

The *Drosophila* Homologue of the Hereditary Spastic Paraplegia Protein, Spastin, Severs and Disassembles Microtubules

Antonina Roll-Mecak and Ronald D. Vale*

The Howard Hughes Medical Institute and
The Department of Cellular and Molecular
Pharmacology

University of California, San Francisco
Genentech Hall, Room N312
600 16th Street
San Francisco, California 94143

Summary

Hereditary spastic paraplegias (HSPs), a group of neurodegenerative disorders characterized by lower-extremity spasticity and weakness, are most commonly caused by mutations in the spastin gene, which encodes a AAA+ ATPase [1, 2] related to the microtubule-severing protein katanin [3]. A *Drosophila* homologue of spastin (D-spastin) was identified recently [4], and D-spastin RNAi-treated or genetic null flies show neurological defects, and protein overexpression decreases the density of cellular microtubules [5, 6]. Elucidating spastin's function and disease mechanism will require a more detailed understanding of its structure and biochemical mechanism. Here, we have investigated the effects of D-spastin, individual D-spastin domains, and D-spastin proteins bearing disease mutations on microtubules in cellular and in vitro assays. We show that D-spastin, like katanin, displays ATPase activity and uses energy from ATP hydrolysis to sever and disassemble microtubules; disease mutations abolish or partially interfere with these activities.

Results and Discussion

Localization and Effects on Microtubules of D-spastin Expressed in *Drosophila* S2 Cells

We first cloned D-spastin from a *Drosophila* Schneider (S2) cell cDNA library, fused GFP to its C terminus, and expressed it in S2 cells in order to establish its subcellular distribution and in vivo activity (Figures 1 and 2). Previous localization studies of mammalian spastin have yielded diverse and often conflicting results, including nuclear localization [7], a mixture of nuclear and cytoplasmic distributions [8–10], and endosomal localization [11]. At low expression levels, we found that D-spastin-GFP was localized to discrete punctate structures that were distributed throughout the cytoplasm, suggestive of membrane vesicle association (Figure 2, first panel). Strikingly, cells expressing even relatively low levels of D-spastin-GFP showed diminished microtubule staining and many small microtubule fragments. Cells expressing higher levels of D-spastin-GFP displayed a nearly complete absence of cytoplasmic microtubules (not shown). These results are consistent with previous studies showing that spastin overexpression results in microtubule disassembly [5,

6, 12–14] and with a recent report showing that spastin is targeted to membrane vesicles [11].

Spastins are composed of three domains: an N-terminal region containing a putative transmembrane-spanning sequence (TM), a microtubule interacting and trafficking (MIT) domain that is well conserved in the spastin family, and an ATP binding AAA domain (AAA) (Figure 1). Because the functions of these domains remain poorly understood, we expressed various truncated D-spastin-GFP constructs in S2 cells (Figure 2). A construct lacking the transmembrane-containing N terminus (Δ TM D-spastin-GFP) was diffusely distributed in the cytoplasm, indicating that the TM domain is required for the vesicular staining pattern observed for the full-length construct. Δ TM D-spastin-GFP was fully active for disassembling cytoplasmic microtubules (Figure 2). In contrast, a construct that lacked the AAA domain (TM + MIT) still localized to punctate structures but did not affect the microtubule cytoskeleton. The MIT or AAA domains alone were diffusely localized and did not disassemble microtubules. Immunoblot analysis confirmed that these GFP fusion constructs expressed intact proteins (see Figure S1 in the Supplemental Data available with this article online). These results indicate that the TM/MIT region is involved in membrane targeting and the MIT/AAA region has activity on the microtubule cytoskeleton.

Recombinant D-spastin Severs and Disassembles Microtubules In Vitro

The cellular expression studies described above and those by others do not prove that spastin is a microtubule disassembly agent because such effects might be indirect or require additional cellular cofactors. We therefore tested whether purified D-spastin is capable of microtubule disassembly in vitro. D-Spastin lacking the TM region was expressed in *E. coli* as a GST fusion protein and was purified to higher than 95% homogeneity (Figure 3A, left panel). We tested the purified D-spastin for ATPase activity and found that it hydrolyzed 1.8 ATP/subunit/s (Figure 3A, right panel), which is similar to the maximal microtubule-stimulated ATPase rate reported for katanin [15]. Thus far, we have not observed a stimulation of the ATPase rate by taxol-stabilized microtubules. When purified D-spastin and ATP were applied to taxol-stabilized, rhodamine-labeled microtubules bound to a glass surface, the microtubules were completely disassembled after 2 min (Figure 3B; Movie S1). At earlier time points, microtubules developed discrete breaks along their length. We also observed severing of non-taxol-stabilized microtubules when D-spastin was added to interphase *Xenopus* extracts containing self-assembled microtubules (not shown). Thus, like katanin [16], D-spastin is capable of severing microtubules. The microtubule-severing reaction required ATP hydrolysis because microtubules remained intact if ATP was omitted from the reaction (Figure 3B, upper panel) or if the nonhydrolyzable ATP analog ATP γ S was added instead of ATP (not shown). Microtubules became resistant to spastin-mediated

*Correspondence: vale@cmp.ucsf.edu

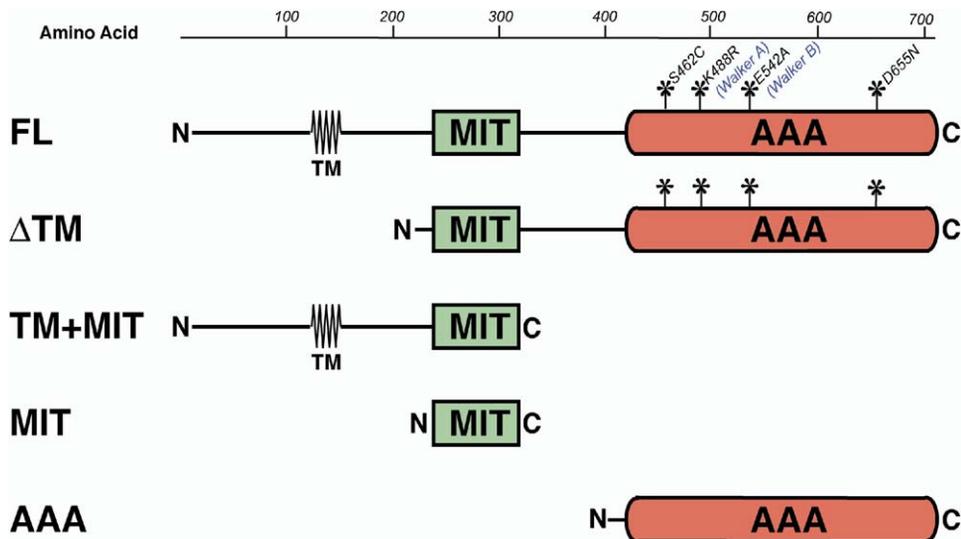


Figure 1. Domain Structure of Spastin

The following abbreviations were used: TM, putative transmembrane region; MIT, microtubule interacting and trafficking domain; and AAA, AAA+ ATPase domain. The positions of disease mutations (S462C, K488R [Walker A motif], E542A [Walker B motif], and D655N) are shown. The precise boundaries of the constructs used in this study are indicated in the [Supplemental Experimental Procedures](#).

severing when the negatively charged C-terminal peptide of tubulin was cleaved by subtilisin digestion ([Figure S2](#)). Subtilisin-treated microtubules are also resistant to severing by katanin [16].

These experiments demonstrate that D-spastin alone can couple ATP hydrolysis to microtubule disassembly, and they confirm our cellular results that the MIT-AAA region is sufficient for this activity. Errico and colleagues [8] reported that the N-terminal TM region in human spastin is crucial for microtubule binding because truncated spastin lacking this region did not cosediment with microtubules. Although it remains possible that the TM region contributes to MT binding affinity, our data show that the N terminus of D-spastin is not required for microtubule binding and severing.

We also attempted to express full-length spastin and the AAA domain in bacteria but found that they were unstable and formed large aggregates. However, the MIT domain could be expressed and purified. This domain did not sever microtubules, and in cosedimentation binding experiments, it bound to microtubules much more weakly ($K_d = 27 \mu\text{M}$) than the ΔTM construct ($K_d < 1 \mu\text{M}$) in the absence of nucleotide. The lack of high-affinity binding is consistent with our finding that the MIT domain expressed in S2 cells did not colocalize with microtubules ([Figure 2](#)). Thus, high-affinity microtubule binding appears to require the combination of the MIT and the AAA domain or at least additional sequence beyond the minimal conserved-MIT domain investigated in this study.

Activity of D-Spastin Mutants That Give Rise to Disease Phenotypes

More than 140 spastin gene mutations have been isolated from HSP patients. Nonsense, frameshift, or splice site mutations are mostly scattered throughout the gene, whereas missense mutations are located almost exclusively in the AAA domain, underscoring the impor-

tance of this ATP binding module for spastin's function. The 28 known missense mutations provide an array of tools with which to probe spastin's function. We decided to examine three such mutations: a critical Walker A residue (K488R) that is predicted to impair ATP binding and mutations situated at the N- (S462C) and C-terminal (D655N) ends of the AAA domain [17] ([Figure 1](#)). On the basis of structure-based sequence alignments, D655 resides in the C-terminal helical subdomain of the AAA module that contributes to nucleotide-dependent conformational changes [18]. In addition to these known disease mutations, we also investigated a mutation in a key Walker B residue (E542A) known to be involved in ATP hydrolysis in other AAA+ ATPases.

When full-length (FL) D-spastin constructs harboring these mutations were expressed in S2 cells, we found that they localized to distinct punctate structures, as seen with wild-type D-spastin. However, for the Walker A (K488R) and Walker B (E542A) mutants, microtubule disassembly was not observed; instead, at moderate to high expression levels, microtubule bundling was observed around the perinuclear region ([Figure 4](#)). Surprisingly, the S462C and D655N mutants still severed microtubules when transfected into cells, although their activity was not as robust as wild-type D-spastin ([Figure 4](#)) because higher levels of expression appear to be needed to disrupt the microtubule network. This result differs from a study showing that expression of the equivalent serine-to-cysteine human disease mutant did not cause microtubule destabilization [11].

In the ΔTM constructs, the Walker A and B site mutants showed very different subcellular localizations. At low expression levels, the ATP-binding-deficient mutant K488R was diffusely distributed, and at high levels it coated and bundled microtubules ([Figure 4](#)). In contrast, the ATP-hydrolysis-deficient mutant E542A colocalized with and bundled only a subset of microtubules, perhaps suggestive of cooperative binding or binding

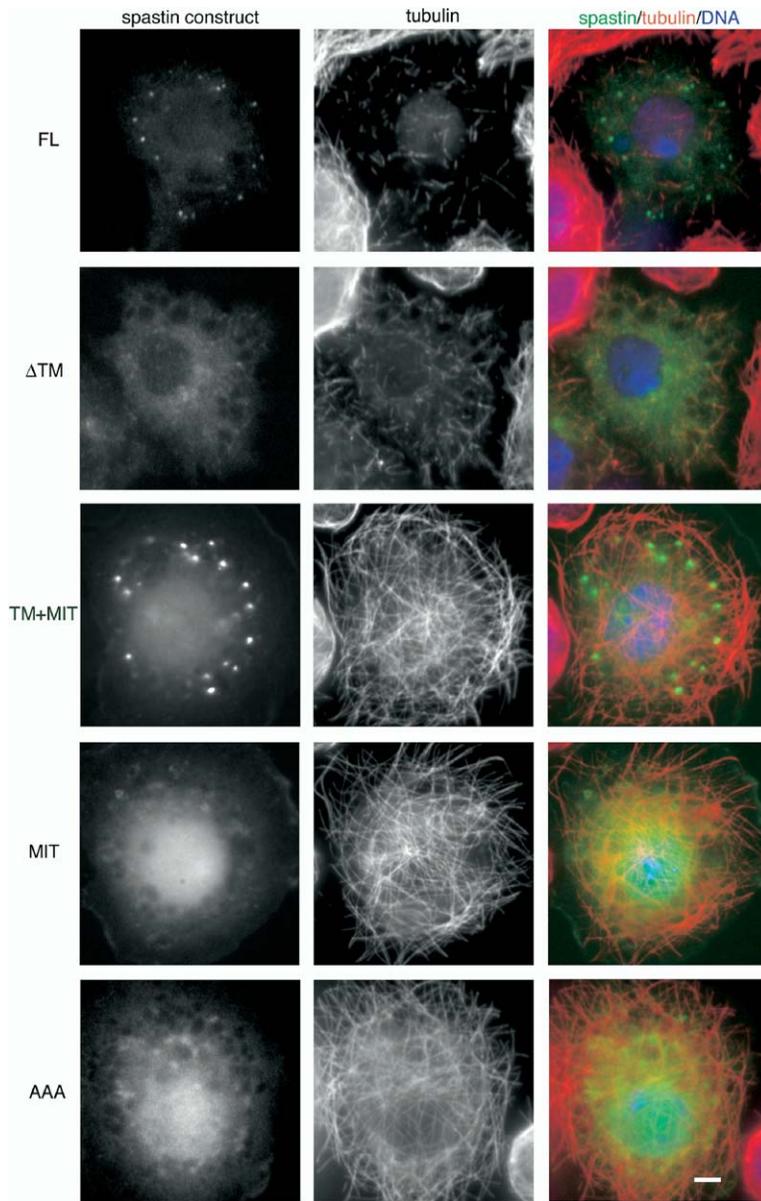


Figure 2. In Vivo Distribution and Microtubule-Destabilizing Activity of D-spastin and Its Domains

Constructs indicated on the left correspond to those shown in Figure 1. Cells were fixed and stained with anti- α tubulin antibodies (red) and Hoechst 33342 (DNA; blue). The distribution of GFP-spastin is shown in the green channel. Microtubule destabilization is evident in the transfected cells expressing full-length (FL) and Δ TM constructs. The scale bar represents 5 μ M.

to a specific subpopulation of microtubules. As noted for the full-length constructs, the expressed Δ TM S462C and D655N mutant proteins also caused partial microtubule disassembly.

We also studied the biochemical activities of purified D-spastin mutant proteins. The Walker A and B mutations, as expected, displayed neither detectable ATPase activity nor in vitro microtubule-severing activity (Figures 3A and 3C). In contrast, the S462C and D655N mutant proteins both showed ATPase activity but were 40% and 80% decreased in maximal activity when compared with the wild-type protein (Figure 3A). Surprisingly, the S462C and D655N mutants displayed a greater impairment in severing taxol-stabilized microtubules than might be expected on the basis of their ATPase activities. Whereas wild-type spastin almost completely disassembled taxol-stabilized microtubules on a surface within 2 min, the S462C mutant protein

generated a comparable degree of disassembly only after >10 min, and the D655N mutant only generated a few microtubule breaks after a 20 min incubation (Figure 3C). These data suggest that the S462C and D655N mutations impair ATPase activity but also produce a defect in the coupling of ATPase activity to microtubule destabilization.

Comparison of Spastin and Katanin

In summary, we show that D-spastin severs and disassembles microtubules both in cells and in vitro. These results provide the first direct biochemical evidence of spastin's ATPase and microtubule-destabilizing activities. It is interesting to compare spastin to katanin, another AAA+ ATPase that severs microtubules. Spastin's AAA domain is highly homologous to katanin, yet these proteins share no sequence similarity in their N-terminal regions. Thus, spastin and katanin appear to have

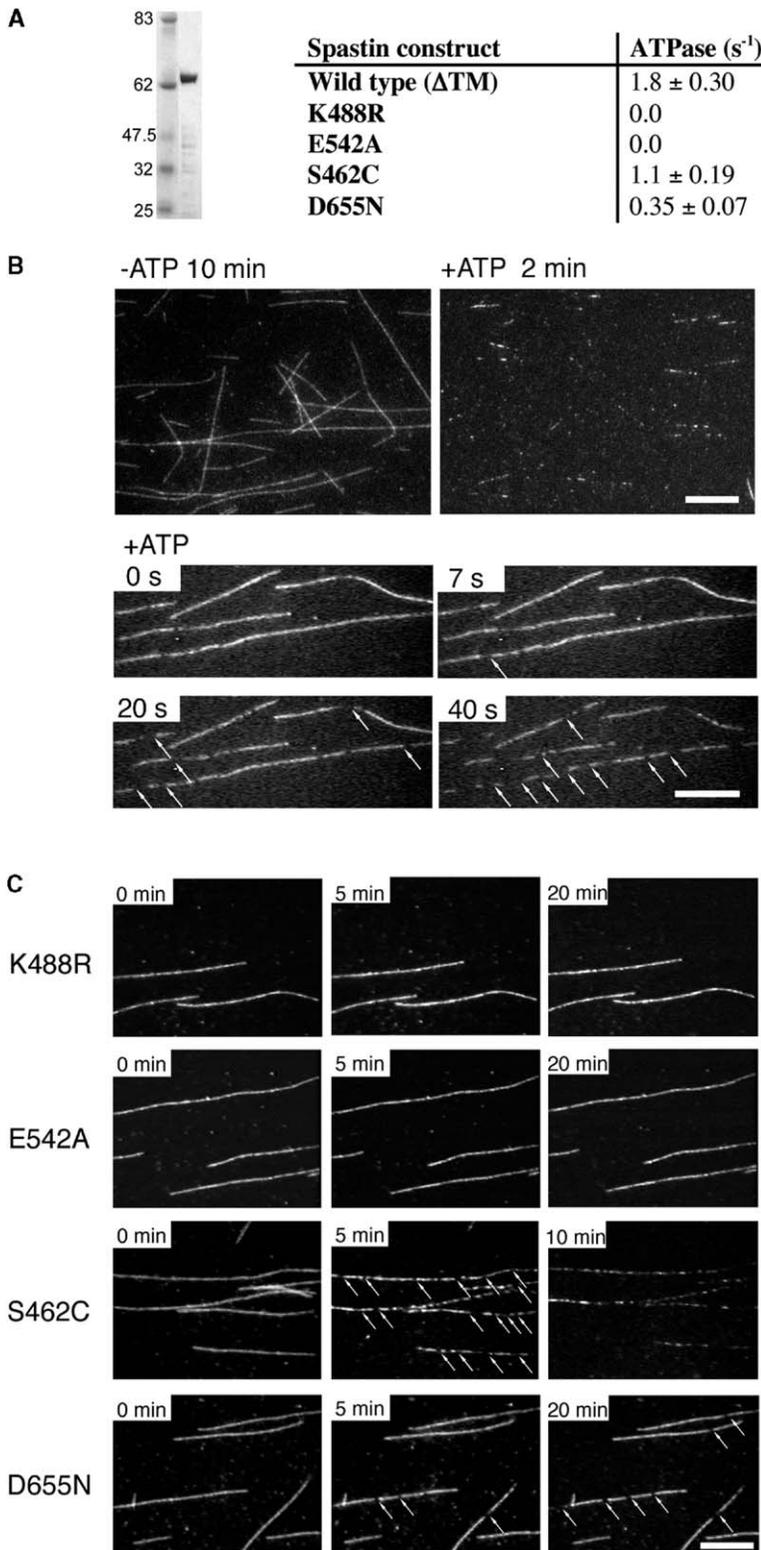


Figure 3. ATPase and Microtubule-Severing Activities of Purified D-spastin In Vitro

(A) Left panel: Coomassie-stained gel of purified GST-D-spastin (Δ TM construct) (left lane, molecular mass standards; right lane, GST-D-spastin). Right panel: ATPase activity of GST-D-spastin and D-spastin mutants (Δ TM construct). ATPase rates (see [Supplemental Experimental Procedures](#)) shown were derived from four measurements (mean and standard deviation).

(B) Severing and disassembly of purified microtubules by D-spastin. Recombinant GST-D-spastin ($0.5 \mu\text{M}$) was introduced to taxol-stabilized, rhodamine-labeled microtubules adsorbed onto the surface of a microscope perfusion chamber in the presence or absence of 1 mM ATP. Arrows indicate breaks in the microtubule.

(C) A similar experiment was performed for GST-D-spastin mutants ($0.5 \mu\text{M}$). The time elapsed after perfusion onto the microtubules is shown. The scale bar represents $5 \mu\text{m}$.

evolved distinct microtubule binding domains that communicate with a similar motor module to bring about the severing reaction. Unlike katanin, which appears to be largely soluble or centrosome associated, spastin appears to associate with vesicular membranes

through its N-terminal domain containing a predicted transmembrane-spanning sequence. This raises the possibility that spastin may be involved in remodeling the microtubule cytoskeleton near membrane surfaces, which may be important for spastin's function in synap-

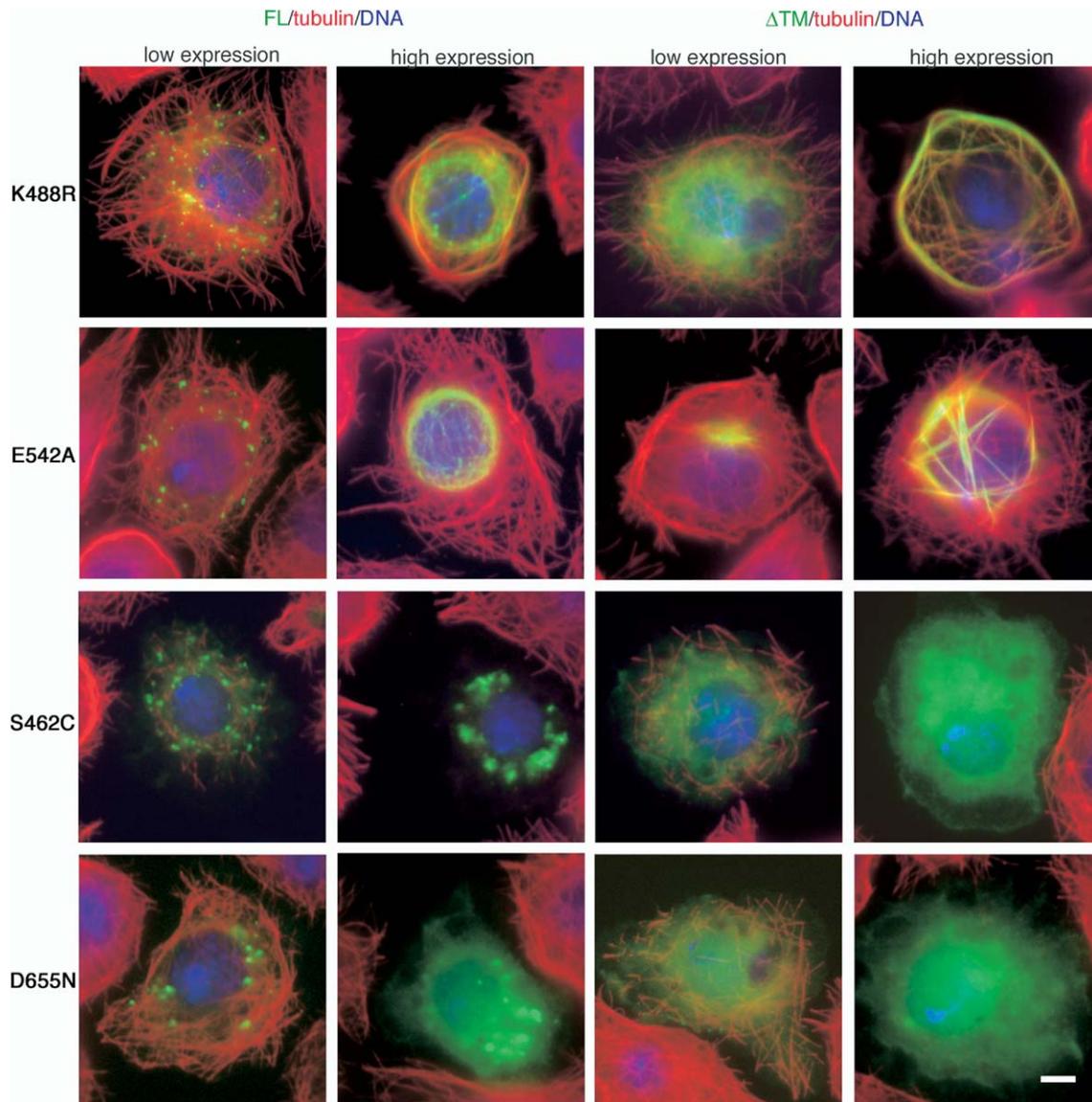


Figure 4. Distribution of D-spastin Mutants in S2 Cells and Effects on Microtubules

S2 cells expressing C-terminally GFP-tagged full-length (FL) D-spastin or D-spastin lacking the TM region (Δ TM) and harboring the indicated mutations. Cells expressing low levels and high levels of these constructs are shown. The S462C and D655N mutants caused microtubule disassembly at higher expression levels. Green, GFP-spastin; red, microtubules; and blue, DNA. Cells were fixed and stained as in Figure 2. The scale bar represents 5 μ M.

tic architecture and transmission [5, 6]. Further investigations linking spastin's enzymatic activity to its cellular function will be required to better understand spastin's role in the nervous system as well as the mechanism by which spastin mutations give rise to the pathology of hereditary spastic paraplegias.

Supplemental Data

Detailed Experimental Procedures, two figures, and a movie are available at <http://www.current-biology.com/cgi/content/full/15/7/650/DC1/>.

Acknowledgments

We are grateful to Nico Stuurman, Adam Douglass, Steve Rogers, Raphael Buencamino, Alex Kelly, Arne Gennerich, and Nick Endres

for technical assistance and helpful suggestions. We thank Julie Hollien for the generous gift of the S2 cDNA library. We thank all members of the Vale and Mullins laboratories and Adrian Ferre-D'Amare for their support and stimulating discussions. A.R.-M. is a Damon Runyon Cancer Research Foundation Postdoctoral Fellow.

Received: December 18, 2004

Revised: January 20, 2005

Accepted: February 3, 2005

Published online: February 17, 2005

References

1. Hazan, J., Fonknechten, N., Mavel, D., Paternotte, C., Samson, D., Artiguenave, F., Davoine, C.S., Cruaud, C., Durr, A., Wincker, P., et al. (1999). Spastin, a new AAA protein, is altered in the

- most frequent form of autosomal dominant spastic paraplegia. *Nat. Genet.* 23, 296–303.
2. Fink, J.K., and Rainier, S. (2004). Hereditary spastic paraplegia: Spastin phenotype and function. *Arch. Neurol.* 61, 830–833.
 3. Frickey, T., and Lupas, A.N. (2004). Phylogenetic analysis of AAA proteins. *J. Struct. Biol.* 146, 2–10.
 4. Kammermeier, L., Spring, J., Stierwald, M., Burgunder, J.M., and Reichert, H. (2003). Identification of the *Drosophila melanogaster* homolog of the human spastin gene. *Dev. Genes Evol.* 213, 412–415.
 5. Trotta, N., Orso, G., Rossetto, M.G., Daga, A., and Broadie, K. (2004). The hereditary spastic paraplegia gene, spastin, regulates microtubule stability to modulate synaptic structure and function. *Curr. Biol.* 14, 1135–1147.
 6. Sherwood, N.T., Sun, Q., Xue, M., Zhang, B., and Zinn, K. (2004). *Drosophila* spastin regulates synaptic microtubule networks and is required for normal motor function. *PLoS Biol.* 2, e429. 10.1371/journal.pbio.0020429
 7. Charvin, D., Cifuentes-Diaz, C., Fonknechten, N., Joshi, V., Hazan, J., Melki, J., and Betuing, S. (2003). Mutations of SPG4 are responsible for a loss of function of spastin, an abundant neuronal protein localized in the nucleus. *Hum. Mol. Genet.* 12, 71–78.
 8. Errico, A., Claudiani, P., D'Addio, M., and Rugarli, E.I. (2004). Spastin interacts with the centrosomal protein NA14, and is enriched in the spindle pole, the midbody and the distal axon. *Hum. Mol. Genet.* 13, 2121–2132.
 9. Beetz, C., Brodhun, M., Moutzouris, K., Kiehnopf, M., Berndt, A., Lehnert, D., Deufel, T., Bastmeyer, M., and Schickel, J. (2004). Identification of nuclear localisation sequences in spastin (SPG4) using a novel Tetra-GFP reporter system. *Biochem. Biophys. Res. Commun.* 318, 1079–1084.
 10. Wharton, S.B., McDermott, C.J., Grierson, A.J., Wood, J.D., Gelsthorpe, C., Ince, P.G., and Shaw, P.J. (2003). The cellular and molecular pathology of the motor system in hereditary spastic paraparesis due to mutation of the spastin gene. *J. Neuropathol. Exp. Neurol.* 62, 1166–1177.
 11. Reid, E., Connell, J., Edwards, T.L., Duley, S., Brown, S.E., and Sanderson, C.M. (2005). The hereditary spastic paraplegia protein spastin interacts with the ESCRT-III complex-associated endosomal protein CHMP1B. *Hum. Mol. Genet.* 14, 19–38.
 12. Errico, A., Ballabio, A., and Rugarli, E.I. (2002). Spastin, the protein mutated in autosomal dominant hereditary spastic paraplegia, is involved in microtubule dynamics. *Hum. Mol. Genet.* 11, 153–163.
 13. McDermott, C.J., Grierson, A.J., Wood, J.D., Bingley, M., Wharton, S.B., Bushby, K.M., and Shaw, P.J. (2003). Hereditary spastic paraparesis: Disrupted intracellular transport associated with spastin mutation. *Ann. Neurol.* 54, 748–759.
 14. Molon, A., Di Giovanni, S., Chen, Y.W., Clarkson, P.M., Angelini, C., Pegoraro, E., and Hoffman, E.P. (2004). Large-scale disruption of microtubule pathways in morphologically normal human spastin muscle. *Neurology* 62, 1097–1104.
 15. Hartman, J.J., and Vale, R.D. (1999). Microtubule disassembly by ATP-dependent oligomerization of the AAA enzyme katanin. *Science* 286, 782–785.
 16. McNally, F.J., and Vale, R.D. (1993). Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell* 75, 419–429.
 17. Fonknechten, N., Mavel, D., Byrne, P., Davoine, C.S., Cruaud, C., Boentsch, D., Samson, D., Coutinho, P., Hutchinson, M., McMonagle, P., et al. (2000). Spectrum of SPG4 mutations in autosomal dominant spastic paraplegia. *Hum. Mol. Genet.* 9, 637–644.
 18. Ogura, T., and Wilkinson, A.J. (2001). AAA+ superfamily ATPases: Common structure—diverse function. *Genes Cells* 6, 575–597.