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Rapid in vivo forward genetic approach for identifying axon death genes in *Drosophila*

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Axons damaged by acute injury, toxic insults, or neurodegenerative diseases execute a poorly defined autodestruction signaling pathway leading to widespread fragmentation and functional loss. Here, we describe an approach to study Wallerian degeneration in the *Drosophila* L1 wing vein that allows for analysis of axon degenerative phenotypes with single-axon resolution in vivo. This method allows for the anatomy of specific subsets of axons followed by examination of progressive axonal degeneration and debris clearance alongside uninjured control axons. We developed new Flippase (FLP) reagents using proneural gene promoters to drive FLP expression very early in neural lineages. These tools allow for the production of mosaic clone populations with high efficiency in sensory neurons in the wing. We describe a collection of lines optimized for forward genetic mosaic screens using MARCM (mosaic analysis with a repressible cell marker; i.e., GFP-labeled, homozygous mutant) on all major autosomal arms (~95% of the fly genome). Finally, as a proof of principle we screened the X chromosome and identified a collection eight recessive and two dominant alleles of *highwire*, a ubiquitin E3 ligase required for axon degeneration. Similar unbiased forward genetic screens should help rapidly delineate axon death genes, thereby providing novel potential drug targets for therapeutic intervention to prevent axonal and synaptic loss.

Significance

The elimination of neurites is a widespread event during the assembly of the nervous system, and in the mature brain after injury or in neurological disease. However, molecular pathways underlying neurite/axon degeneration in any context remain poorly defined. We have developed an in vivo genetic approach with single-axon precision in the *Drosophila* wing, allowing for the observation of the onset, progression, and clearance of axon degeneration alongside control axons. We generated genetic reagents to facilitate rapid in vivo isolation of ethyl methane sulphonate-induced mutants that are defective in axon degeneration. Additional unbiased and genome-wide forward genetic screens will provide novel potential drug targets for therapeutic intervention to prevent axonal and synaptic loss after injury or disease.


Author contributions:


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observe axon death alongside healthy, uninjured axons; and (iii) the ability to initiate and visualize axon death without killing the fly (e.g., thereby allowing for F1-based mutagenesis screens). These criteria led us to explore the Drosophila nerve housed in the marginal (L1) wing vein, which contains both sensory neurons and glia (Fig. 1 and Fig. S1; see also below).

When screening through a number of peripheral nervous system Gal4 drivers, we identified candidate OK371-Gal4, which labels ~40 glutamatergic sensory neurons in the wing (Fig. L4) (16). The cell bodies of neurons expressing OK371-Gal4 are aligned along the length of the L1 vein (arrow in Fig. L4), and project their axons within the L1 vein into the thorax. Some of these axons project through the entire wing and are among the longest axons in Drosophila (17). We focused our imaging of these axons in a region of the L1 vein immediately posterior to the costal vein (Fig. L4, Inset). Hereafter, we will refer to this region as the proximal L1 vein.

The L1 vein allows for a graded level of axotomy, which results in two populations of axons: severed axons, whose cell bodies are distal to the injury site; and intact axons, whose cell bodies are proximal to the injury site. Axotomies were performed with nonlethal surgical cuts through the middle of the L1 vein with microdissection scissors in one wing; the other wing served as an age-matched uninjured control (Fig. LB). Flies were then placed back in vials for 0–7 d. Eventually, both wings were removed, and imaged by confocal microscopy (Fig. 1C). To determine the number of uninjured axons, we counted the number of residual cell bodies that remained in the proximal region of the injured wing. We found that 100% of the severed axons initiated WD (i.e., fragmentation) within 1 d postaxotomy (dpa), and that all axonal debris was absent from the L1 vein by 7 dpa (Fig. 1D). These observations support the model of a dosage-dependent signaling mechanism required for efficient clearance of axonal debris in the L1 vein.

Conserved Molecular Mechanisms Modulate WD in the L1 Wing Vein.

We reasoned that if the L1 wing vein nerve was to be a useful model to study WD, similar molecular programs should be in place as observed in other Drosophila or mouse axotomy models including protection by WldD (18, 19). WldD expression levels correlate with protection/conservation in severed axons, where high levels of WldD result in strong protection (18). Recently, work by Fang et al. showed that axon degeneration in different wing sensory neurons are protected by WldD (15). We tested the glutamatergic neurons for their sensitivity to WldD impaired after olfactory receptor neuron (ORN) ablation (19), and found that GFP+ glial cells were required for active clearance of axonal debris: we assayed homozygous viable draper mutant animals [where clearance of axonal debris in the CNS is fully impaired after olfactory receptor neuron (ORN) ablation (19)], and found that GFP+ glial cell bodies persisted at 7 dpa at similar signal intensities as compared side-by-side to an intact axon (arrowhead in Fig. LE, engulfment-defective). Thus, Draper-dependent signaling mechanisms are required for efficient clearance of axonal debris in the L1 vein.

Glial Cells in the L1 Wing Vein Are Diverse and Engulf Axonal Debris.

The most likely candidate cell types executing engulfment of axon debris are local glial cells, which are known to reside in the Drosophila wing (22). To explore the morphological and functional diversity of glial subtypes in the L1 vein, we combined...
a number of Gal4- and LexA-based drivers, as well as GFP traps that label specific subsets of Drosophila glial cells (23–25). We also took advantage of another binary expression system and generated a number of new QF/QUAS-based tools to differentially label axons and glia side-by-side (Fig. 2A, Fig. S2, and see below).

To examine the histological relationship between neurons and glia, we first labeled all glia (pan-glial driver repo-LexA) and found the entire L1 vein was fully covered by glial membranes (Fig. 2B, Top). Glial cells could be divided into two main subtypes: wrapping glia (mvn-2-GFP protein trap) that ensheathe axons directly, and subperineurial glia (L82-Gal4) that surround both axons and wrapping glia (Fig. 2B, Middle and Bottom). To approximate the density of cells within these populations, we drove a nuclear marker in individual glial subtypes and counted the number of nuclei (Fig. 2C and D). Wrapping glial nuclei were found at a density of 2.6/100 μm, subperineurial glia at 4.1/100 μm, and total glial density was 6.6/100 μm. We suspect that these subsets represent the complete complement of glia in the L1 vein, as the sum of ensheathing and subperineurial glial nuclei is comparable to the number found when all glial nuclei were labeled (Fig. 2C and D).

To determine whether glial cells engulfed axonal debris after axotomy we used RNAi-mediated knockdown of draper using glial-specific Gal4 drivers (Fig. 2E and F). Depleting Draper from all glia suppressed clearance of axonal debris at levels equivalent to those found in homozygous draper-null mutants at 7 dpa. Knockdown of draper in wrapping glial also resulted in a strong suppression of clearance of axonal debris, whereas knockdown in subperineurial glia had a much more modest effect (Fig. 2E and F). Thus, glial cells—and in particular wrapping glia—are the key cell type required for clearance of axonal debris in the L1 wing vein. This tissue represents a histologically simple system in which to visualize axonal degeneration and neuron-glia interactions after injury with high cellular resolution (Fig. 2G).

A Tool Kit for Rapid Genome-Wide Forward Genetic Screens with Single-Axon Resolution. The observation of fine axonal structures within an axon bundle where 40 axons are labeled is challenging. Ideally one would label individual axons. We therefore combined the above-described Gal4-based markers for subsets of glutamatergic neurons with mosaic analysis with a repressible cell marker (MARCM) (26). First, we optimized heat-shock–triggered Flippase expression (hs-FLP), which was sufficient to generate GFP+ MARCM clones with single-axon resolution (Fig. 3A and B and Fig. S3). Based on these initial hs-FLP analyses, we concluded that labeling between three and five axons was optimal for visualizing axonal degeneration. Next, we cloned several promoters of proneural genes: we reasoned that these promoters were highly active during wing sensory neuron development to help pattern sensory organ precursors (SOPs), and therefore they could be excellent candidates for specific FLP expression (Fig. S4A and B). We created transgenic flies harboring three different SOP candidates: promoter-FLP transgenes: senseless-FLP (sense-FLP), achaete-FLP and asense-FLP (sense-FLP), and extramacrochaete-FLP (emc-FLP), which is broadly active in epithelial cells before proneural cluster determination, thus less specific to SOP lineages (Table S1).

We prescreened these transgenic stocks to determine cell-type FLP activity (i.e., neurons, glia, or epithelial cells), and rescreened promising candidates for their relative strength in FLP activity in glutamatergic neurons (Fig. S4C), hs- and emc-FLP sources contained strong and broad (e.g., glial, neuronal, and epidermal) activity, whereas both achaete-FLP and asense-FLP harbored weak, but restricted activity to neurons. However, as-FLPβa and as-FLPβu (inserted on chromosomes 2 and 3, respectively) contained strong neuronal activity (Fig. S4C). We therefore decided to test as-FLPβu in a MARCM background for both arms of chromosome 3. as-FLPβu was sufficient to induce GFP-labeled clones, and the induction of clone numbers was not only dependent on the chromosomal arm used for MARCM (1.2 ± 0.9 vs. 5.2 ± 1.7, FRT2A vs. FRT82B, respectively, in Table S2), but also on the copy number of as-FLPβu, because increased dosage of as-FLP leads to the production of increased numbers of MARCM clones in L1 wing vein sensory neurons (Fig. S5).

Transgene activity in the Drosophila genome is influenced by the genomic area surrounding the transgene insertion site. We therefore mobilized as-FLPβu and as-FLPβu to create random as-FLP insertions. These insertions were screened in a MARCM background to ultimately determine “optimally” paired FLP-sources and FRT lines with which one could generate reliably high (~5) or low (1 or 2) MARCM clone induction rates, using any major chromosome arm (Fig. 3C and D). Each newly isolated FLP source was tested in a MARCM assay (chromosomes 3, 2, and X in Tables S2, S3, and S4, respectively). This established collection of tools allows for genetic access to the vast majority of the Drosophila genome for studies of axon degeneration or basic axon biology using the L1 wing vein.

The ability to label individual axons with GFP in the intact wing allowed us to observe fine structural changes in axonal...


integrity over time after axotomy, and compare them to uninjured control axons in the same preparation (Fig. 3E and F). Within 6 h postaxotomy (hpa), 42% of all severed axons (n ≥ 60) underwent morphological changes that included the formation of spheroids [i.e., local swellings or focal axonal degeneration (27)] and the first signs of fragmentation (Fig. 3E, arrow). At 12 hpa, severed axons began to lose their integrity and contained discontinuous axon shafts, whereas control axons remained morphologically intact. This time course supports previous findings (19), but allows for the unambiguous scoring of breakdown of the integrity of even single axons.

**WD in Wing Sensory Neurons Requires dsarm.** To determine whether axon degeneration in the L1 vein was dSarm-dependent, we generated dsarm mutant MARCM clones in the wing. Whereas in wild-type all severed axons underwent fragmentation and were cleared from the vein by 7 dpa, we found that severed dsarm axons remained intact and were morphologically indistinguishable from adjacent uninjured dsarm axons or control axons (Fig. 4A and B). Thus, axon death in the L1 vein requires dsarm function, similarly to sensory neurons in antennae, and multiple neuronal subtypes in mammals (12).

The wing allowed us also to carefully quantify the persistence of severed but intact dsarm axons by counting the number of cell bodies (i.e., axons that are severed) in the cut off wing: we found that 100% of all severed dsarm axons remained intact at 7 dpa, compared with 0% in wild-type (Fig. 4C). Thus, severed axons remain fully protected in dsarm mutants, at least for 7 dpa.

**Rapid Identification of Dominant and Recessive highwire Alleles Through Forward Genetic Screens in the Wing.** To test the efficiency of the wing as a screening tool, we ethyl methane sulphate (EMS)-mutagenized ~1,500 X chromosomes, and screened those for mutants defective in axon degeneration. We identified several mutants exhibiting potent protection of severed axons at 7 dpa (Fig. 5A). For example, all severed x052 mutant axons remained morphologically intact and undistinguishable from uninjured control axons at 7 dpa (Fig. 5B and C). To determine whether x052 mutants could also protect other Drosophila axons, we axotomized ORNs (19). Unilateral antennal ablation resulted in axon degeneration and debris removal of wild-type ORN axons within 10 d (Fig. 5D, arrow); however, intact severed x052 clones were observed at 10, 30, and even 50 dpa.

To identify the molecular nature in x052 responsible for the axon protective phenotype, we used the Drosophila GEM.app whole-genome sequencing analysis approach followed by complementation tests (28). Through this approach, we identified a premature stop in codon 2791 (CAG → TAG) of the highwire locus, which has recently been shown to be required for axon degeneration (13). We tested for alleleism by performing complementation tests using a large N-terminal deletion allele of hiw termed hiw<sup>ΔN</sup> (29). At 1 dpa, severed wild-type and heterozygous

**Fig. 3.** Genome-wide clonal tool for analyses at single-axon resolution. (A) Examples of heat-shock-induced hs-FLP MARCM axon clones. (Top) GFP<sup>+</sup> glutamatergic axon bundle. (Middle and Bottom) Examples of five and two MARCM clones, respectively. Cross-sections are shown on the right. Heat-shock treatment is shown in Fig. S3. (B) Distribution of heat-shock-induced MARCM clones (chromosome X, FRT19A). Dots indicate clone numbers per wing (n = 30). (C) Examples of ase-FLP MARCM clones on chromosomes 3L and 3R. Detailed distribution is shown in Fig. S5. (D) ase-FLP sources as a tool for genome-wide clonal analysis. ase-FLP source and Ga4 driver combinations induce many or few clones on each chromosomal arm. Individual ase-FLP activity is listed in Tables S2–S4. Data are shown as average ± SD (n = 24). (E) Onset of axon death at single-axon resolution. Examples of uninjured (ctl), as well as injured axon clones 6 and 12 hpa side-by-side of healthy axons, respectively (arrows, pronounced blebbing, swelling, and loss of axonal integrity, respectively). (F) Quantification of severed axons undergoing degeneration (n ≥ 60). (Scale bars, 5 μm.) Genotypes are described in SI Materials and Methods.

**Fig. 4.** dsarm is required for axon death in the wing. (A) Examples of wild-type and dsarm mutant axon clones, uninjured and 7 dpa, respectively. (Scale bar, 5 μm.) (B) Quantification of attached cell bodies and axons (gray and white, respectively) in uninjured control and 7-dpa wings. Data are shown as average ± SEM (n = 24). (C) Potent protection of severed axons in dsarm mutant clones at 7 dpa. First, the number of cut off cell bodies was determined, and then remaining cell bodies/axons scored at 7 dpa. Shown is the percentage of severed axons of wild-type and dsarm mutants that remain morphologically intact a 7 dpa. Genotypes are described in SI Materials and Methods.
mutant axons underwent catastrophic fragmentation; however, hiw\(^{ΔN}\)/x052 axons remained morphologically intact, even at 7 dpa (Fig. 5E). Furthermore, severed x052 clones coexpressing the cDNA of either hiw or gfp::hiw fragmented and their debris was cleared within 7 dpa (Fig. 5F and G). Taken together, these data suggest that x052 is an allele of highwire.

Surprisingly, we identified a total of 10 hiw alleles in the same screen (by similar procedures as described above): hiw\(^{ΔN}\), hiw\(^{ΔE}\), hiw\(^{Δ704}\), hiw\(^{Δ726}\), hiw\(^{Δ352}\), hiw\(^{Δ903}\), hiw\(^{Δ275}\), and hiw\(^{Δ275}\), where the molecular nature was identified, and three alleles, hiw\(^{Δ75}\), hiw\(^{Δ969}\), and hiw\(^{Δ704}\), where the molecular lesion was not determined (Fig. 5H). Highwire belongs to the family of RING domain E3 ubiquitin ligases, where the E3 RING domain is involved in the direct transfer of ubiquitin (Ub) from E2–Ub complex to the target substrate (30). Unexpectedly, we found that two alleles, hiw\(^{Δ352}\) and hiw\(^{Δ726}\), dominantly suppressed axon degeneration (Fig. 5I). Interestingly, genome sequencing revealed that both hiw\(^{Δ352}\) and hiw\(^{Δ726}\) contain mutations in or close to the Ring domain (Fig. 5H). These data argue that specific loss of the Hiw Ring domain—which would lead to a protein predicted to bind its substrate but not transfer ubiquitin—can dominantly suppress axon degeneration. Consistent with this notion, Gal4/UAS-mediated overexpression of Highwire containing a nonfunctional RING domain (Hiw\(^{ΔRING}\)) in wild-type animal phenocopies loss of hiw function in both NMJ and axon degeneration (13, 29). Our observation that both hiw\(^{Δ352}\) and hiw\(^{Δ726}\) fully suppress axon degeneration for 1 wk after axotomy argues for a key role for the Hiw RING domain in axon death signaling.

In summary, the above data show that forward genetic MARCM screens in the Drosophila wing, coupled with whole-genome resequencing, is an extremely approach to isolate axon death signaling genes and, in the case of EMS mutagenesis, generate an allelic series that is informative regarding protein function.

Discussion

Our work helps to further establish Drosophila as a powerful in vivo system in which to explore molecular pathways mediating axon degeneration. Our collection of tools allows for labeling, manipulation, and visualization of single axons in vivo. Individual axons can be severed and the resulting onset and progression of axon degeneration can be observed alongside uninjured neighboring axons. This process allows for counting actual axon numbers in vivo for very precise determination of levels of protection in different mutant backgrounds, rather than simply visualizing a large bundle of axons and scoring remaining GFP intensity (15). When large bundles of GFP\(^{+}\) axons are visualized, one cannot conclusively demonstrate that individual axons have remained morphologically intact.

The newly generated FLP sources driven by the proneural promoters are convenient tools to easily generate MARCM clones early in neural lineages: our collection thus simplifies visualization in different mutant backgrounds, rather than simply visualizing a large bundle of axons and scoring remaining GFP intensity (15). When large bundles of GFP\(^{+}\) axons are visualized, one cannot conclusively demonstrate that individual axons have remained morphologically intact.

It is important to note that although it is a very useful tool for screening, the wing vein is not ideal for some applications essential for studying the cell biology of axons and glia: for example, immunohistochemistry and electron microscopy ultrastructural analysis, based on sectioning issues with a very thin
nerve embedded within a thick cuticle. Nevertheless, the production of additional markers for visualizing live axons, organelles, or even proteins certainly allows for detailed visualization of axonal biology in live or fixed preparations (31). We also note that care must be taken when interpreting phenotypes after L1 vein axotomy: transection of the marginal vein not only severs axons, but also local glial cells. In some cases, axonal debris persisted for over a week close to the injury site, even in controls animals. We suspect this represents an injury-induced impairment of glial engulfment function because of degeneration, apoptosis, or necrosis of glia adjacent to the injury site after axotomy. Indeed, we have observed cell bodies of sensory neurons proximal to the injury site degenerating after axotomy, probably because of local glial loss and, in turn, degeneration of now naked, unsupported neuron cell bodies. However, such problems can be avoided by cutting the wing midway along the L1 nerve and then image axonal degeneration in the most proximal region of the wing (box in Fig. L4).

Finally, by performing a small-scale EMS pilot screen, followed by whole-genome resequencing using the web-based GEM.app tool (28), as well as rescue and complementation tests, the wing allowed us to readily isolate several alleles of highwire, a gene recently identified to be required for axon degeneration both in Drosophila and in mammals (13, 14). Among these hiw alleles, we isolated an early stop at coding position 1312 (hiw137 K1312STOP), as well as two dominant alleles that affect the RING domain (hiw27Rd and hiw 903d). Surprisingly, endogenous expression levels of one copy of hiw predicted to contain a mutation in the RING domain was sufficient to provide potent protection to severed axons. Thus, the Hiw RING domain is a key functional domain required to drive axonal degeneration. It remains to be determined how these dominant hiw alleles block axon degeneration (13, 29).

Loss-of-function screens in the wing vein represent a powerful approach to dissect the molecular basis of axon degeneration. Axonal and synaptic degeneration is a hallmark of every neurodegenerative disease and occurs in peripheral neuropathies (1, 2), but mechanisms promoting axonal self-destruction remain elusive. The identification of new genes that regulate axonal degeneration will advance our fundamental knowledge of a basic biological axon death process, and provide exciting new potential drug targets for intervention in neurodegenerative disease.

Materials and Methods

See SI Materials and Methods for more information. SI Materials and Methods contains detailed descriptions of the wing axotomy assay and subsequent wing microscopy, the wing engulfment assay, and quantification of axon degeneration. It also includes information about the ORN unilateral ablation assay, brain dissection, EMS mutagenesis, and genome-wide sequence analyses.

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