that the G105V mutation in *ZFYVE27* is responsible for the pathological phenotype observed in this family with AD-HSP. It is unclear whether this mutation results in a loss-of-function or a dominant-negative form of *ZFYVE27* that is due to misfolding of the C-terminal FYVE-finger domain. Defects in the spastin-dependent/independent endosomal transport may influence the retrograde transmission and, therefore, contribute to a slow accumulation of pathological processes/substrates in HSP. Identification of *ZFYVE27* as a mutated gene in HSP, therefore, will aid

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Figure 5. *In silico* analysis of the *ZFYVE27* mutation. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

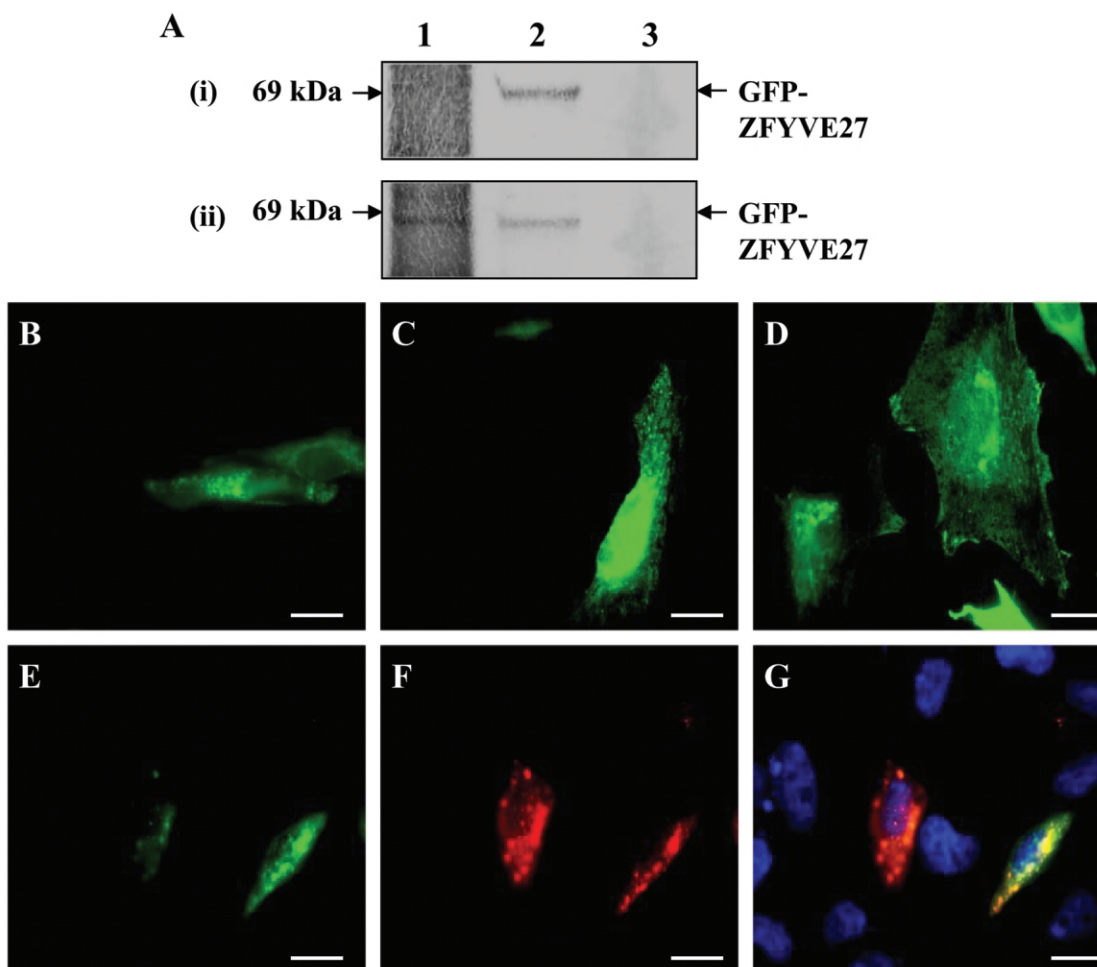


Figure 6. Functional analysis of the ZFYVE27 mutation. *A*, Immunoprecipitation assay between c-Myc-spastin and GFP-ZFYVE27(G105V), with the use of anti-Myc antibody and detection with anti-GFP antibody. *Lane 1*, Immunoprecipitated fraction; *lane 2*, protein standard; *lane 3*, mock precipitation. No band (*i*) or a very weak band (*ii*) corresponding to GFP-ZFYVE27 was detected in the precipitated fraction. *B–G*, Subcellular localization of GFP-ZFYVE27(G105V) and coexpression with c-Myc-spastin in HeLa cells. *B*, Transiently transfected HeLa cells showing a diffuse vesicular expression. However, a significant proportion of cells showed expression in tubular structures arising from the perinuclear region. *C*, GFP-ZFYVE27(G105V) localized to tubular structure in the cytoplasm. *D*, GFP-ZFYVE27(G105V) showing expression also in the plasma membrane. *E–G*, Coexpression of c-Myc-spastin and GFP-ZFYVE27(G105V) in HeLa cells showing a diffuse overlapping pattern of expression. *E*, GFP-ZFYVE27(G105V); *F*, c-Myc-spastin; *G*, superimposition of images E and F. Scale bar is 20 μm (*B–G*).

in the understanding of the axonal neurodegeneration in HSP and suggests that other molecules involved in this process may play a role in the pathogenesis of this group of diseases. Moreover, by elucidating the molecular mechanism and by studying the interacting partner(s) for known HSP proteins, novel genes involved in HSP pathogenesis might be identified. More insight into the pathological mechanisms will help to create new therapeutical options for patients with HSP in the future.

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Web Resources

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

Ensembl, <http://www.ensembl.org>

ClustalW, <http://www.ebi.ac.uk/clustalw>

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for spastin-lacking exon 4 [accession number NM_199436], ZFYVE27-ciso-

form [accession number AK097945], and *ZFYVE27-a* isoform [accession number NM_001002261])
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for spastin, *SPG9*, and *SPG27*)
SOSUI, <http://www.expasy.ch>

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