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Role of Spastin and Protrudin in Neurite Outgrowth

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ABSTRACT

Hereditary spastic paraplegia (HSP) is a neurodegenerative disorder characterized by retrograde axonal degeneration that primarily affects long spinal neurons. The gene encoding spastin has a well-established association with HSP, and protrudin is a known binding partner of spastin. Here, we demonstrate that the *N*-terminal domain of protrudin mediates the interaction with spastin, which is responsible for neurite outgrowth. We show that spastin promotes protrudin-dependent neurite outgrowth in PC12 cells. To further confirm these physiological functions in vivo, we microinjected zebrafish embryos with various protrudin/spastin mRNA and morpholinos. The results suggest that the spinal cord motor neuron axon outgrowth of zebrafish is regulated by the interaction between spastin and protrudin. In addition, the putative HSP-associated protrudinG191V mutation was shown to alter the subcellular distribution and impair the yolk sac extension of zebrafish, but without significant defects in neurite outgrowth both in PC12 cells and zebrafish. Taken together, our findings indicate that protrudin interacts with spastin and induces axon formation through its *N*-terminal domain. Moreover, protrudin and spastin may work together to play an indispensable role in motor axon outgrowth. J. Cell. Biochem. 113: 2296–2307, 2012.

KEY WORDS: PROTRUDIN; SPASTIN; PROTEIN INTERACTION; NEURITE OUTGROWTH

Hereditary spastic paraplegia (HSP) is characterized clinically by spastic gait, mild vibratory sensory impairment, and urinary urgency and pathologically by a retrograde axonopathy and fasciculus gracilis [Hedera et al., 2002; Hattori et al., 2010]. HSP is one of the most genetically heterogeneous diseases, caused by mutations in at least 31 different genes. This means that >0.1% of the human genome can be mutated and result in one predominant neurological outcome: The degeneration of upper-motor-neuron axons [Depienne et al., 2007; Salinas et al., 2008; Stevanin et al., 2008; Blackstone et al., 2011].

The *SPG4* gene is most frequently associated with HSP, with mutations found in ~40% of autosomal dominant pure HSP families [Hazan et al., 1999; Sauter et al., 2002; Proukakis et al., 2011]. *SPG4* encodes spastin, an ATPase belonging to the ATPases Associated with diverse cellular Activity (AAA) family [Hazan et al., 1999; Lumb et al., 2012]. Spastin is involved in microtubule (MT)-severing [Errico et al., 2002; Evans et al., 2005; Roll-Mecak and Vale, 2005; Salinas et al., 2005;], a process by which long MTs can be fractured into shorter tracks. MT-severing plays an important role during meiosis and mitosis to release MTs from the centrosome and is thought to be essential in neurons, where the capacity of a MT to move is strictly related to its length [Baas et al., 2006]. Recent studies identified an important role of spastin in the formation of axonal branches [Yu et al., 2008; Butler et al., 2010]. It is therefore an attractive hypothesis that HSP occurs due to *SPG4* mutations arising through disturbances of the MT dynamics in the long central motor axons. Although previous reports suggested that spastin is principally involved in MT dynamics and severing [Errico et al., 2002; Trotta et al., 2004; Evans et al., 2005; Lacroix et al., 2010], a comprehensive function for spastin has not yet been elucidated. The expression of spastin in punctate vesicles in cultured cells also suggests a role in vesicular trafficking [Reid et al., 2005; Sanderson

Abbreviations: HA, hemagglutinin; HSP, hereditary spastic paraplegia; MT, microtubule; MO, Morpholino; RNAi, RNA interference; siRNA, short interfering RNA.

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et al., 2006]. Furthermore, the *N*-terminal portion of spastin contains a MT-interacting and trafficking (MIT) domain, which is predicted to play a role in the endosomal trafficking process [Ciccarelli et al., 2003]. M1 spastin interacts with the SPG3A protein atlastin-1 and the SPG31 protein REEP1, playing an important role in ER network formation [Park et al., 2010].

Protrudin is a known binding partner of M87 spastin, although the significance of this interaction to biological and disease-related processes is not known [Mannan et al., 2006; Martignoni et al., 2008]. Protrudin belongs to the FYVE-finger family. The majority of FYVE-finger proteins serve as regulators of endocytic membrane trafficking [Stenmark and Aasland, 1999; Stenmark et al., 2002]. Protrudin has been identified as a key regulator of the Rab 11-dependent membrane trafficking during neurite extension [Shirane and Nakayama, 2006; Matsuzaki et al., 2011]. The fact that protrudin is a putative endosomal protein, and its activity is shown to be essential for neurite outgrowth in neuronal cells [Shirane and Nakayama, 2006; Shirane et al., 2008; Saita et al., 2009], combined with the finding of spastin interaction with endosomal protein CHMP1B [Reid et al., 2005], prompted us to investigate the interaction of protrudin with spastin and its physiological relevance to axon outgrowth.

Given the functional similarity and interaction between spastin and protrudin, and the apparent requirement for protrudin function in the motor axon, in this study we tested the contribution of various domains, as well as the previously identified point mutation p.G191V, in protrudin to the interaction with spastin and function on axonal extension. By use of RNAi in vitro and morpholinos in a zebrafish model, we also investigated whether the spastin and protrudin work together in a single cellular pathway to functionally coordinate axonal outgrowth.

MATERIALS AND METHODS

CONSTRUCTION OF PLASMIDS

The cDNAs encoding protrudin and spastin were obtained from a human fetal brain cDNA library. The protrudin cDNA was subcloned into pcDNA3.1 myc/his A (Invitrogen, Carlsbad, CA), pGEX-6p-1 (Amersham Biosciences Corp., NJ), or pEGFP-N1 (Clontech, Palo Alto, CA). Terminal deleted protrudin cDNAs (protruidinΔFYVE or protrudinFYVE) were amplified by PCR using appropriate primers and subcloned into the same vectors as above. The Δexon5 protrudin construct, prepared by recombinant PCR, contained a deletion of the region between nucleotides 453 and 663. To introduce the point mutations V82I and G191V, in vitro mutagenesis was performed using the TaKaRa MutanBEST Kit (TaKaRa Biotech, Co. Ltd., Dalian, China). The spastin cDNA was subcloned into pCMV-HA vector and pET-24a (+) (Invitrogen).

ANTIBODIES AND REAGENTS

The mouse monoclonal antibodies to Myc (9E10) and anti-HA were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies to acetylated α tubulin and β actin were from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC) or rhodamine conjugated goat antibodies to mouse or rabbit immunoglobulin G (IgG) were from Zhongshan Biotech, Ltd. (Beijing, China).

CELL CULTURE AND TRANSFECTION

HEK293A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). HeLa cells were maintained in RPMI 1640 medium (Gibco, Uxbridge, UK) with 10% FBS. Rat pheochromocytoma (PC12) cells were maintained in DMEM high glucose medium (Gibco) with 10% horse serum (Gibco) and 5% FBS (Gibco). PC12 cells were treated with nerve growth factor (NGF, Millipore, Bedford, MA) at 100 ng/ml in DMEM medium supplemented with 1% horse serum. Transfections of HeLa, HEK293A, and PC12 cells with plasmid constructs using Lipofectamine 2000 (Invitrogen) were performed according to the manufacturer's instructions.

IMMUNOPRECIPITATION AND IMMUNOBLOT ANALYSIS

HEK293A cells were transfected with the indicated constructs or empty vector. At 48 h post-transfection, the cells were washed in PBS and then disrupted in lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml leupeptin, 5 mM benzamidine, 1% NP-40). The lysates were clarified by centrifugation, and protrudin-myc was immunoprecipitated from 500 μg of cell lysate for 4 h at 4°C using 5 μg of anti-myc antibody. The immunoprecipitates were analyzed by 12% SDS–PAGE and western blotting. Blots were probed with anti-myc (1:5,000) or anti-HA (1:2,000) antibodies and subsequently probed with secondary goat anti–rabbit or rabbit anti–mouse IgG antibodies conjugated with horseradish peroxidase (Zhongshan Biotech) followed by enhanced chemiluminescence (ECL) detection.

GST PULL-DOWN ASSAY

Recombinant spastin-His was mixed with GST, GST-protrudin, GST-protrudinV82I, GST-protrudinG191V, GST-protrudinΔexon5, GST-protrudinΔFYVE, or GST-protrudinFYVE bound to glutathione–sepharose beads in 0.5 ml of binding buffer [50 mM Tris – HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.3 mM dithiothreitol (DTT), 0.5% NP-40, and protease inhibitor cocktail]. Binding was allowed to occur at 4°C overnight, and the beads were subsequently washed four times with the binding buffer. The bound proteins were eluted in 20 μl of 1× SDS–PAGE sample buffer and analyzed by immunoblotting.

CELL IMMUNOFLUORESCENCE

Cells were grown on glass coverslips (poly-L-Lysine coated for PC12 cells), fixed with 4% paraformaldehyde in PBS for 15 min. Cells were permeabilized in PBS 0.5% Triton X-100 for 10 min and blocked with 3% bovine serum albumin (BSA) in PBS for 60 min. The cells were then incubated with primary antibodies for 3 h, followed by FITC or rhodamine-conjugated secondary antibodies for an additional 40 min. The nuclei were counterstained with 4,6-diamidino-2-phenyl-indole (DAPI, Sigma). Images were obtained with a confocal laser-scanning microscope (Zeiss LSM 510 META, Germany).

GENE SILENCING

Small interfering RNAs (siRNAs) were synthesized by Invitrogen with the following sequences:

Rat protrudin siRNA: 5′-GCUUCUUGAUCCGACUGGAAGUU-3′ [Shirane and Nakayama, 2006];
Rat spastin siRNA 1#: 5′-GCUGUGGAAUGGUAUAAGAUU-3′;
Rat spastin siRNA 2#: 5′-GGAAUCGCGGUUAUAGUUAUU-3′;
Rat spastin siRNA 3#: 5′-CGAAUUUGGUUAUGGCUAAUU-3′;
Non-silencing siRNA: 5′-UUCUCCGAACGUGUCACGUUU-3′.

RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA), and SYBR real-time RT-PCR for rat protrudin and spastin and an endogenous control gene (actin) was then carried out in triplicate using the SYBR PrimeScript real-time reverse-transcriptase PCR kit (Takara, Ohtsu, Japan). Relative RNA concentrations in the mock- and siRNA-transfected samples were calculated using the comparative C_T method, according to the manufacturer's instructions (Applied Biosystems, real-time PCR system, Carlsbad, CA).

FISH STRAIN AND MAINTENANCE

The zebrafish wild-type strain (AB) was used in this study. Zebrafish embryos were collected and raised at 28.5°C according to standard procedures [Nusslein-Volhard, 2002] and staged in hours or days post fertilization (hpf or dpf) according to standard criteria [Kimmel et al., 1995].

INJECTIONS OF MORPHOLINOS, PLASMIDS, AND mRNA INTO ZEBRAFISH EMBRYOS

Zebrafish stocks and embryo cultures were maintained as described previously [Nadauld et al., 2004]. Morpholinos and plasmids were injected at the one-cell stage. Morpholino oligonucleotides were ordered from Gene Tools LLC with the following sequences: Spastinexon7: 5′-GATGTGAAAACAGACCTCTGGACGT-3′, protrudinexon3: 5′-CAAAACACAGGAGTCTCACCTTCAG-3′. The spastinexon7 morpholino was described earlier [Wood et al., 2006]. An irrelevant morpholino (CoMO: 5′-CCTCTTTACCTCAGTTACAATTTATA-3′) was used as control morpholino.

The human protrudin gene was subcloned into pEGFP-N1, and then the resulting plasmid pEGFPN1-protrudin was linearized (ApaI I) and purified using QIAquick PCR Purification Kit (Qiagen) prior to injection. One-cell zebrafish embryos were microinjected with morpholinos (10 ng/embyo) or plasmids (0.1 ng/embryo). Embryos were maintained at 28.5°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM MgSO4, 0.01% methylene blue) and dechorionated with fine forceps prior to fixation. The 5′ capped mRNA was synthesized from siRNA-resistant constructs pcDNA3.1-human protrudin-myc and pcDNA3.1-human spastin-HA linearized by digestion with *Sma*I, using mMESSAGE mMACHINE T7 kit (Ambion Inc., Austin, TX). Approximately 100 pg of RNA purified with the RNeasy Mini kit (Qiagen) was injected into the one-cell stage embryos for the rescue experiment.

RT-PCR

Total RNA was isolated from zebrafish embryos at 48 hpf using Trizol (Invitrogen). A cDNA library was prepared using PrimeScript™ RT-PCR Kit (Takara). The region of spastin spanning exons 5–9 (nt 741–1206) was amplified using the following primers: 5′-GGCCCAAAAATCCTCCCAAATCT-3′ and 5′-GTGGCAGCGCTGATGTTGAAGAAA-3′. The region of protrudin spanning exons 1–5 (nt 101–650) was amplified using the following primers: 5′-

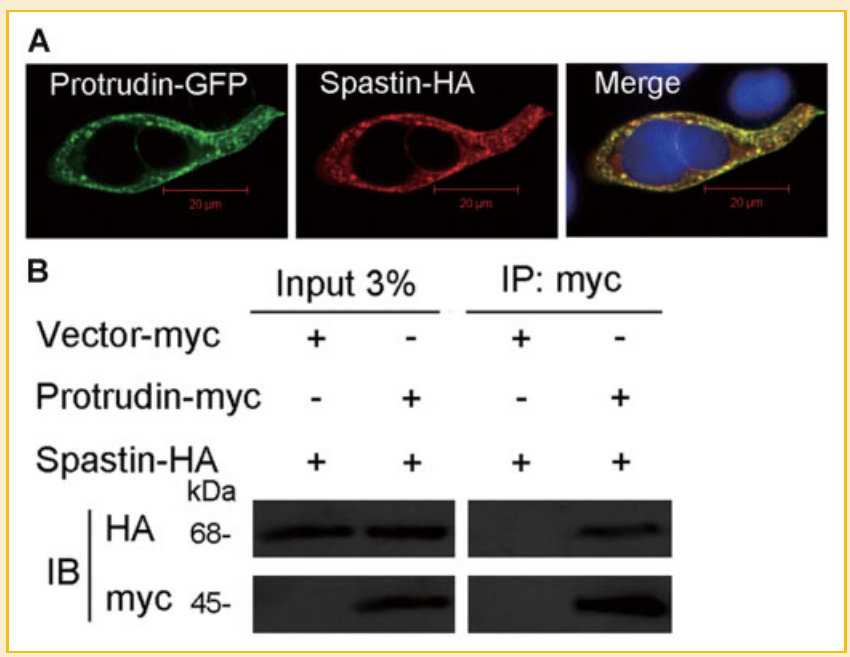


Fig. 1. Protrudin interacts with spastin. A: Co-expression of protrudin-GFP and spastin-HA in HeLa cells. A subset of cytoplasmic vesicles showed co-localization of the two proteins, as observed in the superimposed image. The nuclei of the cells were stained with DAPI. The scale bar equals 20 μm. B: In vivo interaction between protrudin-myc and spastin-HA in HEK293A cells. Immunoprecipitation was performed with a myc antibody, and western blot analysis was performed with an HA antibody. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

GCAGAAGACGGTGTTTTGAGGATT-3′ and 5′-GCTTGGGTCAA-CAGGTCATCTATC-3′.

IMMUNOSTAINING OF ZEBRAFISH EMBRYOS

Whole-mount immunostaining was carried out using standard protocols as previously described [Westerfield, 2000]. The embryos (48 hpf) were incubated in the blocking solution plus anti-znp-1 antibody (1:100 from Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA), or acetylated tubulin monoclonal antibody (1:500, Sigma). The anti-znp-1 antibody was used to detect the cell bodies and axons of primary motor neurons. The anti-acetylated tubulin antibody was used to observe MT integrity [Melancon et al., 1997].

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS16 (SPSS Inc.,Chicago, IL). Fisher's exact test was performed to determine significant differences in embryo motor axons development between experimental groups. One-way ANOVA was used for analysis of PC12 cell extension. P-values < 0.05 were considered statistically significant.

RESULTS

INTRACELLULAR DISTRIBUTION OF GFP-TAGGED PROTRUDIN AND CONFIRMATION OF THE INTERACTION BETWEEN PROTRUDIN AND SPASTIN

The intracellular distribution of protrudin was examined by transfection of the green fluorescent protein (GFP)–protrudin fusion construct into HeLa cells. The results showed that protrudin was predominantly expressed in punctuate vesicles. Examination of HeLa cells co-expressing protrudin–GFP and spastin–HA by immunofluorescence microscopy revealed an obvious co-localization of these proteins in vesicular-like structures in the cytoplasm (Fig. 1A). To investigate the interaction of protrudin and spastin, we performed co-immunoprecipitation studies by co-transfecting HEK293A cells with protrudin-myc and spastin-HA constructs. When protrudin-myc was immunoprecipitated from the total protein lysate of transfected cells, spastin-HA could also be detected in the precipitated fraction (Fig. 1B). These findings are consistent with previously published work [Mannan et al., 2006].

N-TERMINAL REGION OF PROTRUDIN MEDIATES PROTEIN INTERACTION WITH SPASTIN

To determine which domain of the protrudin molecule is responsible for the interaction with spastin, constructs expressing various tagged full-length and deletion mutants of protrudin were generated for analysis in immunofluorescence co-localization, GST pull-down and co-immunoprecipitation assays. We constructed the GFP-, myc-, or GST-tagged expression vectors of full-length protrudin (WT), protrudin with a C-terminal deletion removing the whole FYVE domain (protrudinΔFYVE), protrudin with an *N*-terminal deletion removing the first 300 amino acids (protrudinFYVE), and protrudin with the exon 5 deletion (protrudinΔexon5) removing amino acids 185–220 (Fig. 2A). When these GFP-tagged plasmids were transiently co-expressed with spastin-HA in HeLa cells, the full-length protrudin or protrudinΔFYVE strongly co-localized with spastin in the cytoplasm (Fig. 2B). However, the protrudin mutants with the *N*-terminal or exon 5 deletion did not co-localize with spastin (Fig. 2B). In GST pull-down and co-immunoprecipitation studies with these full-length or deletion protrudin constructs, spastin was shown to interact with protrudinΔFYVE but not with protrudinΔexon5 (Fig. 2D,E), indicating that protrudin mediates interaction with spastin through its *N*-terminal and not the C-terminal FYVE domain. The results of these GST pull-down and co-immunoprecipitation assays were consistent with the co-localization experiments.

The p.G191V mutation of protrudin protein was found in a German family with autosomal-dominant HSP [Mannan et al., 2006]. However, association of the mutation with disease is still in question. Therefore, we wanted to investigate whether the p.G191V mutation of protrudin affects its function and the interaction with spastin, in comparison with WT protrudin and the p.V82I missense mutation found with normal crowd as a control. Immunofluorescence experiments showed a slightly different subcellular localization of the p.G191V mutant version of protrudin from that of the WT and the p.V82I mutant (Fig. 2B,C). However, protein interaction of the p.G191V mutant with spastin was not significantly different in the GST pull-down and co-immunopricipitation experiments (Fig. 2D,E).

FUNCTIONAL CHARACTERIZATION OF PROTRUDIN-MEDIATED NEURITE OUTGROWTH OF PC12 CELLS

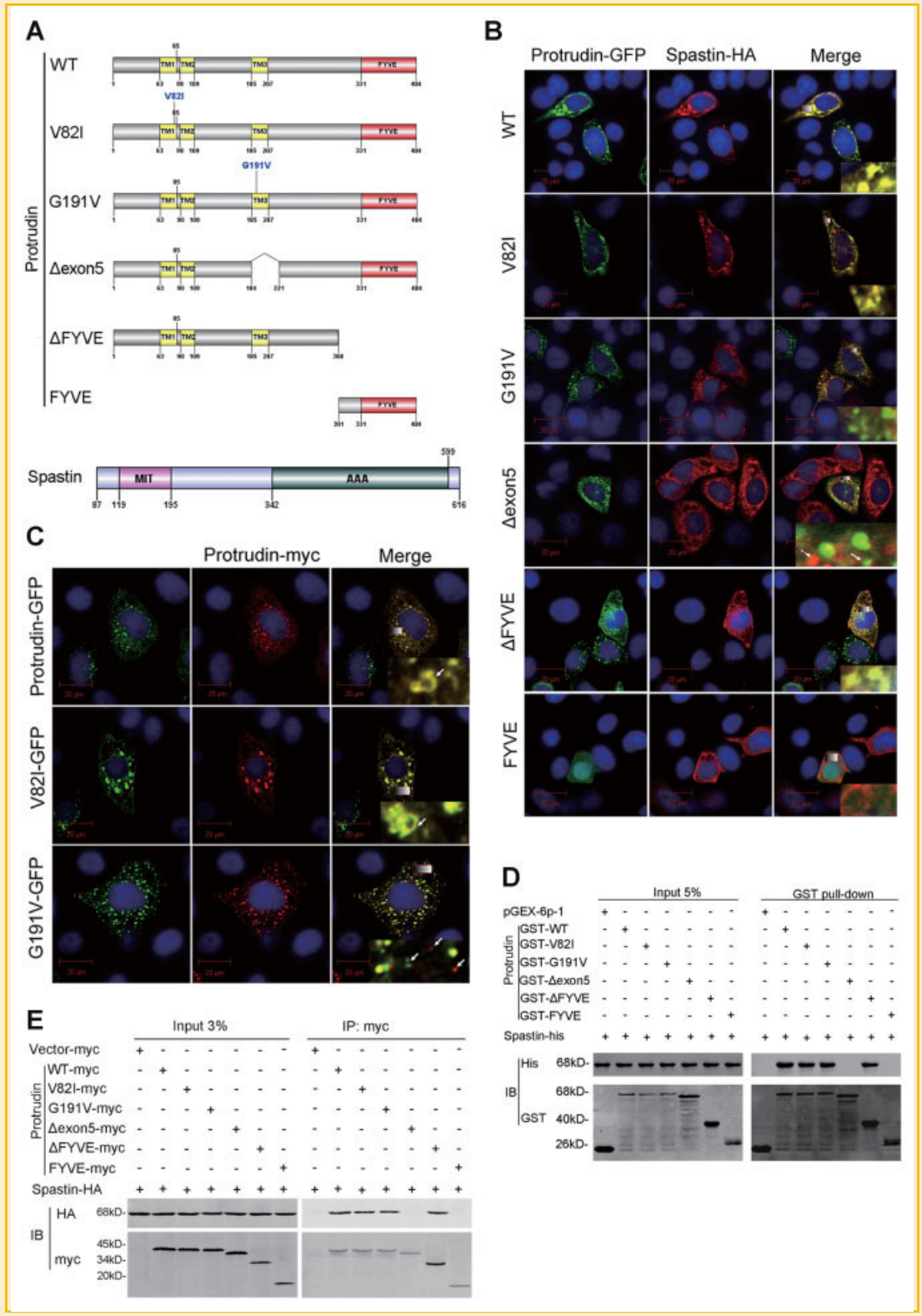
The effect of protrudin-GFP overexpression on neurite outgrowth was observed in PC12 cells, which extends neurites in response to NGF. Six hours after transfection, cells were stimulated with NGF for 40 h, and the proportion of transfected cells with protrusions longer than 20 μm was determined in three different experiments (at least 450 cells were counted per experiment) [Shirane and Nakayama, 2006]. The protrudinΔFYVE-GFP construct still promoted neurite outgrowth but protrudinFYVE-GFP did not, suggesting that the functional domain of protrudin responsible for mediating neurite outgrowth is localized in the *N*-terminal domain and not the FYVE domain. The deletion of exon 5 within the *N*-terminus of protrudin sharply reduced the neurite outgrowth, highlighting the functional importance of this region of the protein (Fig. 3A,B). We also assessed the ability of protrudinG191V and protrudinV82I to mediate outgrowth of PC12 neurites. We found that the ability of the p.G191V protrudin mutant to extend neurite in PC12 cells was slightly decreased, but not significantly different, compared with WT protrudin (Fig. 3A). However, deletion of exon 5 (including the p.G191V HSP mutation) significantly decreased the effect of protrudin on axonal extension (Fig. 3A,B).

To confirm the results above, we next analyzed the effects of depleting endogenous protrudin by RNA interference (RNAi, Fig. 3E) and the potential ability of different protrudin mutants to restore lost function. The results revealed that depletion of protrudin resulted in inhibition of NGF-induced neurite outgrowth, and the rescue effect of protrudinΔFYVE was almost as effective as that of WT protrudin. However, protrudinΔexon5 could only partially rescue the neurite extension, and protrudinFYVE absolutely failed to rescue the neurite outgrowth, which is consistent with results of the

overexpression experiments (Fig. 3C,D). These results indicate that the *N*-terminal domain of protrudin, but not the C-terminal FYVE domain, is necessary for the ability of protein to induce neurite outgrowth.

SPASTIN PROMOTES PROTRUDIN-DEPENDENT NEURITE OUTGROWTH IN PC12 CELLS

We next investigated whether spastin may be necessary for neurite extension of PC12 cells in response to NGF treatment. While



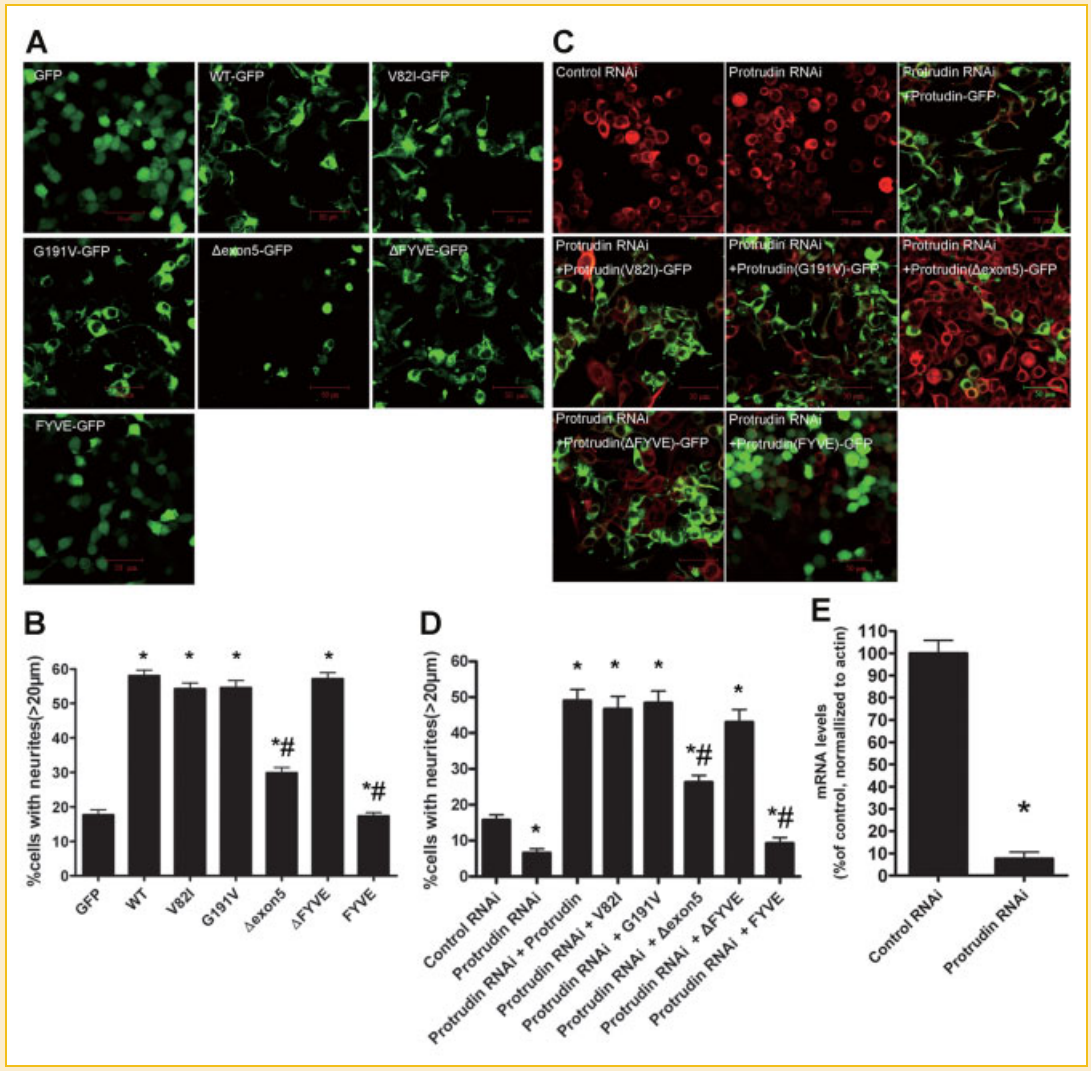


Fig. 3. Enhancement of neurite extension by protrudin and its mutants. A: PC12 cells were transfected with a plasmid expressing GFP only (empty vector) or expressing protrudin, protrudinV82I, protrudinG191V, protrudinΔexon5, or protrudinΔFYVE tagged with GFP at their C-termini. The scale bar equals 50 μm. B: Percentages of cells with neurites longer than 20 μm in different conditions (means of at least three independent experiments ± SEM; at least 450 cells were scored per condition). $^{*}P < 0.01$ (one-way ANOVA) compared with GFP control group; $\#P < 0.01$ (one-way ANOVA) compared with WT protrudin group. C: Ability of human protrudin and its mutants to rescue the inhibition of neurite extension induced by protrudin RNAi. PC12 cells were co-transfected with protrudin siRNA (or control siRNA) and a vector expressing protrudin, protrudinV82I, protrudinG191V, protrudinΔexon5, or protrudinΔFYVE tagged with GFP at their C-termini. The cells were fixed for immunofluorescence staining with acetylated α tubulin antibody (red). The scale bar equals 50 μm. D: Quantitation of neurite formation in cells treated as in (C). Data are means ± SEM of values from three independent experiments. Independent experiments were performed on different days. At least 450 cells were scored per condition. $^{*}P < 0.01$ (one-way ANOVA) compared with control RNAi group; $\#P < 0.01$ (one-way ANOVA) compared with protrudin RNAi + protrudin group. E: Identification of protrudin RNAi effect. After transfection with protrudin siRNA, the total RNA was extracted for real-time RT-PCR. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Fig. 2. The interaction between protrudin and spastin is mediated by the N-terminal region of protrudin. A: Schematic representation of WT and mutant (V82I, G191V, Δexon 5, ΔFYVE, FYVE) forms of human protrudin and the isoform 3 (M87) of human spastin. The p.V82I missense mutation served as the negative control. The p.G191V mutation (putatively associated with HSP in prior studies) is located in exon 5 of the protrudin protein. The FYVE (Fab-1, YOTB, Vac1, and EEA1) domain is a small cysteine rich-Zn^{2+} binding domain of approximately 60 amino acids. Abbreviations: AAA, ATPases Associated with diverse cellular Activity domain; MIT, MT-interacting and trafficking domain; TM, transmembrane domain. B: Subcellular localization of protrudin mutants in HeLa cells. HeLa cells expressing spastin-HA and GFP-tagged protrudin or the indicated mutants were fixed and processed for immunofluorescence staining with anti-HA (red). Merged images are also shown. The scale bar equals 20 μm. C: WT and mutant protrudin formed complexes in transfected cells. Either protrudin (V82I)-GFP or protrudin (G191V)-GFP was co-transfected with WT protrudin-myc in HeLa cells. Both mutated proteins were visible as green signals. Nuclei were stained with DAPI (blue). WT protrudin was detected using a monoclonal anti-myc antibody (red). Arrows indicate the co-localized proteins in merged images. The scale bar equals 20 μm. D: C-terminally His-tagged spastin was expressed in BL21 (DE3) *E. coli.* WT protrudin, protrudinV82I, protrudinG191V, protrudinΔexon5, protrudinΔFYVE, or protrudinFYVE fused at their *N*-termini with GST were also expressed in BL21 (DE3) *E. coli.* Purified His-tagged spastin proteins were mixed with each of the GST-tagged proteins separately. GST pull-down assay was used to detect the in vitro protein–protein interactions. E: WT protrudin, protrudinV82I, protrudinG191V, protrudinΔexon5, protrudinΔFYVE, or protrudinFYVE fused at their C-termini to the myc tag were expressed in HEK293A cells together with HA-tagged spastin. Cell extracts were immunoprecipitated with anti-myc, and the resulting precipitates, as well as a portion of the cell extracts (3% of the input for immunoprecipitation), were subjected to immunoblot analysis with anti-HA or anti-myc. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

overexpression of spastin alone did not affect NGF-induced neurite outgrowth, the co-expression of spastin and protrudin significantly increased the stimulatory effect of protrudin on neurite outgrowth (Fig. 4A,B). Among the three siRNAs tested, the most effective (spastin siRNA 2#) in depletion of spastin mRNA was examined for its effects on NGF-induced neurite outgrowth in PC12 cells (Fig. 4E). We tested the ability of spastin, protrudin, or both proteins together to restore the induction of neurite outgrowth by NGF in PC12 cells depleted of endogenous protrudin and spastin. Whereas protrudin or both protrudin and spastin restored NGF-induced neurite outgrowth to the same extent as shown in the overexpression experiment, spastin alone failed to restore this effect of NGF (Fig. 4C,D). These results thus suggest that spastin promotes protrudin-dependent neurite outgrowth in PC12 cells.

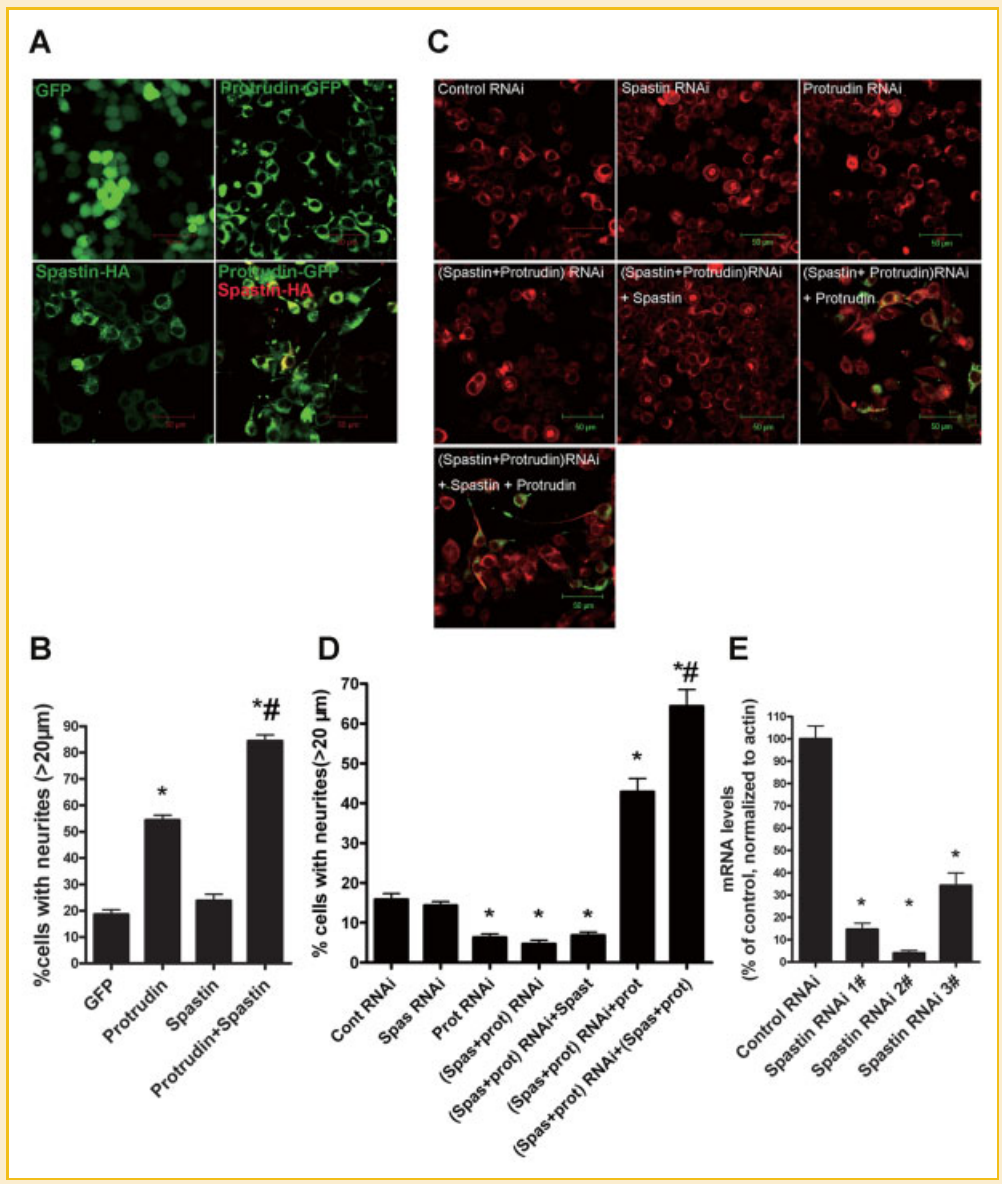


Fig. 4. Spastin promotes protrudin-dependent neurite outgrowth in PC12 cells. A: PC12 cells were transfected with an expression vector for protrudin-GFP, spastin-HA, or both vectors, after which the cells were stimulated with NGF for 40 h. Immunofluorensence analysis was performed with an acetylated α tubulin antibody (red). The scale bar equals 50 μm. B: Quantitation of protrusion formation in cells treated as in (A). The proportion of transfected cells with protrusions (>20 μm) was determined. Data are means $\pm$ SEM of values from three independent experiments, which were performed on different days. At least 450 cells were stored per experiment. $^{*}P < 0.01$ (one-way ANOVA) compared with GFP control group; $\#P < 0.01$ (one-way ANOVA) compared with the protrudin group. C: PC12 cells were transfected with protrudin siRNA, spastin siRNA, or both siRNAs. Human protrudin-GFP, spastin-HA, or both two plasmids were also co-transfected with the above-mentioned siRNAs to identify their recovery effects. The scale bar equals 50 μm. D: Quantitation of protrusion formation in cells treated as in (C). The proportion of transfected cells with protrusions (>20 μm) was determined. Data are means $\pm$ SEM of values from three independent experiments, $^{*}P < 0.01$ (one-way ANOVA) compared with mock RNAi control group; $\#P < 0.01$ (one-way ANOVA) compared with (protrudin + spastin) RNAi + protrudin group. E: Identification of spastin RNAi effects by real-time RT-PCR. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE I. Promotion of Zebrafish Motor Axon Growth by Protrudin Overexpression

	Number of embryos expressing GFP	Embryos showing markedly enhanced motor axon outgrowth[a]
pEGFPN1(control)	15	3
pEGFPN1-protrudin	16	11 ($P<0.05$)
pEGFPN1-protrudinV82I	16	10 ($P<0.05$)
pEGFPN1-protrudinG191V	16	10 ($P<0.05$)
pEGFPN1-protrudinΔexon5	16	6 ($P<0.05$)
pEGFPN1-protrudinΔFYVE	17	11 ($P<0.05$)
pEGFPN1-protrudinFYVE	17	4

[a]Embryos with motor axons 20 μm longer than the controls (Fisher's test).

EFFECT OF PROTRUDIN AND SPASTIN ON MOTOR NEURON AXON OUTGROWTH IN ZEBRAFISH EMBRYOS

In order to determine whether high levels of protrudin could affect growth of spinal motor neuron axons, zebrafish embryos were microinjected at the one-cell stage with the ApaL I lineared pEGFPN1-human protrudin and other mutant plasmids and analyzed by immunostaining with the monoclonal antibody against znp-1 to reveal the overall morphology of differentiating spinal motor neurons. Embryos with motor neurites 20 μm longer than the controls were considered to have enhanced motor axons outgrowth (Table I). Abnormal embryos were defined as those having two or more motor axons exhibiting short or aberrant branches in segments 7–15 (Table II) [Sato-Maeda et al., 2006]. The overexpression of protrudinG191V and protrudinΔexon5 impaired the zebrafish yolk sac extension along with manifestation of a curly-tail phenotype (Fig. 5A). Anti-znp-1 staining revealed that overexpression of WT protrudin, protrudinG191V, protrudinV82I, and protrudinΔFYVE all dramatically promoted outgrowth of motor axons from the spinal cord, with no obvious difference among them (Fig. 5B, Table I). However, overexpression of protrudinΔexon5 and protrudinFYVE did not promote axonal outgrowth (Fig. 5B and Table I). These data confirmed the results found in PC12 cells above.

To validate whether spastin and protrudin work together in promoting motor axon outgrowth, we developed a zebrafish mopholino model. Morpholino oligonucleotide knockdown of spastin or protrudin resulted in an impairment in head and yolk sac extension, along with a curly-tail phenotype, while fish injected with morpholinos specific for both spastin and protrudin showed a more severe phenotype (Fig. 6A). When WT human spastin or protrudin was co-injected with spastin and protrudin double morpholinos, the curly-tail phenotype was partially rescued. However, when both WT human spastin and protrudin were co-injected with spastin and protrudin double morpholinos, the defective phenotype was rescued to levels comparable to that with the control morpholino (Fig. 6A).

Immunofluorescence analysis of anti-znp-1 stained embryos revealed that the motor neurons in the spinal cord did not develop normally and were shorter with abnormal branching in fish injected with spastin morpholino or protrudin morpholino alone (Fig. 6B). Additionally, this phenotype was more severe when both spastin and protrudin morpholinos were injected (Fig. 6B, Table II). When the WT human spastin mRNA, protrudin mRNA, or both were co-injected with double morpholinos of spastin and protrudin, the motor axons were restored at varying degrees, and the double rescue showed better effects (Fig. 6B, Table II). All these findings suggest that spastin and protrudin may work together to play an indispensable role in motor axon outgrowth. RT-PCR was used to confirm that the transcripts were incorrectly spliced with spastin and protrudin morphants (Fig. 6C). Moreover, human spastin and protrudin could compensate for the loss of endogenous zebrafish mRNA, and their expression levels were identified by western blot (Fig. 6D).

TABLE II. Abnormal Motor Axons in Spastin and Protrudin Morphants or Rescued Embryos

	Number of embryos	Aberrant axons[a]
Control morpholino (MO)	27	3
spastin MO	21	15($P<0.05$)
protrudin MO	28	12($P<0.05$)
(spastin + prorudin)MO	23	20($P<0.05$)
(spastin + prorudin)MO + spastin	27	14($P<0.05$)
(spastin + prorudin)MO + protrudin	22	13($P<0.05$)
(spastin + prorudin)MO + spastin + protrudin	24	6

[a]Embryos in which two or more motor axons exhibited short or aberrant branches in segments 7–15 (Fisher's test).

DISCUSSION

Protrudin, as a binding partner of spastin, was found to be mutated in a German family with an autosomal dominant form of HSP (AD-HSP), as reported by Mannan et al. [2006]. Protrudin contains an FYVE domain, which was demonstrated to mediate the association of proteins with phosphatidylinositol 3-phosphate [Takenawa and Itoh, 2006] and was also thought to mediate the interaction with spastin [Mannan et al., 2006]. However, in our study the *N*-terminal domain of protrudin, and not the FYVE domain, was found to mediate the interaction with spastin to regulate neurite outgrowth (Figs. 3 and 4). This finding sheds new light on the molecular function of protrudin, although further study is required to better understand the underlying mechanism.

Protrudins from humans, rats, and zebrafish all have the FYVE homology domain. By the CLUSTAL method using Megalign (Lasergene, DNAATAR, Inc.), the degrees of amino acid homology of protrudin among these speicies were calculated to be 74.3% between rat and human protrudin and 47% between zebrafish and human protrudin. In this study, the rat adrenal PC12 cell line and zebrafish model were selected to examine the role of protrudin and spastin in neurite outgrowth.

Analysis of the function of protrudin in cultured cells reveals that it is an endosomal protein. Studies of its function in the PC12 cells have confirmed that protrudin induces neurite formation by directional membrane trafficking [Shirane and Nakayama, 2006; Saita et al., 2009; Matsuzaki et al., 2011]. In light of the fact that the protrudin-interacting protein spastin regulates axonal growth of zebrafish, we reasoned that protrudin or the protrudin–spastin system may also be important for axonal morphogenesis and

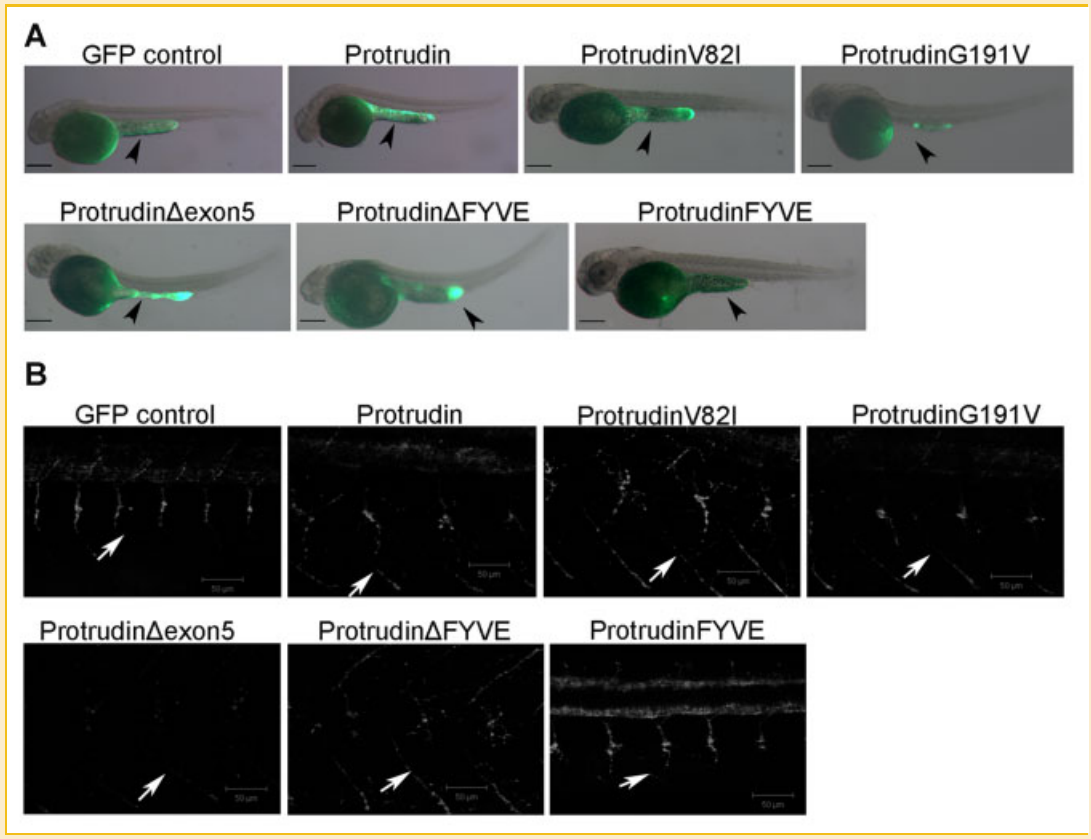


Fig. 5. Overexpression of human protrudin promotes the growth of motor axons in zebrafish. A: ApaLI lineared plasmids pEGFPN1, pEGFPN1-protrudin, pEGFPN1-protrudinV82I, pEGFPN1-protrudinG191V, pEGFPN1-protrudinΔexon5, pEGFPN1-protrudinΔFYVE, and pEGFPN1-protrudinFYVE were microinjected into one-cell stage zebrafish embryos. Arrows indicate yolk sac extensions of zebrafish. Scale bar represents 200 μm. B: At 48 hpf, the embryos were fixed and analyzed by immunostaining with a znp-1 antibody. Arrows indicate the outgrowth of motor axons from the spinal cord. Scale bar represents 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

function in vertebrates. We therefore used morpholino antisense oligonucleotides to knockdown spastin or protrudin function in the developing zebrafish embryo.

Consistent with our hypothesis, reduced protrudin and spastin activity severely impaired outgrowth of spinal and branchiomotor neuronal axons (Fig. 6B, Table II). Protrudin regulates Rab 11-dependent membrane recycling to promote the directional membrane trafficking required for neurite formation [Shirane and Nakayama, 2006]. Moreover, dynamic MTs are required for many aspects of axonal development, such as extension, guidance, growth cone motility, and branching [Kalil et al., 2000; Dent and Gertler, 2003; Gordon-Weeks, 2004]. Our rescue experiments demonstrate that human protrudin and spastin mRNA could restore the defects of early neural development induced by protrudin–spastin double knockdown in zebrafish embryos (Fig. 6B, Table II). Interestingly, the *SPG3A* gene product atlastin, which interacts with spastin [Sanderson et al., 2006], has been shown to promote axon elongation during neuronal development [Zhu et al., 2006], and *SPG3A* mutations are the most common cause of autosomal dominant HSP before 10 years of age [Namekawa et al., 2006]. Taking these studies together with our own findings, axon outgrowth defects can be considered a common feature of HSP, and spastin, atlastin, and protrudin may function in a common pathway to regulate axon outgrowth. Thus, the molecular mechanisms that underlie neurite formation may include both cytoskeletal remodeling and membrane trafficking. However, the mechanism of interaction between protrudin and spastin in regulating neurite outgrowth requires further study.

Mannan et al. [2006] had proposed that the protrudinG191V mutation decreases the strength of interaction with spastin, implying that at the basis of the disease is a failure of protrudin to interact with spastin. However, some of the conclusions of Mannan's group have been called into question by Rugarlin and colleagues. They confirmed the protein–protein interaction but found no difference between interactions of WT and mutated protrudin with spastin. Furthermore, association of the protrudin mutation with disease was doubted since it also appears in the SNP database [Mannan et al., 2006; Martignoni et al., 2008]. At present, the protrudinG191V mutation is not a convincing marker for the disease, and studies from the two groups above disagree about whether this mutation affects the binding properties of protudin. In the present study, the p.G191V mutation also did not significantly affect the protein interaction with spastin in the GST pull-down and co-immunopricipitation assays (Fig. 2D,E). Although protrudinG191V showed a slight change in the subcellular distribution and compromised yolk sac extension of zebrafish (Figs. 2B,C

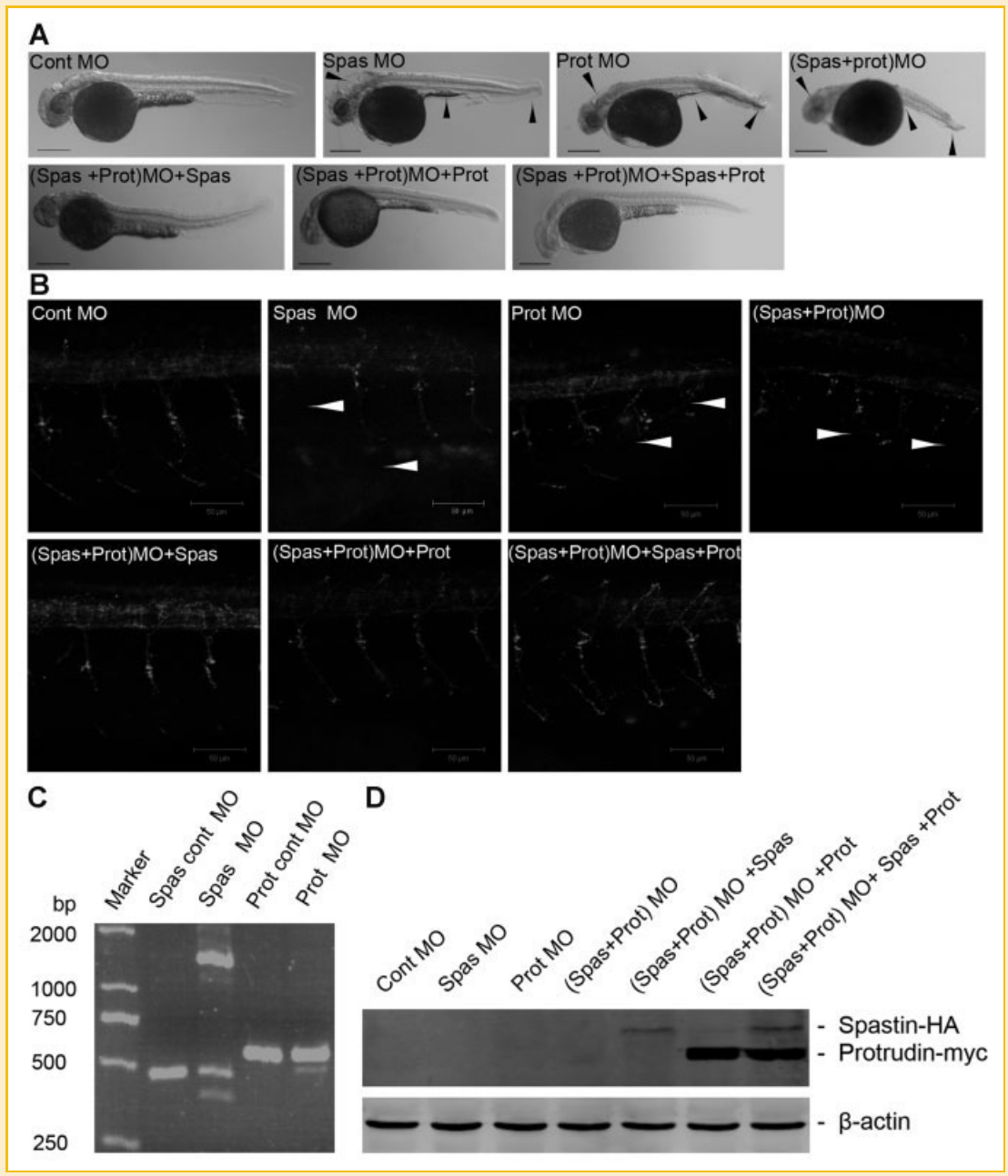


Fig. 6. Effect of the protrudin–spastin interaction on motor neuron axon outgrowth in zebrafish embryos. A: Morphological features of zebrafish at 48 hpf from knockdown and rescue experiments. Arrows indicate the developmental defects in head, yolk sac extension, and tail. Scale bar represents 200 μm. Abbreviations: Cont, Control; MO, Morpholino; Spas, Spastin; Prot, Protrudin. B: Defective motor-neuron axons outgrowth in the reduction of spastin and protrudin. Spinal motor axons were stained with an anti-znp-1 antibody at 48 hpf. Arrows indicate the disturbed outgrowth of motor axons from the spinal cord. Scale bar represents 50 μm. C: RT-PCR analysis suggests that the transcripts were incorrectly spliced with spastin and protrudin morphants. D: Expression of human spastin and protrudin mRNA in rescue experiments. Total proteins were extracted from microinjected embryos, and western blot was used to analyze the expression of spastin-HA and protrudin-myc using anti-HA and anti-myc antibodies in the rescue experiments.

and 5A), we found no obvious difference in the ability of WT and mutant protrudin to extend neurites in PC12 cells (Fig. 4) or to elongate motor neuron axon of zebrafish embryos (Fig. 5B, Table I). Our observations still leaves the real effect of the protrudinG191V mutation in question. The methods to functionally validate this mutation in this study perhaps could not detect weak effects. Thus, more accurate methods for testing genetic effects on neurite extension need to be established.

In summary, our studies at the molecular, cellular, and whole animal levels strongly suggest that the *N*-terminal region of protrudin is the domain responsible for binding with spastin and for promoting neurite formation. In our attempt to understand the role

of protrudin in HSP progression, we found that the protrudinG191V mutation only altered the subcellular distribution and limited the yolk sac extension of zebrafish, but without significant defects in neurite outgrowth both in PC12 cells and zebrafish. Although the real role of the protrudinG191V mutation remains uncertain, our data indicate that protrudin-induced neurite outgrowth is regulated by its binding partner spastin, providing a new insight into the pathomechanism of HSP.

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