The Study of Hereditary Spastic Paraplegia Genes Using Drosophila Homologues: Behavioural Insights

by

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Abstract

Hereditary Spastic Paraplegia (HSP) denotes a heterogeneous group of heritable neurodegenerative disorders predominantly characterized by progressive weakness and spasticity of the legs. Mutations in the gene *SPAST* are by far the most common, while mutations in the gene *ATL1* are the second most prevalent. Recently, a novel gene, *DCNT1*, has been implicated in HSP neuropathology. We used the Drosophila homologues of these three genes—*D-spastin*, *D-atl*, and *Glued*, respectively—to determine whether or not corresponding mutations are associated with locomotion deficits. We employed locomotion assays—i.e., climbing, walking, and larva tracking—and genetic tools available in Drosophila—i.e., genetic mutations and altered gene expression via the GAL4-UAS system—to manipulate the physiological function of these HSP genes at multiple developmental time points. This approach was employed to identify potential locomotion deficits resulting from mutations in single genes or alterations in their expression levels (Experimental Aim 1), or the interactions between different genes (Experimental Aim 2). We show evidence for a functional role of all three genes of interest in Drosophila locomotion, as well as for the pairwise interactions between them. However, the genetic interaction—i.e., the synergistic effect of a combination of two or more genetic manipulations—between *D-spastin* and *D-atl* is relatively inconclusive. The clustering of HSP genes via interaction data will not only aid in elucidating the convergent mechanisms underlying the disorder, but will also facilitate the development of potential treatments for HSP patients.

Dedication

The popularity of sports in modern society is no secret. Participants are highly diverse, including the arguably overpaid professional athletes, the health enthusiasts working hard to improve or maintain their physical fitness, or—perhaps most importantly—those who participate to enjoy themselves and have fun. Fundamentally speaking, I believe that sports, and any other play-based activities—in other words, *fun*—are evolutionary rudiments of human learning.

With that in mind, I would like to dedicate this thesis to Mr. Brad Gilmour, my high school science teacher. Somehow, within the confines of a sometimes painstaking and mundane school system, Mr. Gilmour made learning engaging and fun, and inspired me to pursue further studies in science and health. Mr. Gilmour, thank you. I feel privileged to have had you in my life.

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Symbols and Abbreviations

*	genetic interaction designation (e.g., gene1*gene2)
~	approximately
aa	amino acid
AAA	ATPases associated with diverse cellular activities
AD-HSP	autosomal dominant Hereditary Spastic Paraplegia
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
AOO	age of onset
AR-HSP	autosomal recessive Hereditary Spastic Paraplegia
Arp-1	actin-related protein 1
atl	Drosophila gene *atlastin* and gene symbol for *atlastin* allele designations
Atl	atlastin protein
Atl-1	human atlastin protein, isoform 1
ATL1	human *atlastin* gene, isoform 1
atlastin	Drosophila or human *atlastin* gene
ATP	adenosine triphosphate
ATPase	a family of enzymes that are capable of binding and hydrolyzing ATP

BDSC	Bloomington Drosophila Stock Center
bp	base pair
C	Celsius
C-terminal	carboxy-terminal
CCD	charge-coupled device
cDNA	complementary deoxyribonucleic acid
cis	Latin prefix meaning “on the near side of”
cm	centimeter
Co-IP	co-immunoprecipitation
CO_2	carbon dioxide
CSHL	Cold Spring Harbor Laboratory
D	Drosophila
DCTN1	*Dynactin 1*, a gene that encodes human dynactin-1, also known as $p150^{Glued}$
dsRNA	double stranded ribonucleic acid
e.g.	for example (Latin: *exempli gratia*)
Elav	embryonic lethal, abnormal vision
ER	endoplasmic reticulum
F1	first filial
F2	second filial

FASTA	a text-based software program used for the sequence alignment of either DNA or protein
GAL4	a yeast-derived transcriptional activator
GBP	guanylate binding protein
GCP60	a human Golgi resident protein
Gl	Drosophila *Glued* gene and gene symbol for *Glued* allele designations
Glued	Drosophila *Glued* gene
Glued	Drosophila Glued protein
GST	glutathione S-transferase
GTPase	a family of enzymes that is capable of binding and hydrolyzing guanosine triphosphate
hpRNA	hairpin ribonucleic acid
hr	hour
HSD	Honestly Significant Difference
HSP	Hereditary Spastic Paraplegia
Hu	Humeral
i.e.	that is (Latin: *id est*)
iso	prefix meaning “equal”
katanin 60	katanin 60 protein
Katanin 60	*Katanin 60* gene

kb	kilobase
kDa	kilodalton
$L3^{w}$	third instar wandering larva
LOF	loss of function
min	minute
MIT	microtubule interacting and transport
ml	milliliter
mRNA	messenger ribonucleic acid
N-terminal	amino-terminal
NMJ	neuromuscular junction
P	parental
$p150^{Glued}$	the 150-kD polypeptide subunit of the vertebrate dynactin complex encoded by a gene, *Dynactin 1*, that shares homology with the Drosophila gene *Glued*.
qPCR	quantitative polymerase chain reaction
RNAi	ribonucleic acid interference
RNAi2	a second independent genetic line of RNAi
RT-PCR	reverse transcription polymerase chain reaction
s	second
siRNA	small interfering ribonucleic acid

SLC4A5	Solute Carrier Family 4 (Sodium Bicarbonate Cotransporter), Member 5 gene
spas	gene symbol for *spastin* allele designations
SPAST	human *spastin* gene
spastin	Drosophila or human *spastin* gene
Spastin	Drosophila or human Spastin protein
SPG	spastic paraplegia
T32 EP	an EP element (P element derivative) used to create deletions in the Drosophila *spastin* gene
Tb	phenotypic marker ‘tubby’
TM	transmembrane
TM6B	Third Multiple Six, B structure
UAS	upstream activating sequence
VDRC	Vienna Drosophila RNAi Center
Walker A	protein sequence motif
Walker B	protein sequence motif
WT	wild type
X-HSP	X-linked Hereditary Spastic Paraplegia
Y2H	yeast two-hybrid

CHAPTER 1 - Introduction and Background

1.1. Hereditary Spastic Paraplegia

Hereditary Spastic Paraplegia (HSP) is a designation for a group of clinically and genetically heterogeneous neurodegenerative disorders predominantly characterized by progressive spasticity and weakness of the lower extremities. HSP can exclusively impair locomotion, or cause additional symptoms—e.g., seizure activity and cognitive dysfunction (reviewed in Fink, 2000). Estimates of HSP prevalence vary from 1.2 to 9.6 per 100 000 people (Fink, 2013). Like many aspects of the disorder, age of onset has been shown to be highly variable, with symptoms beginning at any age. HSP is caused by the selective degeneration of the distal segments of the longest spinal cord axons (reviewed in Sherwood et al., 2004). There is no known cure for HSP, due largely to the genetic heterogeneity of the disorder and to a lack of knowledge of how the numerous HSP-associated genetic mutations lead to common neuropathological defects and behavioural phenotypes.

1.2. History of HSP

Adolf Strümpell published the first documented case of HSP in 1880. Strümpell described two brothers diagnosed with spastic paraplegia whose father was deemed “a little lame” (Strümpell, 1880; reviewed in McDermott et al., 2000). This mode of inheritance implied a hereditary nature of the disorder, specifically autosomal dominant transmission. Both Strümpell and Lorrain published similar reports in the following two

decades (Lorrain, 1898; Strümpell, 1893), thereby giving rise to an alternate name for HSP— Strümpell-Lorrain syndrome.

1.3. HSP Heterogeneity

The heterogeneity of HSP became increasingly apparent as the prevalence of HSP reports began to increase in the literature (see McDermott et al., 2000 for review). For example, Pratt (1967) proposed that neurological symptoms above and beyond those originally described by Strümpell and Lorraine be deemed HSP plus syndromes. Subsequently, many HSP researchers began a collective effort to identify and define so-called "pure" HSP, more consistent with the original findings of Strümpell; however, opinions regarding classification criteria varied (Bell & Carmichael, 1939; Bickerstaff, 1950; Holmes & Shaywitz, 1977; Ozsváth, 1968).

In 1981, Harding used data gleaned from a thorough clinical analysis of 22 HSP families to develop the currently adopted criteria and nomenclature for classifying distinct subtypes of HSP. According to the new criteria, pure HSP includes those cases in which the most salient clinical observation is lower limb spasticity due to corticospinal tract dysfunction. Alternatively, those cases with additional neurological symptoms—e.g., deafness, dementia, epileptic activity, and peripheral neuropathy—above and beyond those found in pure HSP are referred to as complicated HSP.

Harding (1981) also originally subdivided pure HSP further into subcategories based on the age of onset (AOO) of the initial symptoms. Specifically, symptoms with an AOO <

35 years of age were classified as type I pure HSP, and symptoms with an AOO > 35 years of age were classified as type II pure HSP. HSP patients flanking the 35-year threshold seemed to display different disease severity and temporal dynamics. Patients with early onset type I pure HSP appeared to have a variable disease time course with slowly evolving symptom severity, whereas those with late onset type II pure HSP displayed an accelerated time course with the appearance of additional symptoms more consistent with a complicated HSP designation—i.e., muscle weakness, and urinary and sensory dysfunction (Harding, 1981; reviewed in McDermott et al., 2000). In spite of Harding's categorical efforts, there still remained HSP patients and families that did not fit easily into the HSP classification system. In fact, these categorical anomalies are now so abundant that the type I versus type II pure HSP distinction is no longer employed in modern neurological practice. Rather, HSP symptoms are now shown to be initially manifest at any age along the continuum from childhood to senescence (Fink, 2000). There are currently greater than 50 genetic types of HSP, with the majority of types being designated by their genetic loci—*"Spastic Paraplegia" (SPG) 1-56*—numbered in order of their discovery (reviewed in Fink, 2013).

Like many aspects of the disorder, the progression of HSP has been observed to be highly variable, including periods of either disorder stabilization—i.e., functional plateauing—or insidious progression (reviewed in Fink, 2013). Early childhood onset HSP is typically characterized by this "relatively non-progressive course" and is most commonly associated with *atlastin* mutations—SPG3A HSP—although it has also been associated with *spastin* mutations—SPG4 HSP (Bien-Willner et al., 2006; de Bot et al., 2010;

reviewed in Fink, 2013). In general, AOO can be highly variable even within a genetic subtype of HSP, although differences emerge when contrasting average AOO between genetic subtypes—e.g., ~6 years for SPG3A HSP versus ~29 years for SPG4 HSP (Fink, 2000).

HSP heterogeneity is further exacerbated by its variable modes of genetic transmission—i.e., autosomal dominant (AD-HSP), autosomal recessive (AR-HSP), or X-linked (X-HSP)—with AD-HSP being most common (Fink, 2000; reviewed in Schüle and Schöls, 2011).

1.4. Neuropathology

The predominant neuropathological feature of HSP is the progressive degeneration of axons within the longest descending and ascending nerve tracts—i.e., the corticospinal tract and dorsal column, respectively—within the human spinal cord (Behan & Maia, 1974). Postmortem analyses consistently show that axonal degeneration is maximal at the distal ends of the corticospinal tract—i.e., the thoracic spinal cord—and at the distal ends of the *fasciculus gracilis* dorsal column fibers—i.e., the cervico-medullary spinal region (Behan & Maia, 1974; Fink, 2002, 2013). Degeneration of spinocerebellar tract fibers has also been described in addition to these canonical neuropathological findings (Bruyn, 1992). Although not as prevalent, reduced numbers of large cortical motoneurons in layer V of the primary motor cortex—i.e., Betz cells—have also been reported in HSP cases (Behan & Maia, 1974; Harding, 1993). In addition, although HSP neurodegeneration has frequently been associated with abnormalities in myelination, the loss of myelin seems to

be consistent with the degree of axonal degeneration, as opposed to a primary demyelination mechanism (Behan & Maia, 1974; Harding, 1993; Schwarz & Liu, 1956).

Any proposed pathophysiological mechanism must account for why the longest neurons of the spinal cord are selectively affected. Support is being garnered for a theory involving microtubule dynamics and axonal transport due to the notion that defects in these processes would selectively affect the distal portions of the longest axons (reviewed in Salinas et al., 2008). According to this theory, most neuropathological observations seem to converge on the same mechanism, centered upon defects in the trafficking of organelles and other intracellular cargoes along microtubules. For example, the mitochondrial abnormalities associated with HSP would affect the adenosine triphosphate (ATP)-dependent transport along the microtubules of long spinal neurons (measuring up to 1 m in length), which would diminish the effectiveness of cargo delivery to distal nerve terminals. It stands to reason that longer axons would be impacted to the greatest extent by such trafficking defects due to the longer transport distance. Logically, the combination of long transport distances and trafficking should impact both anterograde and retrograde transport. Evidence has begun to accumulate implicating retrograde transport defects and the accumulation of cargoes—e.g., organelles and endosomes—at terminal boutons as potential causative mechanisms of HSP (e.g., Holbert et al., unpublished; Lloyd et al., 2012; Rugarli & Langer, 2006).

1.5. Clinical Features

Due to the extensive heterogeneity of HSP as well as its considerable overlap with other disorders sharing various clinical syndromes, it has proven problematic to determine if a collection of syndromes should be included under the category of HSP. Thus, the differential diagnosis of HSP is one of exclusion, in which other diagnoses such as amyotrophic lateral sclerosis, multiple sclerosis, primary lateral sclerosis, and vitamin B12 deficiency are systematically ruled out (reviewed in Fink, 2000). Although the axonal degeneration of the corticospinal tracts and dorsal columns is consistently found during postmortem examinations of HSP patients, HSP is determined predominantly by a combination of clinical and genetic characteristics, as opposed to neuropathological features (reviewed in Fink, 2013).

The onset of HSP can be quite subtle, identified only by leg stiffness or atypical wear of shoe soles (Harding, 1993). The majority of HSP patients present with gait impairment resulting primarily from lower limb spasticity, and often weakness (Fink, 2013). Although those diagnosed with pure (uncomplicated) HSP generally have preserved upper limb functionality—e.g., dexterity, strength, and coordination—and have a normal life expectancy (Fink, 2013), additional pure HSP symptoms can include, e.g., reduced vibration sense and urinary symptoms (Durr et al., 1994; Fink, 2003; reviewed in Salinas et al., 2008). As mentioned previously, complex (complicated) HSP includes those symptoms associated with pure HSP plus a host of additional symptoms such as peripheral neuropathy, cognitive impairment, ataxia, neuroimaging abnormalities, and additional neurologic and systemic abnormalities (reviewed in Fink, 2013).

1.6. Mutation Frequencies and Molecular Convergence

Due to the overwhelming heterogeneity of the disorder, studies dealing with HSP typically have somewhat limited power in terms of the conclusions that can be drawn. This is primarily due to the multitude of genetic influences—many with indeterminate function (see Fink, 2013 for review). The specifics of how these many genes contribute to a common motor phenotype—in terms of their molecular functionality and the effect of mutations—remains largely unknown. This thesis work is designed to circumvent the issue of HSP heterogeneity by two simple, yet powerful, approaches.

The first approach is to focus on the most frequent causes of HSP in order to maximize potential translation to patient care. HSP is most commonly associated with mutations in the gene *spastin* (*SPAST*), which account for approximately 40% of pure AD-HSP cases (Fink, 2000), and approximately 40 to 50% of all AD-HSP cases (Hazan et al., 1999). Mutations in the gene *atlastin* (*ATL1*) are the second most prevalent, have been associated with approximately 10% of pure AD-HSP, and are the most common cause of early childhood onset AD-HSP (Namekawa et al., 2006). These two genes were selected as two of the three genes of interest for further investigation due to their high prevalence relative to the other genetic mutations associated with HSP.

The second approach focuses on circumventing HSP heterogeneity by looking at common behavioural phenotypes resulting from different genotypes. In spite of the multitude of genes that have been implicated in HSP, their putative functions can be grouped into three broad categories: a) genes involved in neuronal recognition and

myelination, b) genes affecting axonal trafficking, and c) genes influencing mitochondrial function (Reid, 2003; Rugarli & Langer, 2006) (see Supplementary Figure 1). These three groups of HSP genes appear to converge further downstream upon microtubule dynamics, which appears to be a key feature of the neuropathology of HSP. Gould and Brady (2004) discuss this molecular convergence and how mutations in the various genes implicated in HSP could collectively produce a similar array of pathological features—e.g., the canonical dying back neuropathy characteristic of HSP. This rationale was used to select the third gene of interest *DCTN1*, and its Drosophila homologue, *Glued*. *DCTN1* has not been previously associated with HSP, but was recently linked to the disorder by the Rouleau laboratory (Holbert et al., unpublished) using clinical data to implicate *DCTN1* as a central component in a HSP protein interactome.

1.7. Gene of Interest 1: *SPAST*

HSP due to *SPAST* mutations is categorized as pure (uncomplicated) autosomal dominant (Hazan et al., 1994; Hazan et al., 1999; Hentati et al., 1994), although additional symptoms such as ataxia, dementia, lower motor neuron involvement, memory impairment, and seizure activity have been described (Fink, 2000). Clinical progression of *SPAST*-associated HSP typically worsens implacably. Genetic penetrance in AD-HSP associated with *SPAST* mutations has been estimated at 85-90% (Durr et al., 1996; Fink, 2000). SPAST-associated HSP has also been shown to be age-dependent with a positive correlation between AOO and disorder severity (Durr et al., 1996).

SPAST has been mapped to the *SPG4* locus on chromosome *2p21-p24* (Hazan et al., 1994). *SPAST* encodes the protein Spastin, which is a 616-amino acid (aa), 67.2-kilodalton (kDa) member of the AAA family of proteins (Hazan et al., 1999). These AAA proteins consist of an approximately 250-aa conserved AAA domain containing Walker A and B ATP-binding motif sequences (for review, see Confalonieri & Duguet, 1995). In Spastin, the AAA domain extends from residue 374 to 510 (Salinas et al., 2007). Spastin also contains two nuclear localization sequences—from residues 4 to 11 and from 309 to 312—as well as a nuclear export signal from residues 50 to 87 (see Supplementary Figure 2 for a schematic of Spastin domain structure). Many AAA proteins, including Spastin (Pantakani et al., 2008), oligomerize into hexameric rings, thereby enabling catalytic activity (Vale, 2000). Spastin belongs to the "meiotic" subcategory of AAA proteins (Frohlich, 2001), which are implicated in vesicle trafficking and microtubule dynamics. In vitro evidence now shows that Spastin uses the energy gained from ATP hydrolysis to disassemble and sever microtubules (Evans et al., 2005; Roll-Mecak & Vale, 2005). It has been proposed that the microtubule-severing activity is enabled by the positively-charged central pore of the hexameric ring of Spastin, which is able bind to the carboxy-terminal (C-terminal) tail of tubulin, inducing a conformation change leading to microtubule breakage (Roll-Mecak & Vale, 2008; White et al., 2000). Spastin also consists of an amino-terminal (N-terminal) hydrophobic region (residues 49-80) as well as a microtubule interacting and transport (MIT) domain (residues 116-194) (Sanderson et al., 2006). The microtubule-severing ability of Spastin is not abolished by the deletion of the MIT domain (White et al., 2007), but is enhanced by its presence (Solowska et al., 2008).

Hazan and colleagues (1999) employed sequence analysis in SPG4-linked pedigrees to show several SPG4 locus DNA alterations such as missense, nonsense, and splice-site mutations. In fact, greater than 150 HSP-associated *SPAST* mutations have been identified to date (Salinas et al., 2008). These loss of function (LOF) mutations, predominantly missense and truncating, affect the conserved ATPases associated with diverse cellular activities (AAA) domain of the Spastin protein (Salinas et al., 2007). HSP-causing *SPAST* mutations have also been shown to include exon deletions—i.e., gene rearrangements (Beetz et al., 2006). Additionally, neuronal *SPAST* mutations seem to be haploinsufficient in that heterozygous splice-site mutations are sufficient to produce the full spectrum of clinical symptoms (Svenson et al., 2001a; Svenson et al., 2001b).

Substantial insight into the function of Spastin has been gleaned over the past decade and a half (reviewed in Salinas et al., 2008). The microtubule-severing activity of Spastin has now been confirmed by a combination of cell culture experiments (Errico et al., 2002), *Drosophila melanogaster*—hereinafter referred to as Drosophila—larval immunohistochemistry (Trotta et al., 2004), and in-vitro assays (Evans et al., 2005; Roll-Mecak & Vale, 2005; Salinas et al., 2005). This activity is abolished by mutations in the conserved AAA domain, the same mutations found in human HSP patients (Errico et al., 2002; Evans et al., 2005; Salinas et al., 2007). In motor neurons, Spastin is enriched within intracellular domains with dynamic microtubules such as the distal axon regions (Errico et al., 2004). Studies that genetically manipulate *spastin* expression in rat hippocampal neurons show the involvement of Spastin in axonal branch formation via microtubule-severing activity (Yu et al., 2008). Additionally, mutations in *spastin* may

also affect microtubule bundling (Salinas et al., 2005; Salinas et al., 2007). In the current thesis work, the function of *spastin* will be investigated by studying its homologous counterpart in Drosophila, which will be discussed in detail below.

1.8. Gene of Interest 2: *ATL1*

Like SPG4 HSP, HSP associated with *ATL1* mutations is categorized as pure (uncomplicated) autosomal dominant (Hazan et al., 1993; Paternotte et al., 1998; Zhao et al., 2001), although lower motor neuron axonal neuropathy resulting in distal wasting has also been described (Fink, 2000). Early-onset HSP is associated with *ATL1* mutations and typically exhibits a relatively non-progressive disease course, which contrasts with the inexorable clinical progression associated with late-onset HSP (Zhao et al., 2001). Like SPG4 HSP, the penetrance of *ATL1*-associated HSP is estimated to be quite high at approximately 90% (Fink, 2003).

ATL1 has been mapped to the *SPG3A* locus on chromosome *14q11-q21*, and includes 14 exons spanning approximately 69 kilobases (kb) (Hazan et al., 1993; Paternotte et al., 1998; Zhao et al., 2001). *ATL1* encodes the atlastin (isoform 1) protein (hereinafter referred to as Atl-1) which is a 558 aa, ~63.5-kDa member of the dynamin superfamily of large GTPases, and shows substantial homology to human guanylate binding protein 1 (GBP1) amongst other interspecies GBPs (Sever et al., 1999; Zhao et al., 2001). The secondary structure of Atl-1 was originally modeled after GBP1, and was thought to include a conserved N-terminal GTPase domain followed by a helical domain (Zhao et al., 2001). The GTPase domain has been shown to extend from residue 30 to 338 (see

Supplementary Figure 3 for a schematic of Atl-1 domain structure). The helical domain of Atl-1 is now interpreted as a flexible linker (residues 337 – 346), which is sequentially followed by a middle domain (residues 346 – 449) and two transmembrane helices (residues 449 – 494) embedded within the endoplasmic reticulum (ER) membrane, leaving the N-terminal GTPase and C-terminal domains exposed to the cytosol (Byrnes & Sondermann, 2011; Sanderson et al., 2006; Zhu et al., 2003).

At least five *ATL1* mutations have been identified that cause pure-AD HSP (Sauter et al., 2004). Additionally, as of 2008, human clinical studies revealed mutations in 20 independent aa's of *ATL* (Namekawa et al., 2006; Zhao et al., 2001). Disease-causing *ATL1* mutations were originally found in exon 7 and 8 (Zhao et al., 2001), but have also been identified at a high frequency in exon 4 and 12 (Namekawa et al., 2006). Mutations in exons 4, 7, and 8 affect the Atl-1 region containing the conserved GTPase domain, whereas mutations in exon 12 affect the Atl-1 region containing the middle domain as well as both transmembrane domains (Byrnes & Sondermann, 2011). In addition, many different *SPG3A* polymorphisms have been described (Sauter et al., 2004).

HSP-associated mutations in the *SPG3A* locus are thought to alter surface amino acids on the globular N-terminal region of the putative Atl-1 protein, which contains the conserved GTPase domain (Zhao et al., 2001). Zhao and colleagues proposed that HSP pathogenesis could be induced by these mutations by disrupting the secondary structure involved in GTPase activity, the interactions between Atl-1 and other proteins—e.g., Spastin—or multimerization function (Schwemmle et al., 1995; Sever et al., 1999).

Atl-1 is primarily involved in ER and Golgi morphogenesis, which is consistent with its intracellular localization mainly to the *cis*-Golgi apparatus and, to a lesser degree, the ER (Namekawa et al., 2007; Zhu et al., 2003; Zhu et al., 2006). The role of Atl-1 in ER and Golgi morphogenesis potentially stems from Atl-1-dependent microtubule disassembly, perhaps through its interaction with Spastin (Lee et al., 2009). The close association of microtubules and ER tubules in mammalian cells strengthens this notion (Waterman-Storer & Salmon, 1998). Atl-1 was originally implicated in intracellular membrane trafficking, predominantly at the interface of the ER and Golgi, due its homology to other members of the dynamin superfamily of large GTPases (Namekawa et al., 2007); however, it has been reported that the atlastin family of proteins does not seem to be involved in anterograde trafficking from the ER to Golgi (Rismanchi et al., 2008). Data from electron microscopy experiments shows that Atl-1 is located on membranes that are being transported along microtubules (Rismanchi et al., 2008).

At the neuronal level, Atl-1, like Spastin has been shown to be enriched in growth cones and is implicated in promoting axonal elongation during neuronal development (Zhu et al., 2006). The role of Atl-1 in neuronal development has been suggested as a potential explanation as to why the onset of SPG3A HSP is so early (Salinas et al., 2008); however, considering the comparatively late onset of other developmentally associated mutations such as in SPG4 HSP, more information is required before such explanations can be validated. In the current thesis work, the function of *ATL1* will be investigated by studying its Drosophila homologue, which will be discussed in detail below.

Further insight into the HSP-specific function of Atl-1 can be gleaned by considering the functions of its overarching dynamin superfamily of large GTPases as a whole. These proteins have been implicated in such processes as vesicle trafficking, synaptic function, maintenance and distribution of mitochondria, and association with microtubules and other cytoskeletal elements (Ochoa et al., 2000; Pitts et al., 1999; reviewed in Zhao et al., 2001). Interestingly, many of these processes have been implicated in HSP neuropathogenesis.

1.9. Gene of Interest 3: *DCTN1*

In contrast to the previous two genes of interest—i.e., *SPAST* and *ATL1*—that were selected primarily based on their mutation frequency in HSP patients, *DCTN1*— i.e., *Dynactin 1*, a gene that encodes dynactin-1, also known as $p150^{Glued}$—has only recently been shown to be mutated in patients with AD HSP (Holbert et al., unpublished). *DCTN1* mutations have been implicated in several neurodegenerative disorders such as distal hereditary motor neuropathy, amyotrophic lateral sclerosis (ALS), and Parkinson's disease (Munch et al., 2004; Munch et al., 2005). The penetrance of *DCTN1* mutations has been shown to be high in some disorders, such as Perry syndrome (Farrer et al., 2009), but not in others, such as ALS (Munch et al., 2004).

DCTN1 has been mapped to the *DCTN1-SLC4A5* locus (Pushkin et al., 2001) on chromosome *2p13.1* (Holzbaur & Tokito, 1996). The *DCTN1-SLC4A5* locus comprises approximately 230 kb of DNA, consists of approximately 66 exons, and separately encodes both a cytoplasmic ($p150^{Glued}$) and a membrane protein—Solute Carrier Family 4

Member 5 (SLC4A5) (Pushkin et al., 2001). Within this locus, the *DCTN1* gene comprises 19.4 kb of DNA and consists of approximately 32 exons (Collin et al., 1998). In an unpublished manuscript, Holbert and colleagues reported three novel *DCTN1* missense mutations, identified in cases of HSP. Two of these mutations result in an increased affinity $p150^{Glued}$ for the HSP-associated protein spatacsin, mutations of which underlie SPG11 HSP. The third *DCTN1* missense mutation results in an increased affinity of $p150^{Glued}$ for Golgi resident protein GCP60, which interacts with the HSP-associated protein spartin (SPG20 HSP). Interestingly, the three novel *DCTN1* missense mutations were identified within two coiled-coil domains that have been implicated in the binding of $p150^{Glued}$ to the dynein intermediate chain (Deacon et al., 2003). *DCTN1* mutations could thus potentially disrupt the interaction between the dynactin complex and cytoplasmic dynein, thereby diminishing the function of dynein in retrograde transport along microtubules. This notion has novel implications regarding HSP considering that the neuropathogenesis of HSP frequently converges upon microtubule dynamics and axonal transport.

As previously mentioned, *DCTN1* encodes the polypeptide $p150^{Glued}$, a 50-kDa polypeptide and the largest of the 10 distinct polypeptide subunits of the dynactin multiprotein complex (Gill et al., 1991; Paschal et al., 1993; Schafer et al., 1994). The dynactin complex enhances the cellular function of cytoplasmic dynein, a microtubule-based, minus-end, motor ATPase involved in retrograde transport of intracellular cargos—e.g., vesicles and organelles—along microtubules (Holzbaur & Vallee, 1994; Zhapparova et al., 2009). The $p150^{Glued}$ polypeptide directly binds to microtubules as well

as dynein (Zhapparova et al., 2009). Furthermore, the interaction between the $p150^{Glued}$ subunit of the dynactin complex and cytoplasmic dynein likely plays a key function in the retrograde axonal transport of intracellular cargos such as vesicles and organelles (Holzbaur & Tokito, 1996). Mammalian $p150^{Glued}$ consists of an N-terminal microtubule binding domain, a dynein intermediate chain binding domain, and an Arp-1 binding domain, which are involved in microtubule interactions, dynein interactions, and dynactin complex formation, respectively (Allan, 1996; King et al., 2003; Vallee et al., 2012) (see Supplementary Figure 4 for a schematic of $p150^{Glued}$ domains). Notably, one of the intracellular regions where a high concentration of $p150^{Glued}$ is observed is at growing microtubule plus-ends (Ligon et al., 2003), which coincides with the intracellular locations of both Spastin and Atl-1, thereby providing an interesting convergence point for a potential neuropathological mechanism of HSP.

1.10. Drosophila Homologues: *D-spastin*, *D-atl*, and *Glued*

1.10.i. *D-spastin*

Importantly, several of the genes that have been implicated in HSP, including *spastin*, maintain a large degree of evolutionary conservation between humans and Drosophila (Sherwood et al., 2004). For example, the AAA domains of human and Drosophila *spastin* (*D-spastin*) are 67% identical (Sherwood et al., 2004). Like human Spastin, D-Spastin contains an N-terminal domain (residues 1-225), a MIT domain (residues 225-311), and an AAA domain (residues 464-758) (see Supplementary Figure 2 for a schematic of D-Spastin domain structure). The D-Spastin AAA domain is divided into a

nucleotide-binding domain and a four-helix bundle domain, which is similar to other AAA proteins (Roll-Mecak & Vale, 2008). Additionally, D-Spastin contains a putative transmembrane (TM) domain between residues 100 and 200 (Roll-Mecak & Vale, 2005), which corresponds to the hydrophobic region in human Spastin. Du and colleagues (2010) demonstrated that human Spastin and D-Spastin are also conserved functionally by showing the equivalent rescue of behavioural and cellular defects resulting from the exogenous expression of either wild type (WT) D-Spastin or human Spastin in *D-spastin* null flies. The behavioural data involved the analysis of fly movement in an enclosed Petri dish, and the cellular data involved third instar larva neuromuscular junction (NMJ) immunohistochemistry. Mutations in *D-spastin* have been shown to affect synapse morphology and function, bouton formation, and the stability of microtubule networks (Sherwood et al., 2004; Trotta et al., 2004). Evidence that *D-spastin* is an essential gene—i.e., a gene that is indispensible for cellular viability—has been gleaned from the pupal lethality of ubiquitous expression of *D-spastin* ribonucleic acid interference (RNAi), although not from pan-neural RNAi expression of null mutations (Sherwood et al., 2004; Trotta et al., 2004). Important to the locomotion-centric behavioural paradigm used in the current group of experiments is that *D-spastin* null mutants have been reported to exhibit severe locomotion impairments similar to the motor weaknesses displayed by HSP patients (Sherwood et al., 2004). In flies, these impairments manifest as an inability to fly or jump, impaired climbing ability, and dragging of the hind limbs. These impairments correspond to the gait impairments and lower limb weaknesses observed in human HSP patients. In contrast, the progression of these impairments with

age, reflecting the neurodegenerative aspect of HSP, has been less well characterized in Drosophila literature.

1.10.ii. *D-atl*

In humans, there exist three members of the atlastin subfamily—Atl-1, Atl-2, and Atl-3—with Atl-1 highly expressed in neural tissues (Rismanchi et al., 2008; Zhu et al., 2003). Contrastingly, Drosophila has a sole ortholog of the mammalian atlastins, D-Atl (Lee et al., 2008). Importantly, D-Atl has high sequence homology with each of the human isoforms, sharing 44% - 49% aa identity, and 61% - 68% aa similarity throughout the full length of the protein. Additionally, this homology is increased after excluding the variable N-terminal and C-terminal regions (Moss et al., 2011). D-Atl is similar to human Atl-1 both in terms of overall length (541 versus 558 aa, respectively) and domain composition (see Supplementary Figure 3 for a schematic of D-Atl domain structure). Also, of the 20 amino acids of *atlastin* that are mutated among human HSP patients, 16 are conserved in *D-atl* (Lee et al., 2008). The human and Drosophila homologues of Atl are also functionally conserved in that they both appear to be involved in ER and Golgi morphogenesis as well as ER membrane trafficking. In fact, D-Atl has been shown to be required for the homotypic fusion of ER membranes and its loss has been associated with ER fragmentation (Orso et al., 2009). In addition, *D-atl* null mutations have been implicated in the age-dependent degeneration of dopaminergic neurons, and the gene has been shown to be essential due to the homozygous lethality of null mutants (Lee et al., 2009). Curiously, *D-spastin* and *D-atl* mutants share extensive phenotypic overlap

including short life spans, locomotor impairments, and age-dependent neurodegeneration (Lee et al., 2008; Orso et al., 2005; Sherwood et al., 2004).

1.10.iii. Glued

The 150-kD polypeptide subunit of the vertebrate dynactin complex was originally sequenced by the characterization of rat complementary DNA (cDNA) clones, and was found to exhibit 31% aa sequence identity and 53% aa similarity relative to the product of the Drosophila gene *Glued* (Holzbaur et al., 1991; Swaroop et al., 1987) (see Supplementary Figure 4 for a schematic of Glued domain composition). The mammalian polypeptide was then aptly termed $p150^{Glued}$ (Paschal et al., 1993). The *Glued* gene was originally identified by the dominant $Glued^{1}$ (Gl^{1}) mutation in Drosophila (Plough & Ives, 1935), which results in severe structural and functional defects in the Drosophila visual system (Garen & Kankel, 1983; Harte & Kankel, 1982; Meyerowitz & Kankel, 1978). Harte and Kankel (1982) employed gene dosage studies to determine that the Gl^{1} mutation is dominant due to a gain of function, as opposed to haploinsufficiency. In fact, the Gl^{1} mutation has now been identified as a dominant negative allele. The Gl^{1} mutation results in a truncated polypeptide that 'poisons' normal dynactin complex function, potentially by disrupting the binding of cargo to the dynein/dynactin complex via an interaction with another dynactin protein subunit, actin-related protein 1 (Arp-1) (Fan & Ready, 1997; Schroer, 1996; Swaroop et al., 1985; Waterman-Storer et al., 1995). Additionally, the *Glued* gene has been shown to be essential for cell viability due to the embryonic lethality of null mutations as well as the lack of recovery following somatic recombination techniques (Harte & Kankel, 1982).

1.11. Genetic Interactions

In a recent manuscript, Holbert and colleagues employed a combination of a yeast two hybrid (Y2H) screen, Western blot co-immunoprecipitation (Co-IP), and clinical data to implicate $p150^{Glued}$ as a central component in a protein interaction network involving several HSP proteins. Spastin and Atl-1 are two of the proteins that have been shown to interact with $p150^{Glued}$, either directly or indirectly (Holbert et al., unpublished), thereby providing a link between the three genes of interest for the current thesis work. Specifically, Holbert and colleagues used Western blotting with $p150^{Glued}$ antibodies directed against Atl-1 immunoprecipitates to confirm the interactions between these two proteins.

The interaction between Spastin and Atl-1 in both humans and Drosophila has been previously established (Evans et al., 2006; Lee et al., 2009; Sanderson et al., 2006), which further supports the inference that these genes are involved in a common HSP-causative neuropathogenic mechanism (refer to Sanderson et al., 2006, Figure 6, for a schematic of the Spastin*Atl interaction). Evans and colleagues (2006) employed a Y2H screen that identified Atl as a Spastin interactor; however, this interaction does not modify the enzymatic function of Spastin. Importantly for the pathogenesis of HSP, this group identified a clinically relevant mutation in Atl, found outside the GTPase domain, which is thought to prevent the proper intracellular interaction with Spastin. Specifically, this group showed that deleting the C-terminal region of human Atl—i.e., residue 496 to the stop codon—abolished the interaction of the resulting Atl transcript with full-length human Spastin. Sanderson and colleagues (2006) also employed a Y2H screen to identify

the interaction between Spastin and Atl, which was subsequently confirmed by Co-IP, and experiments involving glutathione S-transferase (GST) pull-down and co-localization. This group identified the N-terminal region of Spastin (residues 1-80) as the domain required for the Spastin*Atl-1 interaction. This region of Spastin contains a hydrophobic region (residues 49-80) that is only found in the less abundant, full-length, cytoplasmic isoform of Spastin (Claudiani et al., 2005; Sanderson et al., 2006). Human Spastin also contains a stronger second initiation codon just upstream of the MIT domain, which expresses an N-terminal truncated isoform of Spastin that is both nuclear and cytoplasmic (Claudiani et al., 2005; Salinas et al., 2005; Sanderson et al., 2006). Interestingly, it is the full-length isoform of Spastin that is selectively enriched in the human central nervous system (CNS) (Claudiani et al., 2005). Thus, it would seem that an interaction between Atl and the full-length CNS-enriched isoform is important for HSP neuropathology (Claudiani et al., 2005; Evans et al., 2006).

While the interactions in the literature have primarily involved the demonstration of binding partners, the current thesis work intends to expand upon these findings by investigating whether or not corresponding genetic interactions—i.e., the synergistic effect of a combination of two or more genetic manipulations—between the Drosophila homologues of these proteins also exist. To test this, we will investigate the manifestations of these genetic interactions from a behavioural standpoint using Drosophila.

1.12. Drosophila Locomotion and Behavioural Assays

Drosophila melanogaster, the species used throughout this thesis work, has proven to be a valuable research organism for a number of reasons including genetic tractability, high reproduction rate, publically available stocks, and relatively easy to measure, but complex, behavioural patterns. Drosophila locomotion behaviour, specifically, has been used as a means of quantifying and modeling a number of human neurodegenerative disorders (e.g., Hirth, 2010) including HSP (Orso et al., 2005; Sherwood et al., 2004). Drosophila behavioural assays have been employed as a viable research method for over a century (Carpenter, 1905; Manning, 1964), and enable the analysis of a wide variety of behavioural responses including, but not limited to, geotaxis, phototaxis, spontaneous and cued locomotion, and sensory analyses (Benzer, 1967; Dobzhansky & Spassky, 1962; Hadler, 1964; Hirsch, 1962; Vang et al., 2012).

Consequently, Drosophila locomotion was selected in this thesis work as a means of modeling HSP by assaying the cued behaviour of adult flies via a startle-induced negative geotaxis assay (also known as a climbing assay), the spontaneous behaviour of adult flies via a passive walking assay, and the extent of larval locomotion via a larva tracking assay.

1.13. RNAi and Overexpression: the GAL4-UAS System

One of the primary goals of this thesis work is to determine if genetic mutations or altered gene expression of the three genes of interest result in measurable behavioural deficits. A relatively straightforward and effective means of testing this genetic-behavioural link is to employ the GAL4-upstream activating sequence (UAS) system (Brand & Perrimon, 1993) in conjunction with RNAi-based knockdown or gene overexpression techniques. In a landmark study in 1988, Fischer and colleagues demonstrated that expression of GAL4 protein—a yeast-derived transcriptional activator—is capable of stimulating the transcription of a reporter gene under the control of an UAS in Drosophila (see Duffy, 2002, and St. Johnston, 2002, for a detailed review of the GAL4-UAS system). Since then, the *GAL4* gene has been incorporated into numerous positions in the Drosophila genome, thereby generating 'enhancer-trap' genetic lines that permit tissue-specific directed gene expression via the control of nearby enhancers (St. Johnston, 2002). With regard to the experimental paradigms within this thesis work, we employed the GAL4-UAS targeted gene expression system to drive the pan-neural expression of various UAS reporter lines by using the neural-specific embryonic lethal, abnormal vision (Elav)-GAL4 driver (Luo et al., 1994). Specifically we used *Elav-GAL4/UAS-RNAi* lines to direct RNAi-mediated knockdown of *spastin*, *atlastin*, or *Glued* messenger RNA (mRNA) pan-neurally. Alternatively, we employed *Elav-GAL4/UAS-(target gene)* lines to drive the pan-neural overexpression of *spastin* or *atlastin*.

1.14. Experimental Hypotheses

The current thesis work has been divided into two operating experimental hypotheses. These hypotheses are both behavioural in nature and are fundamentally similar in terms of the methods used to test them, differing primarily in terms of the scientific questions being asked and in subsequent interpretation.

> *Experimental Hypothesis 1*: There is a behavioural component associated with HSP genes *SPAST*, *ATL1*, and *DCTN1* that can be identified using genetic manipulations—i.e., mutations and altered gene expression—of Drosophila homologues.

> *Experimental Hypothesis 2*: The Drosophila homologues of the three HSP genes of interest—i.e., *spastin*, *atlastin*, and *Glued*—interact genetically to produce more severe behavioural defects.

To test these hypotheses experimentally, we employed various behavioural assays—i.e., climbing, walking, and larval locomotion—to determine if mutations in single genes (Experimental Hypothesis 1) or a combination of genes (Experimental Hypothesis 2) resulted in a measurable locomotion phenotype in Drosophila. The mutant flies selected to test these experimental hypotheses were heterozygous mutants (e.g., $spas^{5.75}/WT$), transheterozygous mutants (e.g., $spas^{5.75}/spas^{17\text{-}7}$), GAL4-UAS RNAi mutants (e.g., *Elav-GAL4/UAS-spasRNAi*), or GAL4-UAS gene overexpression mutants (e.g., *Elav-GAL4/UAS-spas*).

CHAPTER 2 - Materials and Methods

2.1. Fly Maintenance

Stocks and genetic crosses were maintained on fly medium similar to the Bloomington recipe #4 described by Lewis (1960), and used at Caltech. This recipe is based primarily on cornmeal, dextrose, sucrose, yeast, and agar—also contains potassium sodium tartrate, calcium chloride dehydrate, methylparaben, and antibiotics—and has been specifically optimized for learning and memory research. Flies were segregated according to genotype and both stocks and crosses were maintained in plastic bottles containing fly medium and sealed by porous foam plugs. The developmental period from Drosophila eggs to adult flies—i.e., the eclosion period—varies as a function of factors such as bottle crowding, genotype, and temperature. Under ideal laboratory conditions, this eclosion period has been shown to be 8.5 days at 25°C, or 19 days at 18°C (Ashburner & Thomson, 1978; Ashburner et al., 2005). The developmental eclosion periods observed in our laboratory are approximately 16 days at ~21°C, 14 days at ~22°C, 12 days at ~24°C, or 9 days at ~25°C. For stock maintenance, flies were flipped into fresh bottles weekly to propagate the genetic line. With respect to genetic crosses, parental (P) flies were flipped into fresh bottles approximately every 4 days in order to prevent the first filial (F1) generation flies from mating with P flies. After 1 week, when the fly eggs had developed into third instar larvae, the bottles were stuffed with porous stuffing paper, serving to both increase the surface area upon which flies can lay eggs, and to assist in keeping the food intact as it ages. Throughout the experimentation period, environmental fluctuations

due primarily to seasonal changes resulted in varying humidity levels, with minor temperature fluctuations. In spite of efforts made to maintain environmental conditions at consistent levels—e.g., through the use of humidifiers, incubators, plastic wrap, and heaters—relative humidity levels ranged from approximately 20 to 60 %, and temperature ranged from 20 to 23°C. Fly stocks and crosses were maintained on a light/dark cycle of 8/16, respectively.

The fly stocks that were maintained for experiments often contained an allele created by a genetic mutation—e.g., a deletion mutant such as *spas*$^{5.75}$. Due to issues such as homozygous lethality and sterility, modified balancer chromosomes were used as genetic tools to enable the maintenance of these mutant fly stocks without the need for selection (Muller, 1918). Balancer chromosomes are also used as a tool to help maintain stock integrity by preventing genetic recombination, which is accomplished by a crossover suppressor—often an inversion or inversion complex—within the balancer chromosome (Zheng et al., 1999). Furthermore, balancer chromosomes are subject to homozygous lethality due to the presence of lethal recessive alleles, which simplifies the selection of the genotype of interest.

2.2. Genetic Crosses and Phenotypic Scoring

In addition to both the maintenance of homozygous lethal alleles in a transheterozygous state as well as the prevention of homologous recombination, balancer chromosomes also contain phenotypic markers—e.g., curly wings—that enable the sorting of allelic

combinations of interest during genetic crosses simply by visual inspection. For example, one particular fly stock used throughout these experiments was a *D-spastin* excision line, allele *5.75* (Sherwood et al., 2004). This allele was maintained in a fly stock with the presence of the balancer chromosome Third Multiple Six, B structure (TM6B) (Craymer, 1984). Due to the presence of the *D-spastin* gene on one copy of the third chromosome and the other copy consisting of the TM6B balancer chromosome, in this particular stock, the F1 flies produced by a cross with this stock must inherit either the *D-spastin* or the TM6B balancer chromosome, but not both. This principle is due to a combination of the Mendelian Law of Segregation and the suppression of chromosomal crossover by balancer chromosomes. Ergo, if the F1 flies are selected that lack the TM6B-associated phenotypic marker tubby (Tb, squat larvae and pupae) or Humeral (Hu, dense bristle pattern in the humeral region) dominant markers, then the selected flies will, by exclusion, have the *D-spastin* mutation of interest, provided the stock is not contaminated by a WT allele.

For sorting, flies of interest were first transferred to a hard plastic funnel pre-charged with carbon dioxide (CO_2). After a few seconds, the flies were transferred to a porous anesthetization pad infused with CO_2 and viewed using a dissecting light microscope. Manipulations were performed with a round-tipped paintbrush, while the flies were kept lightly anesthetized by using a minimal dosage of CO_2, as determined by degree of movement. Flies with the genotype of interest were sorted using phenotypic markers, and subsequently transferred to small plastic vials, sealed by a ball of cotton, containing the same fly media used for stock maintenance.

2.3. Stocks and Genetic Lines

Two WT control lines were used throughout these experiments. The first WT strain is a w^{1118} *iso-2; iso-3* isogenic balancer strain, henceforth referred to as iso^{w} (Hoskins et al., 2001).

The second WT control line is known as *2U-Cold Spring Harbor Laboratory (CSHL)—*w^{1118} outcrossed 10 times with *Canton-S* (Kuo et al., 2012). The switch in WT control lines was necessitated by a decrease in performance of the iso^{w} control stock over time. This decreased performance could have be caused by factors such as disease, mite infestation, or genetic drift.

The Sherwood laboratory graciously provided the fly stocks with deletion mutations in the gene *D-spastin* (Sherwood et al., 2004). The two *D-spastin* deletion mutants used were generated by imprecise excision of the T32 EP element (a P element derivative). In the null *D-spastin* allele, $spas^{5.75}$, the entire *D-spastin* sequence is deleted, along with 129 base pair (bp) of the adjacent gene *Rox8*. The Sherwood group was able to demonstrate through rescue experiments that the phenotypes they observed were due to D-Spastin alone. In the hypomorphic allele, $spas^{17\text{-}7}$, the deletion extends only through the first exon and ends within the second intron of the *D-spastin* gene. Importantly, the segment of DNA that encodes the region of D-Spastin protein conserved between humans and Drosophila—i.e., the AAA domain—is maintained after the $spas^{17\text{-}7}$ deletion. However, the N-terminal region corresponding to the region of human Spastin thought to interact with Atl is deleted in the $spas^{17\text{-}7}$ allele (Lee et al., 2009; Roll-Mecak & Vale, 2005).

The *Elav-GAL4* line was maintained in homozygous form in a *2U-CSHL* background. The *Elav* gene encodes a RNA-binding protein found in all neurons. When used as a promoter in conjunction with the GAL4-UAS system, this specificity permits the pan-neural expression of target genes of interest. Homozygous *Elav* females were selected when preparing genetic crosses to avoid hemizygous phenotypes, considering that the *Elav* gene is located on the X chromosome of Drosophila.

Two *D-spastin* RNAi lines were used in these experiments. The first line was procured from the Vienna Drosophila RNAi Center (VDRC) (FlyBase ID: FBst0480549). This construct contains a 508-bp inverted repeat downstream of a UAS promoter that, when expressed, forms hairpin RNAs (hpRNAs) that are processed into small interfering RNAs (siRNAs) directed against *D-spastin* mRNA, thereby providing gene-specific silencing. Of note is that this particular construct is associated with one off-target sequence, that of *D-Katanin 60*, an AAA ATPase with similar function to D-Spastin. The second *D-spastin* RNAi line was procured from the Bloomington Drosophila Stock Center (BDSC, FlyBase ID: FBst0027570) and expresses double-stranded RNA (dsRNA) directed against *D-spastin* mRNA under control of a UAS promoter. Based on the sequence alignment results from a FASTA off-target effect (OTE) analysis, this second *D-spastin* RNAi line is also associated with potential OTE's of *D-Katanin 60*.

One *atlastin* RNAi line was used in these experiments and was procured from the VDRC (FlyBase ID: FBst0470415). This construct contains a 260-bp inverted repeat downstream of a UAS promoter that, upon expression, forms hpRNAs that are processed

into siRNAs directed against *atlastin* mRNA, thereby providing gene-specific silencing. This particular construct is associated solely with its anticipated on-target sequence, with no known off-targets, making it an ideal candidate for targeted gene silencing against *atlastin*.

The Lee laboratory graciously provided the fly stock with a deletion mutation in the gene *atlastin* (Lee et al., 2009). The *atlastin* allele used, atl^2, is a null mutation—i.e., no mRNA produced—generated by imprecise excision of the P-element atl^1 (Lee et al., 2008). This deletion removes approximately 1.6 kb of DNA within the *atl* locus corresponding to exons 3 and 4 of the gene (Lee et al., 2009). Importantly, the atl^2 null mutant allele is pupal lethal, with an adult fly survival rate of only approximately 8% (Lee et al., 2009).

One *Glued* RNAi line was used in these experiments and was procured from the BDSC (FlyBase ID: FBst0027721). This genetic line expresses dsRNA directed against *Glued* mRNA under control of a UAS promoter.

For overexpression experiments, both *D-spastin* and *D-atl* overexpression lines were used. Similar to the RNAi stocks, both of these genetic lines had expression driven by a UAS promoter upstream of the target sequence. Importantly, instead of driving the expression of dsRNA directed against the mRNA of a gene of interest, thereby resulting in RNAi-based knockdown, these lines drive the expression of the gene itself. This

upregulated expression of either *D-spastin* or *D-atl* results in a level of gene activity exceeding physiological baselines—i.e., overexpression.

The fly stock with a mutation in the gene *Glued* was obtained from the BDSC (FlyBase ID: FBst0035524). The specific allele of *Glued* that was used, Gl^{1}, is an amorphic dominant negative allele formed by the insertion of a 9 kb, naturally transposable, roo element at the 3' end of the *Gl* gene, truncating the C-terminus of the resulting protein (Fan & Ready, 1997; Swaroop et al., 1985).

2.4. Virgin Female Collection

When preparing for and performing a genetic cross for experimentation, virgin females of a particular genotype were collected and allowed to mate with males of a different genotype, thereby producing a F1 generation of interest. The selection of virgin females was essential in order to avoid extraneous allelic combinations resulting from sexual interactions between unintended and undesired genotypes. The credence of this procedure is based on the observed behaviour that female flies will generally not be sexually receptive to a male mate until 10-12 hours post-pupal eclosion—i.e., after the time that they emerge from the pupa as a fully developed adult fly (Ashburner & Roote, 2007). The controlled sorting of phenotypic markers in the F1 generation then permitted the isolation the flies with the genotype of interest. Virgin females can be identified by their lack of male sexual characteristics—e.g., sex combs and male genitalia—as well as by general features such as unexpanded wings and lack of abdominal pigmentation (Ashburner & Roote, 2007).

2.5. Behavioural Assay Preparation and Age Synchronization

Adult flies—i.e., mature flies that have eclosed from their pupae—were required for two of the three behavioural assays used in this group of experiments, the walking and climbing assays. Experimenters were blinded with regard to genotype for both of these assays in order to circumvent experimenter bias. Phenotypically, the flies were not all identical at the time of the experiment, with the major phenotypic difference being eye colour. However, due to the small size of the flies, such minor phenotypic differences were not salient enough to affect experimental outcomes. For blinding, a third party coded the genotypes prior to the initialization of an experiment. Codes were broken after completion of the experiment to permit further analysis. A single rater analyzed the experimental results to avoid inter-rater variability. However, minor rater practice effects may have accumulated due to the same rater being used for an extended period of time—i.e., 2 years. Importantly, flies were used from the same source—i.e., the same genetic cross—to perform parallel experiments. For the third behavioural assay, larva tracking, larvae were selected directly from the cross bottles. Flies were housed at 22°C, yielding a 2 week life cycle.

The day that flies from a previously prepared genetic cross began to eclose from their pupae was designated Day 0 of the experimental procedure (Supplementary Figure 5). On Day 0, these F1 flies were "cleared" from emerging culture bottles via transfer into a fly morgue consisting of an Erlenmeyer flask partially filled with 95% ethanol. Clearing the flies in this way without losing the developing larvae or pupae was possible due to the relatively stronger adherence of larvae and pupae to the inside surfaces and stuffing paper

within the plastic bottles. The clearing process was typically performed in the late afternoon to early evening—i.e., 15:00 to 19:00. This late clearing period was selected to minimize ageing variability by synchronizing the developmental window of the test sample cohort as much as possible.

On the following day, Day 1, emerging flies were anesthetized with a minimal dosage of CO_2 and sorted phenotypically via a dissecting light microscope. Flies were segregated according to genotype and collected in groups of 20 per food vial for the climbing assay, and as single flies per Eppendorf tube for the walking assay. Target technical replicate values were set at 10 per genotype per condition for the climbing assay and 20 per genotype per condition for the walking assay. Thus, target values of 10 technical replicates of 20 flies (200 flies total) and 20 technical replicates of one fly (20 flies total) per genotype were set a priori for the climbing and walking assays, respectively. A mixture of male and female flies were randomly selected from the cross bottles, excluding only those flies with severe abnormalities that were not representative of the sample population.

The time necessary for sorting phenotypic markers depends not only on the number of samples being collected, but also on the particular markers being scored. For example, the aforementioned Hu marker associated with the TM6B balancer chromosome requires a considerable amount of time to score due to the microscopic and sometimes unilateral bristle pattern that must be differentiated from the WT pattern. Due to this variability, the amount of time that different genotypes were exposed to anesthetic was normalized,

albeit subjectively, in order to avoid the confounding variable of differential CO_2 exposure. After the flies were collected, the vials and Eppendorf tubes were stored at room temperature until the following morning.

The next day, Day 2, the climbing and walking assays were typically performed between 10:00 and 12:00, although multiple assays and experimental overlap occasionally necessitated that an experiment be concluded as late as 15:00. Accordingly, the flies used in the standard '2-day-old' aging condition were approximately 42 ± 3-hr old, with some as old as 48 hr.

In contrast to the climbing and walking assays, Drosophila larvae were required for the larva tracking assay—as the name suggests—specifically, wandering third instar larvae ($L3^w$). At 22°C, $L3^w$ can be collected from the bottles of genetic crosses approximately one week after eggs are laid. The third instar stage of Drosophila larvae can be subdivided into foraging (early third instar) and wandering (late third instar). The selection of $L3^w$ larvae is simplified by behavioural patterns in that foraging larvae will remain in close proximity to the food source in the bottle consuming a substantial amount of food, whereas wandering larvae can be found moving around the inside walls of the cross bottle. Using these movement and proximity criteria, $L3^w$ were selected and tracked in the larva protocol on the same day.

2.6. Climbing Assay

The climbing assay used in this collection of experiments was performed essentially as described in Palladino and colleagues' 2002 study, with some minor modifications (Supplementary Figure 6). The first adaptation we made was to arbitrarily set the target line at a height of 18.5 cm (190 ml), as opposed to the 17.5 cm (150 ml) used in the Palladino protocol. Although this difference in height is relatively minor, the increase in height was intended to increase the assay difficulty, thereby aiding in the identification of the relatively minor climbing defects. Also, the Palladino group chose to illuminate the top of the cylinder with a fiber-optic lamp, presumably to take advantage of the phototaxic response of adult Drosophila. However, based on observed unexpected fluctuations in performance, we inferred that the overhead light source might have been a source of confusion as a result of light reflection within the cylinder; thus, we opted for the diffuse, overhead, fluorescent light source instead. We also increased the sample size from 10 to 20 flies primarily to increase the throughput of the assay. Initially, we increased this number to as high as 30 flies, although it was subsequently reduced in order to minimize overcrowding and interaction effects between flies. We opted to discard the samples after a single use, as opposed to performing four repeat trials per sample, to eliminate the possibility of acute practice effects. Finally, due to an observed infinite ceiling effect for those flies with severe climbing defects, we did not record the time required for 50% of the flies to cross the target line. Rather, flies were given a duration of 2 min to cross the target line. The number of flies to cross the line was recorded and binned in increments of 10 s, and the resulting value expressed as a percentage.

For each trial, 20 flies were transferred into a 250-ml glass graduated cylinder, one per genotype, which was sealed at the top with Parafilm® M Barrier Film to prevent escape. To begin each trial, the bottom of the cylinder was repeatedly tapped against a foam pad with enough force to displace the flies to the inner bottom surface. Each trial lasted 2 min and was scored as outlined above. The resulting trial profile reflects a running maximum that we believe to be more sensitive than the method used in the Palladino protocol, especially with regard to mutants with moderate to severe locomotion defects. Upon completion of the experiment, cylinders were washed in a Laboratory Glassware Washer G 7883 from Miele Professional.

Trial videos were acquired using a JVC HD Everio GZ-E200 camera. Trial videos were played back using VLC Media Player and analyzed manually upon completion of the assay.

The climbing assay operates primarily on the principle of negative geotaxis, although positive phototaxis towards the overhead ceiling lights also plays a role, especially in the *Drosophila melanogaster* species (Kain et al., 2012). Negative geotaxis—i.e., upward vertical movement—following a startle response has been a relatively common measure of locomotion ability in Drosophila (e.g., Gargano et al., 2005; Simon et al., 2006). The climbing assay employs both of these natural behavioural responses as motivators, which, when combined with the negative geotaxic process being principally a measure of climbing behaviour—as opposed to a mixture of climbing, flying, and jumping—makes this assay a particularly effective measure of locomotion ability (Rhodenizer et al., 2008).

2.7. Walking Assay

The walking assay used in this collection of experiments was simply a means of tracking the spontaneous movement of flies as they move about an acrylic, rectangular prism, tracking chamber (inner dimensions: length = 8.0 cm; width = 1.0 cm; height = 0.5 cm) (Supplementary Figure 7). Flies were tracked using Noldus EthoVision XT video tracking software and trial videos were acquired using a Panasonic WV-BP334 charge-coupled device (CCD) camera. Before an experiment began, settings within the tracking software program were adjusted—e.g., detection and arena settings—to predetermined values in order to maximize the proportion of time that the subject was detected throughout the trial. In spite of these calibrations, a small proportion of each trial—typically less than 10%—remained undetected. In order to circumvent this issue, we employed the data interpolation function built into the EthoVision XT tracking software. This data interpolation procedure was deemed acceptable considering that the undetected samples were typically short in duration, and scattered throughout the trial. Due to the frequency and duration of the missing sample periods, an exclusion criterion of 12.5% was imposed, with any trial exceeding this undetected percentage being excluded from further analysis.

For each trial, a fly of the appropriate age and genotype was loaded singly into the tracking chamber. Fly movement was tracked live using the EthoVision XT tracking software for a trial duration of 60 seconds (s) under overhead, fluorescent, ambient lighting. The raw data file for a given trial was then available for further analysis. The tracking program allows the user to export an array of measures such as total distance

traveled, maximum and mean velocity, and percentage of time spent immobile. After each trial was completed, the fly was discarded into a fly morgue, and a new fly was loaded into the tracking chamber. This process was repeated for all flies in the experiment. Upon completion of the experiment, the chamber was washed according to a carefully designed chamber washing protocol. This protocol involved using water from a Milli-Q® Reference Water Purification System, dish detergent, Q-tips®, and Kimwipes®. Care was taken to completely dry the chambers after washing, and to leave as little soap residue behind as possible because such residue might interfere with fly movement during future experiments.

2.8. Larva Tracking Assay

The larva tracking assay is similar in principle to the walking assay in that the spontaneous movement of $L3^{w}$ is tracked using the same EthoVision XT video tracking software and Panasonic CCD camera (see Supplementary Figure 8). Instead of a rectangular prism tracking chamber, however, $L3^{w}$ were tracked in the lid of a plastic petri dish (diameter = 14.5 cm; depth = 1.0 cm) filled approximately halfway with a solidified aqueous mixture of 1.75% Bacto™ Agar (Thomas Scientific, catalog no. C010Q48) and 5% sucrose. On the day of an experiment, the required number of previously made tracking plates were removed from the 4°C refrigerator and allowed to warm-up for a minimum of 30 min prior to tracking. Similar to the walking assay, detection settings and arena definition parameters were set prior to experimentation, and adjusted accordingly. The detection settings for tracking larvae, as opposed to adult flies, was considerably more difficult due to the nearly insufficient contrast between the

translucent agarose background and the semi-translucent larvae. To circumvent this issue, an X-ray Viewing Box was used to illuminate the larvae from their ventral sides.

For each trial, a $L3^{w}$ of the appropriate genotype was selected with a fine paintbrush according to size and wandering behaviour, as previously discussed. Of note is that many of the phenotypic markers that were used to differentiate the genotypes of adult flies cannot be used for identification in the larval stage. Thus, genetic crosses were strategically designed to avoid this issue. Once an appropriate $L3^{w}$ was selected, it was transferred to the middle of a tracking plate, one per genotype, and given a brief acclimation period of 1 min prior to tracking. Following the acclimation period, the $L3^{w}$ was re-centered, given a 5-s reorientation period, and tracked for 90 s. Due to the slow movement of the $L3^{w}$, sample detection was essentially an all-or-none function based primarily on the trial detection settings. If the sample was not initially detected, the settings were adjusted and the trial restarted. After the trial, the larva was discarded into the fly morgue. This tracking process was repeated for all larvae throughout the duration of the experiment. Upon completion, the agar was scraped off the plate and disposed of. The plastic tracking plates were washed by hand to prevent warping from the excessive heat of the laboratory dishwasher.

2.9. Ageing

Considering that HSP is fundamentally a neurodegenerative disorder, we were interested in testing flies at different developmental stages. In addition to the larval and 2-day-old conditions discussed previously, we chose to analyze behavioural performance at 8, 15, and 22 days. Considering that the fundamentals of life are similar to many species, it has been suggested that the ageing process could consist of similar underlying mechanisms across phylogeny (Longo, 1999; Tissenbaum & Guarente, 2002). However, the direct correspondence between an ageing Drosophila and an ageing human remains poorly understood.

Newly eclosing adult flies were 'cleared' from approximately 2-week-old cross bottles by dumping them into a fly morgue, leaving the developing pupae behind. The flies that eclosed after this clearing process were synchronized in age to within 6-8 hr. As opposed to being tested immediately, as in the 2-day condition, age-synchronized flies were transferred to ageing bottles containing our standard fly media for all ageing protocols. Flies were then left to age until they were tested at the appropriate age condition. Bottles were changed within 2 weeks to circumvent the eclosion of secondary filial (F2) flies, which would severely complicate the interpretation of experimental results.

2.10. Data Analysis

Two-tailed, unpaired, Student's t-tests were used to compare the means of two groups, typically a mutant and control group. One-way analyses of variance (ANOVAs) were used for the omnibus significance tests for the comparison of means between more than two groups. If a significant critical F-value was observed, post-hoc corrections for multiple comparisons were performed using Tukey's Honestly Significant Difference (HSD) test ($\alpha = 0.05$), which compares the means of each group to every other group in the analysis. For ageing profile analyses, the variable distribution was as follows: independent variable 1, genotype; independent variable 2, age; dependent variable, climbing performance. For these analyses, two-way ANOVAs were used for the omnibus significance test. If significant, Bonferonni post-tests were performed to correct for multiple comparisons when comparing each group to every other group in the analysis. All statistical analyses were performed using GraphPad PRISM 5 software.

CHAPTER 3 - Results

3.1. Aim 1: Single Gene Effects

3.1.i. Disruptions of D-spastin Function Result in Severe Defects in Locomotion

We tested flies transheterozygous for both a null and hypomorphic allele of *D-spastin*—i.e., $spas^{5.75}$ and $spas^{17\text{-}7}$, respectively—for motor phenotypes using a climbing and walking assay (Figure 1) due to the homozygous lethality of $spas^{5.75}$ null adult flies. $spas^{5.75}/spas^{17\text{-}7}$ transheterozygous mutants exhibited significant climbing deficits at 2 and 8 days of age relative to the genetically appropriate controls: heterozygous—i.e., $spas^{5.75}/WT$ and $spas^{17\text{-}7}/WT$—and WT (Figure 1, panels A-C). A two-way ANOVA with Bonferroni post-tests revealed overall effects of genotype ($p < 0.001$), age ($p < 0.001$), and the corresponding interaction ($p < 0.001$). Neither group of heterozygous mutants displayed a climbing defect after either 2 or 8 days, although a one-way ANOVA with Tukey's HSD post-tests revealed a climbing defect of the hypomorphic heterozygous group—i.e., $spas^{17\text{-}7}/WT$— at 15 days relative to $spas^{5.75}/WT$ mutants and WT controls (Figure 1, panel D).

In the walking assay, $spas^{5.75}/spas^{17\text{-}7}$ mutants moved a significantly shorter distance than WT controls at both 2 and 8 days of age (Figure 1, panel E). A corresponding two-way ANOVA with Bonferroni post-tests revealed an overall effect of genotype ($p < 0.001$), but not age or the corresponding interaction for distance moved. In addition, $spas^{5.75}/spas^{17\text{-}7}$ mutants spent a greater percentage of time immobile than WT controls at

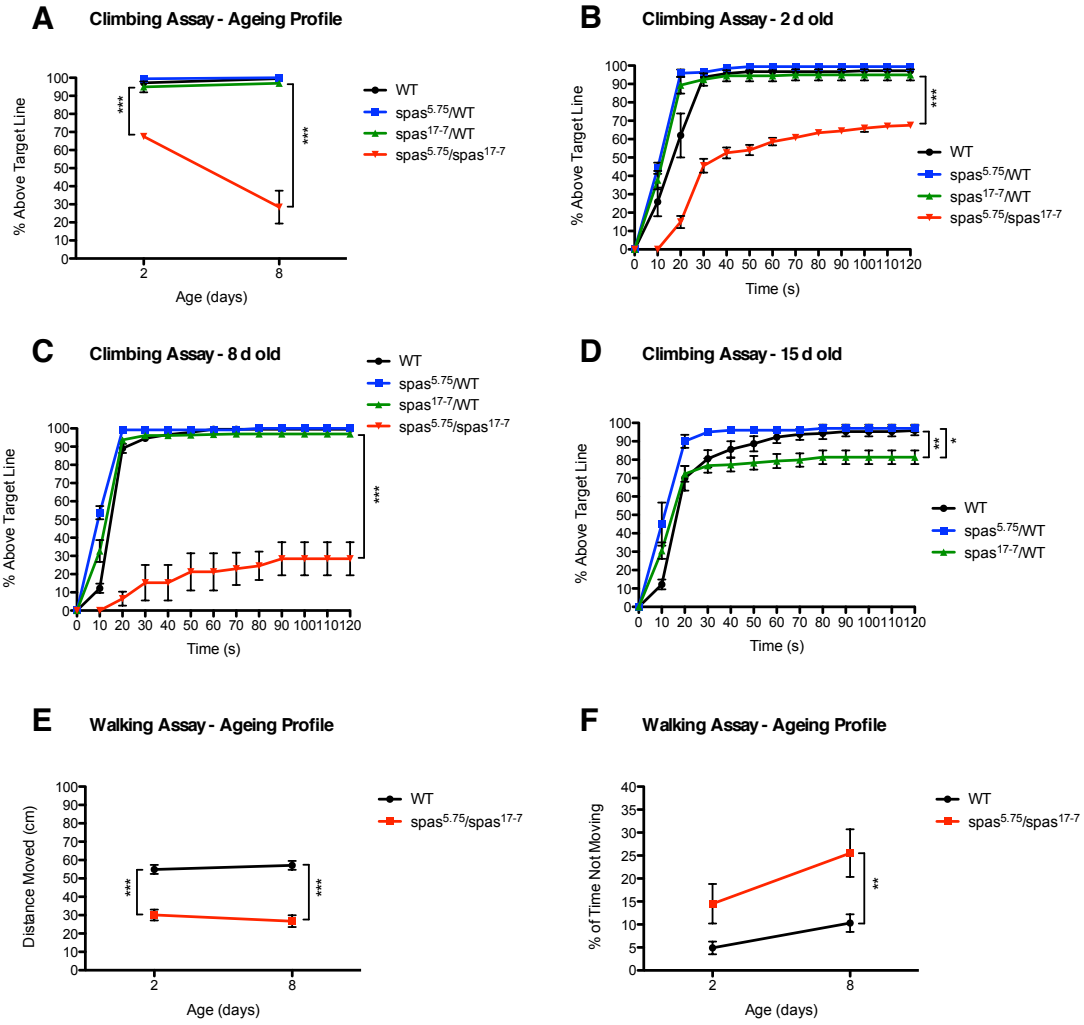

A
Climbing Assay - Ageing Profile
% Above Target Line
Age (days)
WT
spas5.75/WT
spas17-7/WT
spas5.75/spas17-7
B
Climbing Assay - 2 d old
% Above Target Line
Time (s)
C
Climbing Assay - 8 d old
% Above Target Line
Time (s)
D
Climbing Assay - 15 d old
% Above Target Line
Time (s)
E
Walking Assay - Ageing Profile
Distance Moved (cm)
Age (days)
F
Walking Assay - Ageing Profile
% of Time Not Moving
Age (days)

Figure 1 | *spas*$^{5.75}$*/spas*$^{17\text{-}7}$ mutants exhibit compromised climbing and walking ability.

(A) Climbing assay ageing profile. Climbing deficits were observed for 2 and 8-d-old *spas*$^{5.75}$*/spas*$^{17\text{-}7}$ transheterozygous mutants relative to heterozygous—i.e., *spas*$^{5.75}$*/WT* and *spas*$^{17\text{-}7}$*/WT*—and WT controls.

(B), (C), (D) Climbing assay trial profiles—2, 8, and 15-d-old flies, respectively. A climbing defect of spas$^{17\text{-}7}$/WT mutants emerged at 15 d relative to spas$^{5.75}$/WT mutants and WT controls; N_{Climb} WT: 10 (2 d), 12 (8 d), 10 (15 d); *spas*$^{5.75}$*/WT*: 10 (2 d), 6 (8 d), 5 (15 d); *spas*$^{17\text{-}7}$*/WT*: 10 (2 d), 13 (8 d), 10 (15 d); *spas*$^{5.75}$*/spas*$^{17\text{-}7}$: 10 (2 d), 3 (8 d).

(E) Walking assay ageing profile. *spas*$^{5.75}$*/spas*$^{17\text{-}7}$ transheterozygous mutants moved a lesser distance at 2 and 8 d of age relative to WT controls; N_{Walk_Dist} WT: 20 (2 d), 20 (8 d); *spas*$^{5.75}$*/spas*$^{17\text{-}7}$: 20 (2 d), 20 (8 d).

(F) Walking assay ageing profile. *spas*$^{5.75}$*/spas*$^{17\text{-}7}$ transheterozygous mutants spent a greater proportion of time immobile at 8 d of age relative to WT controls; $N_{Walk\_\%NM}$ WT: 20 (2 d), 20 (8 d); *spas*$^{5.75}$*/spas*$^{17\text{-}7}$: 20 (2 d), 20 (8 d).

WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ age for *Elav-GAL4/WT* mutants relative to WT controls.

8 days of age (Figure 1, panel F). A two-way ANOVA with Bonferroni post-tests revealed an overall effect of genotype ($p < 0.001$) and age ($p < 0.05$), but not the corresponding interaction or percentage of time immobile.

We also performed the climbing assay on mutant flies pan-neurally expressing *D-spastin* RNAi—i.e., *Elav-GAL4/UAS-spasRNAi* (Figure 2). We found that these mutants displayed climbing impairments at 2, 8, and 15 days of age relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-spasRNAi/WT*), and WT controls (Figure 2, panels B-D). A two-way ANOVA with Bonferroni post-tests showed overall effects of genotype ($p < 0.001$), age ($p < 0.001$), and the corresponding interaction ($p < 0.001$). In addition to the mutant flies pan-neurally expressing *D-spastin* RNAi, climbing deficits were observed at 8 and 15 days for *UAS-spasRNAi/WT* mutants relative to *Elav-GAL4/WT* mutants and WT controls as shown by a one-way ANOVA with Tukey's HSD post-tests of the trial profile data (Figure 2, panels C & D). Although these defects were significant, they were not as severe as those observed for *Elav-GAL4/UAS-spasRNAi* mutants. We also performed a confirmation experiment in which an alternate group of flies pan-neurally expressing a second line *D-spastin* RNAi—i.e., *Elav-GAL4/UAS-spasRNAi2*—were tested using a climbing assay (Figure 4, panel A). As expected, these flies also exhibited severe climbing impairments at 2 days of age relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-spasRNAi2/WT*), and WT controls.

Next, we tested the locomotion ability of larvae pan-neurally expressing *D-spastin* RNAi (Figure 3). *Elav-GAL4/UAS-spasRNAi* mutant larvae moved a lesser distance ($p < 0.05$),

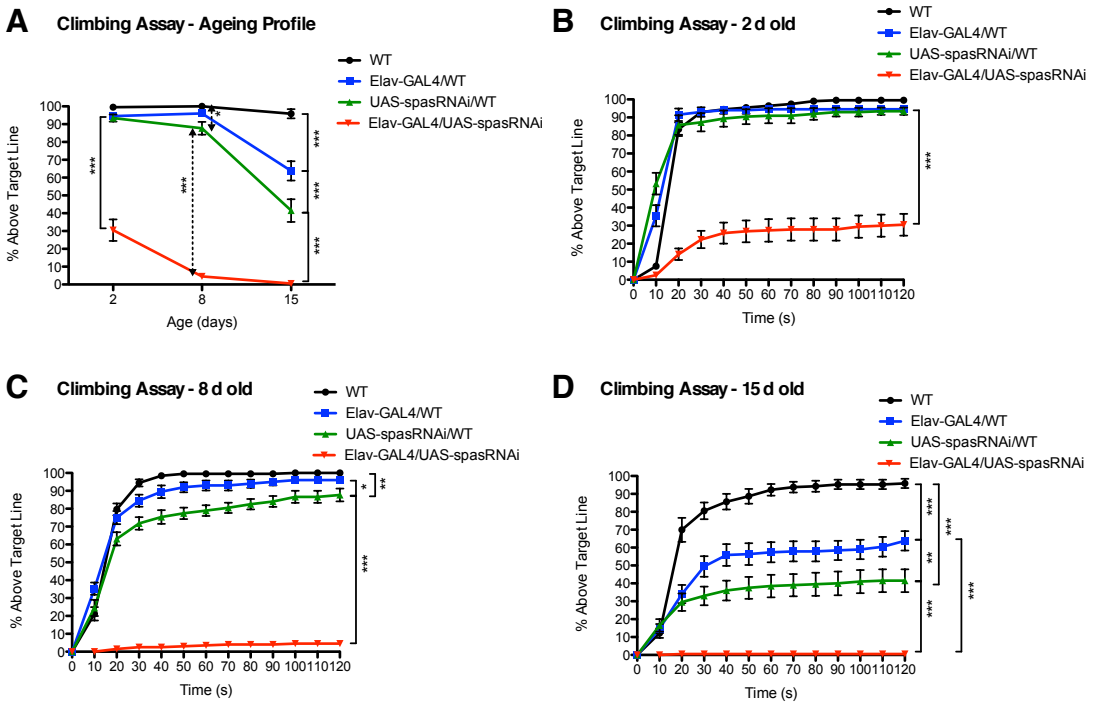

A
Climbing Assay - Ageing Profile
WT
Elav-GAL4/WT
UAS-spasRNAi/WT
Elav-GAL4/UAS-spasRNAi
% Above Target Line
Age (days)
B
Climbing Assay - 2 d old
Time (s)
C
Climbing Assay - 8 d old
D
Climbing Assay - 15 d old

Figure 2 | Pan-neural *D-spastin* RNAi knockdown mutants exhibit climbing defects that are exacerbated by age.

(A) Climbing assay ageing profile. Climbing deficits were observed for 2, 8, and 15-d-old *Elav-GAL4/UAS-spasRNAi* mutants relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-spasRNAi/WT*), and WT controls. Additionally, *UAS-spasRNAi/WT* controls exhibited climbing defects at 8 d relative to WT controls, and at 15 d relative to both driver-only and WT controls. *Elav-GAL4/WT* controls also showed climbing deficits at 15 d relative to WT controls.

(B), (C), (D) Climbing assay trial profiles—2, 8, and 15 d old flies, respectively.

N = 10 (each genotype and age condition); WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

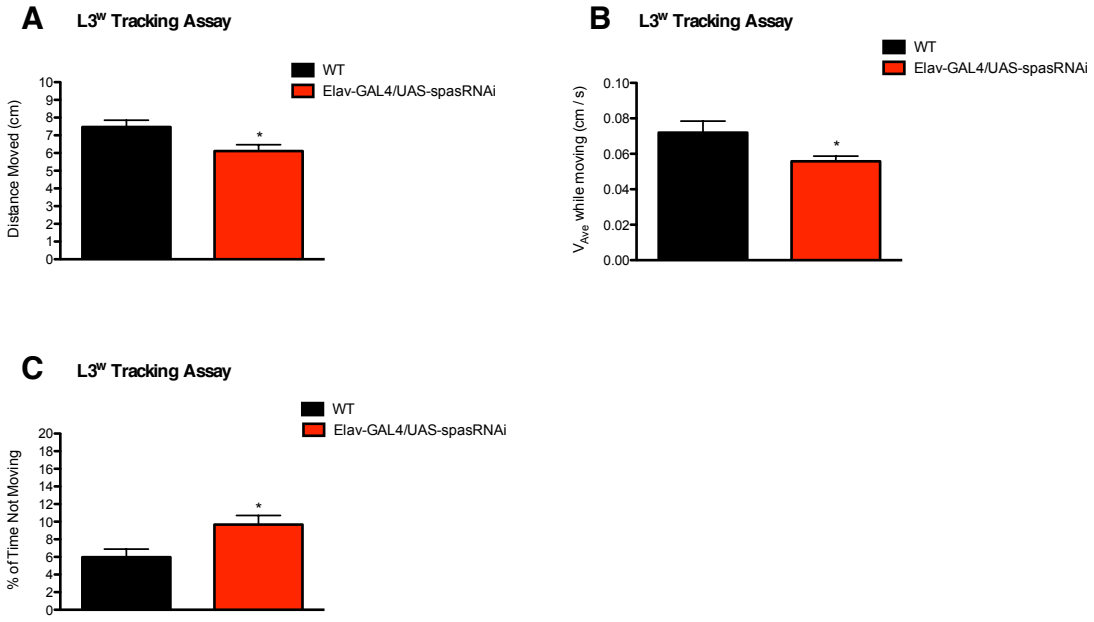

A
L3W Tracking Assay
WT
Elav-GAL4/UAS-spasRNAi
Distance Moved (cm)
0
1
2
3
4
5
6
7
8
9
10
*
B
L3W Tracking Assay
WT
Elav-GAL4/UAS-spasRNAi
VAve while moving (cm / s)
0.00
0.02
0.04
0.06
0.08
0.10
*
C
L3W Tracking Assay
WT
Elav-GAL4/UAS-spasRNAi
% of Time Not Moving
0
2
4
6
8
10
12
14
16
18
20
*

Figure 3 | Pan-neural *D-spastin* RNAi knockdown mutants exhibit locomotion defects in the wandering third instar larval stage.

(A) *Elav-GAL4/UAS-spasRNAi* mutants moved a shorter distance relative to WT controls in a larva tracking assay.

(B) *Elav-GAL4/UAS-spasRNAi* mutants displayed a diminished average velocity than WT controls, excluding time spent immobile, in a larva tracking assay.

(C) *Elav-GAL4/UAS-spasRNAi* mutants spent a greater proportion of time immobile relative to WT controls in a larva tracking assay.

N = 20 (each genotype and age condition); WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

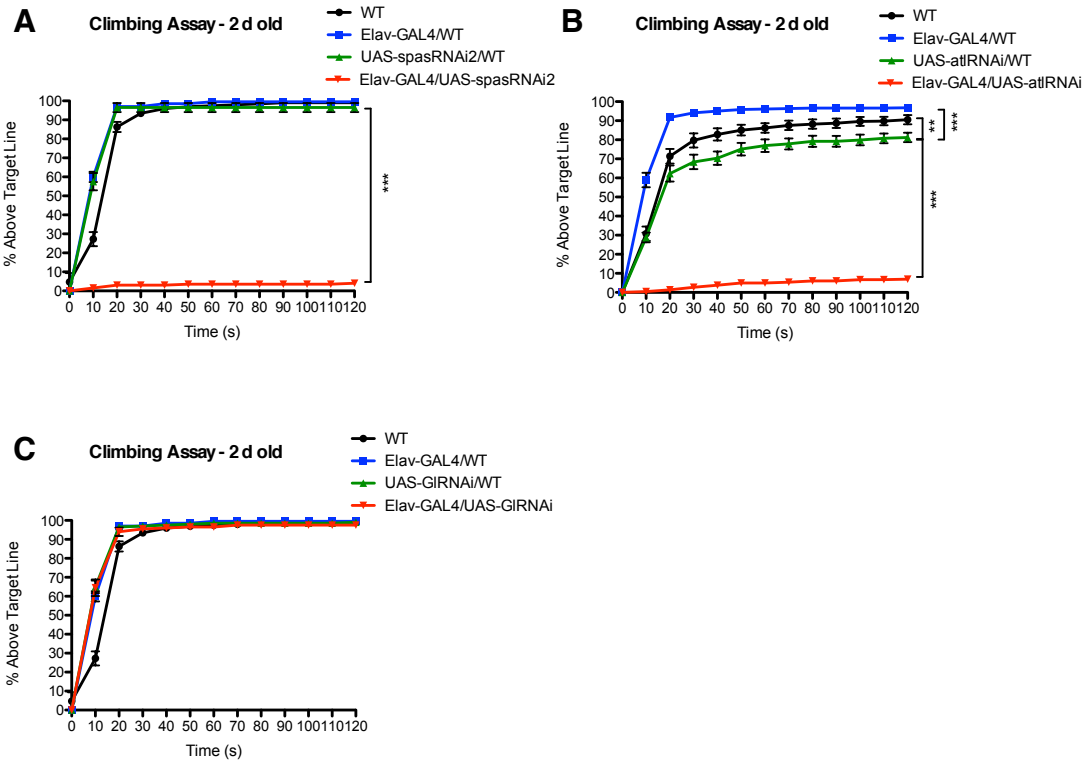

A
Climbing Assay - 2 d old
WT
Elav-GAL4/WT
UAS-spasRNAi2/WT
Elav-GAL4/UAS-spasRNAi2
% Above Target Line
Time (s)
***
B
Climbing Assay - 2 d old
WT
Elav-GAL4/WT
UAS-atlRNAi/WT
Elav-GAL4/UAS-atlRNAi
% Above Target Line
Time (s)
**
***
C
Climbing Assay - 2 d old
WT
Elav-GAL4/WT
UAS-GlRNAi/WT
Elav-GAL4/UAS-GlRNAi
% Above Target Line
Time (s)

Figure 4 | Climbing defects result from pan-neural expression of *D-spastin* and *D-atlastin* RNAi, but not *Glued* RNAi.

(A) Climbing assay trial profile (2nd line of pan-neural *D-spastin* RNAi). Pan-neural expression of *D-spastin* RNAi results in compromised climbing ability at 2 d of age relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-spasRNAi2/WT*), and WT controls; N = 10 (each genotype).

(B) Climbing assay trial profile. Pan-neural expression of *D-atlastin* RNAi results in compromised climbing ability at 2 d of age relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-atlRNAi/WT*), and WT controls. Additionally, *UAS-atlRNAi/WT* controls displayed a climbing defect relative to driver-only and WT controls; N = 15 (each genotype).

(C) Climbing assay trial profile. Pan-neural expression of *Glued* RNAi has no distinguishable effect on climbing ability at 2 d of age relative to driver-only (i.e., *Elav-GAL4/WT*), responder-only (*UAS-GlRNAi/WT*), and WT controls; N = 10 (each genotype).

WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

moved at a lesser average velocity after omitting time spent immobile ($p < 0.05$), and spent a greater proportion of time immobile ($p < 0.05$) relative to WT control larvae. These data were statistically analyzed via two-tailed, unpaired t-tests.

We also used the climbing assay to test the effect of pan-neurally overexpressing *D-spastin* using the GAL4-UAS system—i.e., *Elav-GAL4/UAS-spas*—in adult flies (Figure 5, panel A). A one-way ANOVA with Tukey's HSD post-tests revealed that neural-specific *D*-spastin overexpression resulted in a severe climbing impairment at 2 days of age relative to driver-only (*Elav-GAL4/WT*) ($p < 0.001$), responder-only (*UAS-spas/WT*) ($p < 0.001$), and WT controls ($p < 0.001$).

3.1.ii. Pan-neural Expression of D-atl RNAi Results in Climbing Impairments

Like *D-spastin*, pan-neural expression of *D-atl* RNAi—i.e., *Elav-GAL4/UAS-atlRNAi*—resulted in significant climbing impairments at 2 days of age relative to driver-only (*Elav-GAL4/WT*) ($p < 0.001$), responder-only (*UAS-atlRNAi/WT*) ($p < 0.001$), and WT controls ($p < 0.001$), as revealed by a one-way ANOVA with Tukey's HSD post-tests (Figure 4, panel B). A defect was also observed for the responder-only controls relative to WT controls ($p < 0.01$) and driver-only controls ($p < 0.001$), but not to the same extent as the mutants expressing *D-atl* RNAi.

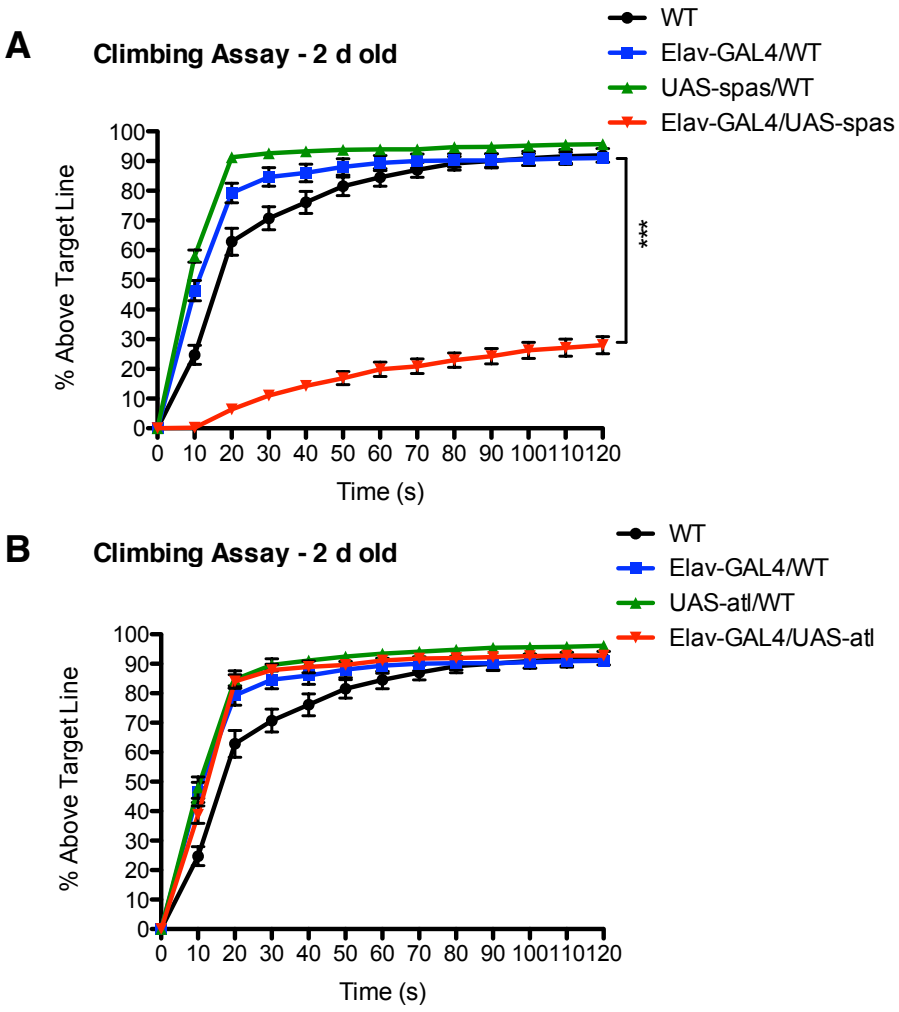

A
Climbing Assay - 2 d old
WT
Elav-GAL4/WT
UAS-spas/WT
Elav-GAL4/UAS-spas
% Above Target Line
Time (s)
***
B
Climbing Assay - 2 d old
WT
Elav-GAL4/WT
UAS-atl/WT
Elav-GAL4/UAS-atl
% Above Target Line
Time (s)

Figure 5 | Climbing defects result from pan-neural overexpression of *D-spastin* but not *D-atlastin*.

(A) Climbing assay trial profile. Pan-neural overexpression of *D-spastin* results in compromised climbing ability at 2 d of age relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-spas/WT*), and WT controls; N = 20 (each genotype).

(B) Climbing assay trial profile. Pan-neural overexpression of *D-atlastin* results in no distinguishable difference in climbing ability at 2 d of age relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-atl/WT*), and WT controls; N = 20 (each genotype).

WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Interestingly, unlike *D-spastin*, neural-specific *D-atl* overexpression—i.e., *Elav-GAL4/UAS-atl*—did not result in any distinguishable climbing deficit in 2-day-old flies relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-atl/WT*), and WT controls, as shown by a one-way ANOVA with Tukey's HSD tests for multiple comparisons (Figure 5, panel B).

3.1.iii. Climbing Ability is Impaired by the Expression of a Dominant Negative Allele of Glued, but not by the Pan-neural Expression of Glued RNAi

Unlike for *D-spastin* and *D-atl*, we found that neural-specific expression of *Glued* RNAi—i.e., *Elav-GAL4/UAS-GlRNAi*—did not result in any distinguishable climbing impairment in 2-day-old flies relative to driver-only (*Elav-GAL4/WT*), responder-only (*UAS-GlRNAi/WT*), and WT controls (Figure 4, panel C).

Climbing defects were observed for mutants heterozygous for the previously published dominant negative *Glued* mutation—i.e., Gl^{1}/WT—at 2 days of age relative to WT controls ($p < 0.01$). These impairments seemed to be exacerbated by age, being progressively worsened at 8 ($p < 0.05$), 15 ($p < 0.001$), and 22 days of age ($p < 0.001$), as shown by a one-way ANOVA with Tukey's HSD post-tests (Figure 6, panels A-E). The climbing defect at 8 days old was identified via a one-way ANOVA followed by Tukey's HSD post-tests, but was not identified in the overall two-way ANOVA. A two-way ANOVA of the overall ageing profile following by Bonferroni post-tests showed overall effects of genotype ($p < 0.001$), age ($p < 0.001$), and the corresponding interaction ($p < 0.001$).

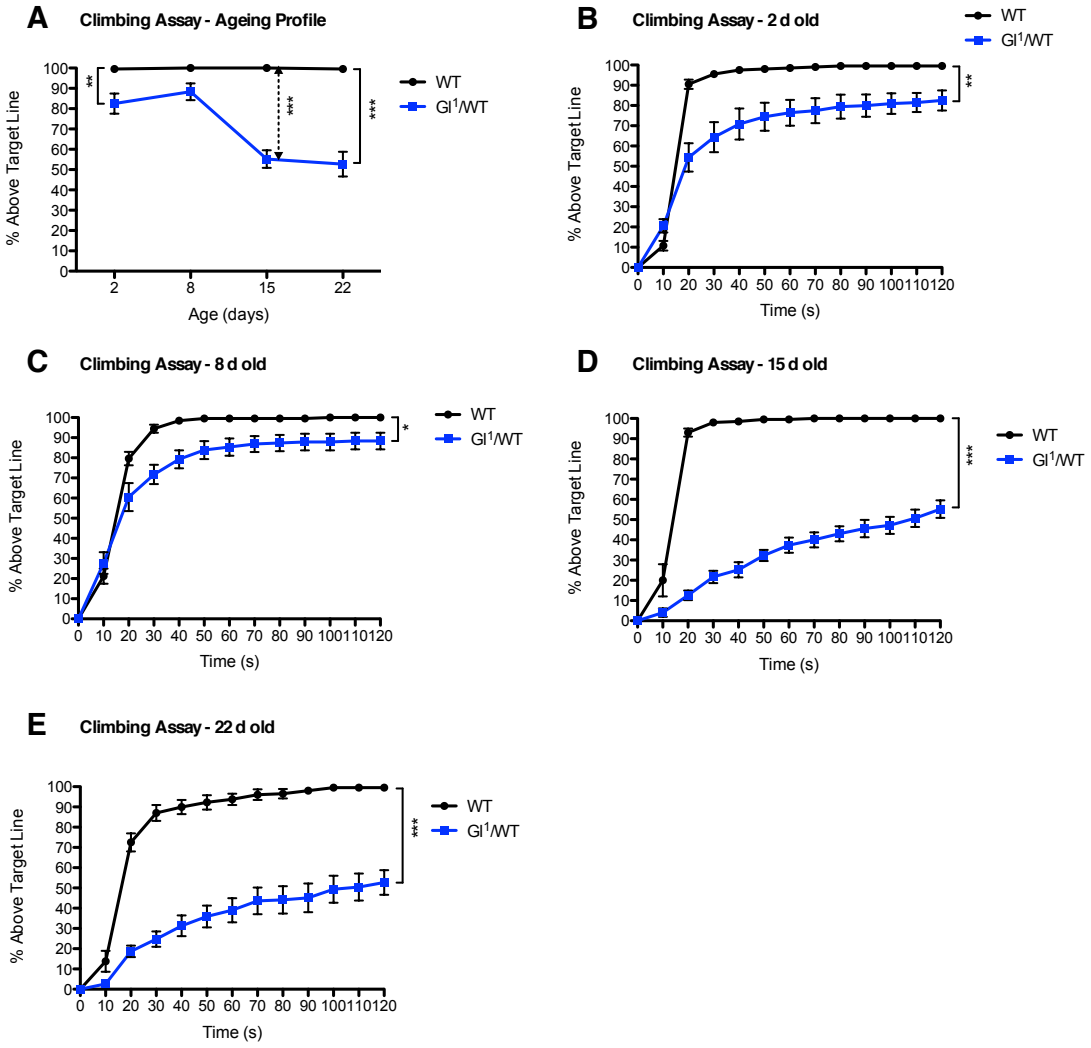

A
Climbing Assay - Ageing Profile
% Above Target Line
Age (days)
WT
Gl1/WT
B
Climbing Assay - 2 d old
Time (s)
C
Climbing Assay - 8 d old
D
Climbing Assay - 15 d old
E
Climbing Assay - 22 d old

Figure 6 | *Gl^{l}/WT* mutants exhibit climbing defects that are exacerbated by age.

(A) Climbing assay ageing profile. Climbing deficits were observed for 2, 15, and 22-d-old *Gl^{l}/WT* mutants relative to WT controls.

(B), (C), (D), (E) Climbing assay trial profiles—2, 8, 15, and 22-d-old flies, respectively. N WT: 10 (2 d), 10 (8 d), 10 (15 d), 11 (22 d); *Gl^{l}/WT*: 10 (2 d), 10 (8 d), 10 (15 d), 10 (22 d); WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.2. Aim 2: Genetic Interactions

3.2.i. D-spastin and D-atl Genetically Interact to Produce Climbing and Walking Impairments

Interestingly, climbing defects were not observed after 2 or 8 days for $spas^{5.75}/atl^{2}$ transheterozygous flies or the corresponding controls (Figure 7, panels B & C). However, a defect emerged in the 15-day-old condition for $spas^{5.75}/atl^{2}$ flies relative to $spas^{5.75}/WT$ heterozygous mutants ($p < 0.01$) and WT controls ($p < 0.001$) (Figure 7, panels A & D). This climbing deficit was identified by the overall two-way ANOVA of the ageing profile followed by Bonferonni post-tests, but not the one-way ANOVA followed by Tukey's HSD post-tests. The overall two-way ANOVA revealed significance for the overall effect of the interaction between genotype and age ($p < 0.05$), but neither effect exclusively.

In the walking assay, transheterozygous mutants ($spas^{5.75}/atl^{2}$) moved a shorter distance relative to $spas^{5.75}/WT$ heterozygous mutants and WT controls at both 2 ($p < 0.001$) and 8 ($p < 0.001$) days of age. Similarly, $spas^{5.75}/atl^{2}$ mutants spent a greater proportion of time immobile relative to $spas^{5.75}/WT$ mutants at 2 ($p < 0.001$) and 8 ($p < 0.01$) days of age, and also relative to WT controls at both 2 ($p < 0.001$) and 8 ($p < 0.001$) days of age (Figure 7, panels E & F); however, no distinguishable differences were observed between $spas^{5.75}/atl^{2}$ mutants and WT controls in either measure at 15 days of age.

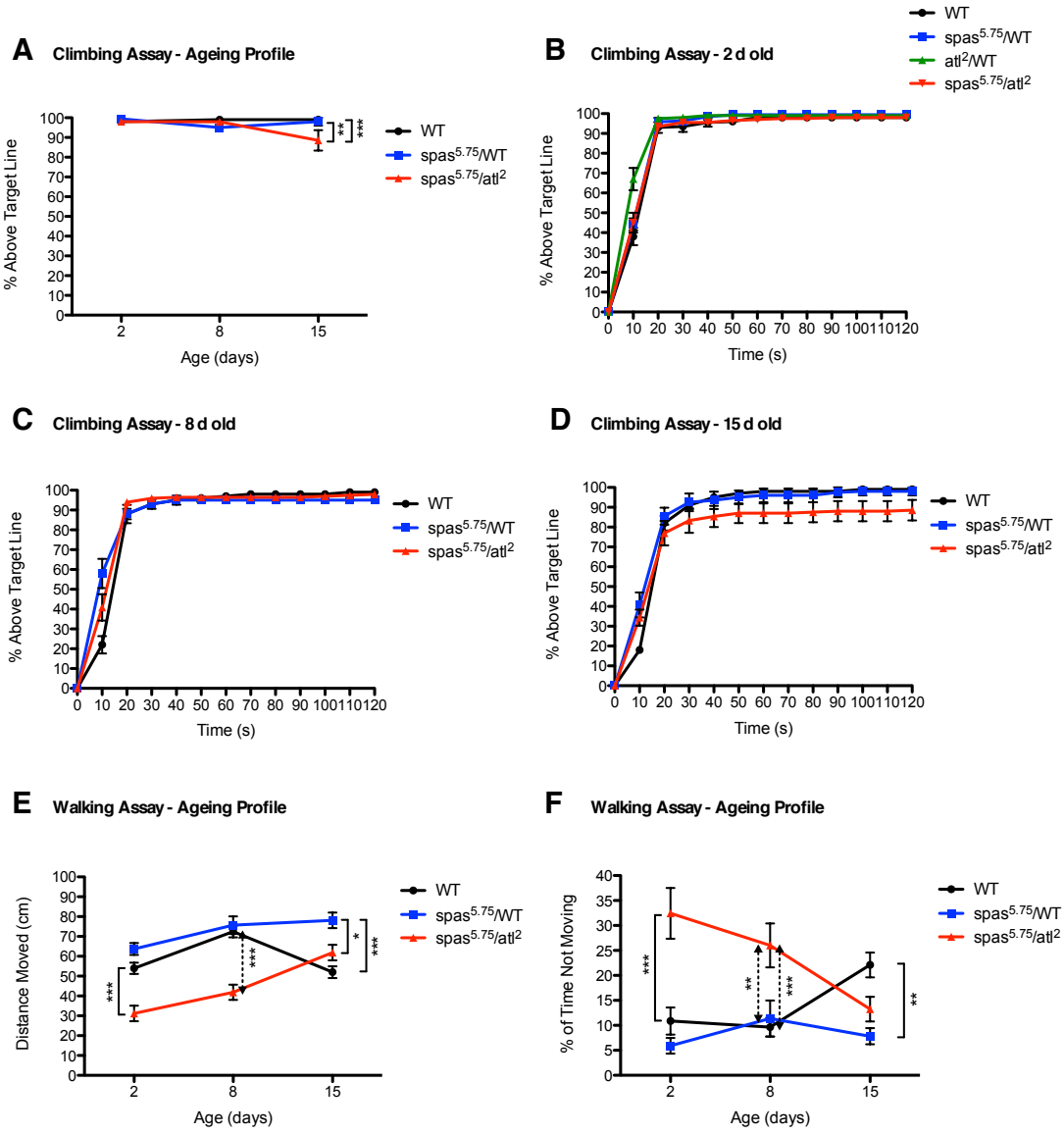

A
Climbing Assay - Ageing Profile
% Above Target Line
Age (days)
WT
spas5.75/WT
spas5.75/atl2
B
Climbing Assay - 2 d old
atl2/WT
% Above Target Line
Time (s)
C
Climbing Assay - 8 d old
D
Climbing Assay - 15 d old
E
Walking Assay - Ageing Profile
Distance Moved (cm)
F
Walking Assay - Ageing Profile
% of Time Not Moving

Figure 7 | *spas*$^{5.75}$/*atl*^{2} mutants exhibit climbing and walking impairments.

(A) Climbing assay ageing profile. Climbing deficits were observed for 15-d-old *spas*$^{5.75}$/*atl*^{2} transheterozygous mutants relative to heterozygous—i.e., *spas*$^{5.75}$/*WT*—and WT controls.

(B), (C), (D) Climbing assay trial profiles—2, 8, and 15-d-old flies, respectively; N_{Climb} WT: 10 (2 d), 11 (8 d), 12 (15 d); *spas*$^{5.75}$/*WT*: 10 (2 d), 10 (8 d), 10 (15 d); *spas*$^{5.75}$/*atl*^{2}: 10 (2 d), 10 (8 d), 10 (15 d).

(E) Walking assay ageing profile. *spas*$^{5.75}$/*atl*^{2} transheterozygous mutants moved a decreased distance at 2 and 8 d of age relative to WT controls, and at 2, 8, and 15 d relative to *spas*$^{5.75}$/*WT* heterozygous controls. Also, WT controls moved a shorter distance than *spas*$^{5.75}$/*WT* mutants at 15 d.

(F) Walking assay ageing profile. *spas*$^{5.75}$/*atl*^{2} transheterozygous mutants spent a greater proportion of time immobile at 2 and 8 d of age relative to both *spas*$^{5.75}$/*WT* mutants and WT controls. Also, WT controls spend a greater proportion of time immobile at 15 d of age relative to *spas*$^{5.75}$/*WT* mutants.

N_{Walk} WT: 30 (2 d), 39 (8 d), 52 (15 d); *spas*$^{5.75}$/*WT*: 21 (2 d), 21 (8 d), 22 (15 d); *spas*$^{5.75}$/*atl*^{2}: 21 (2 d), 21 (8 d), 20 (15 d).

WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

A two-way ANOVA with Bonferonni post-tests regarding distance moved revealed overall effects of genotype ($p < 0.001$), age ($p < 0.001$), and the corresponding interaction ($p < 0.001$). In addition, a similar two-way ANOVA regarding percentage of time spent immobile revealed overall effects of genotype ($p < 0.001$), the interaction between genotype and age ($p < 0.001$), but not the effect of age itself.

Considering the *spas*$^{5.75}$*/WT* heterozygous group separately, no distinguishable differences were observed in climbing ability between *spas*$^{5.75}$*/WT* mutants and WT controls at 2, 8, and 15 days of age, with both groups performing at essentially 100 % throughout. This trend was reflected in the walking assay results, with no distinguishable differences observed between the two groups with regard to distance moved or proportion of time spent immobile at either 2 or 8 days of age; however, at 15 days old, *spas*$^{5.75}$*/WT* mutants moved a greater distance than both *spas*$^{5.75}$*/atl*^{2} mutants ($p < 0.05$) and WT controls ($p < 0.001$), and spent a decreased proportion of time immobile relative to WT controls ($p < 0.01$).

3.2.ii. D-spastin and Glued Genetically Interact to Produce Climbing and Walking Impairments

2-day-old *Gl*^{1}*/WT* heterozygous mutants exhibited significant climbing impairments relative to WT controls ($p < 0.001$) as well as *spas*$^{5.75}$*/WT* ($p < 0.001$) and *spas*$^{5.75}$*/Gl*^{1} mutants ($p < 0.01$), as identified by a one-way ANOVA with post-hoc Tukey's HSD tests (Figure 8, panel A). However, the climbing ability of the spas$^{5.75}$/Gl^{1} transheterozygous mutants was statistically indistinguishable from spas$^{5.75}$/WT mutants and WT controls.

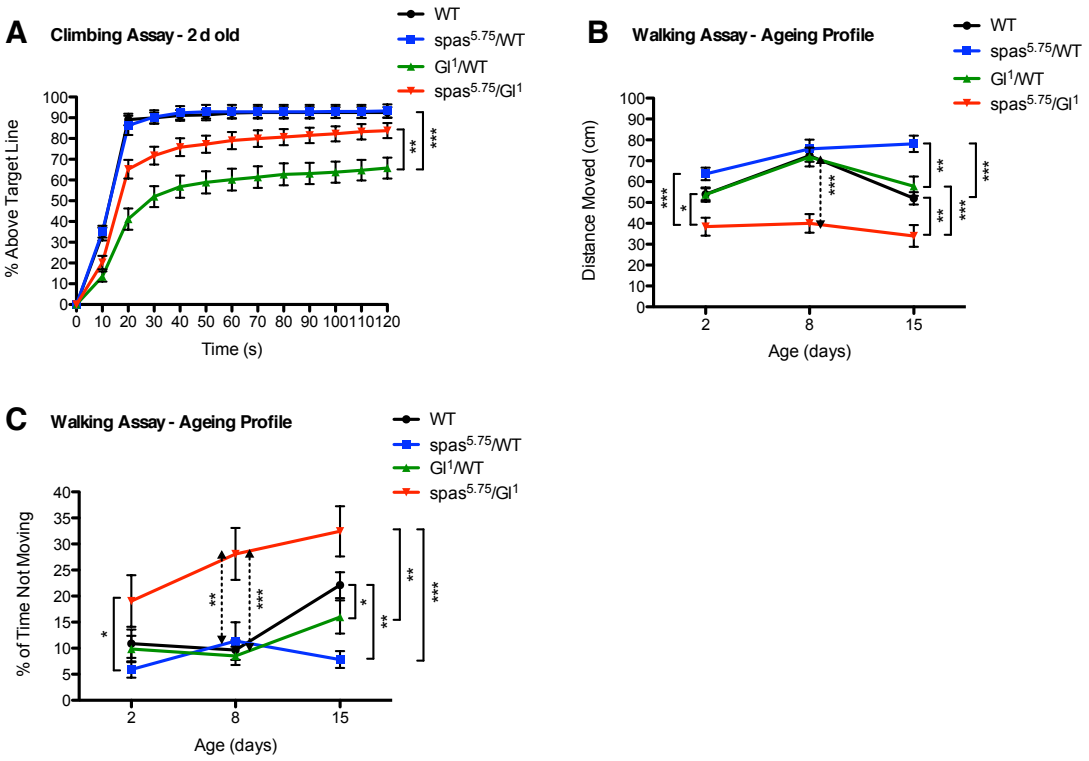

A
Climbing Assay - 2 d old
WT
spas5.75/WT
Gl1/WT
spas5.75/Gl1
% Above Target Line
Time (s)
B
Walking Assay - Ageing Profile
Distance Moved (cm)
Age (days)
C
Walking Assay - Ageing Profile
% of Time Not Moving
Age (days)

Figure 8 | *Gl*^{1}*/WT* mutants exhibit climbing defects, whereas *spas*$^{5.75}$*/Gl*^{1} exhibit walking defects that persist with age.

(A) Climbing assay trial profile. *Gl*^{1}*/WT* mutants display compromised climbing ability in 2-d-old flies relative to *spas*$^{5.75}$*/Gl*^{1} transheterozygous mutants, *spas*$^{5.75}$*/WT* heterozygous mutants, and WT controls; N WT: 21; *spas*$^{5.75}$*/WT*: 20; *Gl*^{1}*/WT*: 20; *spas*$^{5.75}$*/Gl*^{1}: 20

(B) Walking assay ageing profile. *spas*$^{5.75}$*/Gl*^{1} transheterozygous mutants moved a lesser distance at 2, 8, and 15 d of age relative to both heterozygous mutants—i.e., *spas*$^{5.75}$*/WT* and *Gl*^{1}*/WT*— and to WT controls. Also, spas$^{5.75}$/WT moved a greater distance than each of the other groups at 15 d.

(C) Walking assay ageing profile. *spas*$^{5.75}$*/Gl*^{1} transheterozygous mutants spent a greater proportion of time immobile at 2, 8, and 15 d of age relative to *spas*$^{5.75}$*/WT* mutants, at 8 d relative to WT controls, and at 15 d relative to *Gl*^{1}*/WT* mutants. Also, WT controls spent a greater proportion of time immobile at 15 d relative to each heterozygous mutant group—i.e., *spas*$^{5.75}$*/WT* and *Gl*^{1}*/WT*.

N_{Walk} WT: 30 (2 d), 39 (8 d), 52 (15 d); *spas*$^{5.75}$*/WT*: 21 (2 d), 21 (8 d), 22 (15 d); *Gl*^{1}*/WT*: 21 (2 d), 19 (8 d), 21 (15 d); *spas*$^{5.75}$*/Gl*^{1}: 20 (2 d), 22 (8 d), 18 (15 d).

WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The climbing ability of *spas*$^{5.75}$/*Gl*^{1} mutants, however, was found to be statistically unimpaired, although a trend towards significance was observed, thereby exhibiting a partial genetic rescue.

In the walking assay, 2-day-old *spas*$^{5.75}$/*Gl*^{1} transheterozygous mutants moved a lesser distance than *spas*$^{5.75}$/*WT* ($p < 0.001$) and *Gl*^{1}/*WT* ($p < 0.05$) heterozygous mutants as well as WT controls ($p < 0.05$) (Figure 8, panel B). At 8 days of age, *spas*$^{5.75}$/*Gl*^{1} mutants moved a lesser distance relative to each of the other groups ($p < 0.001$). At 15 days of age, *spas*$^{5.75}$/*Gl*^{1} mutants moved a lesser distance relative to *Gl*^{1}/*WT* mutants ($p < 0.001$), *spas*$^{5.75}$/*WT* mutants ($p < 0.001$), and WT controls ($p < 0.01$). A two-way ANOVA with Bonferroni post-tests regarding distance moved revealed an overall effect of genotype ($p < 0.001$), age ($p < 0.001$), and the corresponding interaction ($p < 0.05$).

Additionally, also in the walking assay, *spas*$^{5.75}$/*Gl*^{1} mutants spent a greater proportion of time immobile relative to *spas*$^{5.75}$/*WT* mutants at 2 days ($p < 0.05$); relative to *spas*$^{5.75}$/*WT* mutants ($p < 0.01$), *Gl*^{1}/*WT* mutants ($p < 0.001$), and WT controls ($p < 0.001$) at 8 days; and relative to *Gl*^{1}/*WT* mutants ($p < 0.01$) and *spas*$^{5.75}$/*WT* mutants ($p < 0.001$) at 15 days (Figure 8, panel C). A two-way ANOVA with Bonferroni post-tests regarding proportion of time immobile revealed an overall effect of genotype ($p < 0.001$) and age ($p < 0.01$), but not the interaction.

Considering the *D-spastin* heterozygous group separately, 15-day-old $spas^{5.75}/WT$ mutants moved a greater distance ($p < 0.001$) and spent a decreased proportion of time immobile ($p < 0.01$) relative to WT controls.

Using, instead, the hypomorphic *D-spastin* allele, $spas^{17\text{-}7}$, the climbing ability of 2-day-old Gl^{1}/WT mutants was once again found to be impaired relative to WT controls ($p < 0.05$) as well as $spas^{17\text{-}7}/WT$ heterozygous mutants ($p < 0.05$) (Figure 9, panel A). But this time, $spas^{17\text{-}7}/Gl^{1}$ transheterozygous mutants exhibited severe climbing impairments relative to both heterozygous mutants and WT controls ($p < 0.001$ for all three comparisons). Importantly, this is in direct contrast to the climbing results obtained using the transheterozygous mutants with the null *D-spastin* allele—i.e., $spas^{5.75}/Gl^{1}$.

In the walking assay, 2-day-old $spas^{17\text{-}7}/Gl^{1}$ flies also moved a lesser distance relative to Gl^{1}/WT ($p < 0.05$) and $spas^{17\text{-}7}/WT$ mutants ($p < 0.001$), as well as WT controls ($p < 0.01$), as revealed by a one-way ANOVA with post-hoc Tukey's HSD tests (Figure 9, panel B). Additionally, $spas^{17\text{-}7}/Gl^{1}$ mutants spent a greater proportion of time immobile relative to Gl^{1}/WT mutants ($p < 0.01$), $spas^{17\text{-}7}/WT$ mutants ($p < 0.01$), and WT controls ($p < 0.001$), as revealed by a similarly performed one-way ANOVA (Figure 9, panel C).

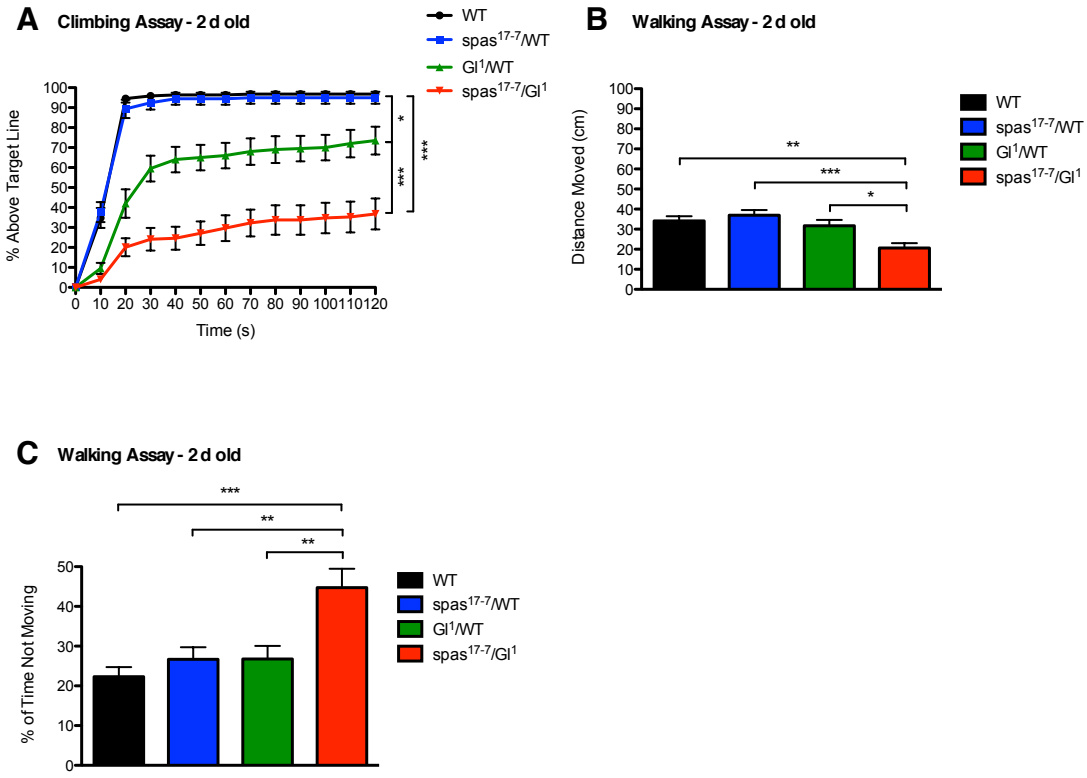

A
Climbing Assay - 2 d old
WT
spas17-7/WT
Gl1/WT
spas17-7/Gl1
% Above Target Line
Time (s)
B
Walking Assay - 2 d old
Distance Moved (cm)
C
Walking Assay - 2 d old
% of Time Not Moving

Figure 9 | *spas*$^{17\text{-}7}$/*Gl*^{1} mutants exhibit climbing and walking defects, whereas *Gl*^{1}/*WT* mutants exhibit climbing defects only.

(A) Climbing assay trial profile. *spas*$^{17\text{-}7}$/*Gl*^{1} transheterozygous mutants display compromised climbing ability in 2-d-old flies relative to each heterozygous mutant—i.e., *spas*$^{17\text{-}7}$/*WT* and *Gl*^{1}/*WT*—and WT controls. Also, *Gl*^{1}/*WT* mutants displayed climbing defects at 2 d relative to *spas*$^{17\text{-}7}$/*WT* mutants and WT controls; N WT: 11; *spas*$^{17\text{-}7}$/*WT*: 10; *Gl*^{1}/*WT*: 10; *spas*$^{17\text{-}7}$/*Gl*^{1}: 10.

(B) *spas*$^{17\text{-}7}$/*Gl*^{1} mutants moved a lesser distance relative to each heterozygous mutant group and WT controls in a walking assay.

(C) *spas*$^{17\text{-}7}$/*Gl*^{1} mutants spent a greater proportion of time immobile relative to each heterozygous mutant group and WT controls in a walking assay.

N_{Walk} WT: 25; *spas*$^{17\text{-}7}$/*WT*: 25; *Gl*^{1}/*WT*: 25; *spas*$^{17\text{-}7}$/*Gl*^{1}: 26.

WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.2.iii. D-atl and Glued Genetically Interact to Produce Climbing and Walking Impairments

Transheterozygous atl^2/Gl^1 mutants exhibit climbing impairments relative to each heterozygous mutant group and WT controls at 2 and 22 days of age ($p < 0.001$ for each comparison in the overall two-way ANOVA) (Figure 10, panels A-C). Once again replicating previous findings, Gl^1/WT mutants exhibit climbing deficits at 2 days relative to atl^2/WT mutants and WT controls ($p < 0.05$) and at and 22 days of age relative to atl^2/WT mutants and WT controls ($p < 0.001$ for each comparison in the overall two-way ANOVA). The 2-day impairment in Gl^1/WT climbing ability was identified via an overall two-way ANOVA with Bonferonni post-tests, but not via a one-way ANOVA followed by Tukey's HSD post-tests. A climbing impairment of atl^2/WT mutants also emerged at 22 days old relative to WT controls ($p < 0.001$), but was not observed at 2 days old. The ageing profile in Figure 10, panel A, clearly shows a decrease in climbing ability for each heterozygous mutant group—i.e., atl^2/WT and Gl^1/WT—as well as atl^2/Gl^1 transheterozygous mutants, but not WT controls. A two-way ANOVA with Bonferroni post-tests revealed an overall effect of genotype ($p < 0.001$), age ($p < 0.001$), and the interaction ($p < 0.001$).

In the walking assay, atl^2/Gl^1 mutants moved a significantly shorter distance and spent a greater proportion of time immobile relative to each heterozygous mutant group and WT controls ($p < 0.001$ for all comparisons), as shown by one-way ANOVAs with Tukey's HSD corrections for multiple comparisons (Figure 10, panels D & E).

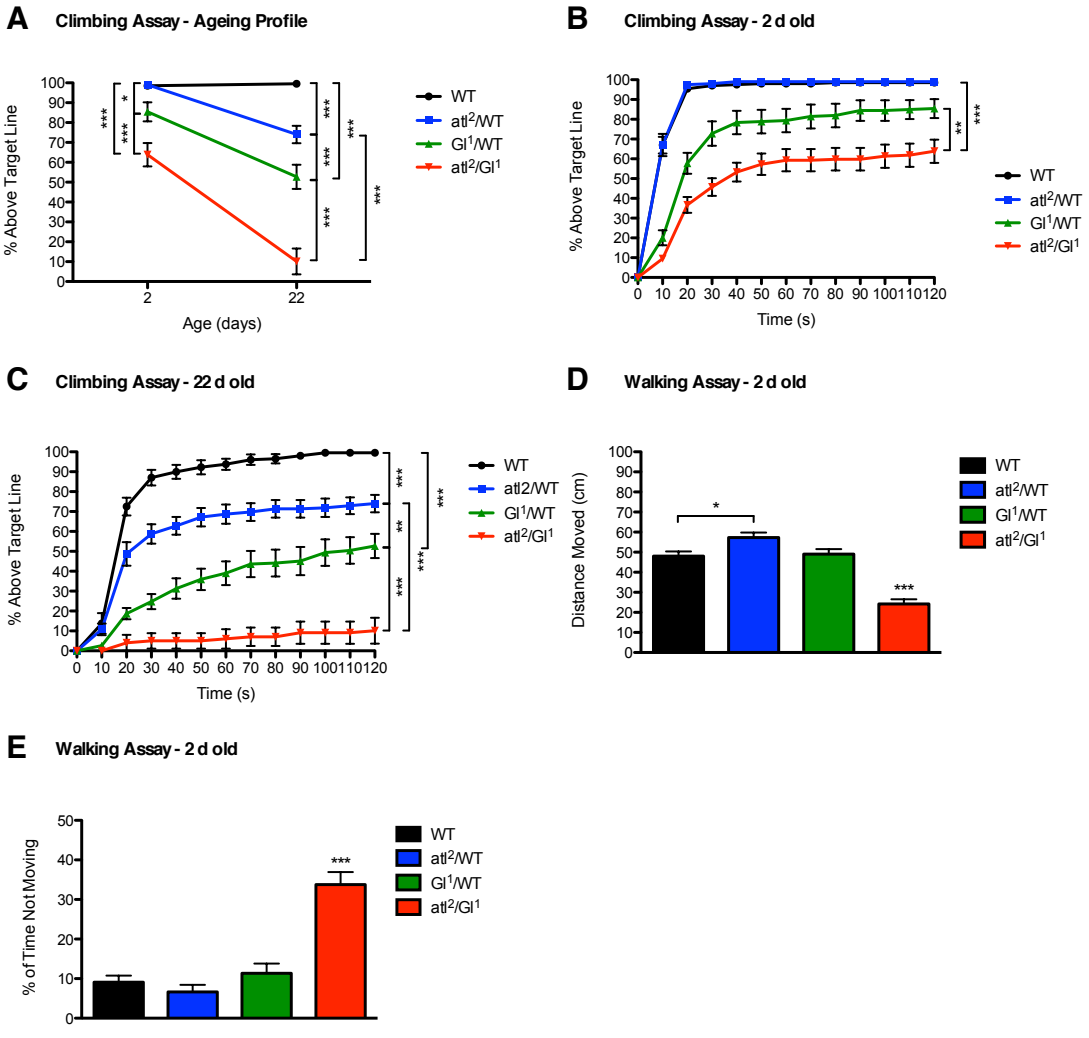

A
Climbing Assay - Ageing Profile
% Above Target Line
Age (days)
WT
atl2/WT
Gl1/WT
atl2/Gl1
B
Climbing Assay - 2 d old
% Above Target Line
Time (s)
C
Climbing Assay - 22 d old
% Above Target Line
Time (s)
D
Walking Assay - 2 d old
Distance Moved (cm)
E
Walking Assay - 2 d old
% of Time Not Moving

Figure 10 | atl^2/Gl^1 and both heterozygous mutants—i.e., atl^2/WT and Gl^1/WT—display climbing defects that worsen with age, whereas only atl^2/Gl^1 mutants exhibit walking defects.

(A) Climbing assay ageing profile. Climbing deficits were observed for 2 and 22-d-old transheterozygous atl^2/Gl^1 mutants relative to each heterozygous mutant group and WT controls, for 2 and 22-d-old Gl^1/WT mutants relative to atl^2/WT mutants and WT controls, and for 22 d atl^2/WT mutants relative to WT controls; N_{Climb} WT: 10 (2 d), 11 (22 d); atl^2/WT: 10 (2 d), 10 (22 d); Gl^1/WT: 10 (2 d), 10 (22 d); atl^2/Gl^1: 10 (2 d), 5 (22 d).

(B) Climbing assay trial profile. atl^2/Gl^1 mutants display compromised climbing ability in 2-d-old flies relative to each heterozygous mutant group and WT controls.

(C) Climbing assay trial profile. atl^2/Gl^1 mutants display compromised climbing ability in 22-d-old flies relative to each heterozygous mutant group and WT controls. Additionally, Gl^1/WT mutants showed climbing defects relative to atl^2/WT mutants and WT controls, and atl^2/WT mutants were impaired versus WT controls.

(D) atl^2/Gl^1 mutants moved a lesser distance relative to each heterozygous mutant group and WT controls in a walking assay. Also, atl^2/WT mutants moved a greater distance than WT controls.

(E) atl^2/Gl^1 mutants spent a greater proportion of time immobile relative to each heterozygous mutant group and WT controls in a walking assay.

N_{Walk} = 31 (each genotype).

WT, wild type (2U-CSHL); error bars ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Like *spas*$^{5.75}$*/WT* heterozygous mutants, *atl*^{2}*/WT* heterozygotes moved further than their WT counterparts ($p < 0.05$). This difference, however, was not reflected in terms of time spent immobile.

For a summary of experimental results, refer to Table 1 for single gene effects and Table 2 for the results of genetic interactions.

Genotype	Defect
spas$^{5.75}$/*spas*$^{17\text{-}7}$	**Climbing** – 2 & 8 d **Walking** – 2 & 8 d **Reason**: *spas* required for locomotion (↓ Spastin → defect)
spas$^{5.75}$/*WT*	**Walking** – 15 d (↑) **Reason**: background effect or deregulated circadian rhythm → ↑ spontaneous locomotion close to death **WA not CA defect?** CA ceiling effect
spastin RNAi	**Climbing** – 2, 8, & 15 d **Larva** (data not shown) **Reason**: *spas* required for locomotion (↓ Spastin → defect)
spastin overexpression	**Climbing** – 2 d **Reason**: *spas* required for locomotion (↑ Spastin → defect)
atl^{2}/*WT*	**Climbing** – 22 d **Walking** – 2 d (↑) **Reason**: CA – *atl* haploinsufficiency @ later ages; failure to sequester Spastin? WA – background effect or deregulated circadian rhythm → ↑ spontaneous locomotion close to death **OR**: CA more difficult
atlastin RNAi	**Climbing** – 2 d **Reason**: *atl* necessary for locomotion (↓ *atl* → defect)
Gl^{1}/*WT*	**Climbing** – 2, 15, & 22 d **Reason**: *Gl* necessary for locomotion (dominant negative) **CA not WA?** CA more difficult

Table 1 | Summary of results of single gene effects.

Genotype	Defect
atl^{2}/*Gl*^{1}	**Climbing** – 2 & 22 d **Walking** – 2 d **Reason**: failure to sequester Spastin at ER membrane & disrupted anterograde transport
spas$^{17\text{-}7}$/*Gl*^{1}	**Climbing** – 2 d **Walking** – 2 d **Reason**: failure to sequester Spastin at ER membrane; rampant truncated *17-7* transcript capable of MT severing & disrupted anterograde transport
spas$^{5.75}$/*Gl*^{1}	**Climbing** – 2 d (↑) **Walking** – 2, 8, & 15 d **WA not CA?** defect → spontaneous locomotion ↓; masked by CA motivation/cueing
spas$^{5.75}$/*atl*^{2}	**Walking** – 2, 8 d **WA not CA?** spontaneous locomotion ↓ masked by CA motivation/cueing **Increases w/ age in WA?** deregulated circadian rhythm → ↑ spontaneous locomotion (close to death)

Table 2 | Summary of results for genetic interactions.

CHAPTER 4 - Discussion

4.1. Experimental Hypotheses and Rationale

The first of two operating hypotheses in the current thesis work is that the Drosophila homologues of three HSP-associated genes of interest—i.e., *D-spastin*, *D-atl*, and *Glued*—are associated with behavioural phenotypes (e.g., locomotion deficits) as a result of genetic mutations. This hypothesis was chosen for several reasons. First, many of the genetic mutants that were used throughout this work have been previously validated in the literature. The two *D-spastin* deletion mutants, $spas^{5.75}$ and $spas^{17\text{-}7}$, were previously used by Sherwood and colleagues (2004) to demonstrate D-Spastin's role in NMJ synaptic bouton formation, NMJ synapse function, locomotion, lifespan, and microtubule severing. Lee and colleagues (2009) used the atl^{2} null mutant to demonstrate D-Atl's role in muscle development, synaptic bouton formation, ER and Golgi morphogenesis, microtubule disassembly, interactions with scaffold proteins, and locomotion (Lee et al., 2008). Thus, by examining the same mutants as other studies, we sought to extend the body of knowledge based on previously published findings. Secondly, mutants that have already been associated with behavioural deficit phenotypes in flies, such as $spas^{5.75}$, can act as positive controls, thereby providing validity to experiments and ensuring that these results can be replicated in our laboratory. Third, once internal validity has been established, these mutants can be tested in a battery of behavioural tests and experimental conditions—i.e., different aging parameters in conjunction with a walking, a larval tracking, and a climbing assay—to expand upon and enrich previous experimental

findings. Fourth, the independent testing of genetic mutations enables a baseline to be established to which interaction data can then be compared. Lastly, the first experimental hypothesis not only serves as a further test of two previously established HSP genetic mutations—i.e., *D-spastin* and *D-atl*—but also incorporates a genetic homologue that has only recently been implicated in HSP neuropathology, *Glued*.

After testing the genes of interest individually, the second experimental hypothesis was investigated. The second hypothesis posits that the three genes of interest genetically interact, and that these interactions can be tested using Drosophila homologues. These interactions were investigated by employing the same battery of behavioural tests and experimental conditions as were used for testing the genes singly. The a priori rationale for our interaction studies was that, should a genetic interaction exist, the transheterozygous mutant flies—e.g., atl^2/Gl^1—would display the most severe defect relative to WT controls, with heterozygous mutants—e.g., atl^2/WT and Gl^1/WT—showing intermediate defects. Thus, the effect of the genetic combination being more severe than either genetic mutation individually would argue for the existence of a genetic interaction. This method is derived from the traditional genetic method of complementation grouping in Drosophila, and has been previously used by our laboratory to identify interacting genes in classical olfaction (Bolduc et al., 2008). Furthermore, being that HSP is fundamentally a neurodegenerative disorder, we reasoned that behavioural deficits should become progressively worse over time under both experimental hypotheses.

A total of three behavioural assays and four ageing conditions were used. The behavioural assays consisted of a climbing, walking, or larva tracking assay, and the ageing conditions were 2, 8, 15, or 22 days. Importantly, while the walking and larvae tracking assays only consisted of a single test subject per technical replicate, the climbing assay consisted of 20 flies per technical replicate. Thus, a technical replicate value of 10 (the target number tested per genotype) in the climbing assay actually corresponds to 200 individual flies, whereas a technical replicate value of 20 in the remaining two assays only corresponds to 20 individual subjects. One caveat to note is that—in the interest of time, data throughput, and the identification of as many defects as possible, should they exist—most of the genetic alleles were tested using a subset of these assays and conditions, with few being subjected to the complete battery.

4.2. Gene 1: *D-spastin*

The first gene investigated with respect to the first experimental hypothesis is also the one most commonly associated with HSP and most established in the literature, *spastin*. Adult flies with mutations in the gene *D-spastin* have been shown to have severe locomotion impairments, including the ability to fly, jump, and climb, and have also been shown to drag their hind limbs (Sherwood et al., 2004). In line with this work, we tested flies with similar alleles—i.e., $spas^{5.75}$ and $spas^{17\text{-}7}$—to replicate and expand upon these behavioural findings. Surprisingly, due to observed homozygous lethality, it was not possible to collect the necessary mutants. To remedy this issue, and to test a novel allelic combination, we opted for testing both alleles in their heterozygous forms—i.e., $spas^{5.75}/WT$ and $spas^{17\text{-}7}/WT$—as well as a transheterozygous group containing both

mutant alleles, *spas*$^{5.75}$/*spas*$^{17\text{-}7}$, which is as close to a homozygous null mutant that we were able to test (see Figure 1).

The climbing assay revealed a defect of the transheterozygous flies at 2 and 8 days, and a minor defect of the *spas*$^{17\text{-}7}$/WT heterozygous flies that emerged at 15 days. These results were replicated in the walking assay for the transheterozygous group at 2 and 8 days. These results support the notion that mutations in *D-spastin* result in climbing deficits in Drosophila. The 8-day climbing data implies that the defects resulting from mutations in *D-spastin* are exacerbated by age, which mimics observations of HSP patients.

In 2004, Trotta and colleagues employed the Elav-GAL4 driver to express *D-spastin* RNAi in a neural tissue-specific manner to examine the resulting effects on microtubule stability. The corresponding neural-specific knockdown of *D-spastin* mRNA resulted in a hyperstabilized microtubule network. In our second experiment, we sought to provide a behavioural correlate to these cellular findings by investigating if mutant flies with the same, or similar, genetic alterations would also exhibit measurable behavioural phenotypes. To this end, we directed RNAi pan-neurally against *D-spastin* mRNA using two independent genetic lines (see Figures 2-4). Severe climbing deficits were observed for 2-day-old flies pan-neurally expressing *D-spastin* RNAi for both independent genetic lines. In addition, the first genetic line of flies pan-neurally expressing *D-spastin* RNAi was used to create an ageing profile (see Figure 2). Not surprisingly, the severe behavioural deficit persisted in all age categories—i.e., 8, 15, and 22 days. Although an overall effect of age was detected in the overall two-way ANOVA, this effect

predominantly reflects the decline with age that is typically observed for the startle-induced negative geotaxis assay (Fernández et al., 1999; Gargano et al., 2005; Le Bourg, 1987; Martin et al., 1999). The impairments of the *Elav-GAL4/UAS-spasRNAi* mutant flies does seem to worsen with age compared to controls, although the identification and interpretation of genotype-specific age effects using the climbing assay are complicated by an upper ceiling effect of the WT controls, and a floor effect for severe mutants such as *Elav-GAL4/UAS-spasRNAi*. In other words, if control flies consistently perform at the highest possible score of 100 %, subsequent decreases in performance of the mutant group may not be reflected as a proportional decrease in the score of WT controls. Similarly, if mutant flies perform at the lowest possible score of 0 %, subsequent decreases in performance will be reflected as a minimum plateau. To address the upper ceiling effect in future experiments, one could increase the target height, decrease the trial duration, or analyze mean velocity as opposed to total distance. To address the floor effect, it might be beneficial to lower the target height, although such a decrease in the overall stringency of the assay would necessarily correspond to decreased assay sensitivity. This decreased sensitivity would be especially true with regard to climbing defects of intermediate severity. The locomotion deficits observed for the *Elav-GAL4/UAS-spasRNAi* were also shown in a corresponding larva tracking assay, in which these mutants moved a shorter distance, displayed a lower average velocity while moving, and spent a greater proportion of time immobile relative to WT controls. Altogether, these results replicate and support previous findings in the literature implicating Spastin as a key player in Drosophila locomotion, as shown by pan-neural *D-spastin* knockdown.

In addition to pan-neurally expressing *D-spastin* RNAi, the Trotta group (2004) also pan-neurally overexpressed the *D-spastin* gene itself, again using the Elav-GAL4 driver. In direct contrast to their *D-spastin* RNAi findings, they were able to show that neural-specific overexpression of *D-spastin* resulted in a severely destabilized microtubule network, to the point of being essentially obliterated. Once again, we sought to provide a behavioural correlate to these cellular findings by testing if flies with the same genetic mutations would exhibit impaired climbing behaviour (see Figure 5, panel A). The results clearly show that, like *D-spastin* knockdown, neural-specific overexpression of the *D-spastin* gene caused a severe climbing deficit.

Altogether, these results demonstrate that D-Spastin function is necessary for proper locomotion behaviour in both larval and adult Drosophila. Curiously, unlike human *spastin* mutations, we observed little evidence for *D-spastin* haploinsufficiency, with the only support for this hypothesis coming from the minor, but significant, decrease in climbing ability of 15-day-old $spas^{17\text{-}7}/WT$ hypomorphic, heterozygous flies. The majority of the time, however, *D-spastin* flies with heterozygous mutations were unimpaired. Rather, severe climbing defects emerged when a null *D-spastin* allele was combined with a hypomorphic allele, which served as our closest approximation to a homozygous null mutant. These defects are not surprising considering the critical intracellular roles D-Spastin plays including, e.g., the regulation of microtubule dynamics. In addition, the RNAi knockdown and overexpression data provide a behavioural correlate for the cellular findings of the Trotta (2004) group in that both manipulations of expression result in robust climbing deficits. Considering that *D-spastin*

RNAi knockdown results in hyperstabilized microtubule networks, and *D-spastin* overexpression results in a nearly complete obliteration of the same networks, these results argue for an optimal dosage level of *D-spastin* in the Drosophila nervous system in order to ensure proper microtubule dynamics. One caveat to note, however, is that the target sequences for both *D-spastin* RNAi lines used are associated with potential OTE's regarding a similar microtubule-severing ATPase, Katanin 60. Thus, it is theoretically possible that the observed results are at least partially contributed to by knockdown of Katanin 60 as well as D-Spastin.

4.3. Gene 2: *D-atl*

Considering the impairments in climbing ability observed when RNAi was directed against *D-spastin*, the gene most commonly associated with human HSP, we sought to determine whether or not a similar behavioural phenotype would be observed for the second most common gene, *D-atl*. Others have shown that a homozygous null mutation in *D-atl*, allele atl^1, is associated with severe climbing deficits at early stages that are exacerbated by age (Lee et al., 2008). However, it is important to note that the atl^1 null allele of *D-atl* is a bang-sensitive allele, and that climbing deficits were observed only after the induction of paralysis following a mechanical shock stimulus—i.e., a 5-s vortex period—with paralysis duration increasing with age. Furthermore, this allele was formed by the insertion of a P-element, atl^1, into the first intron of *CG6668*, the homologue of human *ATL1* (Lee et al., 2008). In a follow-up study, Lee and colleagues (2009) imprecisely excised the atl^1 P-element to form the atl^2 allele, a null allele that is homozygous lethal at the pupal stage. Unlike atl^1, bang-sensitivity has not been observed

for the *atl*^{2} allele (Lee et al., 2009). To evaluate the function of the *D-atl* gene, we obtained the *atl*^{2} mutant flies, and tested them in their heterozygous state—i.e., *atl*^{2}*/WT*. Although these flies were not analyzed independently, they were used as part of a cross testing the potential interaction between D-*spastin* and D-*atl* (see Figure 7, panel B) as well as a cross testing the potential interaction between *D-atl* and *Glued* (see Figure 10). In either case, 2-day-old flies heterozygous for a null mutation in *D-atl*—i.e., *atl*^{2}*/WT*—did not exhibit any climbing impairments relative to WT controls. Interestingly, however, a deficit for *atl*^{2}*/WT* flies did emerge at 22 days relative to WT controls (see Figure 10, panel C). These results imply a haploinsufficient mechanism of *D-atl* that appears in aged but not young flies.

After observing severe behavioural deficits that emerged as a result of the pan-neural expression of *D-spastin* RNAi, we were curious as to whether or not pan-neural expression of *D-atl* RNAi would also result in behavioural deficits (see Figure 4, panel B). Using the climbing assay, we identified severe behavioural deficits in these flies at 2 days of age. Curiously, the responder control line (*UAS-atlRNAi/WT*) displayed slightly diminished climbing ability relative to the other controls, although not nearly to the extent of the flies expressing *D-atl* RNAi (*Elav-GAL4/UAS-atlRNAi*).

We also tested whether or not the pan-neural overexpression of the *D-atl* gene would result in climbing impairments similar to those observed for neural-specific *D-spastin* overexpression (see Figure 5, panel B). Interestingly, we did not observe behavioural deficits in flies pan-neurally overexpressing D-Atl. Further experiments involving

quantitative polymerase chain reaction (qPCR) or western blotting will be required to assess the level of overexpression of atlastin and see if an insufficient level was expressed. In the case where there was efficient overexpression, we could speculate that the level of D-Atl is required to be above a certain threshold for normal locomotion, but that there is a minimal side effect to high levels. This finding would be in contrast to what we and others have observed for D-Spastin.

These results, combined with previous findings in the literature, implicate D-Atl as an important protein in Drosophila locomotion. Here, we use a heterozygous null mutant in *D-atl*, atl^2/WT, to demonstrate that a haploinsufficient mechanism may be involved in older flies. Curiously, although the previous bang-sensitive paralytic *D-atl* mutants, atl^1, exhibited locomotor impairments in young flies more consistent with the early-onset neuropathogenesis of SPG3A HSP (Lee et al., 2008), our results obtained using a different allele do not show this effect. Nonetheless, our experimental paradigm circumvents the potential confound of using a mutant in which the observed behavioural phenotype may be caused by bang-sensitivity. Another potential reason for this discrepancy is that we tested a heterozygous null mutant, as opposed to the homozygous mutant used by Lee and colleagues (2008). The reason for testing the heterozygous mutants is to avoid the issue of homozygous recessive lethality of the atl^2 mutants. Thus, the WT copies of D-Atl could be compensating for the diminished D-Atl levels caused by the atl^2 null mutation, a mechanism that may falter at later developmental stages. It is important to note that the molecular function of these altered polypeptides in human HSP patients is difficult to determine. Although aa mutations can be identified in human

patients, it is difficult to determine how these mutant polypeptides might behave in vivo. Null mutations in flies are used to knock out the function of the gene, thereby providing insight into its function; however, this insight may not be physiologically relevant when extrapolating these findings to a complex human disorder. Nevertheless, a subset of our data agrees with previous findings regarding the early-onset nature of SPG3A HSP considering that pan-neural *D-atl* RNAi expression resulted in robust behavioural deficits in young, 2-day-old, flies. Although D-Spastin and D-Atl have been shown to functionally overlap, physically interact, as well as contribute to a similar HSP neuropathologic mechanism, our results suggest that they differ in terms of dose response. On the one hand, our RNAi and overexpression data dictate that an optimal concentration of D-Spastin is required for normal climbing behaviour in Drosophila. On the other hand, overexpression of D-Atl did not appear to have any impact on climbing ability, thereby suggesting that low levels of D-Atl are detrimental, although an overabundance has no effect. This notion argues for a more ancillary role of D-Atl than D-Spastin in the neuropathologic mechanism of HSP.

4.4. Gene 3: *Glued*

A recent study (Holbert et al., unpublished) has implicated the dynactin-1 protein, also known as $p150^{Glued}$ and homologous to Glued in Drosophila, as a central component in a HSP protein network. Thus, we sought to obtain functional data about the role of its associated gene *Glued* in locomotion using our well-validated method. We wondered if we would observe locomotion defects similar to those observed for both *D-spastin* and *D-atl*. We began by testing mutant flies heterozygous for a previously characterized

dominant negative mutation in *Glued*—i.e., Gl^{1}/WT. The use of a dominant negative mutation is associated with both advantages and disadvantages. One advantage is that the Gl^{1} mutant allele was the original means by which the *Glued* gene was identified, and is therefore physiologically relevant, at least in flies (Plough & Ives, 1935). Secondly, by employing a dominant negative, we were able to better mimic a null state in which the normal function of the *Glued* gene is blocked, thereby simplifying the interpretation of its physiological function. However, the use of a dominant negative allele opens the possibility that the mutant polypeptide, truncated in this case, might disrupt both the function of its WT counterpart, as well as other surrounding proteins. Although these possibilities complicate the interpretation of obtained results, our objective was to assess the putative role of *Glued* in Drosophila locomotion. Future experimentation will therefore be required to tease out the precise mechanism by which these behavioural phenotypes arise. With that said, we did observe climbing deficits for the Gl^{1}/WT mutant flies at all age groups tested—i.e., 2, 8, 15, and 22 days (see Figure 6). These results clearly show a decrease in climbing performance of the Gl^{1}/WT mutant group with age, which was not observed in WT controls. These results support the hypothesis that *Glued* has a role in Drosophila locomotion. Use of a dominant negative allele, however, precludes any conclusions that could be drawn regarding a haploinsufficient versus haplosufficient mechanism of the physiological protein.

In addition to testing whether or not mutant flies heterozygous for a dominant negative allele of *Glued* would exhibit climbing impairments, we also tested whether the neural-specific expression of *Glued* RNAi would result in similar impairments (see Figure 4,

panel C). Curiously, in spite of the positive results obtained for the pan-neural expression of RNAi directed against both *D-spastin* and *D-atl* mRNA (see Figure 4, panels A & B), no climbing impairments resulted from the pan-neural expression of *Glued* RNAi. Negative results from RNAi experiments must be interpreted carefully, however. This is because different genetic lines expressing different RNAi constructs can vary in terms of their penetrance and expressivity. For example, a third genetic responder line expressing *D-spastin* RNAi was used in our experiments (VDRC, FlyBase ID: FBst0459936; data not shown) that did not show climbing defects, which was in stark contrast to the results from our other two *D-spastin RNAi* lines. One possibility for this difference is the potentially low penetrance and expressivity of the third genetic responder line. Other transgenic lines are available and will be tested in the future.

Thus, our results imply that *Glued* function alone is involved in Drosophila locomotion, although this has not yet been proven conclusively with RNAi or with other alleles. Provided that the behavioural deficits in the Gl^{1}/WT mutants are due to disruption of *Glued* function alone, and that the reason these deficits are not observed in the mutants pan-neurally expressing *Glued* RNAi is either low penetrance or low expressivity of the RNAi, these results support the recent findings that human $p150^{Glued}$ acts as a central component in an HSP interactome (Holbert et al., unpublished). Furthermore, these results provide a foundation upon which the function of *Glued* can be further probed. The incorporation of $p150^{Glued}$ into HSP neuropathogenesis provides new and exciting avenues for researchers to explore in terms of the causative mechanism of the disorder,

which consistently converges upon hypotheses involving microtubule dynamics and transport.

4.5. Genetic Interaction 1: *D-spastin*D-atl*

After gleaning evidence implicating the roles of the three genes of interest separately with regard to motor function, the genetic interactions between the genes were investigated. This work began with the interaction between the two genes most commonly associated with HSP, *SPAST* and *ATL1*. We initially sought to test whether the interaction between *spastin* and *atlastin* that was previously identified (Evans et al., 2006; Lee et al., 2009; Sanderson et al., 2006) also has a corresponding behavioural phenotype in flies. To test whether or not these genes genetically interact, transheterozygous null mutants of their Drosophila homologues—i.e., $spas^{5.75}/atl^{2}$—were tested using both the climbing and walking assays at 2, 8, and 15 days of age (Figure 7). Intriguingly, no locomotion defects emerged until 15 days, at which point the $spas^{5.75}/atl^{2}$ transheterozygous mutants began to decline in terms of their climbing ability. Notably, this is the same trend in terms of age-related decline that was observed for $spas^{17\text{-}7}/WT$ and atl^{2}/WT heterozygous mutants (see Figures 1 and 10, respectively), but not for $spas^{5.75}/WT$ heterozygous mutants (see Figure 1). This is curious in that the hypomorphic allele of *D-spastin* displays age-related decline in its heterozygous form, but it is the null allele of *D-spastin* that displays age-related decline as part of an interaction with *D-atl*. Unfortunately, the hypomorphic allele of *D-spastin* was not tested for an interaction with *D-atl* due to the a priori belief that the more severe genetic mutation in *D-spastin* would result in a more severe climbing defect. The age-related decline of the $spas^{5.75}/atl^{2}$

transheterozygous mutant group closely parallels the age-related decline of the *atl*^{2}*/WT* heterozygous mutants, however, suggesting that the observed defect is likely due primarily to the contribution of the *atl*^{2} null allele. Altogether, these results suggest no genetic interaction between *D-spastin* and *D-atl*. Additionally, these results support the finding that in spite of physically interacting with each other, the presence of Atl-1 in humans does not modify the function of Spastin (Evans et al., 2006).

However, a discrepancy exists between the findings of the climbing assay and the findings of the walking assay regarding flies of the same genotype from the same genetic cross. This discrepancy was also found in the *D-spastin*Glued* interaction data (discussed below). Locomotion defects were observed for *spas*$^{5.75}$*/atl*^{2} mutants in the walking assay, suggesting that a component of the 15-day-old deficit observed in the climbing assay could be due to the genetic interaction between *D-atl* and *D-spastin*; however, the trends that emerged with age are opposite those observed in the climbing assay.

These discrepancies between startle-induced negative geotaxis and spontaneous locomotion have been previously reported. For example a 2010 study by White and colleagues investigating the dopaminergic system in Drosophila showed a marked contrast between the ageing trends observed for climbing ability versus walking activity and exploratory behaviour. Although this group of researchers observed an age-related decline in climbing ability typical of locomotion involving startle-induced negative geotaxis (Fernández et al., 1999; Gargano et al., 2005; Le Bourg, 1987; Martin et al.,

1999), they found that the level of walking activity and exploratory behaviour, in contrast, actually increased at later stages in life (White et al., 2010). Although the precise cause of age-related deficits in negative geotaxis is not known (Grotewiel et al., 2005), it is likely related to the functional senescence involved in the deterioration of internal systems with age (White et al., 2010). The opposing increase in walking activity and exploratory behaviour with age, however, is likely due to deregulated circadian motor control in animals approaching death (White et al., 2010). Similar findings have been observed for inbred Drosophila strains (Fernández et al., 1999), old mice (Wax & Goodrick, 1978), and old rats (Martin et al., 1986). Taken together, the findings of these studies suggest that distinct neural circuits, with different susceptibilities to age-related functional decline, likely govern walking activity and startle-induced locomotor behaviour. These neural circuits are potentially involved in the broader category of cued versus non-cued behaviour, a distinction that is often made in rodent research involving spatial learning, memory, and behaviour. In fact, it has been shown in rodents that cued learning depends on the striatum, whereas non-cued spatial learning depends on the hippocampus (Baldan Ramsey & Pittenger, 2010; Morris et al., 1982; O'Keefe & Nadel, 1978; Packard & McGaugh, 1992). Another important finding in the literature is that although spontaneous locomotor activity typically decreases with age in female flies, it has been shown to increase up to the fifth week of age in male flies before it declines (Fernández et al., 1999).

Considering this information, it seems as though the putative interaction between *D-spastin* and *D-atl* has manifest as an effect that shortens the lifespan of the $spas^{5.75}/atl^{2}$

mutants to the point that their circadian motor control becomes deregulated at a more rapid rate than the other groups (as shown by the increase in distance moved in the walking assay in Figure 7, panel E). Notably, the *spas*$^{5.75}$*/WT* group also shows a minor increase in distance moved with age. The previous finding in the literature that *spas*$^{5.75}$ null mutants display a markedly reduced lifespan therefore strengthens the deregulated circadian rhythm hypothesis (Sherwood et al., 2004). Additionally, a shortened lifespan has been shown for *atl*^{1} homozygous mutants (Lee et al., 2008), but lifespan analyses have not yet been performed for the *atl*^{2} allele used in this thesis work. Nevertheless, the question still remains regarding why a genotype-specific deficit in non-cued locomotion would manifest specifically in the *spas*$^{5.75}$*/atl*^{2} group at early ages, a deficit that is not reflected in young flies of the same genotype in terms of their cued negative geotaxis behaviour. It is possible that the lesser distance moved of 2-day-old *spas*$^{5.75}$*/atl*^{2} mutants reflects a diminished propensity to spontaneously explore their environment. This defect is not sufficiently severe, however, that it cannot be overcome by a cue in the startle-induced negative geotaxis—i.e., climbing—assay, a masking effect that deteriorates at later developmental periods.

Also of interest are the trends observed for *spas*$^{5.75}$*/WT* heterozygous mutants in that these mutants show no statistically significant differences in climbing ability relative to WT controls, but a significant increase in walking ability at 15 days of age. The reason for this minor discrepancy between the climbing and walking assays in this case is probably due to an upper ceiling effect in the climbing assay. However, the reason why this genotype-specific discrepancy would exist in the first place is likely due to the fact

that the genetic lines were not completely isogenized prior to experimentation. Thus, these minor discrepancies are likely due to differential background effects between mutant and WT flies.

4.6. Genetic Interaction 2: *D-spastin*Glued*

The next genetic interaction investigated was that between *SPAST* and *DCTN1*. To determine whether the Drosophila homologues of these genes genetically interact, transheterozygous mutants with respect to a null *D-spastin* and a dominant negative *Glued* mutation—i.e., $spas^{5.75}/Gl^{1}$—were tested in both the climbing and walking assays. The climbing assay data procured in this experiment replicated the findings depicted in Figure 6, in that the climbing ability of the Gl^{1}/WT heterozygous mutants was once again found to be impaired relative to WT controls (see Figure 8, panel A). Intriguingly, however, the incorporation of the null *D-spastin* allele in the transheterozygous mutant group—i.e., $spas^{5.75}/Gl^{1}$—resulted in a genetic rescue of climbing ability relative to the heterozygous Gl^{1} mutants, with the transheterozygous group showing no statistical differences relative to WT controls. This effect was replicated in two independent biological replicates—i.e., two distinct genetic crosses—with a total fly count of approximately 400 flies per genotype tested. This suppressor effect ('two wrongs make a right') result is potentially fascinating due to its implications for HSP neuropathology, prognosis, and potential treatments, although the precise mechanism behind this effect is still unknown.

The walking performance for *spas*$^{5.75}$*/WT* mutants was again found to be elevated relative to the other groups, thereby replicating previous findings of this phenomenon (see Figure 8, panel B).

Like the previous data for the interaction between *D-spastin* and *D-atl*, the results between the climbing and walking assays for the *spas*$^{5.75}$*/Gl*^{1} transheterozygous group are seemingly contradictory. This contradiction arises from the genetic rescue of the *spas*$^{5.75}$*/Gl*^{1} mutants relative to the *Gl*^{1}*/WT* mutants in terms of climbing ability, whereas *spas*$^{5.75}$*/Gl*^{1} mutants are markedly impaired relative to both *Gl*^{1}*/WT* and WT controls regarding walking ability. Considering the relatively low variability of the data, the use of experimenter blinding protocols, the use of consistent sources for parallel experiments, and the sufficiently high number of technical replications, it appears that the putative interaction between *D-spastin* and *Glued* has manifest differently in cued (climbing) versus non-cued (walking) locomotion. Consider, for instance, that the *spas*$^{5.75}$*/Gl*^{1} produces a spastic phenotype that either makes movement, or at least its initiation, more difficult. When tested for spontaneous movement, such flies might display a decreased propensity to move relative to controls. However, these same flies could also exhibit a hypersensitivity to a startle stimulus as a manifestation of the spastic phenotype, which may be reflected as an increase in measured climbing performance. Although this hypothesis is speculative and hypothetical, it is an intuitive possibility for what these data could possibly be representing. The notion that cued versus non-cued locomotion are likely regulated by different neurological circuits posits that the results from the two assays need not necessarily coincide.

Interestingly, targeted expression of the same truncated Gl^{1} allele used in this thesis work has been shown to disrupt the formation of synapses of the giant fiber system in Drosophila (Allen et al., 1999), a system that has been shown to mediate the startle response (Sun & Wyman, 1997). Thus, a complex interplay involving giant fiber synapse formation, retrograde axonal transport, and microtubule dynamics underlies how these various factors will manifest as a behavioural response in a startle-induced negative geotaxis assay. Our results suggest that the Gl^{1}-mediated disruption of the startle response, in combination with a null *D-spastin* mutation, has manifest as an increase in hypersensitivity to startle in the case of $spas^{5.75}/Gl^{1}$ transheterozygous mutants.

To further probe the putative genetic interaction between *D-spastin* and *Glued*, the hypomorphic allele of *D-spastin*—i.e., $spas^{17\text{-}7}$—was tested in a similar manner to the null allele, $spas^{5.75}$, in both the climbing and walking assays (see Figure 9). Once again, the climbing ability of Gl^{1}/WT heterozygous mutants was found to be impaired relative to WT controls. Perplexingly, in direct contrast to the behavioural rescue phenotype exhibited by the $spas^{5.75}/Gl^{1}$ mutants, the $spas^{17\text{-}7}/Gl^{1}$ showed a marked decrease in climbing ability relative to all other groups, a defect that was also reflected in the $spas^{17\text{-}7}/Gl^{1}$ walking assay data. Thus, the results show that a decrease in walking activity or exploratory behaviour is exhibited by both the $spas^{5.75}/Gl^{1}$ and $spas^{17\text{-}7}/Gl^{1}$ mutants. However, a decrease in climbing behaviour is only exhibited by the $spas^{17\text{-}7}/Gl^{1}$ mutants, whereas an increase in climbing ability is shown by the $spas^{5.75}/Gl^{1}$ mutants.

It is important to emphasize that these two mutant groups have the same genetic background and differ only in the extent of the *D-spastin* deletion (*spas*$^{5.75}$ is null, whereas *spas*$^{17\text{-}7}$ is N-terminally truncated). To understand this discrepancy, literature regarding interaction domains, deletion regions, and physiological isoforms must be incorporated. However, it is beneficial to first consider the results of the putative *D-atl*Glued* interaction (discussed in section 4.7) before a comprehensive interpretation can be formulated.

Of note is that the impairment observed several times in the climbing ability of the *Gl*^{1}*/WT* mutants is not seen in the walking assay (Figure 9, panels B & C), a result that was also observed in the walking assay data of the *spas*$^{5.75}$*/Gl*^{1} genetic cross (Figure 8, panels B & C). In the case that a defect is identified consistently in the climbing assay, but not the walking assay, a new interpretation must be formulated. It is possible that the climbing assay is a more stringent assay, meaning that some intermediate locomotion deficits will only manifest on the more difficult of the two tasks. Many experiments involving startle-induced negative geotaxis (e.g., Sherwood et al., 2004) use a Drosophila food vial as a climbing apparatus, which is much shorter than the graduated cylinders used in the current thesis work. Thus, by elongating the target climbing height, we have created a situation where an intermediate phenotype—e.g., that of *Gl*^{1}*/WT*—might manifest in the climbing assay, but be washed out by the comparatively less stringent assay involving spontaneous movement on a level plane. An alternative possibility is that the *Glued* mutation affects more specifically structures involved in climbing—e.g., synapses of the giant fiber system—but not as much those involved in walking behaviour.

4.7. Genetic Interaction 2: *D-atl*Glued*

To complete the pairwise analysis of genetic interactions between the three genes of interest—i.e., *D-spastin*, *D-atl*, and *Glued*—the potential interaction between *D-atl* and *Glued* was investigated in both the climbing and walking assays (Figure 10). We used a transheterozygous approach that was similar to our approach with *D-spastin* and *Glued* to assess the genetic interaction between *D-atl* and *Glued*. In contrast to many of the discrepancies observed in earlier data, these data are arguably the most straightforward in that they most closely resemble the expected a priori hypothetical results. As mentioned previously, we initially expected to see the heterozygous mutant groups manifest intermediate behavioural phenotypes, with the double mutant (transheterozygous) groups displaying the most severe behavioural phenotype should a genetic interaction exist. These expectations are reflected remarkably well in the 22-day-old climbing data for the *D-atl*Glued* interaction (see Figure 10, panel C), with the two heterozygous groups—i.e., atl^2/WT and Gl^1/WT—intermediate to the more severe (atl^2/Gl^1) transheterozygous phenotype. Interestingly, although this trend is maintained in the 2-day-old climbing data, the atl^2/WT defect does not manifest until later developmental stages. Note that, once again, the Gl^1/WT climbing deficit was replicated, displaying remarkable intra-assay consistency and low variability. Altogether, the climbing assay data argues strongly for a genetic interaction between *D-atl* and *Glued* in that the behavioural phenotype of the two genetic mutations together is more severe than either individually.

The walking assay data for this genetic cross are also interesting. In this case, the strong atl^2/Gl^1 behavioural phenotype was also reflected in walking ability, which is similar to

the results of the *spas*$^{17\text{-}7}$/*Gl*^{1} genetic cross (see Figure 9). These results suggest that the locomotion deficits of both the *atl*^{2}/*Gl*^{1} and *spas*$^{17\text{-}7}$/*Gl*^{1} transheterozygous mutants are relatively severe, and likely negate any discrepancies that may exist between the two assays, as are observed for more intermediate behavioural phenotypes. Consequently, both a *D-spastin*Glued* and *D-atl*Glued* interaction are argued for.

Once again, the *Gl*^{1}/*WT* mutants were found not to differ from WT controls in terms of their walking ability, replicating previous findings. Also, similar to *spas*$^{5.75}$/*WT* heterozygous mutants, *atl*^{2}/*WT* mutants displayed moved a significantly greater distance in the walking assay relative to WT controls. One potential hypothesis for this elevation in performance is one of differential genetic background effects between the transheterozygous mutants and controls, which would not necessarily be reflected in the climbing assay data due to a ceiling effect. However, considering that the *spas*$^{5.75}$/*WT* and *atl*^{2}/*WT* mutants themselves are from different genetic backgrounds, it seems unlikely that both would exhibit the same performance elevation. More likely is the deregulated circadian rhythm hypothesis. According to this hypothesis, *D-atl* and *D-spastin* mutants, both of which have been shown to have a decreased lifespan (Lee et al., 2008; Sherwood et al., 2004), could exhibit an increase in exploratory behaviour at later developmental ages. Interestingly, this trend was observed for *D-spastin* (see Figure 7, panel E and Figure 8, panel B). On the other hand, the observed increase in exploratory behaviour of the *D-atl* mutants was already observed in young flies (see Figure 10, panel D), suggesting that there are more contributing factors than a deregulated circadian rhythm. Unfortunately, aged *atl*^{2}/*WT* mutants were not tested using the walking assay.

4.8. Conclusions and Future Perspectives

Single gene effects were demonstrated for each of the three target gene homologues—i.e., *D-spastin*, *D-atl*, and *Glued*—in terms of behavioural locomotion defect phenotypes. These phenotypes were reflected primarily by decreases in the proportion of flies climbing passed a threshold height, decreases in spontaneous exploratory activity, and increases in time spent immobile of genetic mutants relative to appropriate genetic controls.

Regarding *D-spastin*, climbing deficits were observed for two independent pan-neural RNAi lines, although these deficits could have been due to OTE's of a similar sequence, that of *D-Katanin 60*. To address this potential issue, the single gene effect of *D-spastin* was also shown in terms of both transgenic GAL4-UAS pan-neural overexpression, and transheterozygous mutants containing both a null and hypomorphic *D-spastin* mutant allele. Defects emerging from both *D-spastin* knockdown and overexpression argues for an optimal in vivo concentration of D-spastin in adult Drosophila. Although a haploinsufficient mechanism has been shown in humans (Svenson et al., 2001a; Svenson et al., 2001b), evidence for haploinsufficiency in our data only emerged after 15 days for the hypomorphic, heterozygous *D-spastin* mutants—i.e., $spas^{17\text{-}7}/WT$. One possible reason for this discrepancy is that human haploinsufficiency has been observed after heterozygous splice-site mutations, which do not correspond to the null and partial deletion mutants used in this thesis work. Altogether, these data suggest that D-Spastin is a crucial player in Drosophila locomotion. Our results also expand the literature by showing an evolution of behavioural impairments with age, a key observation when

studying a neurodegenerative disorder. Moreover, our *D-spastin* data provides a strong foundation for pharmacological interventions, such as those involving the alteration of microtubule stability. Further studies to assess the protein levels in each mutant will be important, as well as the confirmation of mutant alleles and corresponding protein products. Furthermore, confocal imaging of motor neurons and immunolabeling for microtubule acetylation—a marker of stability (e.g., Trotta et al., 2004)—will be instrumental in understanding the intracellular modifications that occur with age. Such experiments may provide fundamental insights into the neuropathological mechanisms of HSP. Finally, this is to our knowledge the first time that *spastin* mutations were tested in Drosophila for their role in spontaneous, non-cued, locomotion.

Regarding *D-atl*, single gene effects were observed for pan-neural knockdown of D-Atl, but not pan-neural overexpression. These results suggest that a minimal concentration of D-Atl is required for normal locomotion in Drosophila, whereas an elevated level of D-Atl does not have a deleterious effect. Single gene effects for *D-atl* were also observed for heterozygous null mutants after 22 days. Altogether, these results suggest that D-Atl function is necessary for normal locomotion in Drosophila, and imply that a haploinsufficient mechanism may be involved at later developmental stages.

Regarding *Glued*, single gene effects were observed for heterozygous dominant negative mutants regarding climbing ability. The climbing ability of these mutants was noticeably impaired as early as 2 days, a defect that was exacerbated by age until the 22-day-old time point. Thus, the function of Glued in Drosophila locomotion is implied by these

data, but has not been shown conclusively due to the observation of a defect using only one dominant negative allele, and one biological replicate—i.e., a single genetic cross. Thus, future experimentation will be required to strengthen this result. These experiments would include testing other mutant alleles and using additional transgenic RNAi lines directed against *Glued*. We would also like to perform western blotting to determine the Glued protein level required for normal locomotion.

In terms of genetic interactions, a slight impairment in climbing ability was observed only after 15 days for mutants transheterozygous for a null mutation of *D-spastin* and of *D-atl*. These data suggest a *D-spastin*D-atl* interaction that manifests at later developmental stages in terms startle-induced negative geotaxis—i.e., the cued locomotion of the climbing assay—although this effect could be due to the influence of the atl^2 allele alone. The results also show that mutants of the same genotype displayed a decreased propensity to spontaneously move at younger developmental stages, with a subsequent increase in walking activity, perhaps arguing for deregulated circadian motor control of dying flies. Altogether, these results imply that a genetic interaction between *D-spastin* and *D-atl* may exist. This is the first in vivo use of transheterozygous mutants for various HSP genes.

Based on our results, the interpretation of the putative genetic interaction between *D-spastin* and *Glued* becomes somewhat complex, but interesting considering the potential implications regarding the extent of the *D-spastin* deletion. We will, therefore, attempt to build a model based on known interactions.

The *spas*$^{5.75}$ allele is null, whereas the *spas*$^{17\text{-}7}$ allele is hypomorphic, which expresses a truncated D-Spastin polypeptide. The level of physiological function of *spas*$^{5.75}$ is thought to be absent, but the level in *spas*$^{17\text{-}7}$*/WT* is somewhat unknown in that the truncated *spas*$^{17\text{-}7}$ transcript could contain some residual functionality (Sherwood et al., 2004), especially considering its preserved, highly conserved, AAA domain.

Both climbing and walking deficits were observed for mutants transheterozygous for a hypomorphic *spas*$^{17\text{-}7}$ allele of *D-spastin* and a dominant negative allele of *Glued*. Walking defects were also observed for mutants transheterozygous for a null *spas*$^{5.75}$ allele of *D-spastin* and the same dominant negative *Glued* allele. However, not only was the climbing ability of *spas*$^{5.75}$*/Gl*^{1} mutants not impaired, it was actually rescued relative to *Gl*^{1}*/WT* heterozygous mutants, and indistinguishable from controls. This is curious in that the only difference between these two transheterozygous groups is the extent of the deletion of the *D-spastin* gene. This suggests that in order to negatively impact locomotion, the *Glued* dominant negative allele requires something that is occurring in the hypomorphic *spas*$^{17\text{-}7}$ allele condition, but not in the null *spas*$^{5.75}$ allele condition.

We wondered if the differential effect of the D-spastin alleles on rescuing the *Glued* mutant phenotype could be related to differential binding to D-Atl via the N-terminal region of D-Spastin. The *spas*$^{17\text{-}7}$ deletion eliminates the entire first exon of the D-Spastin protein and extends from the first start codon to residue 255 of the full-length protein (Sherwood et al., 2004). This deletion eliminates both physiological start codons, and extends into the N-terminal MIT domain (Roll-Mecak & Vale, 2008). Importantly, the

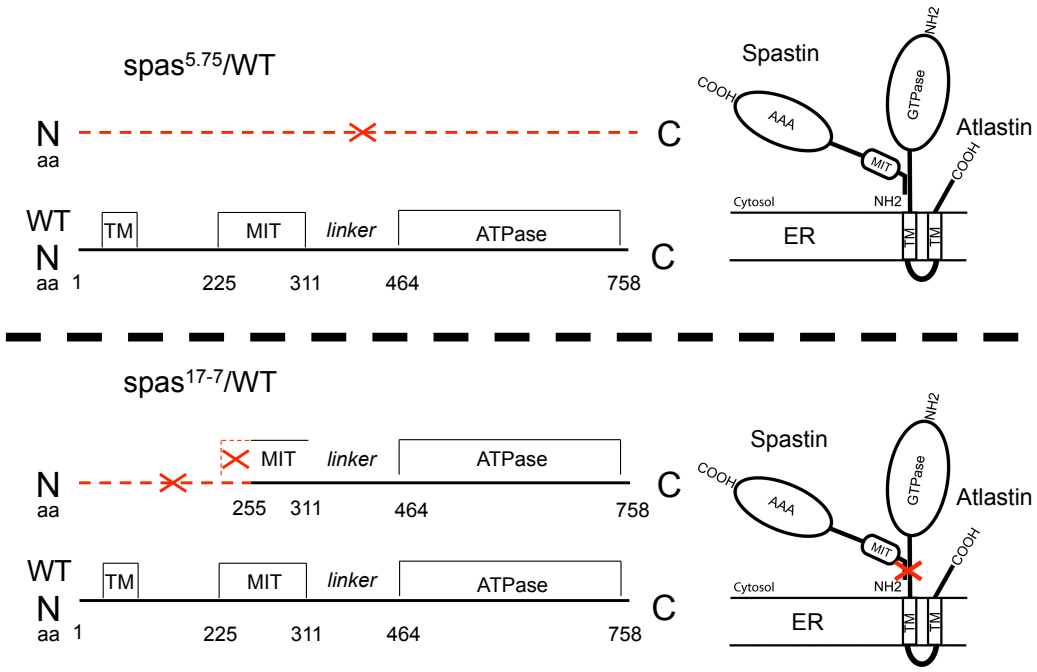
spas$^{17\text{-}7}$ deletion also eliminates the putative N-terminal TM domain shown to be involved in the interaction between D-Spastin and D-Atl via GST pull-down assays (Lee et al., 2009; Roll-Mecak & Vale, 2005). This binding region has also been shown using human proteins in which the hydrophobic region of Spastin (residues 49 to 80) was shown to interact with Atl-1 (Sanderson et al., 2006). This N-terminal hydrophobic region is only present in the full-length, cytosolic isoform of human Spastin, which is the form selectively enriched in the brain and spinal cord (Claudiani et al., 2005; Sanderson et al., 2006). Of course, such CNS-specific enrichment also corresponds to the regions primarily affected by HSP neuropathology. Thus, *spas*$^{5.75}$*/Gl*^{1} transheterozygous mutants would express 50% WT D-Spastin with the potential for further increases in function due to physiological compensatory mechanisms. Importantly, then, all D-Spastin transcripts produced in these mutants would be capable of binding D-Atl. On the other hand, *spas*$^{17\text{-}7}$*/Gl*^{1} transheterozygous mutants would express 50% WT D-Spastin and 50% N-terminally truncated D-Spastin, the latter of which is not capable of binding D-Atl. As mentioned previously, this truncated D-Spastin polypeptide has the potential for residual functionality (Sherwood et al., 2004).

Binding experiments show that Atl-1 interacts with Spastin in the cytosolic region of the Atl-1 protein just N-terminal of the first TM domain (see Figure 6 of Sanderson et al., 2006 for a schematic diagram). D-Atl has been shown to be an ER membrane protein (Orso et al., 2009). Like D-Atl, human ATLs have been shown to be involved in the homotypic fusion of ER membranes (Hu et al., 2009; Rismanchi et al., 2008). Human Atl-1 has also been shown to be membrane-bound with two TM's that leave both the N-

terminal region—containing both the conserved GTPase domain and the region responsible for binding to Spastin—and the C-terminal region exposed to the cytosol (Sanderson et al., 2006; Zhu et al., 2003).

Altogether, this evidence from the literature suggests that the *spas*$^{17\text{-}7}$ truncated polypeptide would not be able to effectively bind to D-Atl, thereby removing a tether of *spas*$^{17\text{-}7}$ to the ER membrane (see Figure 11). Removal of this tether at inopportune times throughout the developmental progress of Drosophila could cause detrimental intracellular effects, such as overabundant microtubule severing, which would reasonably manifest as behavioural phenotypes.

This hypothesis depends on the *spas*$^{17\text{-}7}$ hypomorphic polypeptide retaining some residual function. To examine this possibility, the individual domains, and domain combinations, of D-Spastin must be considered. These experiments were carried out by Roll-Mecak and Vale in a 2005 study, with a follow-up study in 2008. These studies identified the N-terminal region of D-Spastin containing both the TM and MIT domains as responsible for membrane targeting (see Roll-Mecak & Vale, 2008 for a schematic of D-Spastin domains). Deletion of the N-terminal TM domain results in a construct that vigorously severs microtubules (Evans et al., 2005; Roll-Mecak & Vale, 2005; Salinas et al., 2005), displays an ATPase activity similar to the full-length protein (Evans et al., 2005), and binds tightly to microtubules (Roll-Mecak & Vale, 2008). Therefore, the MIT and AAA regions were deemed responsible for microtubule severing. However, the AAA construct



adapted from Sanderson et al., 2006

Figure 11 | The sequestration hypothesis showing differential binding of D-Spastin transcripts to D-Atl at the ER membrane. This hypothesis posits that *spas*$^{17\text{-}7}$*/WT*, but not *spas*$^{5.75}$*/WT* mutants would display locomotion defects due to insufficient sequestration of hypomorphic transcripts at the ER membrane. The *spas*$^{17\text{-}7}$ partial deletion deletes the region of the full-length protein involved in binding to D-Atl, effectively removing a tether of D-Spastin to the ER membrane. Importantly, the *spas*$^{17\text{-}7}$ hypomorphic transcript retains MT-severing functionality due to the preserved AAA domain. Release of this truncated transcript could therefore result in detrimental intracellular MT severing activity. In contrast, all D-Spastin transcripts produced in the *spas*$^{5.75}$*/WT* heterozygous mutant would either be sequestered (i.e., WT allele) or deleted (i.e., *spas*$^{5.75}$ null allele).

alone (lacking the N-terminal region, the MIT domain, and the poorly conserved linker region between the MIT and AAA domains) displayed relatively weaker ATPase activity, microtubule binding, and microtubule severing compared to a construct containing both the MIT and AAA domains (Roll-Mecak & Vale, 2008). The *spas*$^{17\text{-}7}$ deletion extends into the MIT domain, which results in a truncated polypeptide that is intermediate to the AAA-only and MIT+AAA constructs used by Roll-Mecak and Vale (2008). Interestingly, truncation of the linker region between the MIT and AAA domains was shown to abolish microtubule severing while preserving microtubule binding in a construct containing both the MIT and AAA domains.

Sherwood and colleagues determined via reverse transcription polymerase chain reaction (RT-PCR) analysis using *spas*$^{17\text{-}7}$ cDNA that low levels of truncated *spas*$^{17\text{-}7}$ transcripts were still produced. This data, however, was not shown, and therefore the precise nature of the truncated *spas*$^{17\text{-}7}$ transcripts cannot be determined. Nonetheless, based on their results, the *spas*$^{17\text{-}7}$ truncated polypeptide contains part of the MIT domain, all of the linker region, and the AAA domain, provided that the polypeptide is transcribed and translated at an alternative transcription start site proximal to the deletion site.

4.8.i. Sequestration Hypothesis

It is feasible that a mechanism based on the sequestration of D-Spastin during appropriate times in the developmental cycle of Drosophila is involved. Neither transheterozygous *spas*$^{5.75}$*/Gl*^{1} mutants nor heterozygous *spas*$^{5.75}$*/WT* mutants would therefore display a behavioural deficit because the copies of full-length D-Spastin protein would either be

properly sequestered or non-existent as a result of the genetic deletion. This interpretation relies on the observation that evidence of haploinsufficiency is not observed for D-Spastin null deletions, in spite of literature showing the haploinsufficiency of certain human *spastin* mutations (Svenson et al., 2001a; Svenson et al., 2001b). Transheterozygous $spas^{17\text{-}7}/Gl^{1}$ mutants, however, would display a behavioural deficit resulting from the removal of the tether between D-Spastin and D-Atl at the ER membrane, thereby releasing an N-terminally truncated D-Spastin polypeptide with residual function in terms of ATPase activity, microtubule binding, and microtubule severing. This release of truncated polypeptide at inopportune times could reasonably result in excessive microtubule severing within cells that could manifest as behavioural phenotypes. Furthermore, the behavioural deficits observed for the atl^{2}/Gl^{1} transheterozygous mutants could be interpreted as a reduction in D-Spastin sequestration—via the null mutation in *D-atl*—thereby leading to an overabundance of D-Spastin activity (see Figure 12 for an explanatory model based on the sequestration hypothesis). This overabundance of D-Spastin activity is akin to the pan-neural *D-spastin* overexpression results.

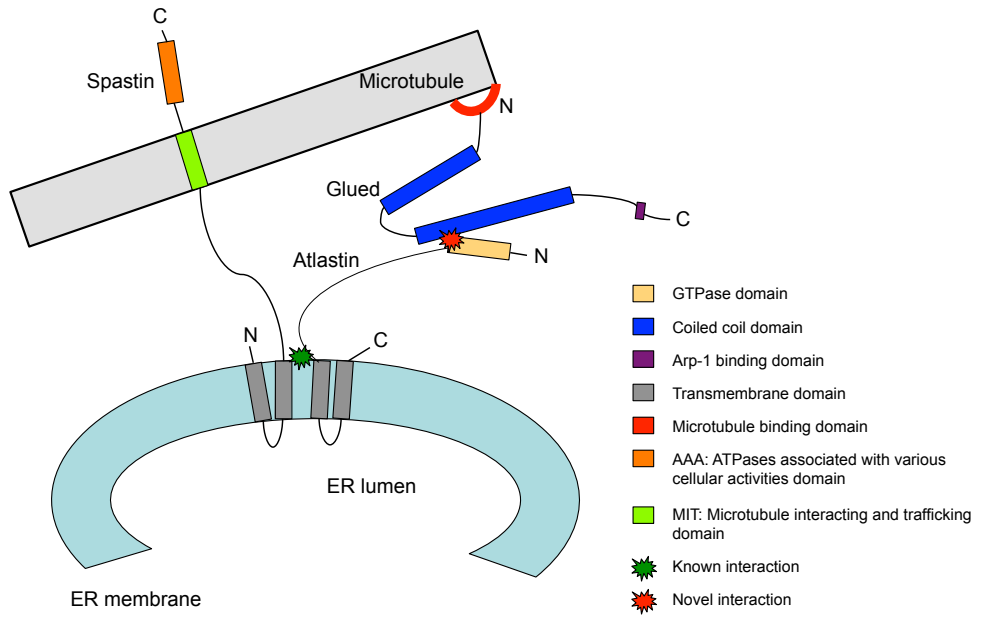
4.8.ii. Concluding Remarks

After interpreting our data using the sequestration hypothesis, the question remains as to why the $spas^{5.75}/Gl^{1}$ mutants would display a behavioural rescue relative to Gl^{1}/WT mutants in terms of climbing ability, but an opposing deficit in spontaneous exploratory behaviour in the walking assay. It appears that the introduction of a null *D-spastin* mutation is beneficial to the climbing ability of genetic mutants. Notably, neither of these

mutants would exhibit a disruption between D-Spastin and D-Atl, thereby largely negating the use of the sequestration hypothesis in this case. Also, without interpreting the *D-spastin* null mutation as rescuing the Gl^{1}/WT phenotype, we would fail to explain why the Gl^{1}/WT climbing deficit does not persist in the $spas^{5.75}/Gl^{1}$ mutant condition. In order to remedy this potentially perplexing conundrum, we look to the data from the walking assay. These data show a diminished walking ability of the $spas^{5.75}/Gl^{1}$ relative to all other groups, a condition that is unlikely if the $spas^{5.75}/Gl^{1}$ mutants were representative of a true genetic rescue. Alternatively, the $spas^{5.75}/Gl^{1}$ transheterozygous mutants could be exhibiting a spastic phenotype that manifests as a decreased propensity for spontaneous locomotion, but a hypersensitivity to startle-induced negative geotaxis. Such an effect is only possible for an intermediate behavioural deficit, with more severe deficits washing out such effects and manifesting in both assays. Thus, further experimentation is necessary to determine the mechanisms underlying this apparent genetic rescue of climbing ability.

This thesis work contains certain caveats and limitations. One limitation is that the presented data set is incomplete in that not all mutants were subject to the full battery of behavioural tests. The particular tests that were performed were determined based on relevant scientific questions on an ongoing basis. Another caveat is that the genetic lines were not isogenized prior to experimentation. The result is that the genetic mutants had a genetic background that was specific to the particular stock center or laboratory that they were obtained from. We opted for incomplete isogenization in order to increase the throughput of our behavioural assays in a limited time frame. Additionally, we

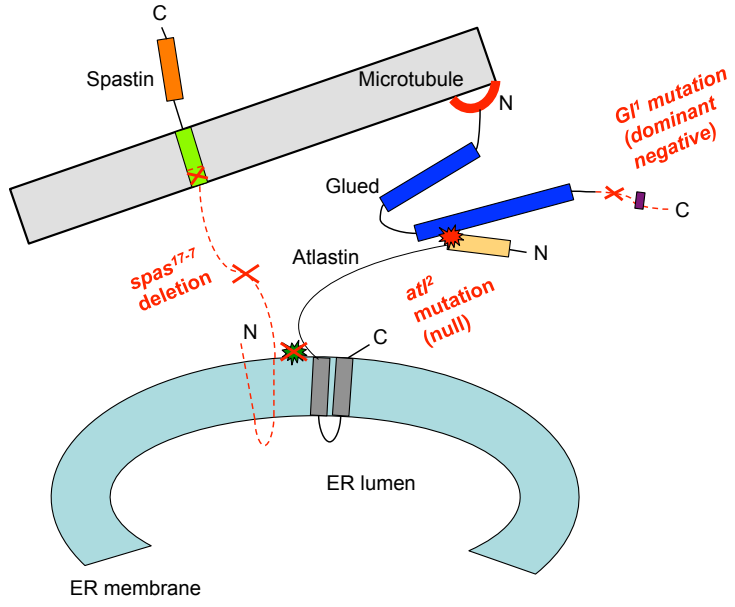
A



*Not to scale

adapted from Holbert et al., unpublished

B



*Not to scale

adapted from Holbert et al., unpublished

Figure 12 | Explanatory model showing relevant experimental mutations.

(A) Explanatory model. This model offers a potential explanation, based on the sequestration hypothesis, as to how the three Drosophila homologues might interact at the endoplasmic reticulum (ER) membrane. Note that D-Spastin is shown interacting with D-Atl at the ER membrane and also with microtubules (MT's) based on known interactions. The interaction between D-Atl and Glued, however, is novel. Protein domains and interactions are colour-coded and shown in the legend to the right.

(B) Illustration of mutations used in experiments. Note that the *spas*$^{17\text{-}7}$ incomplete deletion removes the part of D-Spastin involved in D-Atl interaction, as well as part of the microtubule interacting and trafficking (MIT) domain involved in MT-severing activity. The *spas*$^{17\text{-}7}$ retains severing function and cannot be properly sequestered by D-Atl. The atl2 null mutation disrupts the known interaction between D-Atl and D-Spastin as well as the novel interaction between D-Atl and Glued. The Glued dominant negative C-terminal deletion removes the part of the protein involved in dynactin complex formation, while retaining the coiled coil domains involved in interactions with dynein, and the MT binding domain.

determined that slight differences in locomotion ability between mutant flies of varying genetic backgrounds would not mask the severe locomotion defects of interest. A further caveat was data variability due to seasonal variation and accompanying changes in temperature and humidity. Although efforts were made to control these parameters, environmental chambers may be necessary for future experimentation. The approximately 6 hr developmental window of variability in the ageing procedure (i.e., flies were 42 ± 3 hr old) was an additional source of potential variance. However, efforts made to decrease this window of variability would necessarily decrease assay throughput and was deemed unnecessary for the identification of gross behavioural defects. Additionally, much of the data reflects single biological replicates—i.e., single genetic crosses—and single RNAi lines. These experiments should be repeated to ensure the validity of the reported effects. Another limitation is that the *spastin*atlastin* interaction data is missing a control group, although the missing group was tested as part of a different genetic cross. A dominant negative mutation was used for *Glued*, which has the potential to affect surrounding proteins other than its WT counterpart. Data from *spastin* RNAi experiments is potentially confounded by the possibility of *Katanin 60* OTE's. Being behavioural in nature, these results should also be accompanied by imaging data, protein level quantification and identification, and gene expression profiles. Lastly, Drosophila behavioural data is somewhat limited in terms of the conclusions that can be drawn, and experimental findings can take a long time to translate into the human clinical domain.

Altogether, our data depict single gene effects for all three Drosophila homologues of interest—i.e., *D-spastin*, *D-atl*, and *Glued*—regarding their roles in locomotion behaviour. Our data also support the existence of genetic interactions between *D-spastin* and *Glued* and between *D-atl* and *Glued*. The interaction between *D-spastin* and *D-atl* is implied by our data, but was not conclusively proven. This thesis work embodies a group of experiments that not only test and replicate many findings already shown in HSP literature, but also expand on these findings via novel behavioural approaches and results. This novelty is well represented by the incorporation of *Glued*, the Drosophila homologue of human *DCTN1*, which has only recently been implicated in HSP neuropathology (Holbert et al., unpublished). Considering the human literature, a mutation in the human $p150^{Glued}$ subunit, and the Drosophila Glued subunit by extension, would decrease the binding of the dynactin complex to both microtubules and cytoplasmic dynein, thereby leading to diminished function of dynein and, correspondingly, retrograde transport. Defects in retrograde transport conform to the commonly observed tendency of HSP neuropathology to converge upon some disruption in microtubule trafficking. Our results support the data implicating $p150^{Glued}$ as a central component of a HSP protein interactome by providing a behavioural correlate in Drosophila via the interactions between *D-spastin*, *D-atl*, and *Glued*. Much of our data can be explained by a sequestration hypothesis at the ER membrane, which would likely be upstream of the effects concerning microtubule dynamics and transport. Being fundamentally behavioural in nature, these results are somewhat limited in terms of the conclusions that can be directly drawn from them. However, they do provide a useful expansion to previous findings in the literature while spurring future experimentation into

the precise mechanism regarding how Spastin, Atl-1, and $p150^{Glued}$ functionally interact to produce a common HSP phenotype.

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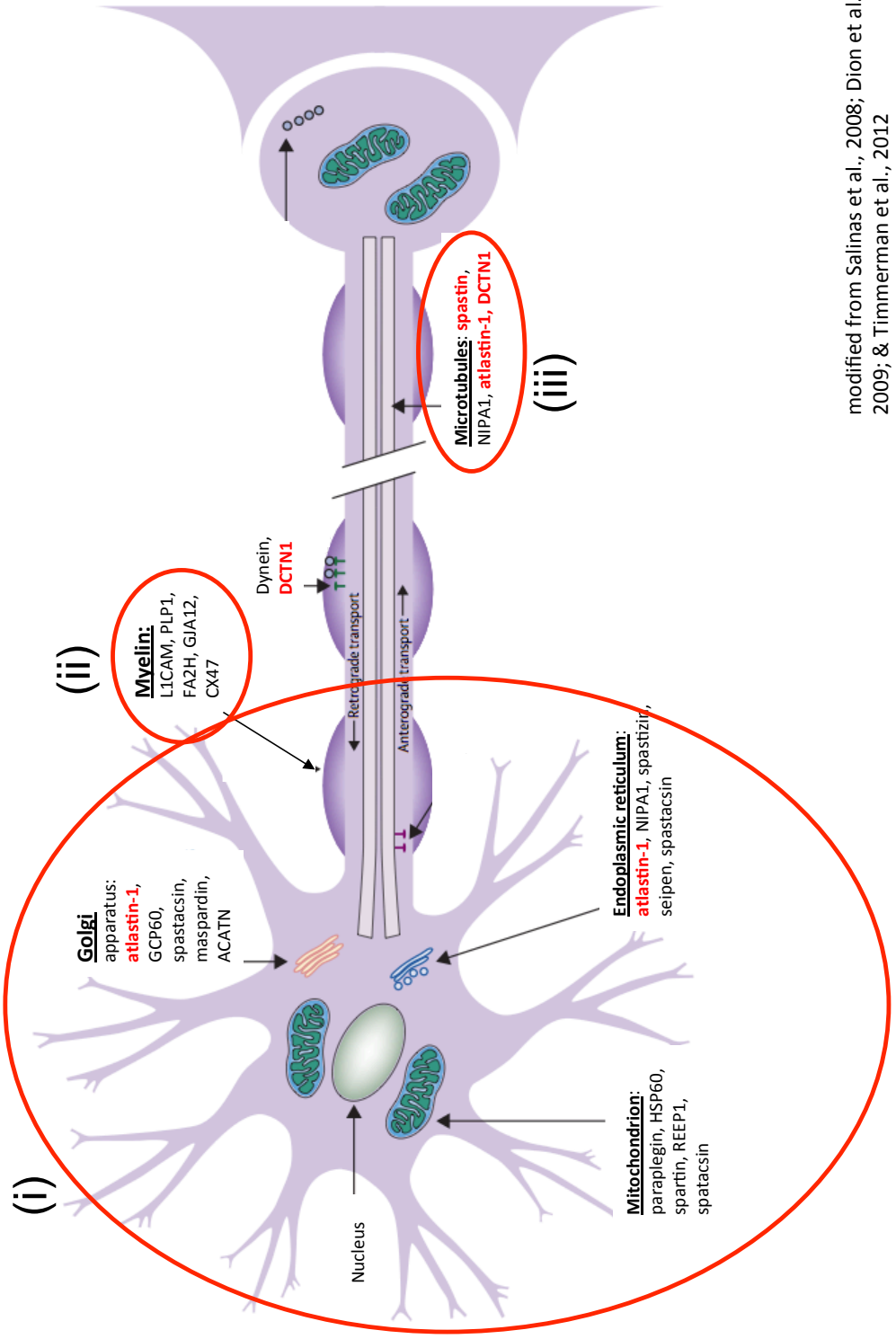
Appendix A - Supplementary Figures

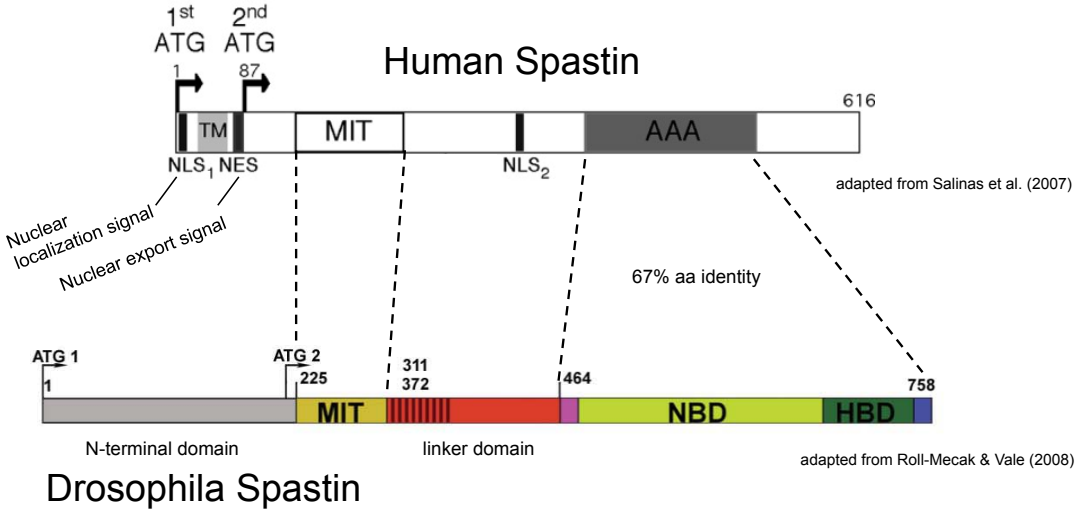


modified from Salinas et al., 2008; Dion et al., 2009; & Timmerman et al., 2012

Supplementary Figure 1 | Molecular convergence of select HSP genes.

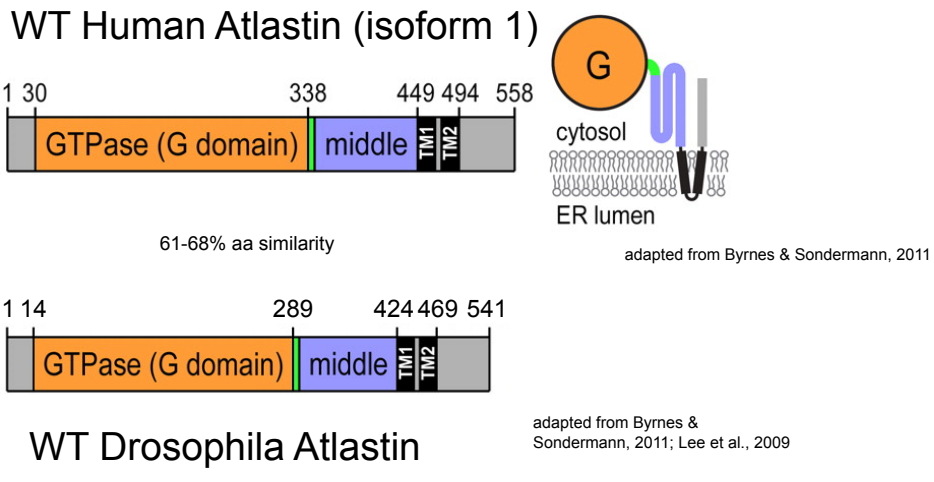
Although there are numerous genes associated with HSP, their molecular functions converge upon three broad molecular pathways: (i) organelle dysfunction, (ii) myelination abnormalities, (iii) defects in microtubule stability and transport. Of the three genes of interest, *ATL1* has been implicated in pathway (i), whereas all three genes of interest—*SPAST*, *ATL1*, *DCTN1*—are involved in pathway (iii). Genes of interest are highlighted in red.

Nuclear role still unknown



Supplementary Figure 2 | Spastin domain composition.

The domain structure of Spastin is well conserved between humans and Drosophila. Both proteins contain an N-terminal hydrophobic domain—thought contain a transmembrane (TM) region and involved in interactions with atlastin—followed by a microtubule interacting and trafficking (MIT) domain, and an ATPases associated with diverse cellular activities (AAA) domain toward the C-terminal end of the protein. The AAA domain is the most highly conserved, sharing 67% amino acid identity between the two proteins. Two physiological start codons are present in both proteins. The Drosophila AAA domain is divided into a nucleotide binding domain (NBD) and a four-helix bundle domain (HBD), whereas the human AAA domain is composed of Walker A and B binding motifs.



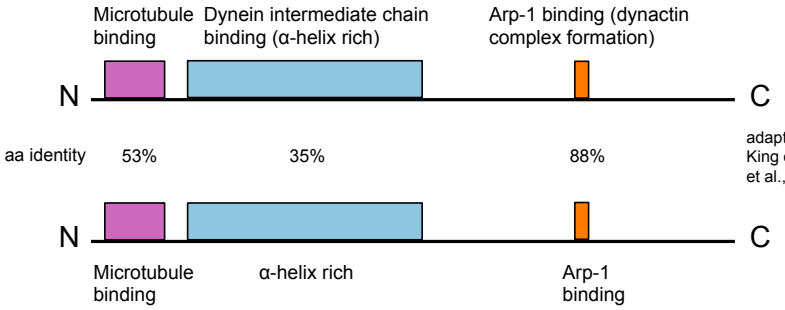
atl^{2}
(functional null allele – no mRNA produced)

Lee et al., 2009

Supplementary Figure 3 | Atlastin domain composition.

Human atlastin, isoform 1 (Atl-1) and Drosophila atlastin (D-Atl) are remarkably similar in terms of both overall length and domain composition. Human and Drosophila atlastin share approximately 61-68% (depending on the isoform of human atlastin) amino acid similarity throughout the length of the protein. Each protein consists of an N-terminal GTPase (G) domain, followed by a middle domain and two C-terminal transmembrane (TM) domains. The top right schematic illustrates how atlastin is embedded in the membrane of the endoplasmic reticulum (ER), leaving both the N-terminal and C-terminal regions of the protein exposed to the cytosol. The atl^2 mutation used in this thesis work is shown at the bottom, which is functionally null due to lack of mRNA production.

WT Mammalian p150^{Glued}



adapted from Allan, 1996; King et al., 2003; & Vallee et al., 2012

Drosophila Glued

adapted from Waterman-Storer & Holzbaur, 1996

Gl^{1} (truncated dominant negative allele)

*binds dynein but not cargo

Swaroop et al., 1985

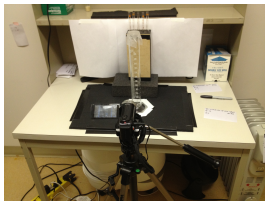
McGrail et al., 1995; Allan, 1996, for review

Supplementary Figure 4 | Mammalian $p150^{Glued}$ and Drosophila homologue Glued domain composition.

Mammalian (rodent) $p150^{Glued}$ and its Drosophila homologue Glued are similar regarding domain composition, with both proteins consisting of an N-terminal microtubule binding domain, a less conserved α-helix rich region, and a C-terminal Arp-1 binding domain. Evolutionary conservation is especially high within the microtubule binding domain and the Arp-1 binding domain, which share 53% and 88% amino acid identity, respectively. The α-helix rich region is thought to be involved in interacting with the dynein intermediate chain, and the Arp-1 binding domain is involved in dynactin complex formation. The dominant negative Gl^{1} mutation used in this thesis work is shown at the bottom. Of note is that the Gl^{1} mutation eliminates the Arp-1 binding domain, thus potentially disrupting dynactin complex formation and, by extension, the retrograde transport of intracellular cargos.

PPE => post-pupal eclosion

~2d old flies, 21°C development

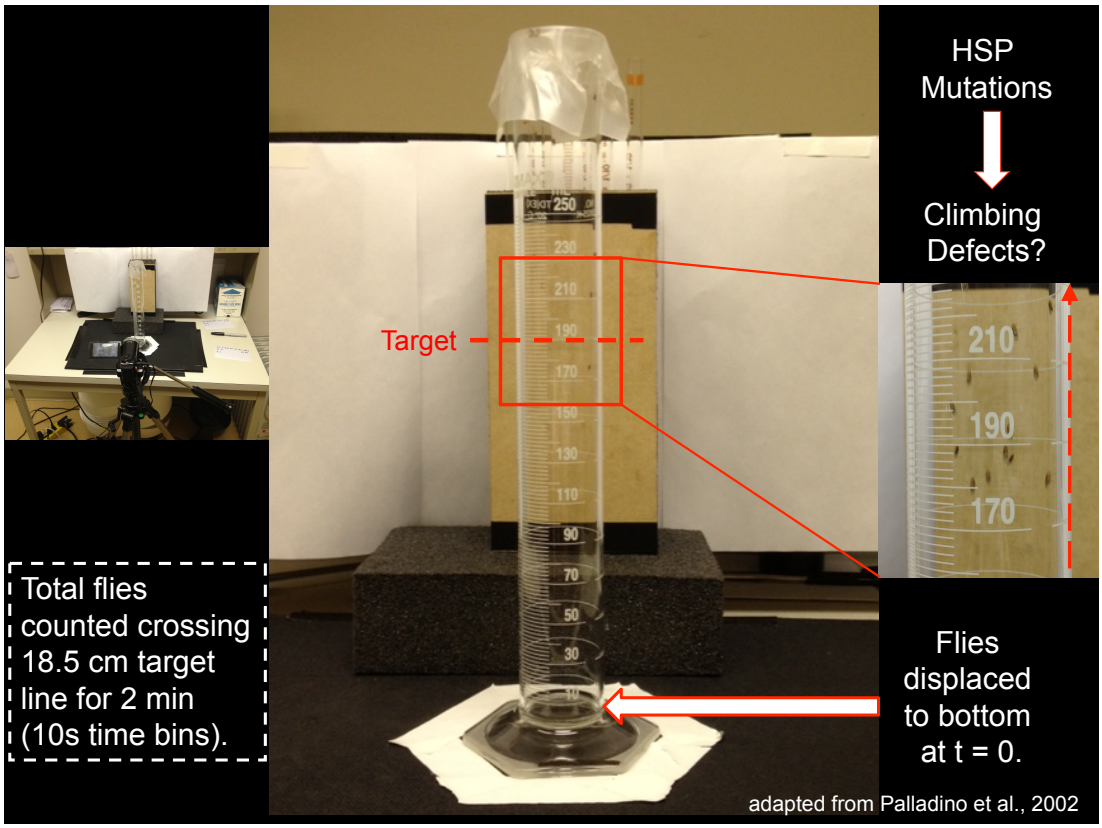


Flies cleared (Day 0 PM) → O/N ~16h → Flies collected at 1d PPE (Day 1 AM) → O/N ~24h → Behavioural Assays (Day 2 AM)

20 flies/vial

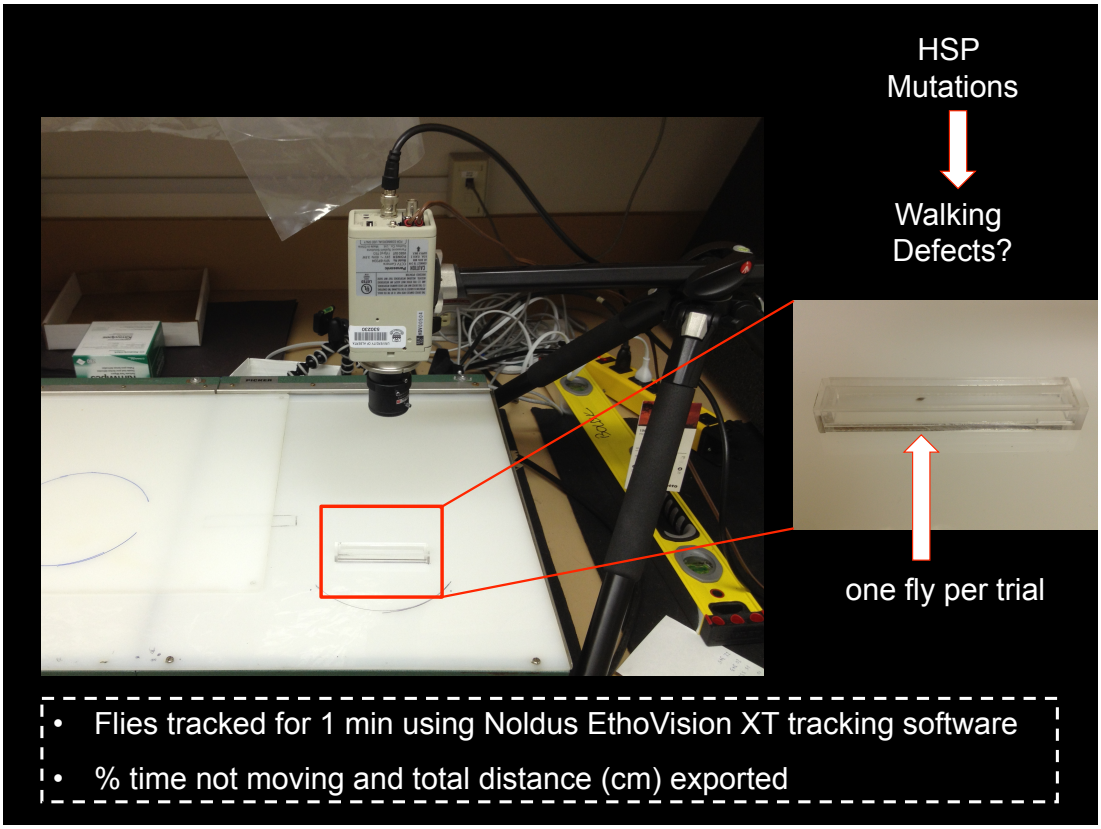
Flies ~ 42 ± 3 hr old

Supplementary Figure 5 | Assay preparation and age synchronization procedures.
In order to minimize the variable of differential developmental stage, flies were age synchronized before the initiation of behavioural experiments. This was done by first clearing newly eclosed flies from stock bottles on Day 0 (bottom left). New flies were allowed to eclose overnight (O/N) and collected the following day, Day 1, using a dissecting light microscope and a CO_2 anesthetization pad (middle bottom). Collected flies were again left overnight to ensure proper recovery from CO_2 anesthetic. Behavioural assays—i.e., climbing or walking—were carried out the following morning, Day 2, using flies that were developmentally synchronized to 42 ± 3 hr post-pupal eclosion (PPE) (top right). The identical procedure was following for ageing experiments except flies were transferred to ageing bottles on Day 2, as opposed to being used for behavioural assays, and aged to the proper ageing condition before being tested.


HSP Mutations
Climbing Defects?
Target
Total flies counted crossing 18.5 cm target line for 2 min (10s time bins).
Flies displaced to bottom at t = 0.
adapted from Palladino et al., 2002

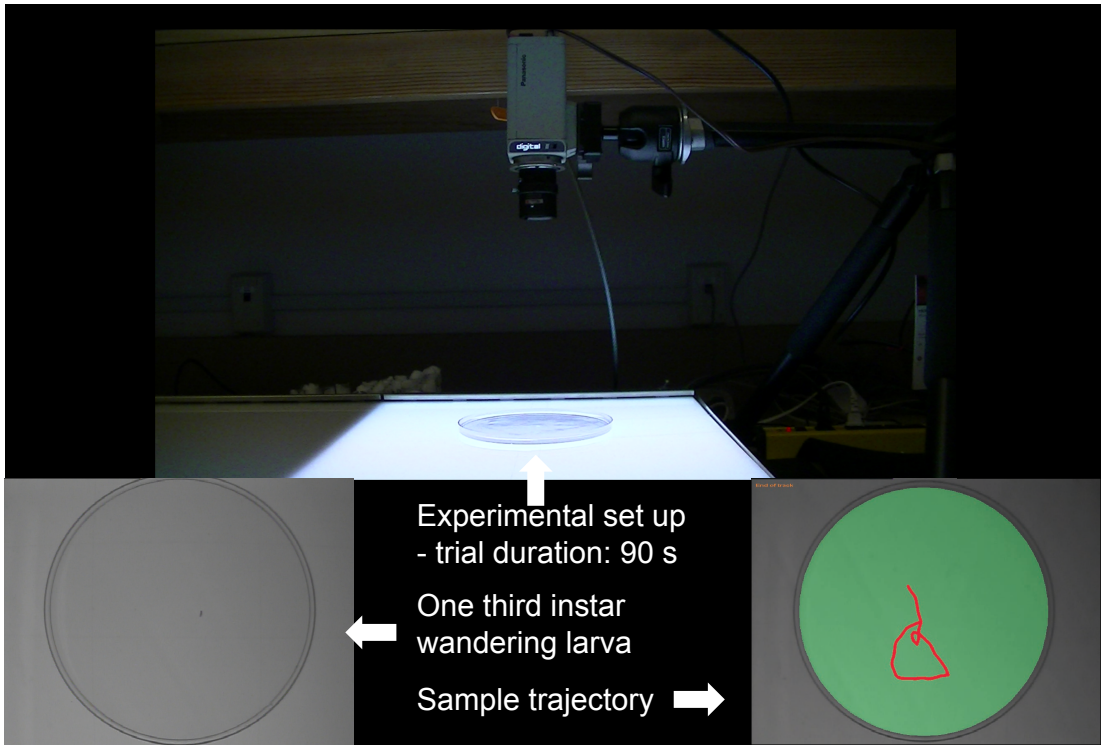
Supplementary Figure 6 | Climbing assay procedure.

The climbing assay was used to assess whether genetic mutations were associated with a corresponding climbing deficit. One of the two primary motivators underlying the assay is positive phototaxis toward diffuse, overhead, fluorescent light sources. The second motivator is startle-induced negative geotaxis. The startle is in response to the light banging used to displace flies to the bottom of the cylinder at the initiation of each new trial, and the negative geotaxis reflects the natural tendency of the Drosophila melanogaster species to travel in a direction opposite the force of gravity. For each trial, flies were allotted 2 minutes to cross a target height of approximately 18.5 cm. The maximum number of flies to cross the target height was recorded in time bins of 10 seconds, and expressed as a percentage of the total number of flies per trial—i.e., 20 flies—after correcting for pre-trial fatalities.


HSP
Mutations
Walking
Defects?
one fly per trial
• Flies tracked for 1 min using Noldus EthoVision XT tracking software
• % time not moving and total distance (cm) exported

Supplementary Figure 7 | Walking assay procedure.

The walking assay was a second method by which we tested the effects of genetic mutations on locomotion ability. Importantly, locomotion in the walking assay is spontaneous (i.e., non-cued) on a level plane, which is quite different from startle-induced negative geotaxis. For each trial, flies were allotted 1 minute to spontaneously explore the walking assay chamber, which measures 8.0 x 1.0 x 0.5 cm. Fly movement was tracked using a dedicated animal tracking software program, and the results were exported for further analysis.


Experimental set up
- trial duration: 90 s
One third instar
wandering larva
Sample trajectory

Supplementary Figure 8 | Larva tracking assay procedure.

The larva tracking assay was a third means by which we were able to assess whether a genetic mutation is associated with a locomotion deficit. For each trial, a wandering third instar larva was selected based on size and wandering behaviour. Following acclimatization, the larva was tracked in a large Petri dish containing solidified agar for 90 seconds. Data acquisition was performed using the same animal tracking software program as the walking assay, rendering the results available for future analysis. Due to the translucent agar and Petri dish, and the semi-translucent body of the Drosophila larva, an X-ray Viewing Box was used to illuminate the larvae from their ventral sides, thereby creating sufficient contrast to permit tracking.