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Research Article   **Neuroscience**

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Disease-related phenotypes in a *Drosophila* model of hereditary spastic paraplegia are ameliorated by treatment with vinblastine

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Hereditary spastic paraplegias (HSPs) are a group of neurodegenerative diseases characterized by progressive weakness and spasticity of the lower limbs. Dominant mutations in the human *SPG4* gene, encoding spastin, are responsible for the most frequent form of HSP. Spastin is an ATPase that binds microtubules and localizes to the spindle pole and distal axon in mammalian cell lines. Furthermore, its *Drosophila* homolog, *Drosophila spastin* (*Dspastin*), has been recently shown to regulate microtubule stability and synaptic function at the *Drosophila* larval neuromuscular junction. Here we report the generation of a spastin-linked HSP animal model and show that in *Drosophila*, neural knockdown of *Dspastin* and, conversely, neural overexpression of *Dspastin* containing a conserved pathogenic mutation both recapitulate some phenotypic aspects of the human disease, including adult onset, locomotor impairment, and neurodegeneration. At the subcellular level, neuronal expression of both *Dspastin* RNA interference and mutant *Dspastin* cause an excessive stabilization of microtubules in the neuromuscular junction synapse. In addition, we provide evidence that administration of the microtubule targeting drug vinblastine significantly attenuates these phenotypes in vivo. Our findings demonstrate that loss of spastin function elicits HSP-like phenotypes in *Drosophila*, provide novel insights into the molecular mechanism of spastin mutations, and raise the possibility that therapy with Vinca alkaloids may be efficacious in spastin-associated HSP and other disorders related to microtubule dysfunction.

Introduction

Hereditary spastic paraplegias (HSPs) are a group of neurodegenerative diseases characterized by progressive weakness and spasticity of the lower limbs (1–4). They are conventionally subdivided into pure and complicated forms, depending on the absence or presence of additional symptoms (2). Pure HSP is the single largest group of HSP pathologies, and the principal histopathological feature of disease appears to be a length-dependent retrograde degeneration of the terminal ends of axons of the corticospinal tracts and fasciculi gracilis (1–4), even though new evidence is emerging of neuropathologic changes outside the motor system (5, 6). Dominant mutations in the human *SPG4* gene, encoding spastin, are responsible for the prevailing form of pure HSP (7). A wide variety of nonsense, missense, and frameshift mutations have been identified in *SPG4* patients and produce clinically indistinguishable phenotypes, suggesting that the molecular mechanism of spastin mutations is haploinsufficiency (8, 9). Nevertheless, cellular expression of pathogenic missense mutations potentially inactivating the ATPase domain of spastin has led to the hypothesis that truncated or missense mutant spastin may cause HSP through a dominant-negative mechanism (10, 11).

SPG4 encodes spastin, a protein belonging to large AAA ATPase family characterized by a conserved domain contain-ing Walker A and B ATP-binding motifs. Spastin N-terminal region contains the MIT domain, a recently identified protein motif found in microtubule-interacting proteins (12). Indeed, human spastin has been shown to bind microtubules in an ATP-dependent fashion (10), suggesting a function in microtubule severing, as was shown for its close relative katanin p60, which is the catalytic subunit of a microtubule-severing protein (13). Experimental evidence that both human and *Drosophila* spastin proteins display ATPase activity and use energy from ATP hydrolysis to sever and disassemble microtubules in vitro has been reported very recently (14, 15).

At this stage, intracellular localization of spastin remains controversial, and both nuclear and cytoplasmic localization has been reported (6, 10, 11, 16). More recently, spastin has been found to be enriched in cell regions containing dynamic microtubules, such as the spindle pole and the distal axon (17).

Robust evidence supporting a role for spastin in microtubule cytoskeleton dynamics in vivo has come from studies in *Drosophila*. *Drosophila* has been shown to contain a highly conserved *spastin* homolog, *Drosophila spastin* (*Dspastin*) (18). In contrast to previous reports that mammalian spastin has a nuclear localization, Dspastin was demonstrated to be a cytoplasmic protein enriched in neuronal axons and synaptic connections. At the neuromuscular junction (NMJ) synapse, loss of *Dspastin* causes morphological undergrowth and loss of synaptic area. Moreover, using antibodies specific for posttranslationally modified tubulin found exclusively in stabilized microtubules, it has been found that *Dspastin* regulates microtubule stability at the NMJ synapse: *Dspastin* overexpression decreases microtubule stability, whereas loss of *Dspastin* enhances microtubule stability (19). Very recently,
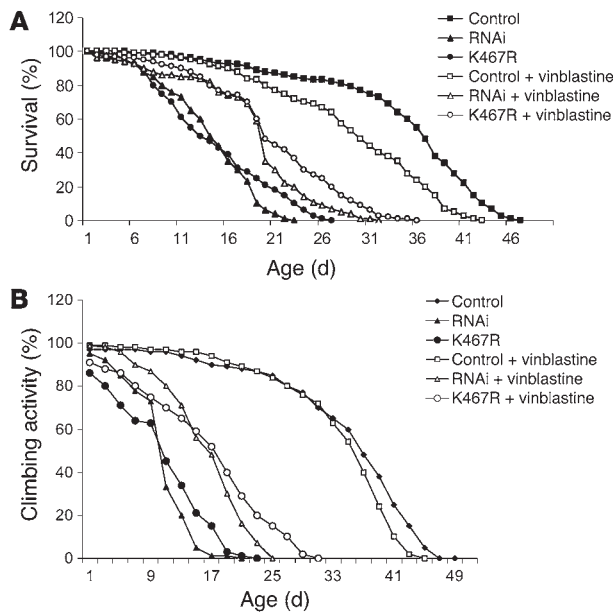
Figure 1
Behavioral phenotypes caused by neuron-specific expression of *Dspastin* RNAi and the *Dspastin* K467R pathogenic mutant are efficiently antagonized by pharmacological treatment with vinblastine. (**A** and **B**) Neuronal transgenic expression of *Dspastin* RNAi and *Dspastin* K467R reduced the longevity of adult flies (**A**) and severely impaired adult locomotor performance (**B**), causing premature loss of climbing ability. Vinblastine treatment increased adult lifespan (**A**) and improved locomotor ability (**B**), as judged by the enhanced performance in the climbing assay, of flies expressing *Dspastin* RNAi and *Dspastin* K467R in neurons. Control genotypes were as follows: Elav-Gal4/+, UAS-*Dspastin*-RNAi/+, and UAS-*Dspastin*-K467R/+. Experimental genotypes were as follows: Elav-Gal4/+;UAS-*Dspastin*-RNAi/+ and Elav-Gal4/+;UAS-*Dspastin*-K467R/+.

Results

Neuron-specific knockdown of Dspastin results in HSP-related phenotypes.
It is unclear how mutations in the *spastin* gene give rise to HSP in humans. However, the dominant inheritance pattern and broad mutational spectrum indicate that the molecular pathogenetic mechanism is likely to be partial loss of spastin function, determined by haploinsufficiency of the *SPG4* locus to which some neurons are acutely susceptible (1–4, 9). The spastin protein is highly enriched within the nervous systems of mammals, although not exclusively confined to neurons (16). Similarly, recent work has demonstrated that *Dspastin* message levels are elevated in the nervous system during *Drosophila* embryonic development (18), and that the Dspastin protein is greatly enriched in larval neurons (19). As in mammals, expression of *Dspastin* is not restricted to the *Drosophila* nervous system, being easily detectable, for example, in the larval musculature.

We therefore sought to determine whether specific neuronal downregulation of *Dspastin* in *Drosophila* might produce phenotypes reminiscent of the human pathology. Although a *Dspastin* loss-of-function mutant is now available, only very few escapers survive, and these die very shortly after eclosion, making adult-onset phenotypes due to neuronal dysfunction impossible to evaluate. We thus employed tissue-specific RNA interference–mediated (RNAi-mediated) knockdown of *Dspastin*, using upstream activating sequence–*Dspastin*–RNAi (UAS-*Dspastin*-RNAi) transgenic lines highly efficient in reducing *Dspastin* mRNA and protein levels (19). To further confirm the efficacy

another study on *Dspastin* has reported a function for this protein in the regulation of synaptic microtubule networks (20). These in vivo data in *Drosophila* strongly support previous in vitro data in other systems, providing a likely cause for the neuronal dysfunction in spastin-associated HSP disease.

Here we report the generation of the first animal model to our knowledge for spastin-linked HSP and show that in *Drosophila*, neural knockdown of *Dspastin* and, conversely, neural overexpression of *Dspastin* containing a conserved pathogenic mutation both recapitulate some phenotypic aspects of the human disease. Moreover, we provide evidence that these phenotypes can be significantly relieved in vivo by the microtubule targeting drug vinblastine. Our findings demonstrate that loss of spastin function elicits HSP-like phenotypes in *Drosophila*, provide evidence that at least a subset of spastin mutations may produce disease via a dominant-negative mechanism, and raise the possibility that therapy with Vinca alkaloids may be efficacious in spastin-associated HSP and other disorders related to microtubule dysfunction.

Figure 2
Reducing *Dspastin* expression produces age-dependent neurodegeneration. (**A**) One-day-old fly with transgenic expression of *Dspastin* RNAi showed no sign of neurodegeneration. (**B**) Twenty-day-old fly with transgenic expression of *Dspastin* RNAi had numerous vacuoles in the cortex and neuropil (arrows). (**C**) Twenty-day-old control fly showed no evidence of neurodegeneration. (**D**) Degenerating cells were TUNEL positive (arrows). (**E**) No TUNEL-positive cells were present in age-matched control flies. Control genotypes were as follows: Elav-Gal4/+ and UAS-*Dspastin*-RNAi/+. The experimental genotype used was Elav-Gal4/+;UAS-*Dspastin*-RNAi/+.
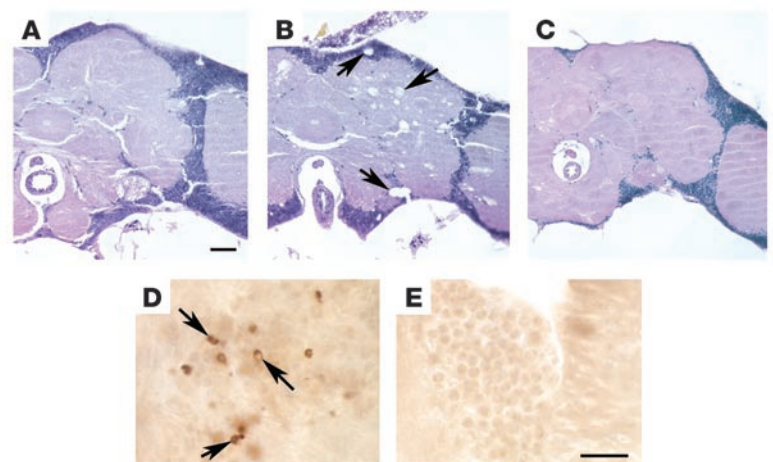
Table 1
Summary of the phenotypic effects of tissue-specific expression of different *Dspastin* transgenes

		Expression pattern	
Transgene	Ubiquitous	Nervous system	Eye
Dspastin RNAi	Pupa lethal with escapers (20%)	Viable, short lifespan, motor dysfunction, neurodegeneration	Normal
Dspastin K467R	Pupa lethal with escapers (4%)	Viable, short lifespan, motor dysfunction	Normal
Dspastin wild-type	Embryo lethal	Embryo lethal	Small, rough eye

To achieve the desired pattern of expression, the following Gal4 driver lines were used: tubulin-Gal4 for ubiquitous expression, Elav-Gal4 for nervous system expression, and GMR-Gal4 for eye expression. Percentages given indicate the percentage of escapers.

of our UAS-*Dspastin*-RNAi transgene, we obtained the *Dspastin*[5.75] allele, a recently described null *Dspastin* mutant (20), and performed a detailed phenotypic comparison between the ubiquitous expression of *Dspastin*-RNAi and *Dspastin*[5.75] mutants. These comparative analyses demonstrate that *Dspastin*[5.75] mutants display behavioral (i.e., eclosion rates) and cytological defects (i.e., decrease of synaptic area and accumulation of stable microtubules at the NMJ synapse) entirely overlapping with those produced by global expression of *Dspastin* RNAi (Supplemental Figure 3; supplemental material available online with this article; doi:10.1172/JCI24694DS1). Our observations demonstrate that RNAi-mediated knockdown of *Dspastin* accurately phenocopies the absence of Dspastin protein.

Expression of UAS-*Dspastin*-RNAi can be targeted to a specific tissue using the heterologous Gal4/UAS binary expression system (21). Use of this system allows for exclusive tissue-specific decrease of Dspastin levels, thus overcoming potential pleiotropic effects due to genetic mutation of *Dspastin*. Expression of UAS-*Dspastin*-RNAi in a pan-neuronal pattern using the Elav-Gal4 driver did not result in lethality prior to eclosion, permitting a detailed analysis of adult-onset phenotypes induced by loss of Dspastin activity. Newly eclosed Elav-Gal4/+;UAS-*Dspastin*-RNAi/+ flies appeared normal as revealed by viability, morphology, and behavior. However, as time progressed, reduction of Dspastin in these animals resulted in a marked shortening of adult lifespan (Figure 1A). Although lifespan is apparently not affected in HSP patients, this result suggests that knockdown of *Dspastin* in *Drosophila* gradually compromises adult nervous system functionality, which may in turn contribute to early lethality.

The principal clinical feature of HSP is progressive spasticity and weakness of the lower limbs, eventually leading to a dramatic motor deficit. To ascertain whether flies with reduced Dspastin expression exhibit similar progressive motor dysfunction, we used the climbing assay. Normal *Drosophila* display a strong negative geotactic response. When tapped to the bottom of a vial they rapidly rise to the top of the vial, and most individuals remain there. As they get older or manifest locomotor dysfunction, normal flies no longer climb to the top of the vial, but instead make short, abortive climbs and fall back to the bottom of the vial. This assay was previously identified as a reliable measure of locomotor performance in *Drosophila* (22–25). When tested for their climbing ability, Elav-Gal4/+;UAS-*Dspastin*-RNAi/+ young adults displayed a normal climbing response. However, over time, their

locomotor performance declined more rapidly than that of controls (Figure 1B). The reduced climbing response of *Dspastin* RNAi flies demonstrates that decreased *Dspastin* dosage in the nervous system causes a significant, progressive motor deficit that is reminiscent of an important feature of HSP and further substantiates a functional conservation between *Drosophila* and human spastin proteins.

To establish whether these behavioral phenotypes were associated with neurodegeneration, we assessed fly brain morphology by examining histologic sections and assaying for apoptotic cells by TUNEL staining. The brain of newly eclosed *Dspastin* RNAi flies showed normal anatomical and histological organization (Figure 2A). In contrast, aged *Dspastin* RNAi flies displayed numerous vacuoles in the neuropil and cortex (Figure 2B, arrows), a hallmark of neurodegeneration in *Drosophila* (26–28). Vacuoles were not present in sections from age-matched control flies (Figure 2C). TUNEL-positive cells were present in the cortex of aged *Dspastin* RNAi flies (Figure 2D), but not in control flies (Figure 2E). These findings are consistent with adult-onset neurodegeneration mediated by reduced levels of Dspastin. These results strongly suggest a causal relationship between the locomotor deficit and shortened lifespan caused by loss of *Dspastin* function in the *Drosophila* adult nervous system and age-dependent brain degeneration.

Mutant Dspastin behaves as a dominant negative in vivo. While nonsense, frameshift, or splice-site mutations in human spastin have
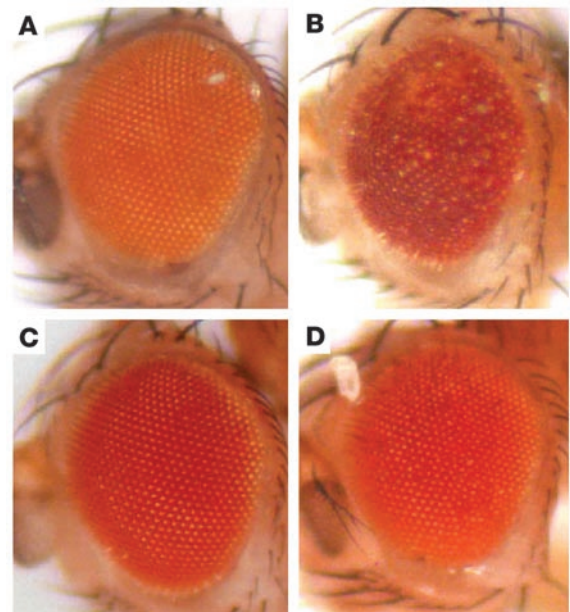
Figure 3
Expression of *Dspastin* K467R in the *Drosophila* eye opposes the phenotypic effects of wild-type *Dspastin* expression. (**A**) Wild-type *Drosophila* eye. (**B**) Eyes expressing low levels of UAS-*Dspastin* under the control of GMR-Gal4 displayed a moderate rough phenotype. (**C**) Expression of UAS-*Dspastin*-K467R alone had no phenotypic consequences in the eye. (**D**) Simultaneous expression of both constructs resulted in a practically normal external morphology, suggesting that *Dspastin* K467R suppresses the rough eye phenotype caused by UAS-*Dspastin* expression.
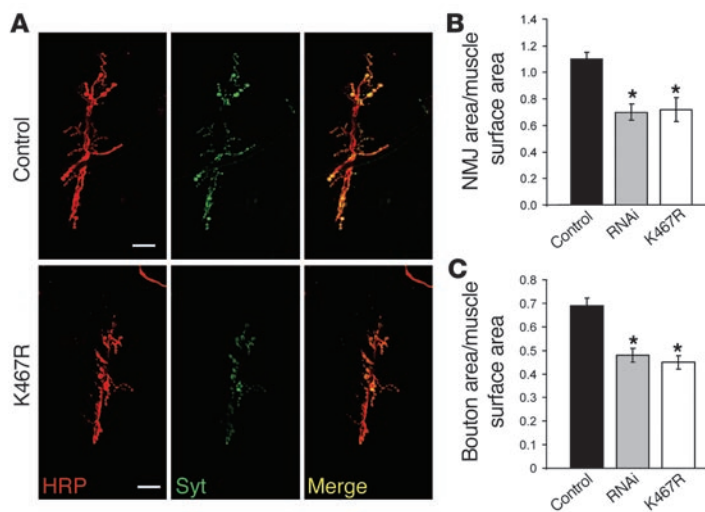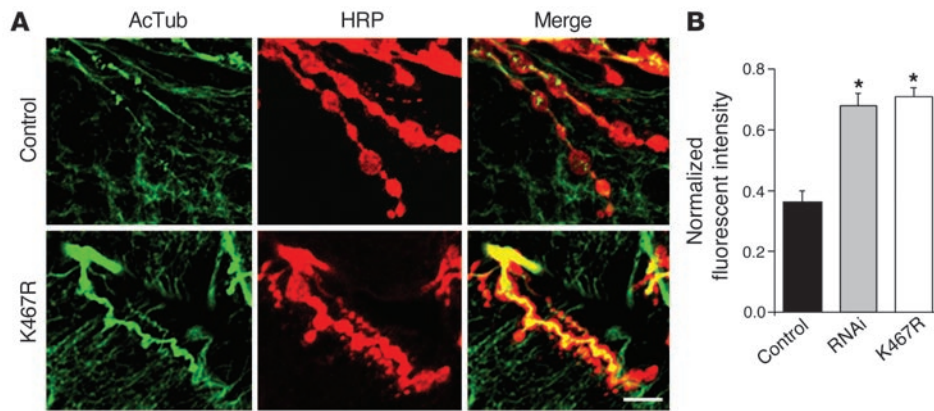
Figure 4
Neuronal expression of pathological mutant *Dspastin* K467R and *Dspastin* RNAi affect synaptic morphology in a comparable fashion. (**A**) Larval NMJ at muscles 6/7, identified by HRP immunocytochemistry (red; left panels). Neural expression of *Dspastin* K467R in a wild-type background caused a reduction of the synaptic area and arborization compared with controls (Elav-Gal4/+ and UAS-*Dspastin*-K467R/+). NMJs were also labeled with anti-synaptotagmin antibody (Syt) to provide an alternative means of measuring synaptic area (green; middle panels). Merged images are shown at right. Scale bars: approximately 20 μM. (**B** and **C**) Quantification of the data using results of both HRP (**B**) and synaptotagmin (**C**) staining shows that the observed decrease in synaptic area was statistically significant and similar to that detected in NMJs expressing *Dspastin* RNAi. *$P < 0.0001$.

suggested a haploinsufficiency mechanism for the human disorder, cellular expression of pathogenic missense mutations potentially inactivating the ATPase domain of spastin has led to the hypothesis that the disease may also be caused by a dominant-negative mechanism. To address the pathogenetic mechanism underlying spastin mutations, we generated transgenic flies bearing a K467R substitution that corresponded to pathogenic mutation K388R in the human spastin protein. We chose this mutation because in mammalian cell lines, spastin K388R has been shown to bind microtubules constitutively (10), and analogous amino acid changes in ATPases belonging to the same family are known to abrogate ATP binding and elicit dominant-negative effects (29, 30). To examine the functional significance of the K467R mutant, we expressed it in a wild-type background (i.e., in flies with normal amounts of Dspastin), a genetic combination closely resembling that of *SPG4* patients, where wild-type and mutated spastin are simultaneously present. We reasoned that a dominant-negative mutant should give rise to phenotypes similar to those produced by downregulation of Dspastin but distinct from those caused by Dspastin overexpression. Consistent with this hypothesis, eye-specific expression of both UAS-*Dspastin*-RNAi and UAS-*Dspastin*-K467R had no phenotypic effects, while expression of UAS-*Dspastin* gave rise to external eye roughness (Table 1 and Figure 3, B and C), suggesting that replacement of lysine 467 with arginine leads to inactivation of the protein. Moreover, similar to the expression of UAS-*Dspastin*-RNAi, ubiquitous and nervous system–specific expression of UAS-*Dspastin*-K467R resulted in partial lethality at the pupa stage and shortening of adult lifespan, respectively. In contrast, UAS-*Dspastin* driven ubiquitously by tubulin-Gal4, or expressed in a neuronal pattern by Elav-Gal4, consistently caused early embryonic lethality (Table 1). Immunohistochemical analyses using an anti-HRP antibody to highlight the larval NMJ synapse, an anti-synaptotagmin antibody to label exclusively synaptic boutons, and an anti–acetylated α-tubulin antibody to specifically identify stabilized microtubules revealed that expression of the K467R mutation in wild-type neurons resulted in a decrease in synaptic area (Figure 4) as well as the accumulation of hyperstable microtubules at the NMJ presynaptic terminal (Figure 5) as was previously demonstrated for neuronal *Dspastin* knockdown (19), suggesting that these synaptic defects are produced by loss of endogenous Dspastin activity mediated by expression of Dspastin K467R.

We next assessed adult vitality, climbing response, brain morphology, and extent of neuronal apoptosis in Elav-Gal4/+;UAS-*Dspastin*-K467R/+ flies. Reduction of lifespan and impairment of locomotor activity were remarkably similar in *Dspastin* RNAi and K467R mutant flies (Figure 1, A and B). Although progressive neurodegeneration in K467R-expressing brains was modest (Supplemental Figure 2B), when K467R was expressed in the presence of reduced levels of Dspastin (Elav-Gal4/+;UAS-*Dspastin*-K467R/+; *Dspastin*[5.75]/+), significant degeneration was seen, particularly in the medulla (Supplemental Figure 2C). The observation that knockdown of *Dspastin* and overexpression of the *Dspastin* K467R mutant consistently elicited similar phenotypes suggests that the K467R mutation acts through a dominant-negative mechanism by interfering with the function of endogenous Dspastin.

Corroboration for a dominant-negative role of the K467R mutation has also come from a comparison of the phenotype caused by co-overexpression of *Dspastin* and *Dspastin* K467R with those caused by overexpression of each alone. In the *Drosophila* eye, moderate ectopic expression of *Dspastin* produced a mild rough eye phenotype, whereas overexpression of Dspastin K467R had no phenotypic consequences (Figure 3, B and C), indicating that this pathologic amino acid substitution causes loss of spastin protein activity. However, simultaneous expression of both transgenes resulted in essentially normal external eye morphology, strongly suggesting that the K467R mutant causes suppression of the rough eye induced by ectopic expression of Dspastin (Figure 3D). Taken together, our findings demonstrate that in *Drosophila* overexpression of *Dspastin* K467R and *Dspastin* RNAi cause largely overlapping phenotypes reminiscent of HSP pathology, and that pathogenic mutation K467R acts as a dominant negative.

Administration of vinblastine attenuates disease-related phenotypes in vivo. Dspastin has been recently shown to play a role in destabilizing the microtubule cytoskeleton in neurons. Loss of this Dspastin activity leads to the accumulation of excessively stable microtubules in the larval NMJ, thereby affecting synaptic neurotransmission (19). Synaptic function, however, can be restored by treating *Dspastin* RNAi dissected larval preparation with nocodazole (19). This observation prompted us to investigate whether pharmacological treatment targeted at microtubules could counteract in vivo the phenotypic effects of expressing *Dspastin* RNAi and *Dspastin* K467R. Feeding has been previously shown to be an efficient means of administering

A

Figure 5
Neuronal expression of pathological mutant *Dspastin* K467R and *Dspastin* RNAi induce an analogous hyperstabilization of microtubules. (**A**) Images of NMJ immunocytochemistry using an anti-HRP antibody (red; middle panels), a neuronal maker of the presynaptic arbor, and an anti–acetylated α-tubulin antibody (AcTub, green; left panels), present only in stable microtubules (33), demonstrate that nervous system expression of Dspastin K467R induced accumulation of stabilized microtubules, as indicated by the dramatic increase of acetylated α-tubulin signal compared with controls (Elav-Gal4/+ and UAS-*Dspastin*-K467R/+). (**B**) Quantification of the data in **A** revealed that this increase was statistically significant and comparable to that caused by RNAi knockdown of *Dspastin*. Values were normalized as described in Methods. *$P < 0.0001$. Scale bar: approximately 10 μM.

the NMJ presynaptic terminal (Figure 7, A–C) and the increase in acetylated α-tubulin (Figure 8) of flies expressing *Dspastin* RNAi and *Dspastin* K467R in the nervous system, thus specifically rescuing the cytological defect caused by loss of Dspastin activity (Figures 4 and 5). We also tested the ability of vinblastine to suppress the NMJ phenotypes observed in *Dspastin*[5.75]-null mutants. In these individuals, synaptic boutons are more numerous, and synaptic area is reduced compared with controls (Supplemental Figure 3). However, administration of vinblastine produced a moderate but statistically significant increase in total synaptic area and a decrease in bouton number (Figure 7, D–G), suggesting that this drug is capable of ameliorating the phenotypes associated with loss of *Dspastin* function in the complete absence of spastin protein from neuronal as well as muscle cells.

drugs to flies (31–35). Thus, we monitored the effects of feeding flies with nocodazole and vinblastine, 2 structurally and mechanistically diverse microtubule destabilizing drugs (36, 37). These drugs were assayed for their ability to suppress both the lethality induced by ubiquitous expression of *Dspastin* RNAi and *Dspastin* K467R and the motor and adult lifespan phenotypes observed in Elav-Gal4/+;UAS-*Dspastin*-RNAi/+ and Elav-Gal4/+;UAS-*Dspastin*-K467R/+ flies. Surprisingly, in vivo treatment with nocodazole did not rescue any of the above phenotypes (data not shown). In contrast, administration of vinblastine resulted in a suppression of both *Dspastin* RNAi– and *Dspastin* K467R–induced phenotypes. Global expression of *Dspastin* RNAi and *Dspastin* K467R normally results in lethality with a few escapers; however, treatment with vinblastine led to a substantial increase in the eclosion rate of flies ubiquitously expressing both RNAi and the K467R mutant (Figure 6). Similarly, a greater proportion of *Dspastin*[5.75]-null mutant *Drosophila* eclosed in food containing vinblastine than in food lacking this drug (Figure 6), even though the effect of vinblastine on *Dspastin*[5.75] mutants appears to be less pronounced than that on tubulin-Gal4/+;UAS-*Dspastin*-RNAi/+ individuals, possibly because the positive effects of vinblastine were aided by a residual presence of Dspastin protein in RNAi-treated flies. Consistent with these observations, vinblastine-fed Elav-Gal4/+; UAS-*Dspastin*-RNAi/+ and Elav-Gal4/+;UAS-*Dspastin*-K467R/+ *Drosophila* displayed a moderate but statistically significant improvement in both locomotor ability and adult lifespan compared to untreated controls ($P < 0.05$; Figure 1). It is to be noted, however, that vinblastine treatment was associated with small levels of toxicity (Figure 1 and Supplemental Figure 4). We then determined whether suppression of these behavioral phenotypes correlated at the cellular level with arrest of neuronal degeneration and rescue of synaptic area reduction as well as aberrant microtubule accumulation at the larval NMJ. While administration of vinblastine did not rescue terminal neurodegeneration of Elav-Gal4/+;UAS-*Dspastin*-RNAi/+, it was remarkably capable of reversing the reduced area of

Discussion

The most common form of HSP, accounting for approximately 40% of all cases, is caused by mutations in the *SPG4* gene, which encodes the spastin protein. To date, no vertebrate or invertebrate animal models for spastin-linked HSP have been developed to allow a better understanding of the basic pathophysiologic mechanisms responsible for this disease. Although human spastin is widely expressed, the nervous system appears to be specifically vulnerable to the partial loss of spastin protein observed in *SPG4* patients. Likewise, *Dspastin* expression is not restricted to the nervous system, indicating that an analysis of neuronal specific phenotypes due to loss of *Dspastin* may be hampered by pleiotropic effects caused by the lack of *Dspastin* in other tissues. We have
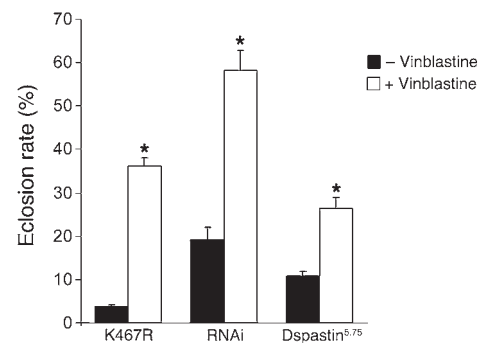
Figure 6
In vivo consequences of vinblastine administration. Addition of vinblastine to the food significantly increased the eclosion rate of *Dspastin*[5.75]-null mutants and that of flies ubiquitously expressing *Dspastin* RNAi and *Dspastin* K467R compared with genotypically identical flies raised in the absence of vinblastine. Experimental genotypes were as follows: tubulin-Gal4/+;UAS-*Dspastin*-RNAi/+, tubulin-Gal4/+;UAS-*Dspastin*-K467R /+, and *Dspastin*[5.75]/*Dspastin*[5.75]. *$P < 0.0001$.
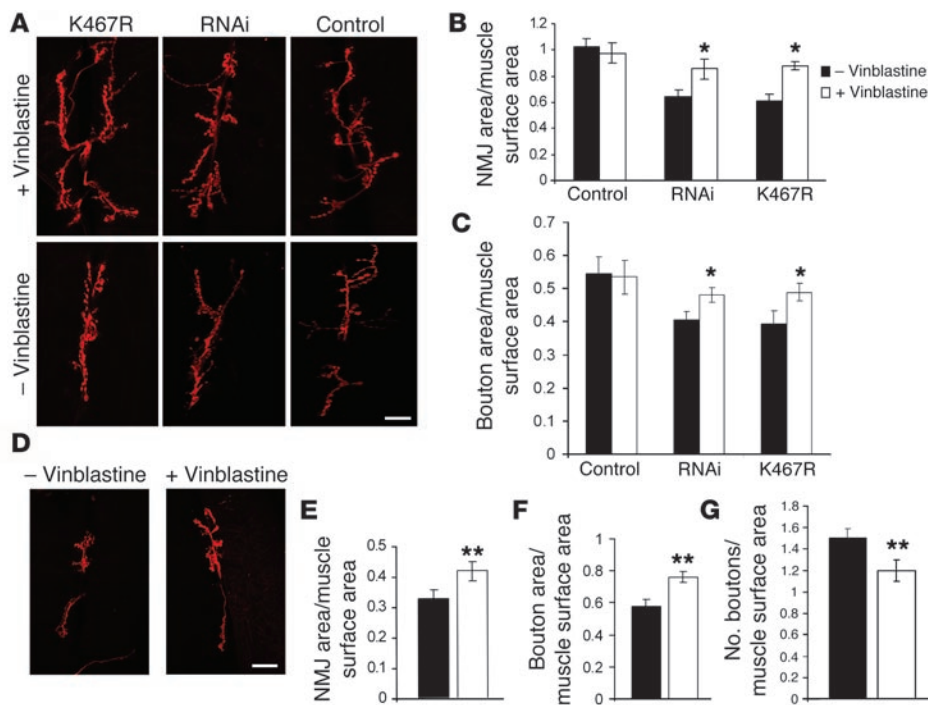
Figure 7
Administration of vinblastine reverts the loss of synaptic area caused by nervous system–specific expression of *Dspastin* RNAi and *Dspastin* K467R and ameliorates NMJ phenotypes of *Dspastin*[5.75] mutants. (**A** and **D**) Representative images of NMJ synapses at muscles 6/7 revealed with anti-HRP antibody (red) show that vinblastine treatment partially restored synaptic area in Elav-Gal4/+; UAS-*Dspastin*-RNAi/+ and Elav-Gal4/+;UAS-*Dspastin*-K467R/+ (**A**) and *Dspastin*[5.75] *Drosophila* (**D**). Control genotypes were as follows: Elav-Gal4/+, UAS-*Dspastin*-RNAi/+, and UAS-*Dspastin*-K467R/+. Scale bars: approximately 20 μM. (**B**, **C**, **E**, and **F**) Quantification of synaptic area restoration, determined both by anti-HRP (**B** and **E**) and anti-synaptotagmin (**C** and **F**) immunoreactivity, shows that phenotypic rescue by vinblastine was statistically significant. (**G**) Vinblastine treatment produced a statistically significant decrease in bouton number of *Dspastin*[5.75] third instar larva NMJ. **P < 0.05; *P < 0.0001.

therefore employed a tissue-specific transgenic RNAi strategy for the selective elimination of Dspastin from the fly nervous system in order to create a spastin-linked HSP disease model in *Drosophila* that allowed us to examine the phenotypic consequences of neuron-specific removal of Dspastin.

Recently, a study has been published describing phenotypes for *Dspastin*-null mutant flies that contrast with our present data (20). For instance, the authors report that in *Dspastin*-null mutants, there are fewer microtubule bundles within the NMJ, in contrast to the accumulation of hyperstabilized microtubules that we observed at the NMJ upon expression of *Dspastin* RNAi. A direct comparison of our results with those presented by Sherwood et al. is not possible, because we have knocked down *Dspastin* specifically in the nervous system, while the null allele used by Sherwood et al. affects all tissues in which Dspastin is normally expressed (20). However, to rectify these apparent discrepancies, we have carried out a detailed comparative analysis between *Dspastin*[5.75]-null mutant *Drosophila* and *Drosophila* expressing *Dspastin* RNAi ubiquitously (see Supplemental Discussion). We found that eclosion rates were similar between *Dspastin*[5.75] and *Dspastin* RNAi flies, that synaptic area was similarly decreased, and that the levels of acetylated α-tubulin were analogously increased in synaptic terminals of *Dspastin*[5.75] and *Dspastin* RNAi flies (Supplemental Figure 3).

Moreover, in *Dspastin*[5.75] mutants and in flies globally expressing *Dspastin* RNAi, NMJ synaptic boutons are smaller, more numerous, and more clustered than in wild-type flies (Supplemental Figure 3).

These phenotypic similarities strongly argue for the efficiency and specificity of our RNAi transgene in eliminating Dspastin protein expression by demonstrating its ability to phenocopy a *Dspastin*-null allele. The observation that some cytological NMJ phenotypes seen in the *Dspastin*-null allele (i.e., decreased synaptic area and increased acetylated α-tubulin levels) corresponded largely to those caused by neuronal expression of *Dspastin* RNAi provide strong support for our model, which relies on the elimination of Dspastin specifically in the nervous system. Despite these similarities, some divergence must be noted between global and neuronal loss of *Dspastin*. For instance, neuronal expression of RNAi does not cause an increase in bouton size, number, and clustering as seen in *Dspastin*-null mutants. However, these discrepancies can be rationalized because in *Dspastin*[5.75]-null mutants, this bouton phenotype is likely to arise from the simultaneous loss of the protein from the presynaptic as well as the postsynaptic muscle compartment, where Dspastin has been shown to be enriched (19, 20).

Interestingly, *Drosophila* lacking Dspastin exclusively within their nervous systems displayed mutant phenotypes that resembled observations made in HSP patients. First, there was no lethality associated with neuronal loss of Dspastin activity, and flies eclosed into apparently normal young adults. Indeed, HSP is not a lethal condition, and patients present variable ages of onset, indicating that there are no obvious developmental defects linked to spastin mutations or, if present, these defects become manifest only after birth. Second, as time advanced, these flies progressively developed severe movement defects and within around 15–20 days of birth stopped moving altogether. Progressive locomotor dysfunction and impaired gait are the hallmark of HSP pathology. In addition to these disease-related abnormalities, a marked shortening of adult lifespan was also associated with loss of Dspastin in *Drosophila* that is not observed in *SPG4* patients. Third, HSP is thought to be a progressive neurodegenerative disorder. Consistent with this notion, nervous system–specific reduction of *Dspastin* function resulted in progressive neurodegeneration of the fly brain with obvious presence of neurons undergoing apoptosis. Although in *SPG4* patients the most prominent neuropathological finding is a progressive retrograde degeneration of corticospinal tracts without obvious loss of anterior horn cells, new evidence is emerging that neuronal involvement in *SPG4* HSP is not always limited to distal axons of long spinal cord neurons, but may be
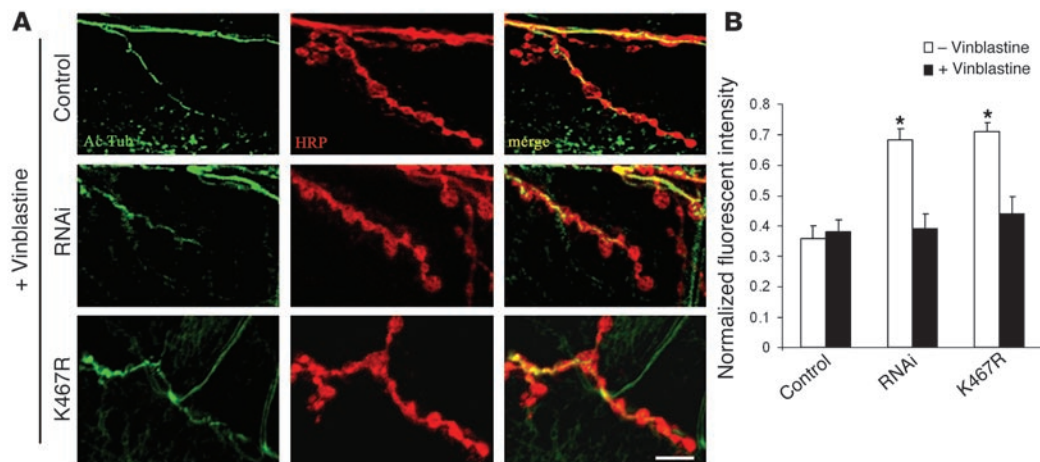
Figure 8
Administration of vinblastine suppresses the accumulation of stabilized microtubules caused by nervous system–specific expression of *Dspastin* RNAi and *Dspastin* K467R. (**A**) Confocal images of NMJ synaptic terminals labeled with the neuronal marker anti-HRP antibody (red) and an anti–acetylated α-tubulin antibody (green), a marker for stable microtubules (33), demonstrate that in vinblastine-treated Elav-Gal4/+;UAS-*Dspastin*-RNAi/+ and Elav-Gal4/+;UAS-*Dspastin*-K467R/+ individuals, acetylated α-tubulin levels were reduced compared with those of untreated animals and similar to those of controls raised in vinblastine. Control genotypes were as follows: Elav-Gal4/+, UAS-*Dspastin*-RNAi/+, and UAS-*Dspastin*-K467R/+. Scale bar: approximately 10 μM. (**B**) Quantification of the decrease of acetylated α-tubulin signal shown in **A** demonstrates that phenotypic rescue by vinblastine was statistically significant. *$P < 0.0001$.

more widespread and affect neurons outside the motor system (5, 6). An alternative explanation for the greater severity of the phenotypes observed in flies is that the neurodegeneration and cell loss seen in our *Drosophila* model may reflect the dramatic reduction of Dspastin levels we achieved through our RNAi-knockdown approach compared with the more moderate partial loss of spastin function thought to underlie HSP.

The mechanisms by which spastin mutations produce dominant HSP in humans are controversial. The broad mutational spectrum observed has suggested that the molecular mechanism is likely to be haploinsufficiency; however, it has also been proposed that truncated or missense mutant spastin may function as a dominant negative. The outstanding sequence homology between human spastin and Dspastin proteins has allowed us to use our model system to investigate the mode of action of pathogenic spastin mutations. Our results demonstrate that neuronally targeted expression of the Dspastin K467R mutant (corresponding to pathologic mutation K388R in humans) in a fly containing wild-type levels of endogenous Dspastin caused behavioral phenotypes that largely overlapped with those produced by the specific removal of Dspastin from neurons. These phenotypes include adult-onset progressive locomotor impairment and neurodegeneration in the context of reductions in Dspastin levels. Likewise, we provide evidence that at the molecular level, neuron-specific knockdown of *Dspastin* and neuron-specific expression of the *Dspastin* K467R mutant induced an analogous reduction of synaptic area and accumulation of hyperstabilized microtubule cytoskeleton at the neuromuscular synapse. The observation that loss of *Dspastin* activity and overexpression of a pathogenic *Dspastin* mutant caused analogous phenotypic consequences strongly suggests that in both instances, the observed abnormalities are mediated by loss of endogenous *Dspastin* activity, and thus at least some disease mutations act through a dominant-negative mechanism. These results further suggest that aberrant microtubule stabilization in neurons is likely to be the underlying cause of *SPG4* HSP in humans.

Since human spastin containing the K388R substitution (corresponding to the K467R mutation in *Drosophila*) has been shown to bind microtubules as effectively as wild-type spastin, the simplest mechanistic explanation for the dominant-negative effect of Dspastin K467R is that spastin binding sites on microtubules are titrated out by the inactive mutant, thus preventing the action of wild-type spastin. This explanation may also account for the low levels of neurodegeneration observed in *Dspastin* K467R brains compared with *Dspastin* RNAi brains. While expression of *Dspastin* RNAi dramatically reduces the levels of endogenous Dspastin, its function is likely to be only partially blocked by overexpression of the dominant-negative K467R mutant. In neurons expressing *Dspastin* K467R, greater residual Dspastin function could result in histologically more subtle, but physiologically important, phenotypes. This interpretation is supported by the observation that expression of the K467R mutant in a *Dspastin*[5.75]-null heterozygous background, where the levels of endogenous Dspastin are reduced by 50%, resulted in obvious degeneration.

The dominant-negative pathogenic mechanism of the K467R mutant could be identical for missense and other mutations that may alter the function of the ATPase domain of spastin, leaving its functional microtubule binding region intact. However, pathogenic frameshift mutations have been identified that result in truncation of the spastin protein upstream of the microtubule-binding domain (38, 39). Therefore, spastin mutations may cause HSP pathology by 2 distinct mechanisms: loss of wild-type spastin function mediated by a dominant-negative effect or decrease in spastin dosage through haploinsufficiency (16).

The generation of a disease model with features reminiscent of the human disease and whose biochemical and cytological defects are well characterized has allowed us to focus on the identification of pharmacological agents capable of suppressing the phenotypes induced by loss of *Dspastin* function in vivo, whether direct or mediated by the expression of the *Dspastin* K467R mutant. Using this approach, we have shown that in *Drosophila* the microtubule

destabilizing agent vinblastine, but not nocodazole (another potential candidate drug), significantly attenuated disease-related abnormalities in vivo, and this attenuation correlated with a robust suppression of the cytological defects of the neuromuscular synapse cytoskeleton associated with these abnormalities. At low concentrations, vinblastine is thought to exert its action by modulating microtubule dynamics rather than by decreasing tubulin polymer mass (40). Therefore, our findings imply that vinblastine treatment rescues the pathological effects of loss of Dspastin function in vivo by pharmacologically compensating for an increase in microtubule stability caused by downregulation or complete loss of *Dspastin* and further confirm that misregulation of microtubule dynamics is likely responsible for the pathogenesis of *SPG4*. Since no pharmacological treatment is currently available for this disorder and vinblastine, as well as several other Vinca alkaloid family members, are currently widely used clinically in cancer chemotherapy, these drugs could be rapidly tested as potential therapeutic agents for spastin-linked HSP and related diseases.

In summary, we have created the first *SPG4* animal model to our knowledge and shown that in *Drosophila*, RNAi-mediated knockdown of *Dspastin* and overexpression of the *Dspastin* K467R pathogenic mutant reproduce salient features of the human disorder: adult onset, progressive motor dysfunction, and progressive neurodegeneration. Our *Drosophila* model demonstrates that a dominant-negative mechanism is implicated in the pathogenesis of spastin-associated HSP and provides compelling evidence that pharmacological modulation of microtubule stability in vivo effectively counters the pathological phenotypes arising from loss of *Dspastin* function, strongly supporting the hypothesis that alteration of microtubule dynamics underlies the human disease.

Despite the apparent parallels underscored by the data presented, *Drosophila* can mimic the human disease only in part due to the obviously much simpler anatomical and physiological organization of flies. However, existing data indicate that the underlying biochemical and cytological defects, both in mammalian cells and in flies, are traceable to the misregulation of microtubule stability in nerve terminals caused by loss of spastin function.

Almost half of all HSP disease cases are linked to microtubule motor proteins or other proteins with microtubule interacting domains (e.g., KIF5A, spartin, and spastin) (12, 41, 42). The availability of a genetically tractable animal model for spastin-associated HSP may lead to new insights into this group of diseases by helping to identify common pathways through which alterations in microtubule cytoskeleton formation, stability, and/or function can cause different forms of HSP.

Methods

Drosophila stocks and crosses. The UAS-*Dspastin*, UAS-*Dspastin*-RNAi, and *Dspastin*[5.75]-null *Drosophila* used in this study were described previously (19, 20). Site-directed mutagenesis was employed to generate the UAS-*Dspastin*-K467R construct. Nine independent transgenic lines were derived for the K467R mutant, and all were tested for protein expression by immunohistochemistry using different Gal4 driver lines. The 2 lines with the highest expression levels, which consequently displayed the most severe phenotypes, were chosen for the studies reported here.

The Gal4 activator lines used in this study were Elav-Gal4, tubulin-Gal4, and glass multiple reporter–Gal4 (GMR-Gal4; Bloomington Stock Center, Indiana University). To increase protein and double-stranded RNA expression, all experimental crosses were performed at 28°C.

Histology. Heads from adult flies were fixed in formalin and embedded in paraffin, and 4-μm frontal sections were prepared. Serial sections were cut through the entire brain and placed on a single glass slide. Hematoxylin and eosin staining was performed using a standard protocol. Apoptosis was detected with the TUNEL assay using a commercially available kit (TdT FragEl; Oncogene).

Immunohistochemistry. NMJ immunostaining was performed on wandering third instar larvae reared at 28°C. Larvae were dissected in standard saline and fixed in 4% paraformaldehyde for 45 minutes. Preparations were washed in PBS containing 0.5% bovine serum and labeled overnight with anti-HRP antibody (1:1000; Sigma-Aldrich), anti-synaptotagmin (1:100; Developmental Studies Hybridoma Bank, University of Iowa), and anti–acetylated α-tubulin (1:1000; Sigma-Aldrich). Secondary antibodies were from Invitrogen Corp. Third instar larva NMJs on abdominal muscle 6/7 from thoracic segment 2 were assayed for total synaptic area, highlighting areas of HRP and synaptotagmin immunoreactivity. Total synaptic area, determined either by anti-HRP or anti-synaptotagmin staining, was normalized to the surface area of the corresponding muscle. Quantification of immunohistochemistry was achieved by normalizing the intensity of the acetylated α-tubulin signal to that of HRP, as previously described (19). At least 10 individual NMJs were analyzed for each experiment. Images were acquired with a Nikon C1 confocal microscope and analyzed using either Nikon EZ-C1 (version 2.10) or NIH ImageJ (version 1.32J) software.

Adult behavioral analysis. Adult climbing test was used to measure *Drosophila* locomotor function. For each genotype tested, 30 individual flies, maintained at 28°C, were collected and placed into an empty vial with a line drawn 2 centimeters from the bottom of the tube. After a 1-hour recovery period from anesthesia, flies were gently tapped to the bottom of the tube, and the number of flies that successfully climbed above the 2-cm mark after 20 seconds was noted. Fifteen separate and consecutive trials were performed, and the results were averaged. At least 300 flies were tested for each genotype. Lifespan experiments were performed starting with 300–400 animals for each genotype. Flies were collected at 1 day after eclosion and placed in vials containing 50 animals. The animals were maintained at 28°C, transferred to fresh medium every 2 days, and the number of dead flies was counted. Adult climbing and lifespan experiments were repeated at least 3 times.

Vinblastine treatment. Vinblastine sulfate (Sigma-Aldrich) was added to regular *Drosophila* food, and concentrations ranging from 0.01 μM to 1 μM were tested. To perform the experiments presented in this study, we selected the lowest effective concentration (0.05 μM).

Statistics. Statistical analyses were performed using unpaired 2-tailed Student's *t* tests and were carried out using Prism (version 3.03; GraphPad) or Microsoft Excel software.

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