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Pranav Yalamanchili April 10, 2024

The RNA binding protein Nab2 regulates level of RhoGEF Trio protein isoforms to govern mushroom body axon development

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Abstract

The RNA binding protein Nab2 regulates level of RhoGEF Trio protein isoforms to govern mushroom body axon development

By Pranav Yalamanchili

Precise spatiotemporal regulation of gene expression is critical for normal brain development and function. Important neurodevelopmental events including neuronal differentiation, axon guidance, and synaptic plasticity are governed in part by post-transcriptional processing of mRNAs. Critically, these mechanisms rely on the actions of RNA binding proteins (RBPs) which associate with nascent mRNA molecules to regulate key aspects of RNA fate including stability, localization, and alternative splicing. Although these RBPs are generally ubiquitously expressed, loss of RBP function disproportionately results in significant neurological impairments, illustrating the enhanced reliance of the nervous system on post-transcriptional regulation of gene expression. Notably, loss of function mutations in the RBP *ZC3H14* have been linked to a form of non-syndromic, autosomal recessive human intellectual disability (NS-ARID). To study the function of ZC3H14 in the nervous system, we utilize *Drosophila Melanogaster* as a model system. *Drosophila* have a well conserved ortholog of ZC3H14, known as nuclear polyadenosine RNA binding protein 2 (Nab2). The loss of Nab2 function in *Drosophila* causes behavioral impairments, short-term memory deficits, reduced survival, and disruptions in mushroom body morphology. Here, we demonstrate that Nab2 dictates *Drosophila* neurodevelopment through regulating splicing of the 5' introns in the *trio* mRNA transcript, a gene shown to be conserved in humans. The *Trio* gene encodes a Rho guanine nucleotide exchange factor (RhoGEF) that controls axon development within the *Drosophila* nervous system. Specifically, these data identify that

Nab2 controls cell-type specific expression of two opposing function Trio-GEF domains to rescue defects caused by the *Nab2*^{null} mutation including mushroom body morphology, viability, and lifespan.

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Table of Contents

Introduction:

Intellectual disability refers to a broad class of neurodevelopmental disorders defined by limitations in intellectual function and adaptive behavior (Vissers et al 2016). Diagnostically, these criteria are met by an IQ less than 70, reduced adult autonomy, and general learning deficits (WHO Europe 2020). Intellectual disability is genetically heterogeneous affecting approximately 1% of the world population (Maulik et al 2011). Due to the disorder's heterogeneity, genetic presentation can range from monogenic forms of intellectual disability to increasingly more complex genetic presentation. To date, over 700 genes have been linked to intellectual disability (Ilyas et al 2020) and this complexity makes finding treatment and therapies uniquely difficult. Importantly however, numerous studies have uncovered that many genes linked to intellectual disability converge upon limited number of molecular pathways (Ilyas et al 2020). Thus, the study of monogenic intellectual disabilities could reveal the underlying molecular dysfunction contributing to various forms of intellectual disability.

Interestingly, many of the genes linked to intellectual disability encode RNA binding proteins (RBP) (Bardoni et al 2012). RNA binding proteins regulate every aspect of RNA processing and gene expression through a multitude of mechanisms including polyadenylation, nuclear transport, and the regulation of splicing and translation (Olesnicky and Wright 2018). Moreover, RNA binding proteins are critical for the development of highly specialized cells such as neurons which require enhanced spatiotemporal regulation of gene expression for proper development (Olesnicky and Wright 2018, Bardoni et al 2012). Thus, proper RBP function is critical for proper neurodevelopment and neuronal function; therefore, exploring the molecular functions of RBPs is essential to discover pathways implicated in diverse neurodevelopmental pathologies.

One group of RNA binding proteins that specifically interacts with the poly adenosine RNA and regulates post-transcriptional RNA processing events is known as polyadenosine binding

proteins (Pabs) (Rha et al 2017). A subset of Pabs utilize zinc finger (ZnF) domains instead of RNA recognition motifs (RRMs) to bind RNA and downstream processing events (Tanaka Hall 2005). One such RNA binding protein is known as ZC3H14 (Zinc finger Cys-Cys-Cys-His-type containing 14) and loss of this ubiquitously expressed RBP results in a form of severe non-syndromic autosomal recessive intellectual disability (NS-ARID), implicating elevated roles for this RBP within the nervous system (Pak et. al 2011). NS-ARID presents as an autosomal recessive genotype in populations of consanguineous cultures in Asia and the Middle East. Non-syndromic specifically highlights that ID is the sole symptom of the disorder (Kaufman et al 2010).

Drosophila melanogaster has proven to be a powerful model organism for the study of these forms of intellectual disability (Oortveld et al 2013). To preface, fruit flies are easily manipulated and genetically tractable. Furthermore, studies have shown that approximately 70% disease-linked genes (Prübing et al 2013) and 75% of human intellectual disability-linked genes have orthologues in *Drosophila* (Inlow and Restifo 2004, Oortveld et al 2013). Similarly, ZC3H14 has a well conserved orthologue in *Drosophila* known as nuclear polyadenosine binding protein 2 (Nab2) and our labs have extensively exploited this *Drosophila* model to uncover molecular and behavioral roles for this conserved RBP (Pak et al 2011, Fasken et al 2019).

Importantly, studies in *Drosophila* reveal that Nab2 is critical for proper neuronal development (Corgiat et al 2021). In order to elucidate the role of Nab2, a researcher in our lab found that removing the Nab2 protein would yield interesting results. To remove the Nab2 protein, the Nab2 gene was targeted using a transposable recognition molecule known as a P-element. The P-element was inserted into the promoter region of Nab2, and imprecisely excised, which lead to the $Nab2^{null}$ genetic line (Pak et al 2011). Previous studies have demonstrated a role for Nab2 in the regulation of poly(A) tail length, compaction, packaging of mature transcripts for export, and splicing (Green et al 2002, Hector et al. 2002, Kelly and Bienkowski et al 2016, Jalloh and Lancaster et al. 2023). Specially, Nab2 also plays a role in *Drosophila* sex determination through control of splicing and regulation of N6-methyladenosine (m^6A) levels on *Sxl* (Jalloh and

Lancaster et al 2023). Studies of *Nab2*^{null} fly behavior further reveal that Nab2 regulates *Drosophila* locomotion, lifespan, and viability (Pak et al 2011). Moreover, loss of Nab2 causes defects in the morphology of a structure within the fly brain known as the mushroom body (Kelly and Bienkowski et al 2016). The mushroom body is a twin neuropil structure that regulates *Drosophila* associative olfactory learning and memory and is homologous to the human hippocampus (Heisenberg 2003) (**Supplemental 3**).

Unbiased RNA sequencing analysis of mutant *Nab2*^{null} heads revealed that Nab2 regulates the splicing of select neuronally-enriched transcripts within the fly brain. One specific transcript of interest with retention of a 5'UTR intron upon loss of Nab2 is known as *trio. Trio* encodes a Rho guanine nucleotide exchange factor (GEF), that promotes the exchange of inactive GDP for active GTP on select small GTPases (Bircher and Koleske 2021). Mutations in *Trio* are linked to intellectual disability (Park et al 2018) and previous studies demonstrate that Trio has critical roles in both human and *Drosophila* neurodevelopment. Trio regulates actin dynamics (Park et al 2018), axon pathfinding (Newsome et al 2000), mushroom body morphology (Awasaki et al 2000), and dendritic outgrowth (Iyer et al 2012, Bateman et al 2000). One method through which Trio regulates neurodevelopment is through its guanine nucleotide exchange factor (GEF) domains. Trio has two unique domains GEF1 and GEF2 (**Supplemental 4**), which activate Rac1 and Rho1/RhoA, respectively (Kempers et al 2021). Rac1 and Rho1/RhoA have opposing neuronal functions (Soriano et al 2021, Iyer et al 2012). For example, Trio GEF1 actives Rac1, which promotes axon branching and neurite outgrowth (Iyer et al 2011, Bai et al 2015). On the other hand, Trio GEF2, which activates Rho1/RhoA has relatively unexplored function, but recent research shows GEF2 plays a role in neuron collapse (Iyer et al 2011, Tao et al 2019, Bircher and Koleske 2021). Trio has multiple isoforms, and the two implicated variants are known as Trio long (Trio L) and Trio medium (Trio M) (McPherson et al 2005). Trio L has both a GEF1 and GEF2 domain and, recent work suggests that Trio L primarily acts through its GEF1 domain (Iyer et al

2012). However, Trio M, which is less studied, contains only a GEF2 domain. We determined that these domains were key to the function of the Trio and Nab2 molecular pathways.

Interestingly, previous experiments performed in the lab show that loss of Nab2 causes a significant reduction in the levels of Trio M within the *Drosophila* head. Given that Trio M contains only a GEF2 domain, we postulated that the loss of Trio M, and therefore GEF2 function, may underly *Nab2*^{null} phenotypes such as defects in mushroom body morphology and reductions in viability, lifespan, and locomotion. Specifically, due to the loss of Trio M in *Nab2*^{null} heads, we hypothesized that overexpressing the GEF2 domain of Trio using the Gal4-UAS system in a *Nab2*^{null} fly will rescue the observed behavioral and mushroom body morphology defects. Using the Gal4-UAS system we genetically overexpressed Trio-GEF2 within mushroom body neurons. Here we demonstrate that overexpression expression of Trio-GEF2 within mushroom body neurons, rescues α and α' lobe formation in *Nab2*^{null} flies. On the other hand, overexpression of GEF1 in the mushroom body leads to significant morphological defects when present in the complete structure and lethal when expressed in the prime-lobes of the mushroom body within healthy flies and *Nab2*^{null} flies, suggesting proper ratios of GEF1 and GEF2 are critical for proper mushroom body development. Along with morphology improvements, the overexpression of GEF2 rescues both viability and locomotion within *Nab2*^{null} flies. This data implies that both the Trio and Nab2 function in a common neurodevelopment pathway critical for axon guidance.

Methods:

***Drosophila melanogaster* stocks and genetics**

Drosophila melanogaster were raised on cornmeal agar and maintained in humidified incubators at 25°C with 12 circadian cycles. Crosses were performed in similar vials by adding yeast to promote mating. Lines used in experiment are shown below.

Genetic lines
UAS-mcd8::gfp/c305a-Gal4 (control)
UAS-mcd8::gfp/c305a-Gal4;Nab2^{null}/Nab2^{null}
UAS-mcd8::gfp/c305a-Gal4;UAS-trio-GEF1,Nab2^{null}/Nab2^{null}(experimental)
UAS-mcd8::gfp/c305a-Gal4;UAS-trio-GEF2,Nab2^{null}/Nab2^{null} (experimental)
UAS-mcd8::gfp/c305a-Gal4;UAS-trio-GEF2/TM6B
UAS-mcd8::gfp/c305a-Gal4;UAS-trio-GEF1/TM6B

***Drosophila* brain dissection, immunohistochemistry, visualization, and statistical analysis**

For mushroom body morphology imaging, brains were dissected using procedure originally described in Kelly *et al*. (2017) Approximately 25 *Drosophila* are submerged in .1% PBS-T. Using fine forceps, the proboscises are removed to allow for penetration of .1% phosphate buffer saline with Triton X-100 (PBS-T), which is a detergent. The cuticle and trachea are peeled away revealing the brain (**Supplemental 1**). Brains were submerged in 1X PBS on ice until all brains were dissected. The dissected brains are fixed in 4% paraformaldehyde for 30 minutes and permeabilized in .3% PBS-T on ice for 30 minutes. Brains are transferred to .5 ml Eppendorf Tubes in .1 % PBS-T. They are incubated on a rocker at 4°C in the primary antibodies, m-αTrio and r-bα-GFP, supplemented with NGS (normal goat serum). The brains are washed with 1x PBS to clear primary. The secondary antibodies, g-α-mouse Cy3 and g-α-rb 488, supplemented with NGS are introduced. Incubation times and dilutions are shown in table below (Probably add figure count). Immunostained brains are mounted on SuperFrost Plus slides in 40 mL of Vecta-shield (Vector Laboratories) using a coverslip bridge. Brains were imaged on Nikon A1R confocal microscope. Image alterations were performed using Fiji ImageJ.

Antibody	Incubation Time	Dilution
Mouse monoclonal 9.4A anti-Trio	48-72hr	1:50
Mouse monoclonal 1D4 anti-Fasciclin II	48-72hr	1:50
Rabbit Polyclonal Anti-Green Fluorescent Protein (GFP)	Overnight-24hr	0.125:100
Alexa Fluor® 488 AffiniPure Polyclonal Goat Anti-rabbit IgG	Overnight-24hr	1:100
Cy™3 AffiniPure Polyclonal Goat Anti-Mouse IgG	Overnight-24hr	1:100

Viability and Lifespan Assay

Viability was measured at 25°C by counting eclosion rates of 100 wandering L3 larvae collected for each experimental genotype. At least 3 replicates per genotype were tested for significance. Rates of hatching were recorded for 5-6 days. Viability was calculated as the number eclosed compared to total eggs. Data was analyzed using group analysis on GraphPad. Lifespan was tested at 25°C as described in Morton *et al*. (2020). Essentially, 10 newly eclosed flies were placed in standard cornmeal agar vials. The flies were transferred to new vials weekly. The number of surviving flies was counted daily. Each genotype was replicated three times for significance. Data was analyzed using group analysis on GraphPad.

Locomotion assay

Drosophila melanogaster have previously been shown to have a negative geotaxis by Morton *et al.* (2020). Testing the negative geotaxis represents a good method to test the locomotive ability of the flies. Essentially, newly eclosed and age matched flies were collected and raised for 2-5 days. Groups of 10 age-matched flies were placed in 25 mL graduated cylinders for analysis. Graduated cylinders were tapped, which dropped flies to bottom of vial, and the rates of ascension were measured at 5, 10, 15, and 30 seconds. 3 replicates were performed for each genotype. (**Supplemental 2**)

Statistical Analysis

Statistical analysis of biological triplicate data sets was performed using One-way or Two-way ANOVA on Prism Graph Pad. The statistical significance, calculated with p values is denoted on graphs using asterisks (*, $p \leq 0.05$, **,$p \leq 0.01$,***,$p \leq 0.001$,****,$p \leq 0.0001$). Statistical significance is generally not used in the imaging when looking at brain morphology defects because the data is represented as a percentage. However, due to our data's biological repeats, the rescue shown is most likely accurate.

Results:

Overexpression of Trio-GEF2 in mushroom body lobes rescues α' lobe development along with defasciculation in *Nab2^{null}* mushroom bodies

Previous studies in the lab illustrate that loss of Nab2 causes reduced levels of the Trio M protein in *Nab2^{null}* heads. Given the established roles for both Nab2 and Trio in regulating mushroom body morphology (Kelly and Bienkowski et al 2016, Awasaki et al 2000), we postulated whether overexpression of the catalytic GEF1 or GEF2 domains of Trio within mushroom body neurons of a *Nab2^{null}* fly would mitigate the *Nab2^{null}* mushroom body morphology defects. To determine whether the loss of Trio-GEF1 or -GEF2 contribute to *Nab2^{null}* mushroom body morphology defects we utilized the Gal4-UAS system to overexpress Trio-GEF1 and Trio-GEF2. Gal4 is a transcriptional activator that dictates the expression of any protein under the control of a UAS sequence. Here we utilized the mushroom body *Ok107-Gal4* driver to drive overexpression of either *UAS-Trio-GEF1* or *UAS-Trio-GEF2* within all mushroom body neurons. Using the α-FasII antibody to visualize the α/β/γ lobes of the mushroom body, these data show that overexpression of the Trio-GEF2 domain in the mushroom body rescues α lobe formation (**Figure 1B**). On the other hand, we did not observe any changes in β lobe structure (**Figure 1C**).

We next tested whether overexpression of the Trio-GEF1 or -GEF2 domains in the prime-lobes of the mushroom body mediated the prime-lobe morphology of *Nab2^{null}* flies. The prime lobes are essential to *Drosophila* neurodevelopment as they form during the larval stage prior to the other lobes (Poppinga et al 2022). Once again, we utilized the Gal4-UAS system to drive overexpression of either *UAS-Trio-GEF1* or *UAS-Trio-GEF2* within neurons of the α' and β' lobes mushroom body neurons (*C305a-Gal4*). We also drove overexpression of *UAS-mcd8::GFP* to visualize the α' and β' lobes. *Nab2^{null}* mutant mushroom bodies had defects in both α' and β' structures and a distinct defasciculation, stringy neuron, phenotype(**Figure 1D**). Interestingly, the overexpression of Trio-GEF1 in isolation or in a *Nab2^{null}* background was lethal. However, overexpression of Trio-GEF2 in the background of a *Nab2^{null}* fly rescues both defects in α' structure and defasciculation (**Figure 1E**). Trio-GEF2 overexpression in isolation has no effect on mushroom body structure. This data suggests that proper levels of Trio-GEF1 and GEF2 may be required for proper mushroom body development and that the loss of Trio-GEF2 contributes to *Nab2^{null}* mushroom body defects.

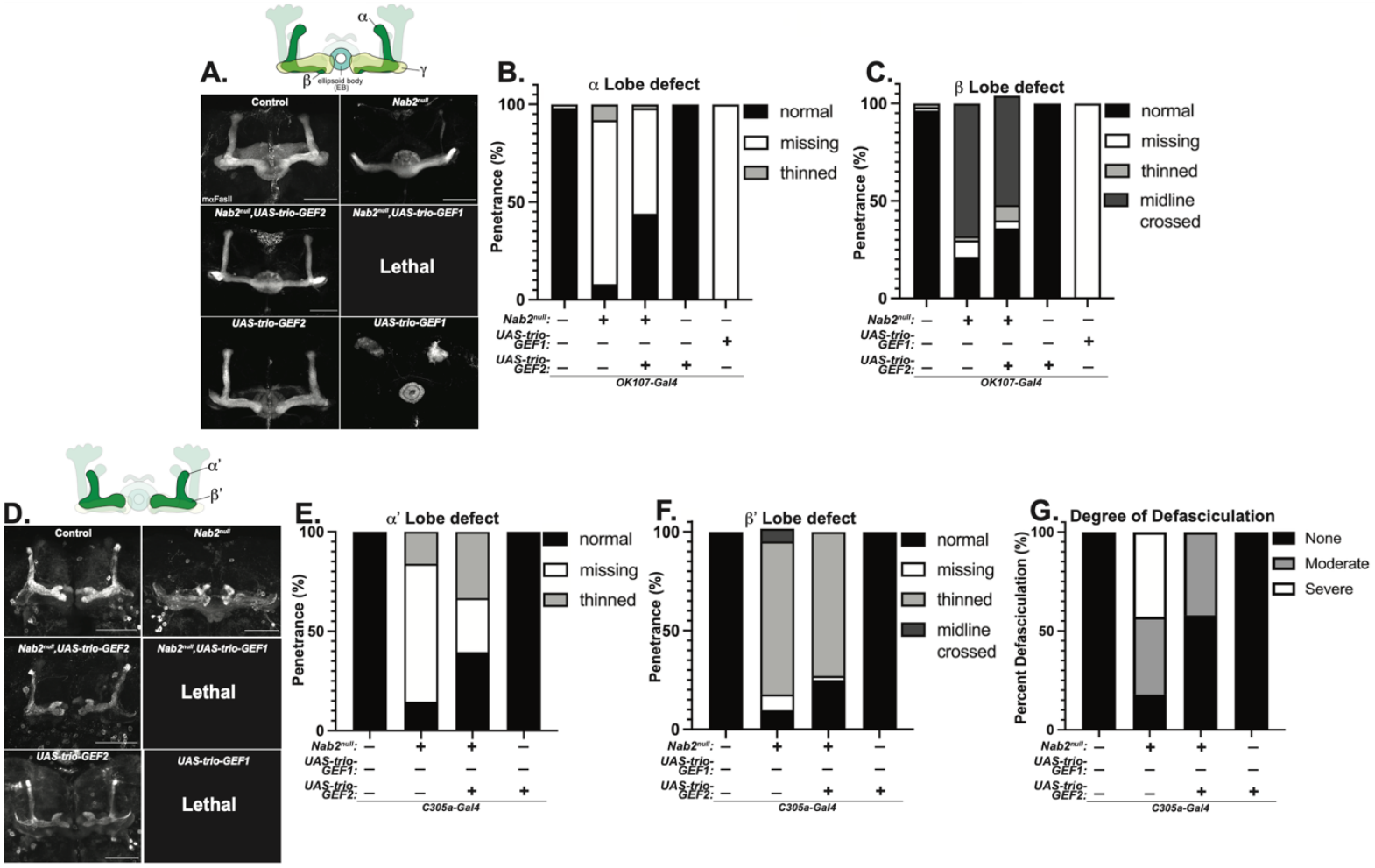


Figure 1. Overexpression of Trio-GEF2 rescues α and α' *Nab2*^{null} mushroom body morphology defects. **A**. Visualization of mushroom body with overexpression of GEF domains within entire mushroom body using *OK107-Gal4* driver. **B**. Segmented bar graph depicting morphological defect prevalence within fly genotypes in the α lobe. **C**. Segmented bar graph depicting morphological defect prevalence within fly genotypes in the β lobe (midline crossed highlights an abnormally connected set of β lobes). **D**. Visualization of mushroom body with overexpression of GEF domains using *C305a-Gal4*, a prime specific driver. **E**. Segmented bar graph depicting morphological defect prevalence within fly genotypes in the α' lobes. **F**. Segmented bar graph depicting morphological defect prevalence within fly genotypes in the β' lobes. **G**. Segmented bar graph depicting the percent of the lobes that showed the stringy, defasciculation, phenotype.

Overexpression of Trio-GEF2 rescues *Nab2*^{null} defects in lifespan and viability

Previous findings have shown that the loss of Nab2 causes a reduction in *drosophila* lifespan, locomotion, and viability (Pak et al 2011). Given the loss of Trio M within *Nab2*^{null} heads, we tested whether overexpression of the GEF domains (*UAS-Trio GEF1* or *UAS-Trio-GEF2*)

using the *Ok107-Gal4* mushroom body driver could mitigate these negative behavioral phenotypes. The loss of Nab2 causes reductions in viability, lifespan, and locomotion relative to control flies (**Figure 2B**), as expected. Experiments revealed that overexpression of Trio-GEF2 rescues the *Nab2^{null}* viability (**Figure 2A**) by nearly 40%. Furthermore, the completion rate of the locomotive ability test increased by approximately 40% at the end of the 30 second time period in the GEF2 genotype (**Figure 2B**). The rate of travel up the graduated cylinder (**Supplemental 2**) seemed to stay constant between the various genotypes (**Figure 2B**). Lifespan, however, is not altered by the overexpression of GEF2 (**Figure 2C**). Flies repeatedly died of natural issues instead of accidents such as getting stuck in the food. As predicted due to other data, the overexpression of Trio-GEF1 in the mushroom body is lethal (**Figure 2A**). The flies simply do not eclose, so viability can not be calculated (**Figure 2A**).

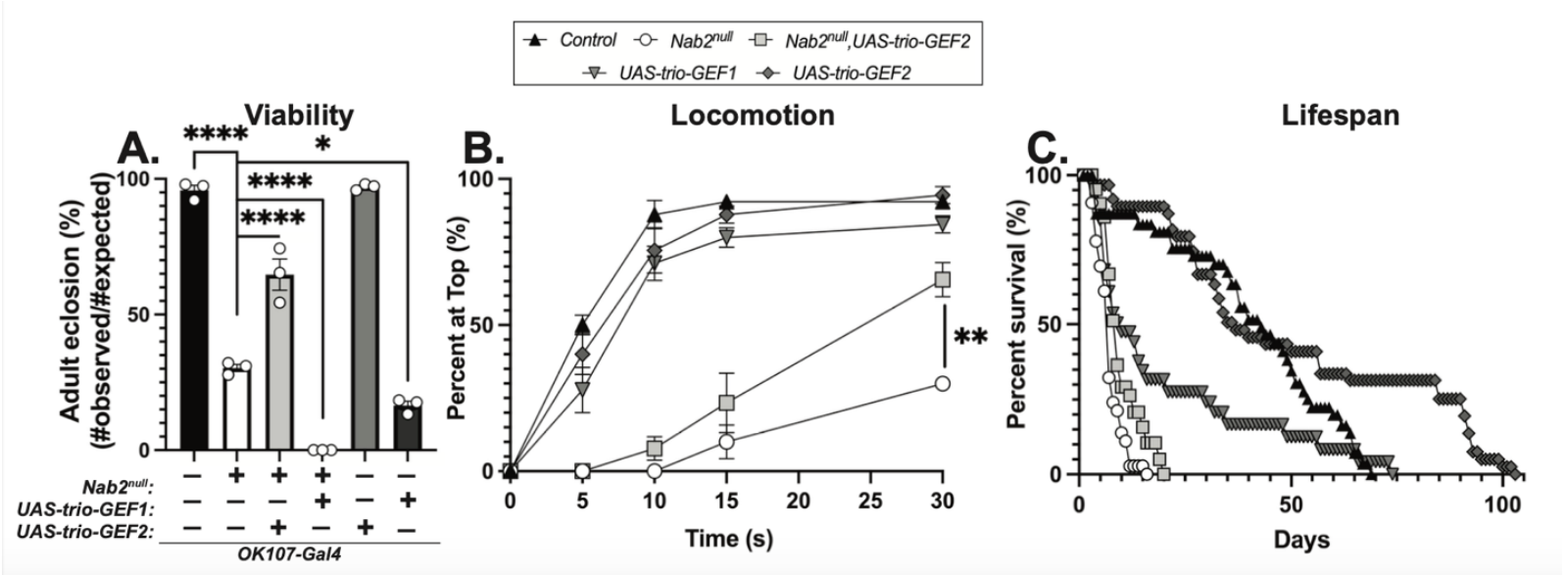


Figure 2. Overexpression of Trio-GEF2 rescues *Nab2^{null}* defects in viability and locomotion. (**A**) Percent of Control, *Nab2^{null}*, *Nab2^{null} UAS-Trio-GEF2, UAS-Trio GEF2,* or *UAS-Trio-GEF1* that eclose as viable adults (calculated as #observed/#expected) using the *Ok107-Gal4* mushroom body driver. (**B**) Negative geotaxis of age-matched adult flies of indicated genotypes over time in seconds (s). (**C**) Survival of age matched adult flies of the indicated genotypes over time in days. Significance values are indicated (*p≤0.05,**p≤0.01,****p≤0.0001).

Discussion:

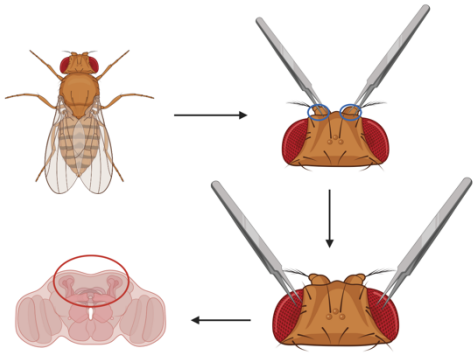
Our findings reveal a critical role for *Drosophila* Nab2 in regulating mushroom body morphology, viability, and locomotion via Trio-GEF2. These experiments also highlight a novel importance of Trio-GEF2 for brain development. Here we demonstrate that overexpression of trio-GEF2 within the prime lobes (*C305a-Gal4*) and the mushroom body as a whole (*Ok107-Gal4*) rescue the morphological and defasciculation defects caused by loss of Nab2. On the other hand, Trio-GEF1 overexpression in the mushroom body (*OK107-Gal4*) causes loss of axon projection altogether and is lethal in a $Nab2^{null}$ background (**Figure 1A**). Moreover, our data show that overexpression of Trio-GEF1 within the prime lobes (*C305a-Gal4*) is lethal in isolation and in a $Nab2^{null}$ background (**Figure 1D**). Previous studies illustrate that Trio-GEF1 acts on Rac1 to support neurite outgrowth (Iyer et al 2011, Bai et al 2015), while Trio-GEF2 acts on Rho1/RhoA to aid in neuron collapse (Iyer et al 2011, Tao et al 2019, Bircher and Koleske 2021). Thus, it is possible that loss of Nab2 disrupts the ratio of Trio-GEF1 to Trio-GEF2 within the head thereby disrupting mushroom body development.

Intriguingly, overexpression of Trio-GEF2 only rescues the α and α' lobes and not the β and β' lobes. This pattern suggests that different Nab2-regulated pathways govern the development of the α/ α' and β/ β'. It is possible that Trio M, and therefore GEF2, regulates pathways that governs the formation of the α/ α' lobes. Future experiments will focus on determining Nab2-regulated pathways that govern development of the β/ β' lobes. Furthermore, the data presented here reveal that the overexpression of GEF2 in the primes autonomously rescues the morphology of the α' prime lobe. Future experiments will also address whether overexpression of Trio-GEF2 in the α'/ β' autonomously rescues α lobe formation, which is a logical assumption due to the order of neurodevelopment as previously stated (Poppinga et al 2022).

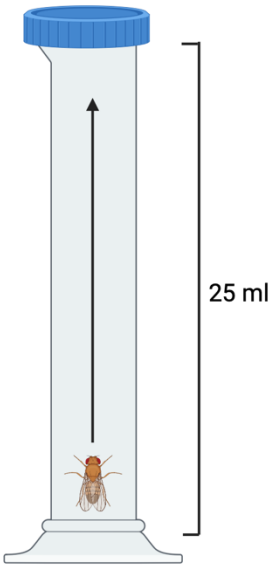
A novel finding of our experiment was the presence of the defasciculation phenotype in the primes upon loss of Nab2. This defasciculation phenotype presented in approximately 80% of the *Nab2*^{null} flies (**Figure 1D**). Excitingly, the overexpression of Trio-GEF2 rescued the neuronal defasciculation in the primes. This indicates that Trio-GEF2 function within the primes is critical for the proper fasciculation of mushroom body neurons.

In the broader physiological picture, the overexpression of Trio-GEF2 in mushroom body neurons improves viability and locomotion but not lifespan in the *Nab2*^{null} flies. Nab2 regulates a large number of transcripts which may or may not be involved in elongating the lifespan the flies. In order to validate these ideas, future directions include testing the effects of other Nab2-target transcripts with roles in nervous system development, such as those present in the peripheral nervous system, which could have more widespread results for the health of the flies such as locomotive ability and appendage movement. Another validating direction would be to quantitatively check the importance of the ratios of GEF1 and GEF2 present within the brain, which could provide a possible experimental direction for repairing Nab2 based neurological issues. This route would involve the overexpression of both GEF1 and GEF2 concurrently, which would allow us to separate the importance of the ratio of the GEF domains and the general amount expressed. Our work reveals that Nab2 controls fly behavior and neurodevelopment through the regulation of Trio M, and therefore GEF2 levels in the mushroom body. These results provide insight into how Nab2 and Trio dysfunction could impact human neurodevelopment.

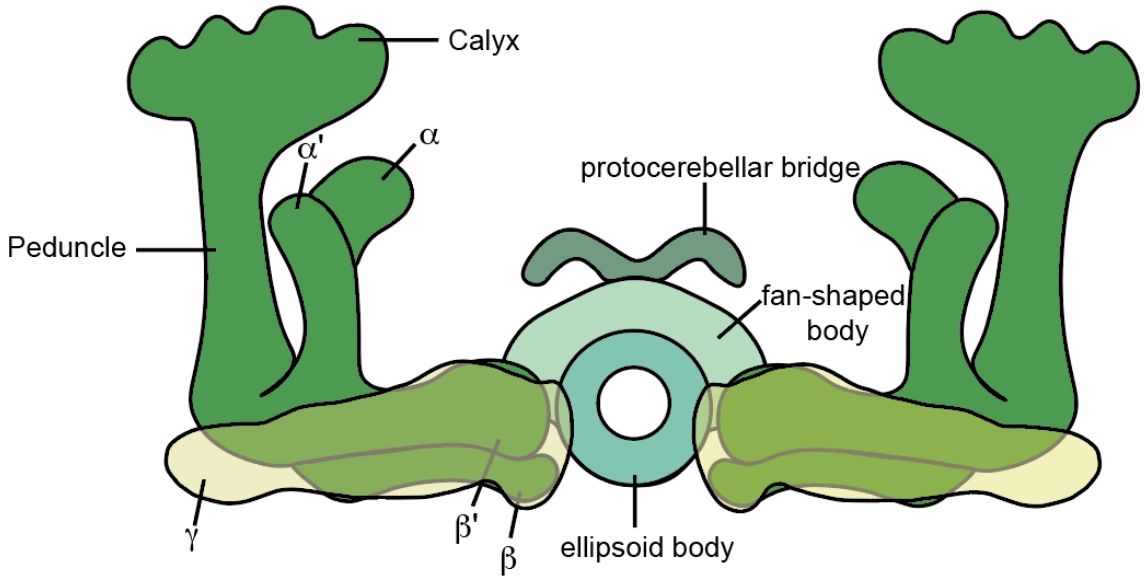
Supplemental Figures:



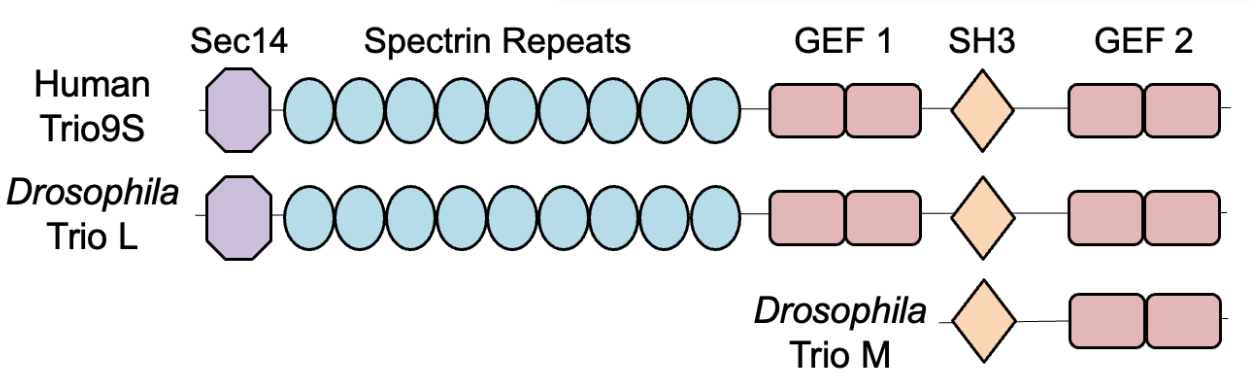
Supplemental 1. Approximated drawing showing process of fly brain dissection. First step is to remove antennae, which allows for penetration of PBS. Next is to isolate the central part of brain by placing tweezers in antennae holes and peeling off the eye casings. The central part, containing the mushroom body, should be revealed unharmed.



Supplemental 2. Diagram showing negative geotaxis of flies used to test locomotive ability. 10 flies are tapped to the bottom of a 25 mL graduated cylinder. They are timed for their ability to rise to the top of the tube.



Supplemental 3. Diagram showing structure of healthy mushroom body. Mushroom body is a twin neuropil structure composed of 10 distinct lobes as shown above. There are projections on either side known as the peduncle and calyx, which are the locations from which the mushroom body neurons grow.



Supplemental 4. Diagram showing the structure of the domains present in Human Trio isoform and *Drosophila* Trio L and Trio M. Notice the GEF 1 and GEF2 domains present in the Trio L isoform, while Trio M only contains a GEF 2 domain.

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