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Arjolyn Penas March 29, 2024

Investigating the Role of Fascin-1 in Axonal Development of *Drosophila*

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Abstract

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During early neurodevelopment, neurons produce axonal projections that are directed to their specific targets for synaptic connections, a process known as axon guidance. One of the key axon guidance events occurs in the embryonic spinal cord where select sets of sensory and motor neurons send their axons across the midline to the contralateral sides of the spinal cord. Axon guidance depends on the motile tip of axons called the growth cone. The motility of the growth cone depends on the actin cytoskeleton and its regulation by actin-binding proteins. Fascin is a family of actin filament bundling proteins that is found in growth cones and concentrates in actin-based membrane protrusions called filopodia, but how it functions in axon guidance remains unknown. Therefore, studying how fascin molecules contribute to the ability of the growth cone to navigate to its target location is an important question, especially as neurological diseases have been linked to defects in axon guidance. To study the role of fascin in axon development and guidance, we used *Drosophila melanogaster* embryos as a model system to specifically investigate the formation of the ventral nerve cord. Not only do flies have ventral nerve cords that undergo similar processes to the human spinal cord, but *Drosophila* also contains a homolog of fascin actin-bundling protein 1 (*FSCN1*) called *singed*. Taking advantage of the available genetic tools in *Drosophila,* we performed the knockdown of *singed* in *Drosophila* embryos to determine whether the axonal projections of the ventral nerve cord were altered. Our results showed that *singed* knockdown appears to have some detrimental effects on overall survival of the embryos, and neuronal specific knockdown of *singed* resulted in altered axonal projections of the ventral nerve cord. Due to the limited numbers of embryos obtained for *singed* knockdown, future experiments are still needed to establish the role of *singed* in axon development in *Drosophila*.

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Introduction

Background on Axon Development

Axon guidance is an important part of neuronal development where a diverse group of extracellular factors guide the direction of axonal projections, leading to subsequent wiring of neuronal circuitry. Axon guidance is primarily achieved through the motile tip of the axon: the growth cone. Four major mechanisms affect growth cone movement: long-range chemoattraction, long-range chemorepulsion, contact-mediated chemoattraction, and contact-mediated chemorepulsion (Comer et al., 2019). Attractive and repulsive signals help ensure axons synapse onto the correct location during development. One of the hallmark axon guidance events is midline crossing, during which selected groups of axons cross the midline to project to the contralateral sides for synaptic connections. The presence of attractive and repulsive molecules helps direct these axons to cross the midline while preventing others from crossing. A critical structure for axon guidance is the floor plate, which is found in the central nervous system at the ventral midline; it helps separate which axons will cross the midline to innervate the other side of the body through the presence of different guidance cues (Comer et al., 2019). Midline crossing is important as it has been found to affect sensory information integration, information processing, and neuromuscular control of both sides of the body (Howard et al., 2019).

Improper crossing over can result in neurological diseases, such as horizontal gaze palsy with progressive scoliosis (HGPPS) and congenital mirror movements (CMM). HGPPS results from improper crossing over at the midline in the hindbrain, while CMM is caused by incomplete crossing over in the hindbrain, additional ipsilateral projections in the corticospinal tract, and incorrect contralateral branching in the spinal cord (Comer et al., 2019). As a result, it is important to examine how changes in protein composition can affect axon movement during growth to gain

a better understanding of overall neuronal development, which can eventually provide support for studying and developing therapies for neurological diseases that initially stem from improper midline crossing and incorrect axon guidance.

The Protein of Interest

This project focused on a protein called fascin-1 (FSCN1), a 55 kDa actin-bundling protein abundantly found in growth cones. Growth cones form two main actin-based structures called filopodia and lamellipodia that project from the ends of axons. Filopodia are finger-like protrusions that are important for sensing the environment and interacting with other cells and molecules, while lamellipodia are flat sheet-like projections that are important for the actual movement of the axons (Vignjevic et al., 2006). Fascin is concentrated in filopodia, helping to crosslink and bundle actin filaments and contributing to growth cone morphology and motility (Vignjevic et al., 2006). In a more recent study, fascin has also been found in the actin meshwork of lamellipodia, contributing to the elasticity of growth cones (Tanaka et al., 2019). While there are three isoforms of fascin in the mammalian genome, this project focused on FSCN1, which is widely found in mesenchymal and nervous tissue (Jayo & Parsons, 2010). Specifically, during human embryonic and fetal development, FSCN1 is expressed mainly in the nervous system and is thought to play a significant role in cell migration during these developmental periods (Lamb & Tootle, 2020). Because fascin-1 is a prominent molecule found in both major actin structures of growth cones, this suggests that fascin-1 is a good marker to use for tracking axon guidance in embryos. Furthermore, previous studies have found that downregulation of fascin-1 in the thalamus of mice has been linked to disturbances in neuronal circuitry, causing absence seizures, while upregulation in the hippocampus has been linked to impaired learning and memory in mice with Down's syndrome (Hashimoto et al., 2011). Therefore, interfering with fascin-1 appears to have some

correlation with detrimental effects in the overall functioning of the nervous system, which is why examining fascin-1's function during neurodevelopment is an important goal. As such, this project looked at the development of the ventral nerve cord (VNC) in *Drosophila* embryos to serve as a model for investigating the role of fascin-1 in axonal motility and structural formation of the nervous system; our findings can help contribute to understanding the overall process of neuronal development and what correlations the protein may have with neurological diseases.

Drosophila melanogaster as a Model Organism

Drosophila melanogaster was used as a model organism for this project because axon guidance is relatively conserved between the *Drosophila* VNC and human spinal cord. Similar to human development, commissural axons in the *Drosophila* ventral cord also respond to the same family of guidance cues, such as the attractive molecule netrin1 and repulsive protein slit (Comer et al., 2019). Additionally, while crossing over is seen at the floor plate in humans, a similar process is seen at the midline of the ventral nerve cord of *Drosophila* embryos. In addition to the similarities to humans, flies are a good model to use because of their small size, fast developmental time from embryo to mature adult, large number of offspring, repeating segmented pattern in embryos, relatively transparent membrane of embryos, large database of knowledge for genetic manipulation, and low cost for maintaining lines. Furthermore, fascin-1's function in migration and cell movement is conserved across many organisms, including in *Drosophila. Drosophila* expresses a homolog of the human fascin actin-bundling protein 1 (*FSCN1*) gene called *singed (sn)*. The Singed protein shares a similar function to human fascin-1's role in bundling actin filaments (Cant et al., 1994). Previous studies have found that hemocytes in *Drosophila* embryos and migratory border cells of *Drosophila* ovaries all express fascin for the purpose of cell movement (Lamb & Tootle, 2020). As a result, this study focused on manipulating the presence of

the Singed protein in the ventral nerve cord to evaluate whether Singed is important for axonal crossing over during development.

Specifically, this project used *Drosophila* embryos to study how neural development may change when the expression of the protein is manipulated. *Drosophila* life history consists of four stages: embryo, larva, pupa, and adult. Embryogenesis lasts about 24 hours. After 24 hours, the embryos develop into larvae and go through three instar stages, including feeding and going up the walls to search for a place to undergo pupation before reaching adulthood; the entire process from embryo to adult takes about 10 days (Fernández-Moreno et al., 2007). Though reorganization of the nervous system occurs during the pupal period, new axons are grown during embryogenesis and maintained into adulthood (Sánchez-Soriano et al., 2007). Therefore, examining fundamental changes that occur during axonal development in embryos can provide insight into neural development throughout the lifespan of the fly.

The *Drosophila* VNC is made up of 14 repeating segments, where each segment is divided by the midline into two hemisegments with each hemisegment containing about 300 neurons (Rossi et al., 2021). Within each segment, anterior and posterior commissural tracts extend across the midline, contributing to the contralateral projections seen in the VNC (Araújo & Tear, 2003). *Drosophila* embryos go through seventeen embryonic stages before entering the larval stages, taking around 22 hours (Brody, 1995). The eggs, on average, have a length of 500 micrometers and a diameter of 180 micrometers (Campos-Ortega & Hartenstein, 2013). A large part of embryogenesis also involves the development of the gut. The hindgut and midgut begin folding early in development during stages 7-9; during stage 12, the midgut reorganizes, and by stage 15, the midgut closes ventrally and dorsally (Hartenstein, 1993). Therefore, presence of the gut can be an important marker of development in *Drosophila* embryos.

Neurogenesis begins during stages 9-10 and continues until stage 17. In stage 13, neuronal differentiation begins, where neurons start laying down a scaffold of fibers on the dorsal area of the central nervous system; these give rise to longitudinal and transversal fibers that form the ipsilateral and commissural tracts of the embryo's ventral nerve cord (Hartenstein, 1993). By stage 14, the VNC will begin to condense until stage 17, where the VNC has retracted to about 60% of the egg length (FlyMove, n.d.). As such, we collected embryos that were in the last stages of embryogenesis to ensure development of the VNC had occurred.

Hypothesis

This project aimed to understand the role Singed protein plays during early axon development. By using *Drosophila* embryos as the model organism, significant genetic techniques and previous methodologies are available, allowing for manipulation of the expression of the Singed protein and visualization of the neurons in the ventral nerve cord. The hypothesis of this study is that Singed plays an essential role in axon development, in particular, the midline crossing of developing axons. We expected that there would be a difference in axon growth and crossing over, such as incomplete connections during development due to either the presence or absence through knockdown of *singed*, indicating the importance of the Singed protein during neuronal development.

Materials and Methods

Drosophila melanogaster

Stocks of the flies are kept in vials and bottles at 25ºC and transferred to new food every 7-14 days. When creating the genetic crosses, to ensure that the embryos were of the intended cross, female virgins were collected. After eclosion, female flies are generally not sexually active until around after 10 hours, and they can be identified by the presence of the meconium, a dark greenish spot, on their body. After combining collected virgin females with males, they were allowed to mate before collection of embryos occurred. The males and females were transferred to new vials every 2-7 days to ensure that the offspring of those crosses did not develop and affect the intended crosses.

Embryo Collection

To collect embryos, we used fly cages that have mesh wiring at the top for air flow and an opening on the bottom. A grape juice plate with yeast paste is secured to the bottom, which the flies lay their embryos on. After putting the flies in the fly cage for approximately four hours, they were removed, and the embryos on the agar plate were allowed to age overnight for approximately 16-22 hours so that the embryos were in stages 16-17. The agar plates were made of grape juice, agarose, and sucrose.

Preservation of Embryos

The embryos were then transferred to a strainer where they were dechorionated using 50% bleach for 2-3 minutes. The chorion membrane, which can be visually confirmed by the presence of dorsal appendages, must first be removed because it is difficult to image the inside of an embryo when the chorion is still intact. The embryos were then fixed using 4% formaldehyde, phosphate-buffered saline (PBS), heptane, and methanol (Bashaw, 2010). The embryos were transferred to a

container containing 4% formaldehyde diluted in PBS and heptane, which will separate into two layers with the formaldehyde at the bottom layer. After rocking for 15 minutes, the majority of the embryos float at the interface. After removing the lower formaldehyde phase, methanol was added and shaken for about a minute. After letting the liquids settle, embryos that sink to the bottom are properly fixed and can be collected into another tube. Unfortunately, a sizable number of embryos are lost at this step, as not all embryos will sink after adding the methanol. The embryos were then rinsed with methanol two or three times and stored in -20ºC or stained.

Staining

For staining, two main protocols were used, depending on the antibodies used. Generally, the staining process involved the following steps: permeabilization, blocking, incubation in primary antibodies, washes, incubation in secondary antibodies, and then final washes. For the crosses that stained for Singed, tubulin, and green-fluorescent protein (GFP), a protocol adapted from a double label immunohistochemistry of adult fly central nervous system was used, taking about 7-10 days to complete (FlyLight, n.d.). For embryos that were stained for BP102 and/or GFP only, a shorter protocol was used that took about 2-3 days to complete (Bashaw, 2010).

For the longer protocol, washes and permeabilization were done with either 0.5% or 0.1% Triton X-100 in PBS (PBST) four times for 10 minutes each. Then, embryos were blocked with 2% donkey serum in PBST at room temperature on a rotator. After 1.5 hours, the blocking serum was removed, and the primary antibodies diluted in the blocking serum were added. The primary antibodies were mouse anti-Singed (DSHB sn-7c), sheep anti-a/b tubulin (1:200, Cytoskeleton, Inc ANT02), and rabbit anti-GFP (1:200, Invitrogen A11122). Primary antibodies were incubated at room temperature with rotation for four hours and then incubated overnight at 4ºC. The primary antibodies were removed and washed with PBST four times for 15 minutes each. Then, the

secondary antibodies diluted in the blocking serum were added. The secondary antibodies had to be added in two separate groups to avoid an interaction between the goat-derived secondary antibody and the donkey-targeting sheep antibody. Therefore, the Alexa 568 donkey anti-sheep secondary antibody (1:400, Invitrogen A21099) was added first and incubated for three nights at 4ºC. Then, the Alexa 647 goat anti-mouse (1:400, Invitrogen) and Alexa Fluor 488 donkey anti-rabbit (1:400, Invitrogen A21206) antibodies were added and incubated for three more nights.

For staining for BP102, which labels all central nervous system axons, and/or GFP, the embryos were first washed twice with either 0.5% or 0.1% PBST, then permeabilized for 5 minutes in PBST. Then, 2% or 5% of goat blocking serum was added and rocked for 5-10 minutes. Primary antibodies were then added and incubated overnight at 4ºC. The primary antibodies were mouse anti-BP102 (1:100, DSHB) and rabbit anti-GFP (1:500, Invitrogen A11122). After rinsing three times and washing two more times for 5 minutes each, the secondary antibodies (1:250, Alexa Fluor 488 goat-anti-mouse [Invitrogen A11001], Alexa 594 goat anti-mouse [Invitrogen A11032], and/or Alexa Fluor 488 donkey anti-rabbit [Invitrogen A21206]) were added for 30 minutes at room temperature. After, the embryos were washed and mounted in *SlowFade* Gold mounting media (ThermoFisher S36936).

Imaging

After staining, the embryos were mounted on a slide for imaging (Mir et al., 2018). We used a Nikon Ti laser confocal microscope and took z-sections focused on the ventral nerve cord to get 3D images. We also imaged live embryos in stages 16-17 to check whether the genetic crosses produced viable embryos that were able to develop past the early stages. Because the gut autofluoresces green under the microscope and development of the gut indicates progression to

later stages of embryogenesis, the number of live embryos that developed were viewed and counted through the Hammamatsu widefield microscope.

GAL4/UAS System

We used the Gal4-UAS system to induce expression of certain genes in a controlled manner. Specifically, the upstream activation sequence (UAS) gene line is found in all cells but will not be expressed unless there is a Gal4 transcription factor that binds to the UAS. Once the Gal4 is expressed and binds to the UAS binding site, then the sequence linked to UAS will be expressed. Gal4 is expressed in specific tissues only, so the UAS genes will also only be expressed in those particular tissues (Elliott & Brand, 2008). In this project, three Gal4 lines were used: *elav*-Gal4, which is expressed in all neurons; *Act5*-Gal4, which is linked to the production of actin, a cytoskeletal protein found in all cells; and *eagle*-Gal4 (*eg*-Gal4), which is expressed only in two clusters of neuron groups per abdominal section (Pollitt et al., 2020). The two neuron clusters are the EG neurons and the EW neurons (Higashijima et al., 1996). Specifically, the EG neuron cluster is composed of 10-12 cells that cross the midline in the anterior commissure, while the EW neuron cluster is composed of four cells that cross the midline in the posterior commissure of the adjacent segment (Garbe et al., 2007; Higashijima et al., 1996). The UAS responders used were *UAS-tau-GFP* gene line, which expresses GFP to fluoresce green under the microscope linked specifically to microtubule proteins; *UAS-myr-GFP* gene line, which also expresses GFP to fluoresce green but instead localized to the membrane; *UAS-CD8-GFP;UAS-snRNAi,* which expresses both GFP and knockdown of *singed*; and *UAS-Dicer2;UAS-snRNAi,* which enhances the knockdown of *singed*.

snRNAi and Dicer Enhancement of the Knockdown

To induce knockdown of gene expression in embryos, we used genetic lines that expressed RNA interference (RNAi) constructs that specifically target the mRNA encoding for the translation of the Singed protein, which will be referred to as snRNAi from here on. Because RNAi targets the mRNA that its sequence is complementary to, it does not knockout the *singed* gene from being completely transcribed and translated, which means there is still a potential for low levels of expression of *singed*. However, knocking down the mRNA is still useful because it can block a large portion of expression of the protein (Lee et al., 2004). Furthermore, the knockdown effect of snRNAi can be enhanced through overexpression of the enzyme Dicer. Dicer is a required part of the processing of pre-RNAi into mature RNAi, specifically by cleaving the pre-RNAi molecules and participating in formation of RNA-induced silencing complexes (Lee et al., 2004). To enhance the snRNAi effect, we used a *UAS-Dicer-2;UAS-snRNAi* line. *UAS-Dicer-2* has been shown to be able to enhance RNAi potency when co-expressed with RNAi, allowing for a stronger knockdown of the intended gene (Dietzl et al., 2007). Figure 1 shows preliminary confirmation that *UAS-Dicer2;UAS-snRNAi* successfully reduces expression of the Singed protein and causes phenotypic differences compared to wild type.

Additionally, we did not use flies that carry a X-linked *singed* null mutant genotype (*singed*^{X2}) because homozygous females are sterile (Cant et al., 1994) Therefore, genetic crosses would require heterozygous females that contain a balancer chromosome that has a wild-type copy of the *singed* gene. Due to maternally deposited mRNA that is present during the early stages of animal development (Atallah & Lott, 2018), there is a chance that embryos will contain maternally derived mRNA for the Singed protein from the balancer chromosome, thus making early development look phenotypically like the wild type. Therefore, we instead used lines that knocked down the mRNA transcript to combat any maternal transfer of the protein. Because the snRNAi

flies are not sterile when homozygous, the females with both copies of the knockdown can be used. Also, knockdown will reduce all expression of the protein, regardless of if it is maternally or zygotically expressed.

Genetic Lines

To determine whether Singed protein is actually expressed in the VNC of the embryos, we used two different crosses: *elav*-Gal4 crossed with *UAS-tau-GFP* and *eagle*-Gal4 crossed with *UAS-myr-GFP.* Because the *elav*-Gal4 gene is found on the X chromosome, only virgin females from that line can be crossed with males of the *UAS-tau-GFP* line to ensure the embryos receive the intended genes. However, the *eagle*-Gal4 gene is found on an autosome, so either males or females can be used to cross with the *UAS-myr-GFP* line. The two crosses mentioned above were stained with GFP, fascin, and tubulin antibodies to check that the Singed protein is actually expressed in the embryos' ventral nerve cords (FlyLight, n.d.).

To determine whether knocking down expression of *singed* would cause any noticeable defects in the ventral nerve cord of the embryos, we used a couple of different crosses, especially as one of the crosses did not produce viable embryos, which will be touched upon later. The crosses were *Act5*-Gal4 with *UAS-Dicer2;UAS-snRNAi*, *eg*-Gal4 with *UAS-CD8-GFP;UAS-snRNAi,* and *elav*-Gal4 with *UAS-Dicer2;UAS-snRNAi*. Controls included the *UAS-CD8-GFP;UAS-snRNAi* line that was over a balancer, *UAS-Dicer2;UAS-snRNAi* line over a balancer, and *UAS-myr-GFP* with *eg*-Gal4, as all of them should express the wild type for *singed*.

Some of the genetic lines were over a balancer. Balancer chromosomes can maintain expression of the intended gene by preventing recombination that often occurs during meiosis through incorporation of multiple inversions on one of the homologous chromosomes of a pair. Furthermore, balancers have phenotypes that are identifiable on the adult fly and are also recessive

lethal or sterile, preventing the balancers from displacing the intended gene from the population (Stocker & Gallant, 2008). Two of the genetic lines we used were over a balancer: *UAS-Dicer2/Cyo;UAS-snRNAi/TM2* and *UAS-CD8-GFP/Cyo-dfd-GFP;UAS-snRNAi/TM6B.* Therefore, when collecting either males or virgin females from those two lines, homozygous individuals were required, meaning they did not have the balancer phenotypes. This would manifest as either straight-winged flies for those maintained with the *Cyo* balancer, fewer and longer bristles on the shoulder area of the fly for those maintained with the *TM6B* balancer, or the presence of no bristles on the halteres, the small external organs that are derived from ancestral hindwings, for those maintained with the *TM2* balancer (Chyb & Gompel, 2013).

Statistical Analysis

The proportion of number of embryos that survived to stage 16/17 was calculated and compared using a chi-squared comparison of proportions to evaluate if there are statistical differences between the mutant embryos and the controls. Measuring distances between each segment was done in ImageJ.

Results

Although the Singed protein is known to be concentrated in neuron growth cones, we first confirmed that Singed is also present in the VNC of *Drosophila* embryos. Using the *elav*-Gal4 crossed with *UAS-tau-GFP,* we generated images that stained for Singed and confirmed the presence of the protein in the VNC (*Figure 2*). Furthermore, we also collected a cross between *eg*-Gal4 and *UAS-myr-GFP* to be able to look at the presence of Singed in a specific set of neurons in the VNC. The protein appears to be present in the cell bodies, though due to how widespread the staining of the protein is, it is difficult to confirm its presence in the axons (*Figure 3*).

In order to figure out whether the crosses actually produced viable embryos, the proportion of developed embryos were compared (*Figure 4A*). Because all the embryos were collected in stages 16-17, the development of the gut should be highly visible. Therefore, the presence of the gut, which autofluoresces green, was used as a predictor for whether embryos successfully developed past the early stages of embryogenesis and would likely produce surviving adults (*Figure 4B*). *UAS-Dicer2;UAS-snRNAi* flies (n=119, two replicates, 90.76%) were used as controls for comparison to the *Act5*-Gal4 and *UAS-Dicer2;UAS-snRNAi* cross (n=82, two replicates, 76.83%) and the *elav*-Gal4 and *UAS-Dicer2;UAS-snRNAi* cross (n=141, three replicates, 45.39%). Because the *UAS-Dicer2;UAS-snRNAi* control does not include a Gal4 driver, the resulting progenies do not express the knockdown of *singed* and, therefore, represent the wild type. There is a statistically significant difference between the control and the *Act5*-Gal4 cross ($p=0.0065$) and between the control and the *elav*-Gal4 cross ($p<0.0001$).

The second set of comparisons was between the *UAS-CD8-GFP;UAS-snRNAi* flies (n=103, two replicates, 88.35%) and the *eg*-Gal4 and *UAS-CD8-GFP;UAS-snRNAi* cross (n=29, two replicates, 3.45%). Because the *UAS-CD8-GFP;UAS-snRNAi* embryos do not include a Gal4

driver, the resulting progenies do not express the knockdown of *singed* and, therefore, represent the wild type. There is a statistically significant difference in embryo survival ($p<0.0001$). Because the *eg*-Gal4 and *UAS-CD8-GFP;UAS-snRNAi* cross consistently showed little to no survival of any embryos, this suggested something was wrong with the *UAS-CD8-GFP;UAS-snRNAi* line. Thus, the set of comparisons involving the *UAS-CD8-GFP;UAS-snRNAi* line was not continued, and the focus shifted to the crosses involving *UAS-Dicer2;UAS-snRNAi.* Comparisons between the two controls were also calculated, as even though they are two different lines, there should not be a difference in survival because they are both expressing the wild type for *singed*. No statistical difference was found between the two proportions ($p=0.56$).

Because of the low yield of imageable embryos after collection, fixation, and staining, only preliminary results were collected on the differences between the crosses of *elav*-Gal4 with *UAS-tau-GFP* (control), *elav*-Gal4 with *UAS-Dicer2;UAS-snRNAi*, and *Act5*-Gal5 with *UAS-Dicer2;UAS-snRNAi*. There were three embryos (50% of the six total embryos) from the cross that induced Dicer-enhanced knockdown of *singed* in all neurons (*elav*-Gal4 x *UAS-Dicer2;UAS-snRNAi*) shown in Figure 6 that appear to have significant defects, including missing segments, large gaps in between each segment, and incomplete connections. However, other embryos collected from the same cross did not show the same defects. Therefore, more replicates with a larger sample size will need to be done before making any conclusive results. The embryos from the cross that expressed the Dicer-enhanced knockdown in all cells (*Act5*-Gal5 with *UAS-Dicer2;UAS-snRNAi*) did not show any obvious defects (*Figure 7*), though again more samples will need to be collected to see if there any differences, especially considering there was a significant number of embryos that did not completely develop (*Figure 2*). Figures 8 and 9 provide a summary of the number of segments and average distance between segments for each embryo of

the three crosses mentioned above. No clear trend can be determined due to the low sample size. If there were more embryos, the number of segments and average segment distances could be compared between the control and mutant embryos to see whether knocking down *singed* has widespread effects on overall VNC structure.

Discussion

By examining whether reducing the levels of Singed can detrimentally affect VNC development, we aimed to determine how functionally important the protein is for correct axonal projections and wiring of neuronal circuitry, especially given there are neurological diseases that have been found to be correlated with improper crossing over events. In order to understand what role Singed plays during early neurodevelopment, we manipulated the expression of *singed* through different genetic crosses and visualized the VNC in *Drosophila* embryos to see if there were any visible defects in axonal midline crossing. The first experiments we did were to confirm that Singed is present in neurons of the VNC. Using *elav*-Gal4 crossed with *UAS-tau-GFP*, which tags the expression of GFP to microtubules in all neurons, and staining for Singed, GFP, and tubulin, we found that the embryos' VNCs appear to show the presence of Singed. As an additional confirmation, we crossed *eg*-Gal4 with *UAS-myr-GFP*, which localized GFP expression to the membranes of a subset of neurons in the VNC. Though less clear, Singed also appears to be expressed in the cell bodies of the neuron clusters (*Figure 3*). It is widely accepted that fascin-1 is present in filopodia and lamellipodia of axonal growth cones from the beginning of growth cone formation and retained even during morphogenesis (Cohan et al., 2001). Therefore, we know that Singed is in both cell bodies and axonal growth cones of *Drosophila* embryo VNCs.

Although the preliminary results cannot be analyzed for statistical differences due to the low yield of embryos, there appears to be some differences that may suggest reducing *singed* expression in neurons can affect morphology of the ventral nerve cord during embryogenesis. For example, there appears to be some missing segments in some of the embryos that expressed Dicer-enhanced knockdown of *singed* in all neurons (*Figure 6*). The first image of Figure 6 has abnormally longer distances in between each segment, along with a reduced number of segments.

The second image appears to have segments that are missing and potentially twisted to the side, and the third image shows that some segments in the VNC did not properly connect to neighboring segments. These images suggest that Singed plays a role in the correct formation of connections between VNC segments. On the other hand, the embryos collected from the cross that globally expressed Dicer-enhanced knockdown (*Act5*-Gal4 x *UAS-Dicer2;UAS-snRNAi*) do not have any defects that are definitively noticeable (*Figure 7*). This is an interesting result because the expectation is that reducing the amount of Singed protein globally in all cells should have more of an effect than only in neurons. However, more replicates and a larger sample size will need to be collected, as we were only able to collect four imageable embryos. With more samples, we may find clearer defects in both crosses. Furthermore, a potential explanation for this discrepancy is the differential strengths of the Gal4 drivers. Therefore, follow-up experiments can first measure the relative strength of the Gal4 drivers to verify whether that may also be affecting how effective the knockdown responder is. This can be done by measuring the amount of protein being expressed from each line and by using other procedures like Goentoro et al.'s (2005) assay for assessing the relative levels of expression in live embryos.

As seen in Figure 2, both crosses that express Dicer-enhanced knockdown of *singed* in either all neurons (*elav*-Gal4) or all cells (*Act5*-Gal4) have a statistically lower proportion of embryos that survive compared to the control. This potentially implies that suppressing the expression of *singed* can have detrimental effects on the ability of embryos to complete embryogenesis. This implication for survival has also been found in other animals. Hashimoto et al. (2011) note that gene knockout of *FSCN1* in mice is not embryonically lethal but does cause 48% neonatal lethality in inbred stains and reduced weight in surviving mice. This suggests that disrupting fascin function may negatively impact the health of both mice and *Drosophila.* However,

the survival rate results generated in Figure 2 are tentative at best considering that the cross using a driver that expressed the knockdown in all cells appears to have a higher rate of survival than the cross that drives expression only in neurons. Furthermore, the stark contrast in survival in the *UAS-CD8-GFP;UAS-snRNAi* crosses is likely due to problems with the line itself rather than a product of the knockdown.

It is interesting though that 76.83% of the embryos from the *Act5*-Gal4 cross and 45.39% of the embryos from the *elav*-Gal4 cross still survive. The fact that around half or more of the embryos still survive could be due to a couple of factors. One could be due to other actin-bundling proteins that rescue some effects of the knockdown of *singed*. For example, Khaitan et al. (2022) have found that Vinculin is another actin-binding protein that shares a similar role with Singed in regulating border cell migration during development; in their study, they found that knocking down either *singed* or *vinculin* separately did not produce significant border cell migration defects, but knocking down both did have defects. Therefore, other actin-bundling proteins may help to substitute for some of the roles of the Singed protein. Another possible explanation is that we are only knocking down some of the protein production; therefore, differential levels of expression between individual embryos are still possible as the *singed* gene is still present and active, allowing some embryos to survive. As shown in Figure 1, the Dicer-enhanced knockdown of *singed* does not completely stop all expression of the protein. Because the number of surviving embryos is statistically different, this suggests that reducing the amount of Singed protein is potentially affecting important developmental processes that are needed for the survival of the embryos. However, more embryos will need to be collected and visualized to see what kinds of defects are occurring in the ventral nerve cord.

Additionally, another surprising result is that basically all of the embryos from the *eg*-Gal4 x *UAS-CD8-GFP;UAS-snRNAi* cross did not survive. This is peculiar because the expectation is that the Dicer-enhanced knockdowns will have a stronger negative effect than the regular knockdown; additionally, *eg*-Gal4 is expressed only in a subset of neurons, which we would expect to have a weaker effect than the crosses where the knockdown is expressed in all neurons and cells. Because this was the first time using the *UAS-CD8-GFP;UAS-snRNAi* line, it would be helpful for future experiments to cross this line with other drivers, such as the *elav*-Gal4 line and the *Act5*-Gal4 line to see if it is a universal problem with that specific line of flies. Furthermore, because we were not able to collect any viable embryos from this cross, we could not analyze specific midline crossing patterns of individual neurons, as was the original intention by using the *eg*-Gal4 line. Also, because the *UAS-Dicer2;UAS-snRNAi* line does not have a GFP tag, we could only stain for BP102, which labels all neurons, in the crosses that had Dicer-enhanced knockdown of *singed*. Therefore, it would be useful to develop a line that has a GFP tag in the *eg*-Gal4 line so that they can be crossed with *UAS-Dicer2;UAS-snRNAi* and stained for GFP, allowing for the specific visualization of a subset of neurons.

Another limitation we found during this study was the difficulty in collecting enough embryos that would be imageable by the end of the fixation and staining protocols. Not only did only a portion of embryos survive during development (*Figure 2*), but many embryos are also lost during the fixation and staining process; the yield after fixation is usually only around half of the embryos. Additionally, after staining, some of the embryos appear to break down despite being fixed. As a result, other methods can be explored, such as using a glyoxal-based fixation protocol rather than fixing with formaldehyde (Amin et al., 2023), to improve the number of embryos for analysis.

Future experiments can also expand the study to look at the motor behavior of adult flies or larvae that express a knockdown of *singed* by measuring fly climbing rate or larva locomotion to see if changes in axonal development has widespread effects on motor abilities past embryogenesis. Additionally, we hope to develop a stable and efficient noninvasive in vivo methodology for tracking axon movement that can be used for future studies, allowing for a better visualization of what is occurring naturally in real time. Unfortunately, we did not have time to do imaging of live movement of the axons in knockdown embryos, but once the crosses are reevaluated, it would be useful to look at spatiotemporal dynamics of axonal projections in live embryos to see when exactly development stops and if there are any defects during midline crossing.

Tables and Figures

Figure 1

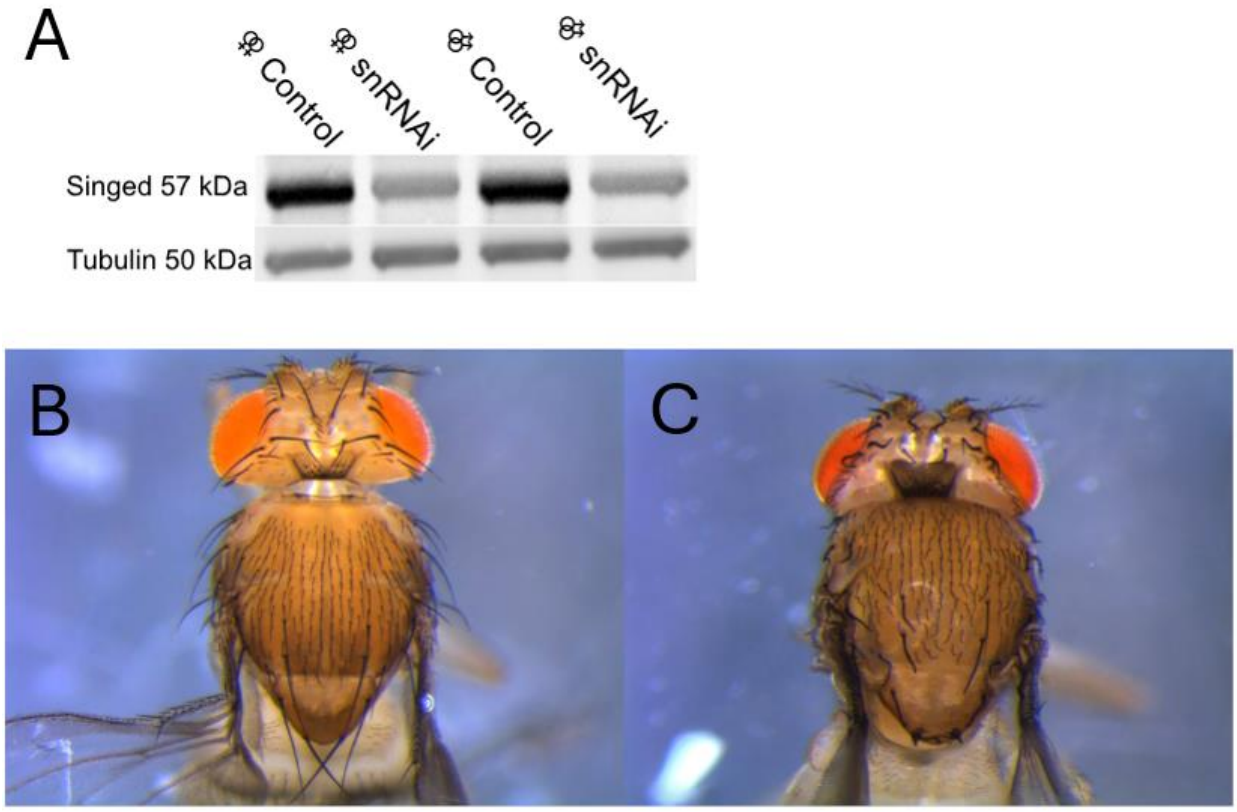


Figure 1. RNAi-based Knockdown of *Singed* (snRNAi). (A) Western blot showing the effectiveness of snRNAi knockdown. Here, the *Act5*-Gal4 driver was used to drive the expression of snRNAi together with Dicer for *singed* knockdown. (B) Wild type with no bristle defects. (C) Gnarled bristle defects from snRNAi knockdown. Courtesy of Kate Hardin.

Table 1

Genetic Crosses	General Details
UAS-CD8-GFP;UAS-snRNAi x *UAS-CD8-GFP;UAS-snRNAi*	Control for comparing proportions of embryos that survived in the crosses involving *UAS-CD8-GFP;UASsnRNAi,* expresses wild type for *singed*
UAS-Dicer2;UAS-snRNAi x *UAS-Dicer2;UAS-snRNAi*	Control for comparing proportions of embryos that survived in the crosses involving *UAS-Dicer2;UAS-snRNAi,* expresses wild type for *singed*
UAS-myr-GFP x *eg*-Gal4	Control for visual comparison of VNC, expresses wild type for *singed,* tags the membrane of the *eg* neurons with GFP
Act5-Gal4 x *UAS-Dicer2;UAS-snRNAi*	Expresses Dicer-enhanced knockdown of *singed* in all cells (the driver is linked to the production of actin)
elav-Gal4 x *UAS-Dicer2;UAS-snRNAi*	Expresses Dicer-enhanced knockdown of *singed* in all neurons
eg-Gal4 x *UAS-CD8-GFP;UAS-snRNAi*	Expresses knockdown of *singed* in a subset of neurons tagged with GFP, did not produce viable offspring
elav-Gal4 x *UAS-tau-GFP*	Expresses GFP tagged to microtubules in all neurons

Table 1. Summary of Genetic Crosses.

Figure 2

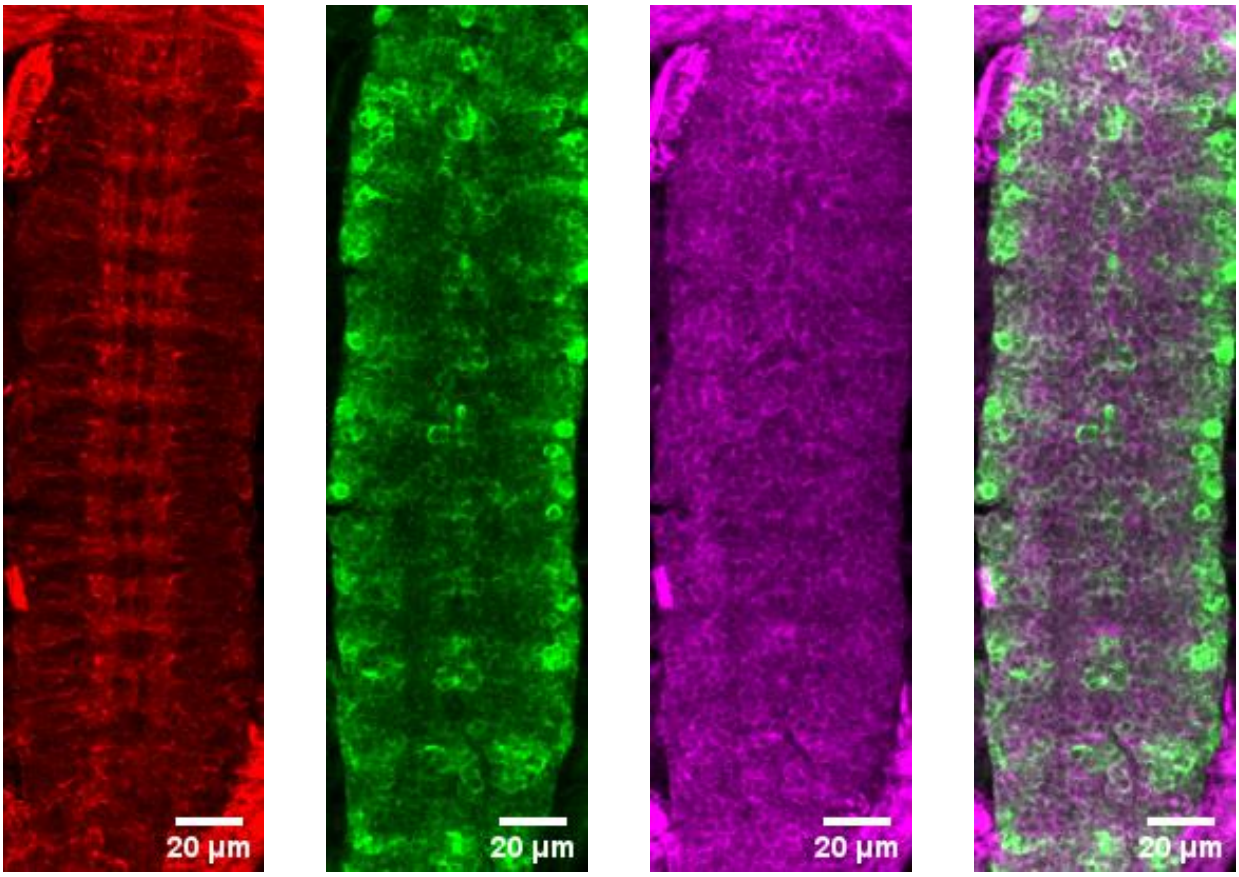


Figure 2. Singed is Expressed in the Ventral Nerve Cord. Representive images of *Drosophila* embryos stained for tubulin (red), GFP (green), and Singed (magenta). The last image on the right shows the IF of GFP and Singed. Here, *elav*-Gal4 was used to drive the expression of tau-GFP in neurons.

Figure 3

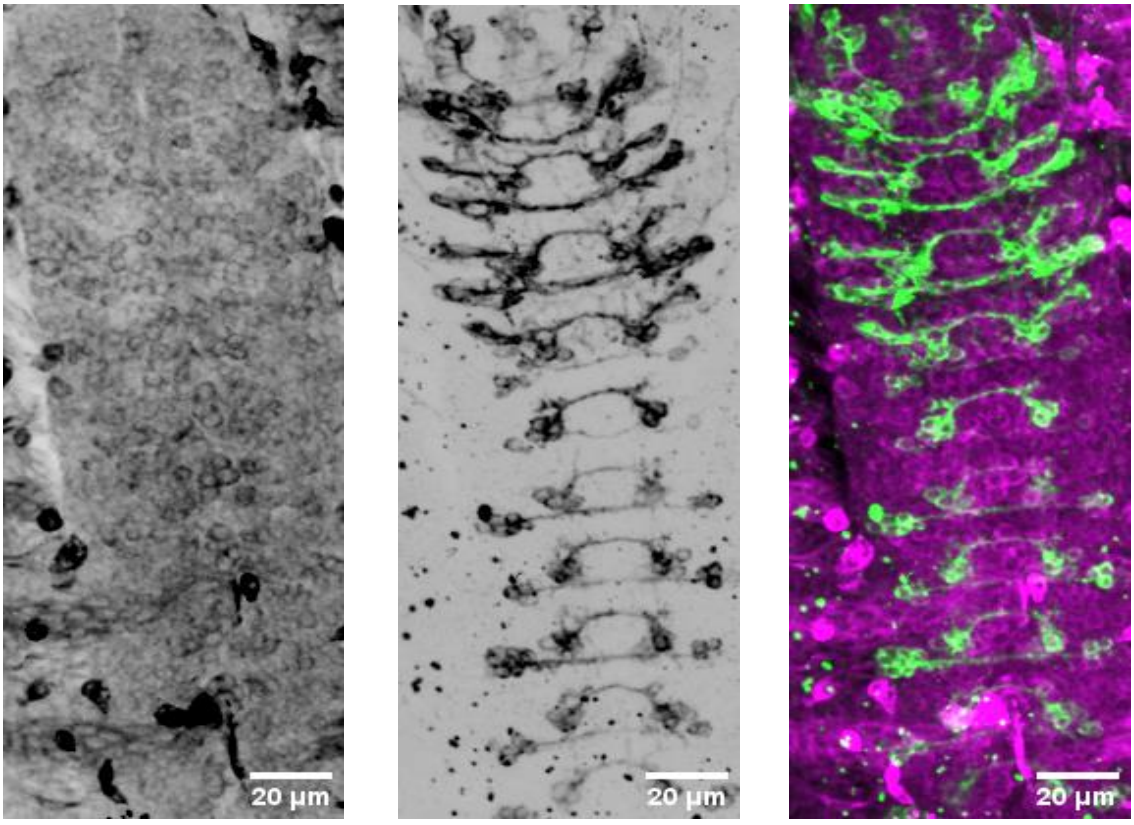
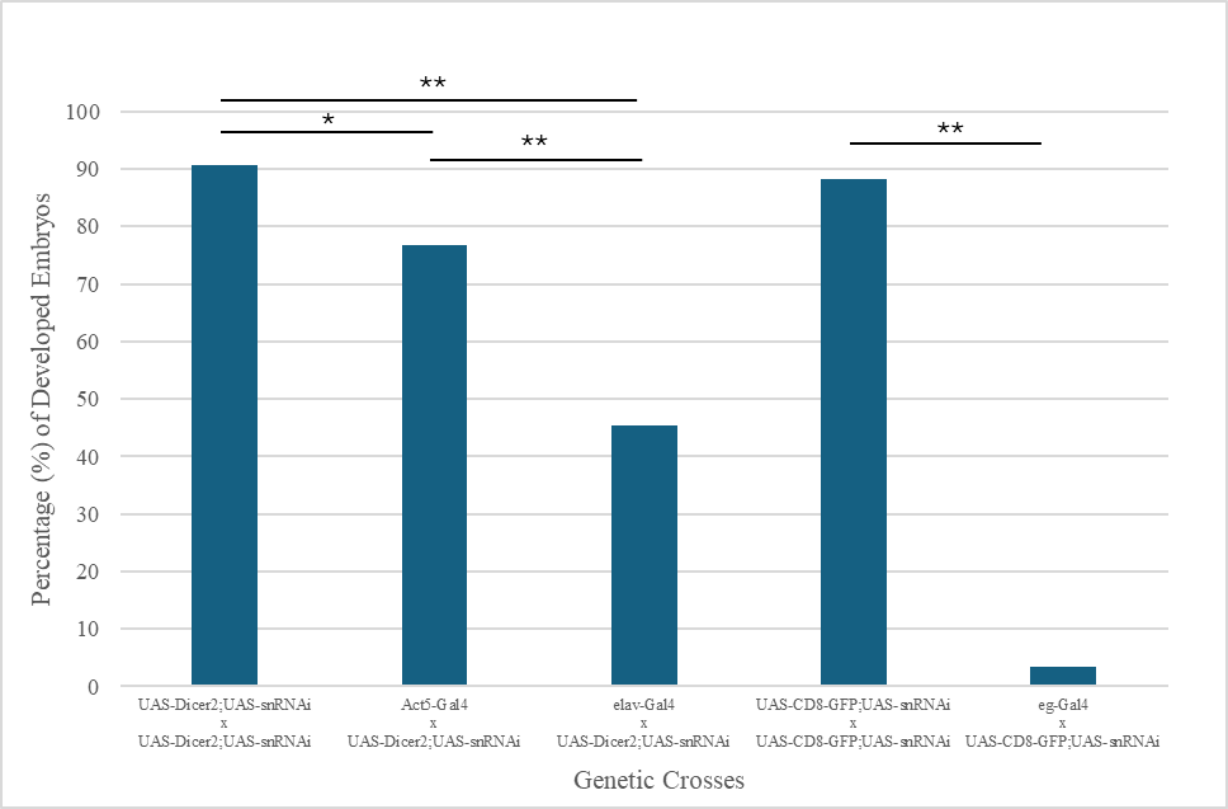


Figure 3. Singed is Present in the Cell Bodies of a Subset of Neurons. Images of *Drosophila* embryo VNC stained for Singed (left) and GFP (middle). The last image on the right shows the IF of GFP (green) and Singed (magenta). Here, *eg*-Gal4 was used to drive the expression of myr-GFP in a subset of neurons.

Figure 4

A


**
*
**
**
100
90
80
70
60
50
40
30
20
10
0
Percentage (%) of Developed Embryos
UAS-Dicer2;UAS-snRNAi x UAS-Dicer2;UAS-snRNAi
Act5-Gal4 x UAS-Dicer2;UAS-snRNAi
elav-Gal4 x UAS-Dicer2;UAS-snRNAi
UAS-CD8-GFP;UAS-snRNAi x UAS-CD8-GFP;UAS-snRNAi
eg-Gal4 x UAS-CD8-GFP;UAS-snRNAi
Genetic Crosses

B

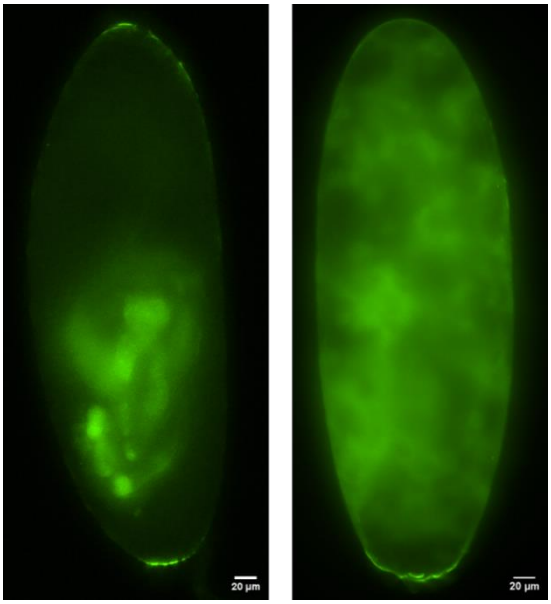

20 µm
20 µm

Figure 4. Proportion of Embryos that Successfully Developed. (A) *UAS-Dicer2;UAS-snRNAi x UAS-Dicer2;UAS-snRNAi* (n=119, two replicates, 90.76%) and *UAS-CD8-GFP;UAS-snRNAi x UAS-CD8-GFP;UAS-snRNAi* (n=103, two replicates, 88.35%) are the controls used for comparisons. *Act5*-Gal4 x *UAS-Dicer2;UAS-snRNAi* (n=82, two replicates, 76.83%, p=0.0065) and *elav*-Gal4 x *UAS-Dicer2;UAS-snRNAi* (n=141, three replicates, 45.39%, p<0.0001) crosses show a statistically significant difference in comparison to the control. There is also a significant difference between *eg*-Gal4 x *UAS-CD8-GFP;UAS-snRNAi* survival (n=29, two replicates, 3.45%) and the control (p<0.0001). *p<0.05; **p<0.0001 by chi-squared comparison of proportions. (B) Representative images showing developed (left) vs. non-developed (right) embryos based on gut fluorescence in stages 16-17.

Figure 5

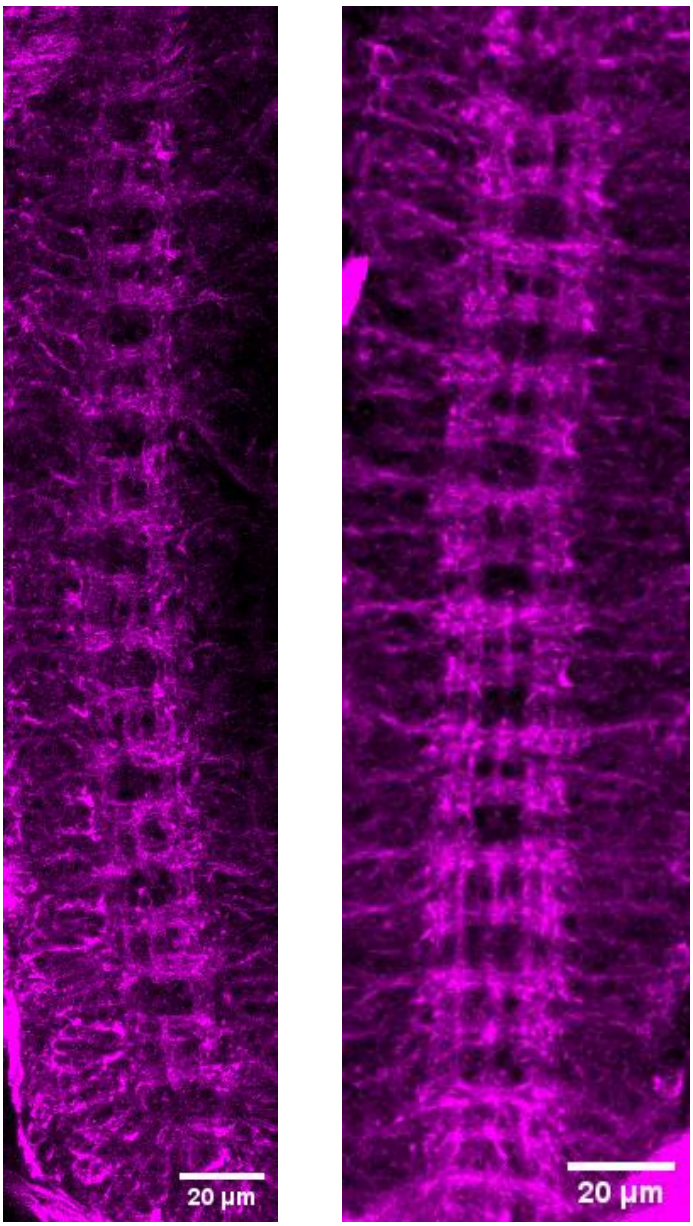


Figure 5. Control Embryos for Comparison to the Crosses that had Dicer-enhanced Knockdown of *Singed*. Here, *elav*-Gal4 was used to drive the expression of tau-GFP in neurons, which should express the wild-type amount of Singed in its cells. The magenta indicates labelling for tubulin, which is distributed throughout all cells and in neurons as shown above.

Figure 6

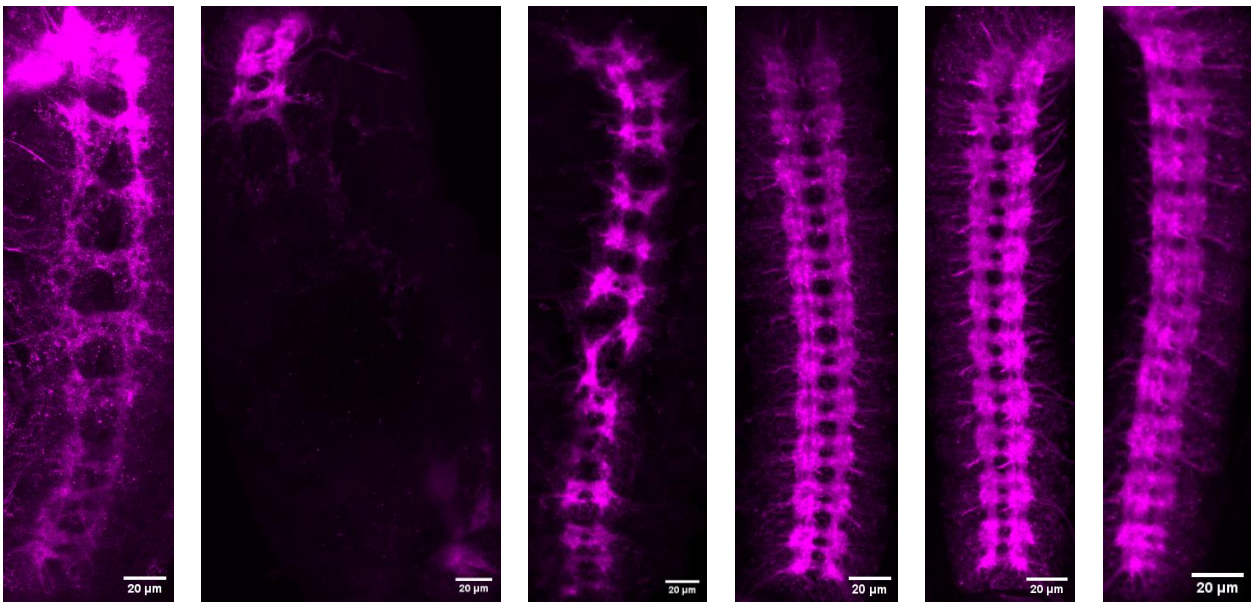


Figure 6. Embryos that Expressed Dicer-enhanced Knockdown of *Singed* in All Neurons. *elav*-Gal4 was used to drive the Dicer-enhanced knockdown of *singed*. Embryos were stained for BP102. The three images on the left show abnormalities in the VNC, including missing segments and large separation between segments The VNC in the 6th image also appears to be abnormal compared to the normal appearance of VNC segmented axonal projections (the 4th and 5th images, see also *Fig. 5*), though embryo orientation during imaging may also play a factor.

Figure 7

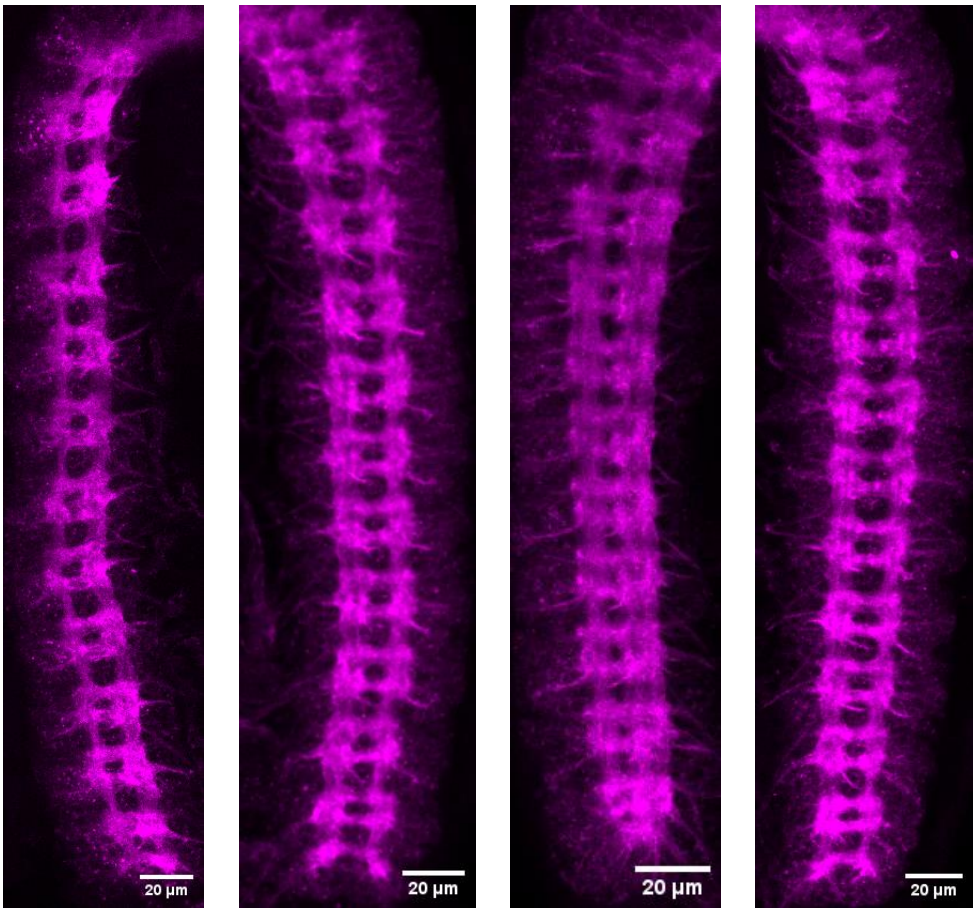


Figure 7. Embryos that Expressed Global Dicer-enhanced Knockdown of *Singed*. *Act5*-Gal4 was used to drive the Dicer-enhanced knockdown of *singed*. Embryos were stained for BP102. No drastic defects are visible among the four embryos globally expressing the knockdown of *singed* when compared to the control embryos (see *Figure 5*).

Figure 8

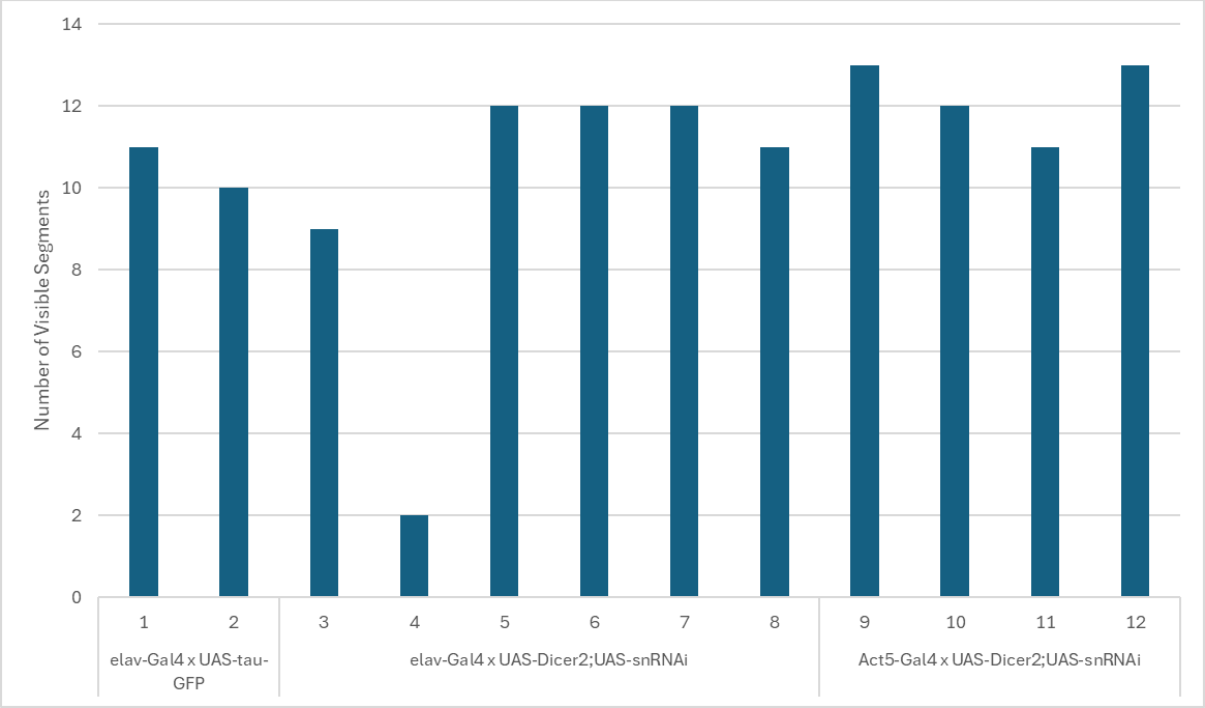


Figure 8. A Comparison of the Count of Visible Segments for Each Embryo. 1 and 2 on the graph represent two control embryos from the cross of *elav*-Gal4 with *UAS-tau-GFP,* expressing wild type for *singed*. Embryos 3 through 8 represent the six embryos shown in Figure 6 that are expressing Dicer-enhanced knockdown of *singed* in all neurons. Embryos 9 through 12 represent the four embryos shown in Figure 7 that are expressing global Dicer-enhanced knockdown of *singed.* There is no obvious trend in number of visible segments between the three crosses, except for Embryos 3 and 4, which correspond to the first two embryos in Figure 6 that clearly have a lower number of segments. For all other embryos, their number of segments appear to resemble the number of segments seen in the control embryos. The varying numbers between individual embryos is potentially due to the curved nature of embryos such that some segments were not visible in the 2D images.

Figure 9

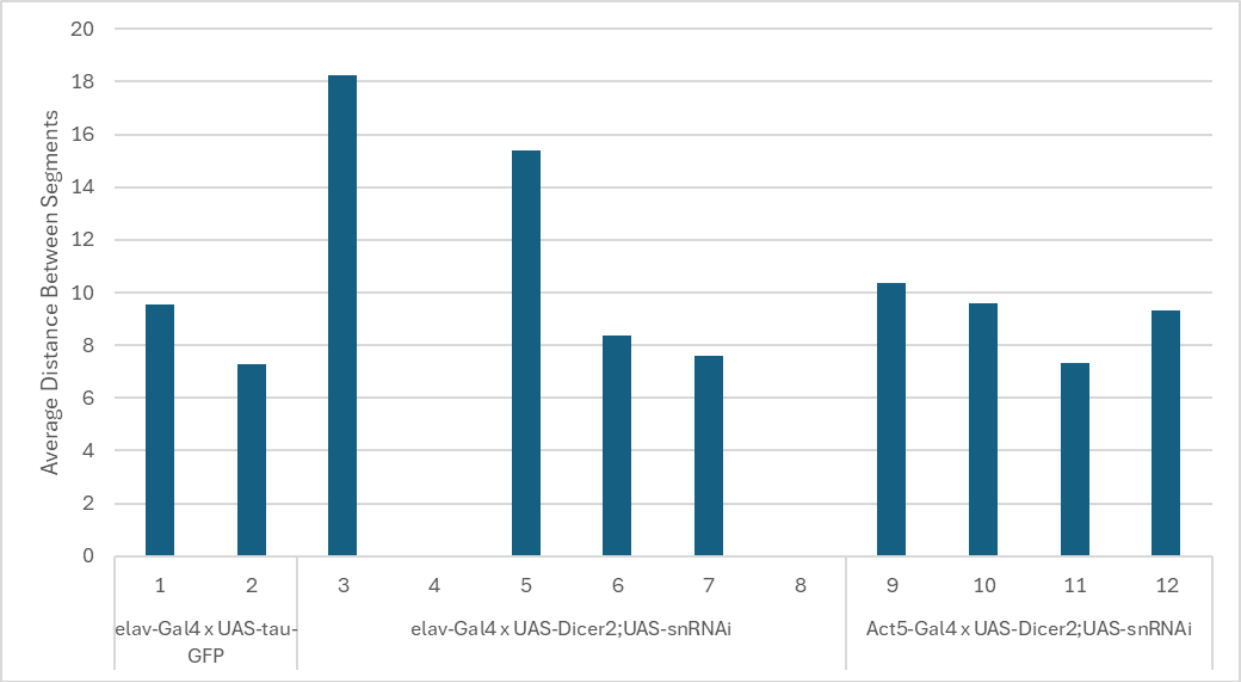


Figure 9. A Comparison of the Average Distance Between Segments for Each Embryo (measured in pixels on ImageJ). Embryo 4 was not included due to a large number of missing segments, and Embryo 8 was not included due to a VNC orientation that made it difficult to analyze. A larger distance value, as seen in Embryos 3 and 5, indicates that each segment is spaced further apart from its neighboring segment in the VNC than compared to the VNC of the other embryos with a lower average distance. This suggests that defects in the formation of the VNC might occur through less accurate neuronal connections that visually manifests as larger spacing in between each segment, though a larger sample size is needed to account for individual differences.

References

Amin, S., Basu, M., Buzinova, V., Delgado, A., Mahadevan, T., Mishra, S., Zaida, S., Wang, X. & Sokac, A. M. (2023). Glyoxal-based fixation of *Drosophila* embryos for immunofluorescence staining and RNA in situ hybridization. *STAR Protocols*, *4*(3), 102385. https://doi.org/10.1016/j.xpro.2023.102385

Araújo, S. J., & Tear, G. (2003). Axon guidance mechanisms and molecules: lessons from invertebrates. *Nature Reviews Neuroscience*, *4*(11), 910-922. https://doi.org/10.1038/nrn1243

Atallah, J., & Lott, S. E. (2018). Evolution of maternal and zygotic mRNA complements in the early *Drosophila* embryo. *PLoS Genetics*, *14*(12), e1007838. https://doi.org/10.1371/journal.pgen.1007838

Bashaw, G. J. (2010). Visualizing axons in the *Drosophila* central nervous system using immunohistochemistry and immunofluorescence. *Cold Spring Harbor Protocols*, *2010*(10), pdb-prot5503. https://doi.org/10.1101%2Fpdb.prot5503

Brody, T. B. (1995). *Stages of Development and Mitotic Domains.* The Interactive Fly. https://www.sdbonline.org/sites/fly/aimain/2stages.htm

Campos-Ortega, J. A., & Hartenstein, V. (2013). *The embryonic development of Drosophila melanogaster*. Springer Science & Business Media.

Cant, K., Knowles, B. A., Mooseker, M. S., & Cooley, L. (1994). *Drosophila* singed, a fascin homolog, is required for actin bundle formation during oogenesis and bristle extension. *The Journal of Cell Biology*, *125*(2), 369-380. https://doi.org/10.1083/jcb.125.2.369

Chyb, S., & Gompel, N. (2013). Atlas of *Drosophila* morphology: Wild-type and classical mutants. Academic Press.

Cohan, C. S., Welnhofer, E. A., Zhao, L., Matsumura, F., & Yamashiro, S. (2001). Role of the actin bundling protein fascin in growth cone morphogenesis: localization in filopodia and lamellipodia. *Cell Motility and the Cytoskeleton*, *48*(2), 109-120. https://doi.org/10.1002/1097-0169(200102)48:2%3C109::AID-CM1002%3E3.0.CO;2-G

Comer, J. & Alvarez, S. & Butler, S. & Kaltschmidt, J. (2019). Commissural axon guidance in the developing spinal cord: from Cajal to the present day. *Neural Development, 14*. https://doi.org/10.1186/s13064-019-0133-1

Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., Couto, A., Marra, V., Keleman, K., & Dickson, B. J. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*, *448*(7150), 151-156. https://doi.org/10.1038/nature05954

Elliott, D. A., & Brand, A. H. (2008). The GAL4 system: A versatile system for the expression of genes. In C. Dahmann (eEs.), *Drosophila methods and protocols* (pp. 79-95). Humana Press.

Fernández-Moreno, M. A., Farr, C. L., Kaguni, L. S., & Garesse, R. (2007). *Drosophila melanogaster* as a model system to study mitochondrial biology. *Mitochondria: Practical Protocols*, *372,* 33-49. https://doi.org/10.1007/978-1-59745-365-3_3

FlyLight. (n.d.). *Double Label IHC for Adult CNS*. Janelia. https://www.janelia.org/sites/default/files/Project%20Teams/Fly%20Light/FL%20Protocol%20-%20Adult%20IHC%20-%20Double%20Label.pdf

FlyMove. (n.d.). *Stage 17*. http://flymove.uni-muenster.de/Stages/Stage17/Stg17page.html?http&&&flymove.uni-muenster.de/Stages/Stage17/Stg17txt.html

Garbe, D. S., O'Donnell, M., & Bashaw, G. J. (2007). Cytoplasmic domain requirements for Frazzled-mediated attractive axon turning at the *Drosophila* midline. *Development, 134*(24), 4325-4334. https://doi.org/10.1242/dev.012872

Goentoro, L. A., Yakoby, N., Goodhouse, J., Schüpbach, T., & Shvartsman, S. Y. (2006). Quantitative analysis of the GAL4/UAS system in *Drosophila* oogenesis. *Genesis*, *44*(2), 66-74. https://doi.org/10.1002/gene.20184

Hartenstein, V. (1993). *Atlas of Drosophila Development*. The Interactive Fly. https://www.sdbonline.org/sites/fly/atlas/0809.htm

Hashimoto, Y., Kim, D. J., & Adams, J. C. (2011). The roles of fascins in health and disease. *The Journal of Pathology*, *224*(3), 289-300. https://doi.org/10.1002/path.2894

Higashijima, S. I., Shishido, E., Matsuzaki, M., & Saigo, K. (1996). *eagle*, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the *Drosophila* CNS. *Development*, *122*(2), 527-536. https://doi.org/10.1242/dev.122.2.527

Howard, L. J., Brown, H. E., Wadsworth, B. C., & Evans, T. A. (2019, January). Midline axon guidance in the *Drosophila* embryonic central nervous system. *Seminars in Cell & Developmental Biology*, 85, 13-25. https://doi.org/10.1016/j.semcdb.2017.11.029

Jayo, A., & Parsons, M. (2010). Fascin: a key regulator of cytoskeletal dynamics. *The International Journal of Biochemistry & Cell Biology*, *42*(10), 1614-1617. https://doi.org/10.1016/j.biocel.2010.06.019

Khaitan, V., Shill, K., Chatterjee, P., Mukherjee, S., & Majumder, P. (2023). Singed and vinculin play redundant roles in cell migration by regulating F-actin. *Developmental Dynamics*, *252*(7), 986-1008. https://doi.org/10.1002/dvdy.585

Lamb, M. C., & Tootle, T. L. (2020). Fascin in cell migration: more than an actin bundling protein. *Biology*, *9*(11), 403. https://doi.org/10.3390/biology9110403

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., & Carthew, R. W. (2004). Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, *117*(1), 69-81. https://doi.org/10.1016/S0092-8674(04)00261-2

Mir, M., Reimer, A., Stadler, M., Tangara, A., Hansen, A. S., Hockemeyer, D., Eisen, M. B., Garcia, H., & Darzacq, X. (2018). Single molecule imaging in live embryos using lattice light-sheet microscopy. *Nanoscale Imaging: Methods and Protocols*, 541-559. http://dx.doi.org/10.1007/978-1-4939-8591-3_32

Pollitt, S. L., Myers, K. R., Yoo, J., & Zheng, J. Q. (2020). LIM and SH3 protein 1 localizes to the leading edge of protruding lamellipodia and regulates axon development. *Molecular Biology of the Cell*, *31*(24), 2718-2732. https://doi.org/10.1091/mbc.E20-06-0366

Rossi, A. M., Jafari, S., & Desplan, C. (2021). Integrated patterning programs during *Drosophila* development generate the diversity of neurons and control their mature properties. *Annual Review of Neuroscience, 44,* 153-172. https://doi.org/10.1146/annurev-neuro-102120-014813

Sánchez-Soriano, N., Tear, G., Whitington, P., & Prokop, A. (2007). *Drosophila* as a genetic and cellular model for studies on axonal growth. *Neural Development, 2*(9), 1-28. http://www.neuraldevelopment.com/content/2/1/9

Stocker, H., & Gallant, P. (2008). Getting started: An overview on raising and handling *Drososphila.* In C. Dahmann (Eds.), *Drosophila methods and protocols* (pp. 27-44). Humana Press.

Tanaka, M., Fujii, Y., Hirano, K., Higaki, T., Nagasaki, A., Ishikawa, R., Okajima, T., & Katoh, K. (2019). Fascin in lamellipodia contributes to cell elasticity by controlling the orientation of filamentous actin. *Genes to Cells*, *24*(3), 202-213. https://doi.org/10.1111/gtc.12671

Vignjevic, D., Kojima, S. I., Aratyn, Y., Danciu, O., Svitkina, T., & Borisy, G. G. (2006). Role of fascin in filopodial protrusion. *The Journal of Cell Biology*, *174*(6), 863-875. https://doi.org/10.1083/jcb.200603013