



miR-51 regulates GABAergic synapses by targeting Rab GEF GLO-4 and lysosomal trafficking-related GLO/AP-3 pathway in *Caenorhabditis elegans*

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ABSTRACT

A deficit of GABA (γ -aminobutyric acid) transmission will lead to epilepsy and other cognitive disorders. Recent evidence has shown that neuronal miRNAs affect various synapses, including GABAergic synapses. However, the miRNAs that control GABAergic synapses remain not fully understood. Here, we identified miR-51, a member of *Caenorhabditis elegans* miR-99/100 family, as a key regulator of GABAergic synapses. Loss of *mir-51* increased PTZ (Pentylentetrazole) and aldicarb hypersensitivities, and decreased the number of GABAergic synapses and abundance of GABA_A receptors. A Rab guaninenucleotide exchange factor (GEF) GLO-4, a well-known component in lysosomal trafficking-related GLO-4/GLO-1/AP-3 (GLO/AP-3) pathway, was discovered to be the direct target of miR-51. Rescue experiments showed that GLO-4 expressed in GABAergic motor neurons functioned as a suppressor of miR-51. Disruption of *glo-1* or AP-3 gene *apm-3* attenuated the defects of GABAergic synapse in *mir-51* mutants, suggesting miR-51 regulated GABAergic synapses through GLO/AP-3 pathway. The present study implies the essential roles of miRNAs on the nervous pathologies characterized by mis-regulated GABA signaling, such as epilepsy.

1. Introduction

The balance between neuronal excitation and inhibition is critical for information processing in brain. Synapses are the specialized structures required for communication between neurons and their targets. GABAergic synapses control the higher cognitive functions of brain by mediating the majority of the neuronal inhibitory signals (Fagioli and Hensch, 2000; Tao and Poo, 2005). These inhibitory synapses consist of a presynaptic terminal where GABA (γ -aminobutyric acid) neurotransmitter is released from synaptic vesicles (SVs), and an opposing postsynaptic terminal where GABA binds to its receptors to initiate the signals (Waite et al., 2005). When GABA transmission is impaired, neurological disorders such as epilepsy, schizophrenia, alcoholism and anxiety will occur (Prosser et al., 2001; Schuler et al., 2001; Yuen et al., 2012). Therefore, to screen and identify the genes in regulating GABAergic synapses is important to get insights into the circuits' dysfunction diseases.

Caenorhabditis elegans (*C. elegans*) is a powerful system to screen the crucial genes required for synaptic function by using behavioral and pharmacological selection criteria. Most synaptic genes discovered in *C. elegans* are highly conserved to mammals, offering an opportunity to study these genes in this relatively simple system (Bargmann, 1998; Richmond, 2005). *C. elegans* body muscles receive both excitatory (acetylcholine, ACh) and inhibitory (GABA) inputs. Therefore, treatment of aldicarb (an acetylcholinesterase inhibitor) results in accumulation of ACh at neuromuscular junctions (NMJs). Hypersensitivity to aldicarb-induced paralysis is caused by mutations with loss of GABA transmission, or failure to negatively regulate ACh release (Sieburth et al., 2005; Vashlishan et al., 2008). Pentylentetrazole (PTZ) is a GABA receptor antagonist and epileptogenic agent in mammals (Rocha et al., 1996). In *C. elegans*, the mutant worms with defects in GABAergic synaptic function display seizure-like convulsions in response to PTZ. However, wild-type worms (N2), as well as those with specific defect in ACh transmission, do not exhibit this kind of

Abbreviations: GABA, γ -aminobutyric acid; PTZ, pentylentetrazole; SNB-1, Synaptobrevin 1; GEF, guaninenucleotide exchange factor; SVs, synaptic vesicles; ACh, acetylcholine; NMJs, neuromuscular junctions; miRNAs, microRNAs; GluR, glutamate receptor; nAChR, neuronal acetylcholine receptor; MEF-2, myocyte enhancer factor 2; Limk1, Lim-domain-containing protein kinase 1; LROs, lysosome-related organelles; UTR, untranslated regions; DNC, dorsal nerve cord; RPM-1, Regulator of presynaptic morphology 1

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sensitivity (Kowalski et al., 2014; Williams et al., 2004). Therefore, measuring both aldicarb and PTZ hypersensitivities is a good approach to identify genes in regulating GABAergic synapses.

microRNAs (miRNAs) are a class of non-coding ~ 22-nucleotide RNAs that control gene expression by blocking translation or destabilizing target mRNAs (Huntzinger and Izaurralde, 2011). Increasing evidence has documented miRNAs influence various synapses (Corbin et al., 2009; Schratt, 2009; Siegel et al., 2011). In *Drosophila*, the abundance of glutamate receptor subunits GluRA and GluRB at NMJs is changed by miR-284 (Karr et al., 2009). In *C. elegans*, miR-1 modulates the muscles' responsiveness to acetylcholine (ACh) by targeting two nAChR receptor subunits (UNC-29 and UNC-63) and transcription factor MEF-2 (Simon et al., 2008).

A few of miRNAs also affect GABAergic synapses. Overexpression of mice miR-33 significantly reduced the mRNA level of GABA_A receptors *Gabra4* and *Gabra2* (Jovasevic et al., 2015). Rat miR-500 decreased glutamate decarboxylase GAD67 expression and contributed to neuropathic pain (Huang et al., 2016). In *C. elegans*, miR-84 inhibited *hbl-1* and influenced GABAergic DD motor neurons remodeling (Thompson-Peer et al., 2012). The expression levels of many miRNAs are up- or down-regulated in the patient tissues or mice model of epilepsy and other GABA signaling-misregulated cognitive diseases, such as Schizophrenia (An et al., 2005; Li et al., 2009; Moreau et al., 2011; Yen et al., 2011). These suggest that some miRNAs that play crucial roles in GABAergic synapse regulation are still undiscovered.

Here, we identified *C. elegans* miR-51 as a modulator of GABAergic synapses by aldicarb and PTZ hypersensitivity assays. In GABAergic motor neurons, miR-51 repressed the expression of a Rab GEF GLO-4 by binding to its 3'UTR, which lead to the increase in the number of GABAergic synapses and the abundance of GABA_A receptors. GLO-4 plays roles in GLO/AP-3 pathway by interacting with target Rab GLO-1 and lysosomal cargo sorting protein AP-3. We found loss of *glo-1*, or *apm-3* (AP-3 gene) partially decreased the defects of GABAergic synapses in *mir-51* mutants. These findings imply that miR-51 regulates GABAergic synapses by inhibiting its direct target GLO-4 in GABAergic motor neuron, and then GLO/AP-3 pathway in *C. elegans*.

2. Materials and methods

2.1. Genetics

C. elegans strains were grown and maintained by standard methods. Alleles used in this study included the following (provided by the *Caenorhabditis* Genetics Center): *mir-51(n4473)*, *mir-52(n4114)*, *mir-53(n4113)*, *mir-54-56(nDf58)*, *mir-51 mir-53(nDf67)*, *glo-4(ok623)*, *glo-1(zu391)*, *apm-3(tm920)*, *unc-25(e156)*. Strains *mir-51(n4473)*, *mir-52(n4114)*, *mir-53(n4113)*, *mir-54-56(nDf58)* were all outcrossed to wild type N2 Bristol for three times before used. *glo-1* and *glo-4* mutants were recognized by their lack of gut granules (Glo) and partially penetrant dumpy (Dpy) body shape. *mir-51(n4473)* was crossed to *mir-52(n4114)*, *mir-54-56(nDf58)*, *glo-4(ok623)*, *glo-1(zu391)* and *apm-3(tm920)*, respectively. Double and triple mutants were constructed following standard procedures and confirmed by PCR genotyping. The transgenic reporter genes used in this study were *juIs1[Punc-25::SNB-1::GFP]* and *oxIs22[Punc-49::UNC-49::GFP]*.

2.2. Pentylentetrazole (PTZ) and aldicarb hypersensitivity assays

PTZ hypersensitivity assays were performed by adding 10 mg/mL (PTZ) (Sigma) to NGM plates seeded with OP50 bacteria. 30 young adults for each mutant strain were placed onto the drug plates and observed for a period of 30 min. Worms were scored positive for convulsions if they demonstrated repetitive body contractions ('tonic-clonic' convulsions), which characterized by anterior contractions with posterior immobilization, or simultaneous contraction of both the anterior and posterior occurred.

For aldicarb hypersensitivity assays, 30 young adults for each mutant strain were transferred into NGM plates containing 1 mM aldicarb (Sigma), and continuously scored for paralysis after 30 min, 60 min, 90 min, 120 min, 150 min and 180 min, respectively. Negative (wild-type N2), and positive (*unc-25* mutant) controls were randomly included during every assay. All scoring was done blindly. The assays for each strain were repeated more than three times.

2.3. Fluorescence microscopy and quantitative analysis

SNB-1::GFP and UNC-49::GFP were imaged in the region near the anterior gonad bend on an Olympus FV-1000 confocal microscope. Image stacks were captured and maximum intensity projections of Z-series stacks were made by Olympus Fluoview software. Identical camera gain, exposure settings, and fluorescence filters were used in all the quantitative studies for a particular integrant. Line scans of 100 μm dorsal cord fluorescence were analyzed by the software of microscope to estimate the densities of SNB-1::GFP and UNC-49::GFP. The intensities of SNB-1::GFP and UNC-49::GFP punctal fluorescence were then analyzed by Image-Pro Plus.

2.4. Transgenes

The constructs for miR-51 rescue were performed according to previous publication (Shaw et al., 2010). For *glo-4* rescue experiments, *glo-4* gene with *unc-25* promoter or *myo-3* promoter was cloned into pPD95.75, whose *unc-54* 3'UTR was replaced by *glo-4* 3'UTR, to generate plasmids *Punc-25::glo-4::gfp::glo-4* 3'UTR and *Pmyo-3::glo-4::gfp::glo-4* 3'UTR. After all of the cloned sequences were confirmed, 10 ng/μL of each construct was injected into their target strains, along with 50 ng/μL *ord-1*. The plasmid *Punc-25::gfp::glo-4* 3'UTR was generated by cloning *unc-25* promoter (about 2 kb) into pPD95.75. The *glo-4* 3'UTR sequence of this plasmid was mutated (MUT) or deleted (DEL) at miR-51 binding sites within 4 bp according to the protocol of QuikChange (Stratagene). 10 ng/μL of wild-type (WT), mutated (MUT) or deleted (DEL) plasmids were injected into N2 or *mir-51(n4473)* mutant, along with 50 ng/μL *rol-6*. The primers used in transgenes were shown in Table S1.

2.5. Mammalian cell culture and luciferase reporter assays

miR-51 gene, including its upstream and downstream sequences with 731 bp, was cloned in pmR-mCherry overexpression plasmid between *XhoI* and *BamHI* sites. The 3'UTR sequence of each potential target gene for miR-51 was inserted in pGL3-Control Vector at 3'UTR of luciferase reporter gene via *FseI* site. The primers used in clone were shown in Table S1. The mutation (MUT) and deletion (DEL) of *glo-4* 3'UTR in reporter plasmids were generated by using the protocol of QuikChange (Stratagene).

Human 293T cells were grown in DMEM (Invitrogen) with 10% FBS in 5% CO₂ at 37 °C, and seeded into 24-well plates. Cells were transfected 24 h later with PEI according to the manufacturer's protocol with luciferase reporter vectors and *mir-51* expression plasmid, followed by 24 h incubation. Dual-Glo (Promega, Madison, WI) luciferase assay kit was used according to manufacturer's protocol to detect firefly and Renilla luciferase activity. Illuminescence was detected with a Glomax luminometer (Turner BioSystems, Sunnyvale, CA). Renilla luciferase activity was used as the internal reference.

3. Results

3.1. miR-51 plays key functions in modulating PTZ and aldicarb hypersensitivities

To identify miRNAs in regulating GABAergic synapses, six miRNAs were selected for hypersensitivity assays in response to PTZ

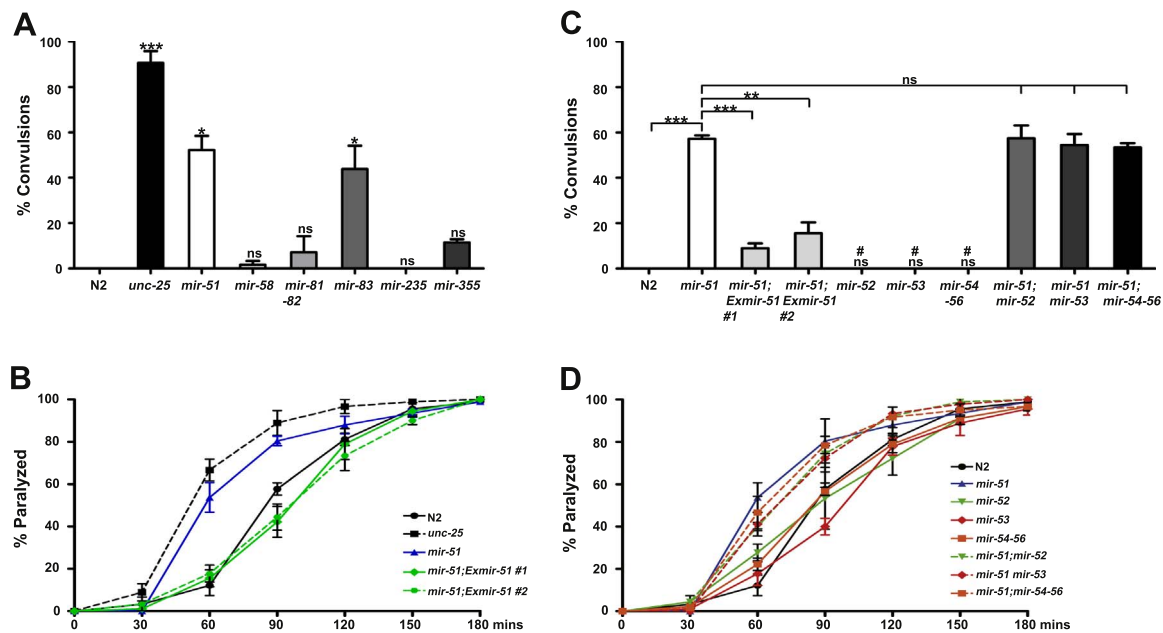


Fig. 1. *C. elegans* miR-51 plays key roles in regulating PTZ and aldicarb hypersensitivity. (A) PTZ induced seizure-like convulsions of *mir-51* mutant. Six miRNA genes, each of which has been predicted to target about 5–10 synaptic related genes according to previous algorithms analysis (Table S2), were selected for PTZ hypersensitivity assay, including *mir-51* mutant. Wild-type worms (N2) and *unc-25(e156)* mutant were used as a negative and positive control, respectively. For each strain, the assays were repeated more than three times. *p* values were calculated by Student *t*-test compared with N2. ns, no significance; *, *p* < 0.05; ***, *p* < 0.005. (B) Recovery of miR-51 expression attenuated the increase in the aldicarb hypersensitivity of *mir-51* mutant. #1 and #2 mean the two independent extrachromosomal arrays of transgenic worms expressing *mir-51* promoter-activated miR-51 in *mir-51* mutant worms. The percentage of paralyzed worms was scored from 30 min to 180 min in each assay. For each strain, the assays were repeated more than three times. Error bars represented the SEM. (C–D) miR-51 was a key member of miR-51 family to regulate hypersensitivities in response to PTZ and aldicarb. Transgenic worms #1 and #2 utilized in C were the same as those in B. PTZ (C) and aldicarb (D) hypersensitivity assays were performed in the single mutants of each miR-51 family member and *mir-51;mir-52*, *mir-51;mir-53*, *mir-51;mir-54–56* double mutants, respectively. The assays were carried out and analyzed as the legends of A and B. ns, no significance; **, *p* < 0.01; ***, *p* < 0.005. #ns mean no significance compared with wild-type (N2). Error bars represented the SEM.

(Pentylenetetrazole) (Table S2), since each of which has been predicted to target 5–10 synaptic related genes by algorithms according to previous publication (Sun et al., 2006). The mutant lacking of GABA synthesis gene *unc-25* was used as a positive control (Williams et al., 2004). Results showed that PTZ induced convulsions in *mir-51* or *mir-83* mutants, but not other four miRNAs mutants (Fig. 1A).

miR-51 is a member of *C. elegans* miR-51 family, which is conserved to miR-99/100 family from cnidarians, nematodes, flies, to humans (Fig. S1). Therefore, we then selected miR-51, but not miR-83, for aldicarb hypersensitivity assay, and found loss of *mir-51* increased the percentage of paralyzed worms, compared to wild-type (N2) (Fig. 1B). By utilizing transgenic technique, we found recovery of miR-51 expression driven by *mir-51* promoter attenuated the hypersensitive phenotypes of *mir-51* mutant in response to both PTZ and aldicarb (Fig. 1B–C).

There are six genes in miR-51 family, including *mir-51*, -52, -53, -54, -55, and -56, of which *mir-54/55/56* (*mir-54–56*) is likely derived from the same transcript (Shaw et al., 2010). We found *mir-52*, *mir-53* and *mir-54–56* single mutants had no hypersensitive phenotypes in response to PTZ and aldicarb, which was similar to wild-type N2 (Fig. 1C and D). Furthermore, loss of *mir-52*, *mir-53* or *mir-54–56* did not enhance the PTZ and aldicarb hypersensitivities of *mir-51* mutants (Fig. 1C and D). These data suggest that miR-51 is the key member of miR-51 family to modulate GABAergic synapses in *C. elegans*.

3.2. miR-51 increases the number of GABAergic synapses and abundance of GABA_A receptors

Synaptogenesis and abundance of neurotransmitter receptors are two of important factors to influence synapse function. To further investigate how miR-51 regulates GABAergic synapses, GFP-tagged synaptic vesicle protein SNB-1/Synaptobrevin in *juIs1[Punc-25::SNB-1::GFP]* was utilized to examine the change of GABAergic synaptogen-

esis in *mir-51* mutant. The expression of SNB-1::GFP creates a continuous pattern of fluorescent puncta in the dorsal nerve cords (DNC), where each punctum corresponds to a single synaptic site. Therefore, the density of GFP-tagged SNB-1 is widely regarded as the number of synapses, and the fluorescence intensity of each punctum is considered as the number of synaptic vesicles (SVs) at a synapse (Sieburth et al., 2005; Vashlishan et al., 2008). The abundance of GABA_A receptors in the posterior DNC at NMJs was assessed by utilizing *oxIs22[Punc-49::UNC-49::GFP]* transgenic worms expressing GFP-tagged GABA_A receptor subunit UNC-49.

Results showed that the densities of SNB-1::GFP puncta, but not the intensities of these punctal fluorescence were decreased in *mir-51* mutant, compared to those of wild-type (wt) worms (Fig. 2A–C). Loss of *mir-51* also reduced both densities and fluorescence intensities of UNC-49::GFP puncta (Fig. 2D–F). Rescue of miR-51 expression driven by *mir-51* promoter in *mir-51;Exmir-51* transgenic worms attenuated the decrease in the densities of SNB-1::GFP and UNC-49::GFP puncta of *mir-51* mutants (Fig. 2A–B and D–E). These *mir-51;Exmir-51* transgenic worms even showed higher fluorescence intensities of UNC-49::GFP puncta than those of wild-type (wt) worms (Fig. 2D and F). These data indicate that miR-51 increases both the number of GABAergic synapses and abundance of GABA_A receptors in *C. elegans*.

3.3. miR-51 represses *glo-4* expression in GABAergic motor neurons

miRNAs generally prevent their target mRNAs' expressions (Huntzinger and Izaurralde, 2011). Therefore, it is necessary to explore the direct targets of miR-51. By combining the predictions via three algorithms of "TargetScan", "miRBase" and "PicTar" together (Griffiths-Jones et al., 2006; Lall et al., 2006; Lewis et al., 2005), we obtained an overlap of 25 genes for miR-51 targets (Fig. 3A). Then, 8 of them were selected for luciferase reporter assay, according to their neuronal expression and functions documented previously (Table S3).

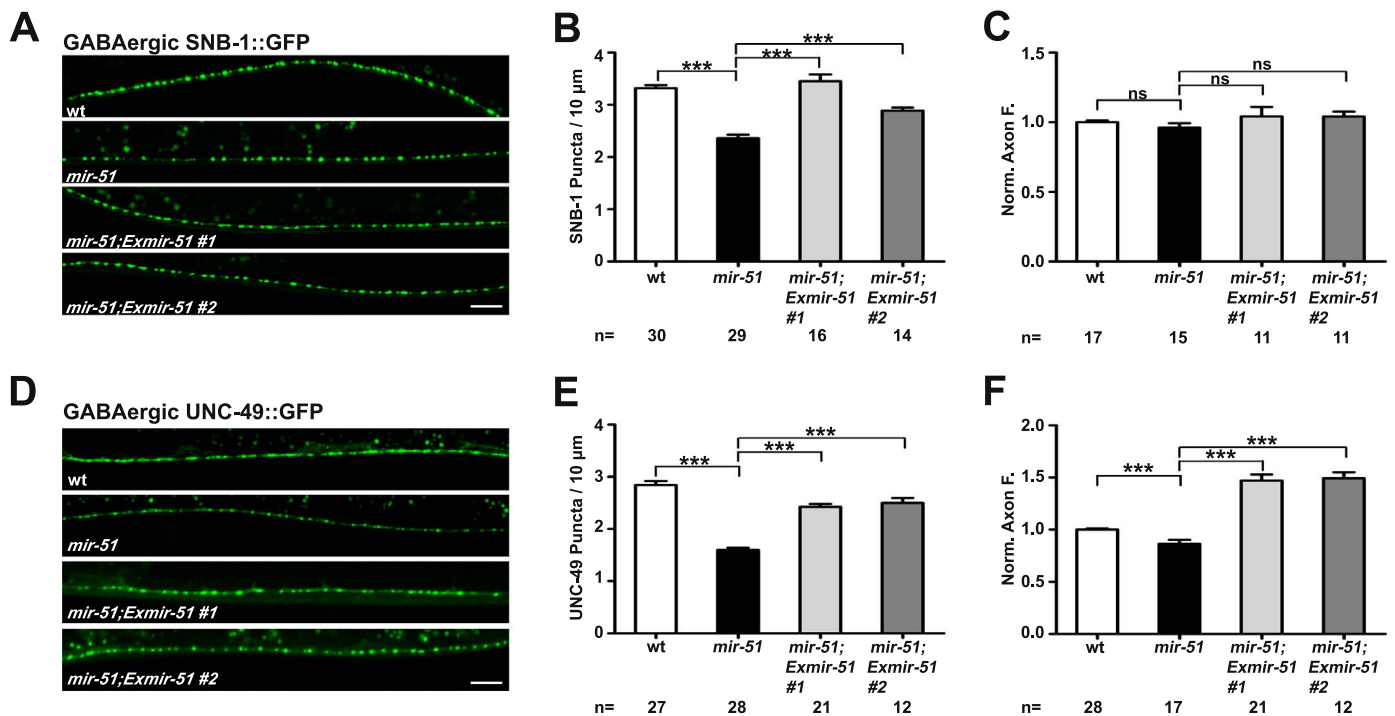


Fig. 2. miR-51 increases the number of GABAergic synapses and the abundance of GABA_A receptors. (A) Representative images of SNB-1::GFP distribution. *juIs1[Punc-25::SNB-1::GFP]* was utilized for detecting SNB-1::GFP distribution in the dorsal nerve cord. #1 and #2 mean the two independent extrachromosomal arrays of *mir-51;Exmir-51* transgenic worms that rescue miR-51 expression driven by *mir-51* promoter in *mir-51(n4473);juIs1*. wt, wild-type. Scale bar, 10 μm. **(B) Rescue of miR-51 expression attenuated the decrease in density of SNB-1::GFP puncta of *mir-51* mutants.** Quantification of SNB-1 puncta per unit length in dorsal nerve cord of GABAergic motor neurons was analyzed on L4 animals grown at 20 °C. n, the number of worms used in quantitative analysis. p values were calculated by Student t-test. *** p < 0.005. **(C) The intensity of SNB-1::GFP punctal fluorescence was rarely influenced by miR-51.** The intensity of SNB-1::GFP punctal fluorescence was analyzed by Image-Pro Plus, and then normalized to that of wild-type (wt). n, the number of worms used in quantitative analysis. p values were calculated by Student t-test. ns, no significance. **(D) Representative images of UNC-49::GFP distribution.** *oxIs22[Punc-49::UNC-49::GFP]* was utilized for monitoring UNC-49::GFP distribution in post-synaptic dorsal body wall muscle. #1 and #2 mean the two independent extrachromosomal arrays of *mir-51;Exmir-51* transgenic worms that rescue miR-51 expression driven by *mir-51* promoter in *mir-51(n4473);oxIs22*. wt, wild-type. Scale bar, 10 μm. **(E) Recovery of miR-51 expression attenuated the decrease in density of UNC-49::GFP puncta of *mir-51* mutants.** The density of UNC-49::GFP puncta were analyzed as legend of B. n, the number of worms used in quantitative analysis. p values were calculated by Student t-test. *** p < 0.005. **(F) Overexpression of miR-51 in *mir-51* mutant showed higher intensity of UNC-49::GFP punctal fluorescence than that of wild-type (wt).** The intensity of UNC-49::GFP punctal fluorescence was analyzed as legend of C. n, the number of worms used in quantitative analysis. p values were calculated by Student t-test. *** p < 0.005.

Results showed that the 3'UTR of *glo-4*, but not other 7 genes, conferred a significant inhibition of translation by overexpression of miR-51 (Fig. 3B). We analyzed the conserved site of *glo-4* 3'UTR that binds to the seed sequence of miR-51 family 5' region, then deleted or mutated this binding site within 4 bp (Fig. S2). Results showed that miR-51 failed to repress reporter gene expression when this binding site was disrupted (Fig. 3C). These indicate that *glo-4* is a direct target of miR-51.

To investigate whether miR-51 represses *glo-4* expression in GABAergic motor neurons. A reporter plasmid *Punc-25::gfp::glo-4* 3'UTR was constructed by inserting *unc-25* promoter and *glo-4* 3'UTR to 5' and 3' sites of GFP gene, as *unc-25* promoter specifically triggers gene expression in GABAergic motor neurons (Jin et al., 1999). By generating transgenic worms, we found depletion of *mir-51* increased GFP expression in GABAergic motor neurons (Figs. 3D–E, S3). Mutation and deletion of *glo-4* 3'UTR sequence at predicted miR-51 binding sites elevated GFP expression of N2 (Figs. 3D–E, S3). These data illustrate that miR-51 prevents *glo-4* expression by binding its 3'UTR in GABAergic motor neurons.

3.4. miR-51 regulates GABAergic synapses by inhibiting GLO-4 expression in GABAergic motor neurons

To address whether GLO-4 negatively mediates the function of miR-51 in GABAergic synapse control, we generated *mir-51;glo-4* double mutants, and found loss of *glo-4* attenuated the increases in aldicarb and PTZ hypersensitivities of *mir-51* mutants (Fig. 4A–B). The percentage of PTZ-induced convulsion and aldicarb-mediated

paralysis in *glo-4* single mutant were similar to those of wild-type (N2) (Fig. 4A–B). Consequently, the densities of SNB-1::GFP puncta, and both the densities and fluorescence intensities of UNC-49::GFP puncta of *mir-51;glo-4* double mutants were higher than those of *mir-51* single mutant (Fig. 4C–F). These suggest that miR-51 increases the number of GABAergic synapses and abundance of GABA_A receptors by inhibiting GLO-4.

C. elegans GLO-4 is enriched in both motor neurons and body wall muscles (Grill et al., 2007), whereas miR-51 is expressed in neuron, but not in muscle (Shaw et al., 2010). To explore whether GLO-4 expressed in GABAergic motor neurons or muscle functions as a suppressor of miR-51, we generated transgenic worms expressing GLO-4 driven by *unc-25* promoter (GABAergic motor neurons-specific) or *myo-3* promoter (body wall muscle-specific) in *mir-51;glo-4* double mutants.

Results showed that recovery of GLO-4 expression in GABAergic motor neurons (but not in body wall muscles) increased the PTZ and aldicarb hypersensitivities of *mir-51;glo-4* double mutants (Fig. 4A–B), decreased their densities of SNB-1::GFP and UNC-49::GFP puncta, and reduced the intensity of UNC-49::GFP punctal fluorescence in this double mutants as well (Fig. 4C–F). These imply that miR-51 regulates GABAergic synapses by inhibiting GLO-4 expression in GABAergic motor neurons.

3.5. miR-51 controls GABAergic synapses by targeting GLO/AP-3 pathway

GLO/AP-3 pathway is composed of GEF GLO-4, Rab GLO-1 and lysosomal cargo sorting protein AP-3 (encoded by *apm-3*) (Hermann

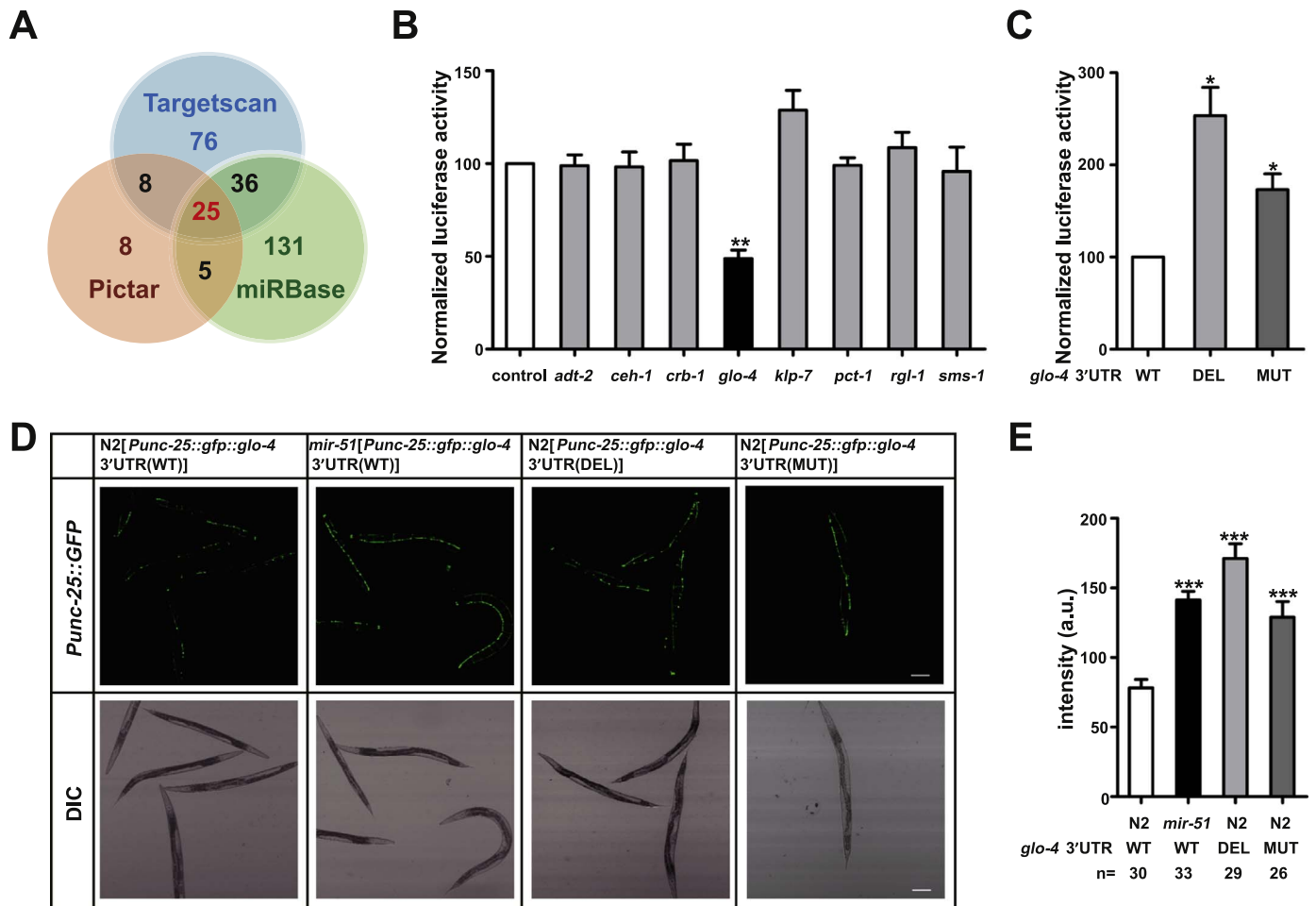


Fig. 3. miR-51 repressed *glo-4* 3'UTR in GABAergic motor neurons. (A) Venn diagram showed the overlapped target genes of miR-51 predicted by three different algorithms "Targetscan", "miRBase" and "PicTar". (B) The 3'UTR of *glo-4* was inhibited directly by miR-51. The luciferase reporter constructs containing 3'UTR sequences of each potential targets of miR-51 were co-transfected with the empty vector (control) or miR-51 overexpression plasmid in human 293T cells, followed by luciferase assay. The Firefly luciferase activity was normalized to empty vector (control). Renilla is used as the internal reference. **, $p < 0.01$. (C) miR-51 failed to suppress *glo-4* 3'UTR by disruption of their binding sites. The luciferase reporter constructs containing wild-type (WT), mutant (MUT) or deleted (DEL) *glo-4* 3'UTR were co-transfected with miR-51 overexpression plasmid in human 293T cells, respectively. The Firefly luciferase activity was normalized to wild type (WT) *glo-4* 3'UTR. Renilla is used as the internal reference. *, $p < 0.05$. (D–E) The 3'UTR of *glo-4* was repressed by miR-51 in GABAergic motor neurons. The representative images of *Punc-25::gfp::glo-4* 3'UTR (WT/MUT/DEL) transgenic worms on N2 and *mir-51* mutant background were shown in D. All photos were taken at L4 stage with identical camera gain, exposure settings and fluorescence filters. GFP reporter protein driven by *unc-25* was expressed specifically in GABAergic motor neurons. Scale bar, 100 μ m. The fluorescence intensity analysis of images was shown in E. p values were calculated by Student t -test compared with N2 [*Punc-25::gfp::glo-4* 3'UTR(WT)]. *** $p < 0.005$.

et al., 2005). This pathway is documented to regulate axon termination and synapse formation in mechanosensory neurons (Grill et al., 2007). To explore whether GLO/AP-3 pathway is required for miR-51 influencing GABAergic synapses, we generated *mir-51;glo-1* and *mir-51;apm-3* double mutants. Results showed that loss of *glo-1* or *apm-3* attenuated the increase in PTZ and aldicarb hypersensitivities of *mir-51* mutants (Fig. 5A–B). The percentages of PTZ-induced convulsion and aldicarb-mediated paralysis in *glo-1* and *apm-3* single mutants were similar to those levels of *glo-4* single mutant and wild-type (N2) (Fig. 5A–B). Accordingly, loss of *glo-1* or *apm-3* upregulated the SNB-1::GFP puncta's densities, and UNC-49::GFP puncta's densities and fluorescence intensities and of *mir-51* mutants (Fig. 5C–D, and E–F). These data suggest that GLO/AP-3 pathway is pivotal for the role of miR-51 in GABAergic synapses.

4. Discussion

microRNAs (miRNAs), a class of small non-coding RNAs, play key roles on controlling synapse development and function by preventing the expressions of synaptic relevant proteins (Cohen et al., 2011; Gao, 2008; Schratt, 2009). Here, we found that miR-51 regulates the

number of GABAergic synapses and abundance of GABA_A receptors by inhibiting its direct target GLO-4 and lysosomal trafficking-related GLO/AP-3 pathway. As miR-51 is highly conserved to miR-99/100 family in mammals (Shaw et al., 2010), our study provides a key clue to the investigation of physiological and pathological function of miR-99/100 family in mammalian synapses in the future.

It has been reported that loss of single gene in miR-51 family induces no typical phenotype, whereas depletions of multiple members in miR-51 family result in synthetic phenotype, including pharynx unattachment, slow growth and mating defective (Shaw et al., 2010). This implies that the members of miR-51 family might act redundantly. Here, we found loss of *mir-52*, *mir-53* or *mir-54–56* had no effect on the PTZ and aldicarb hypersensitivities of *mir-51* mutants, suggesting miR-51 controls GABAergic synapses without the redundant function of other family members. Evidence has shown that all miR-51 family members are expressed in neurons (Shaw et al., 2010). Therefore, whether these members redundantly play roles in other types of neurons is worth exploring.

In this study, we identified a Rab GEF GLO-4 as the direct target of miR-51. GLO-4 is reported to regulate GABAergic synapse formation, as a part of RPM-1 (Regulator of Presynaptic Morphology 1) signaling

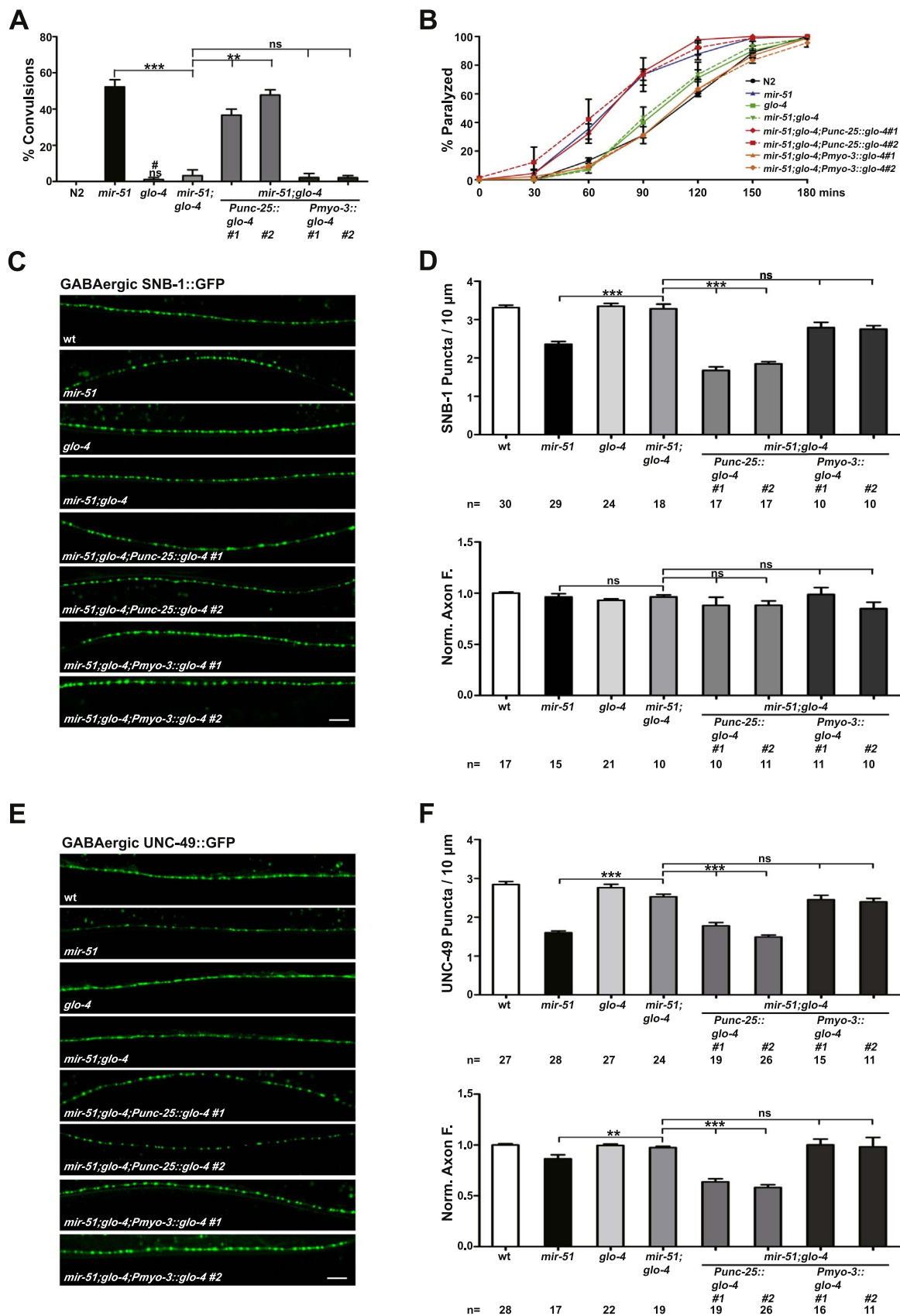


Fig. 4. miR-51 regulates GABAergic synapses by inhibiting GLO-4 expression in GABAergic motor neurons. (A–B) Recovery of GLO-4 expression in GABAergic motor neurons increased the PTZ and aldicarb hypersensitivities of *mir-51;glo-4* mutants. #1 and #2 mean the two independent extrachromosomal arrays of transgenic worms that rescue GLO-4 expression driven by *unc-25* or *myo-3* promoter in *mir-51;glo-4* double mutants. PTZ hypersensitivity assays (A) and aldicarb hypersensitivity assays (B) were performed as legends of Fig. 1. ns, no significance; **, $p < 0.01$; ***, $p < 0.005$. #ns mean no significance compared with wild-type (N2). Error bars represented the SEM. (C–F) The GABAergic synapse number and GABA_A receptor abundance of *mir-51;glo-4* mutants were decreased by rescue of GLO-4 expression in GABAergic motor neurons. The rescue experiments were performed by similar methods in A and B. The representative images of SNB-1::GFP and UNC-49::GFP distribution were shown in C and E. wt, wild-type. Scale bar, 10 μ m. The densities and fluorescence intensities of SNB-1::GFP (D) and UNC-49::GFP puncta (F) were analyzed as legends of Figs. 2B–C and 2E–F. ns, no significance; **, $p < 0.01$; ***, $p < 0.005$.

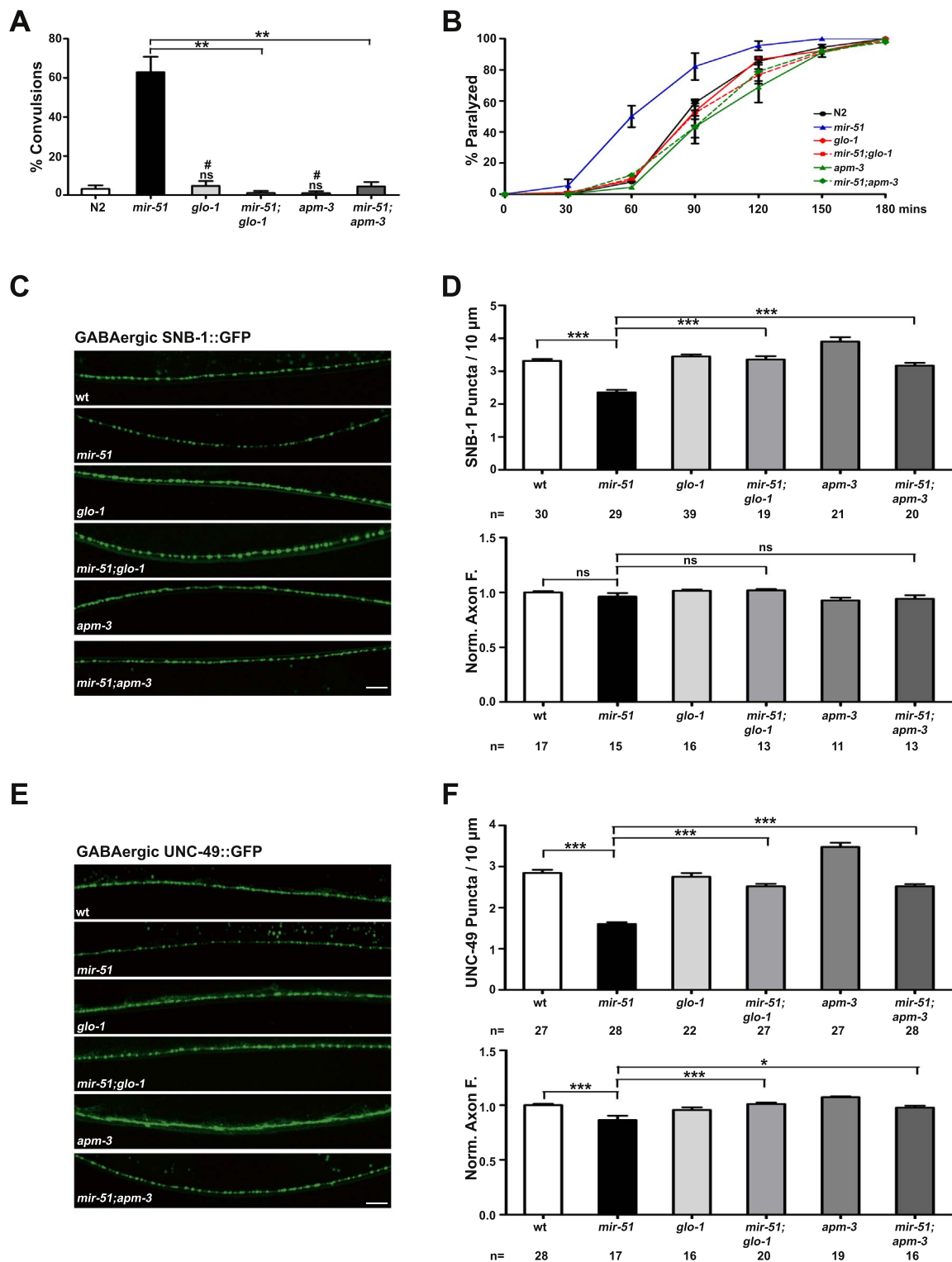


Fig. 5. miR-51 regulates GABAergic synapses through GLO/AP-3 pathway. (A–B) Loss of *glo-1* or *apm-3* decreased the PTZ and aldicarb hypersensitivities of *mir-51* mutants. PTZ hypersensitivity assays (A) and aldicarb hypersensitivity assays (B) were performed as the legends of Fig. 1. **, $p < 0.01$; #ns, mean no significance compared with wild-type (N2). Error bars represented the SEM. **(C–F) Loss of *glo-1* or *apm-3* increased the GABAergic synapse number and GABA_A receptor abundance of *mir-51* mutant.** The representative images of SNB-1::GFP and UNC-49::GFP distribution were shown in C and E. wt, wild-type. Scale bar, 10 μm. The densities and fluorescence intensities of SNB-1::GFP (D) and UNC-49::GFP puncta (F) were analyzed as legends of Figs. 2B–C and 2E–F. ns, no significance; *, $p < 0.05$; ***, $p < 0.005$.

network (Grill et al., 2007). *C. elegans* RPM-1 is a component of a Skp/Cullin/F-box (SCF) protein complex containing SKR-1, CUL-1, and the F-box synaptic protein FSN-1 (Liao et al., 2004). This complex has essential roles in presynaptic differentiation and function in rodents, flies and worms (Blackwell and Walker, 2008; Boag et al., 2005;

Olahova et al., 2008). Evidence shows that loss of *glo-4* enhances the defects in GABAergic synapse formation of *fsn-1* mutants, suggesting lacking of GLO-4 impairs GABAergic synaptogenesis as an enhancer of FSN-1 (Grill et al., 2007).

Here, we found the increasing GLO-4 expression level of *mir-51*

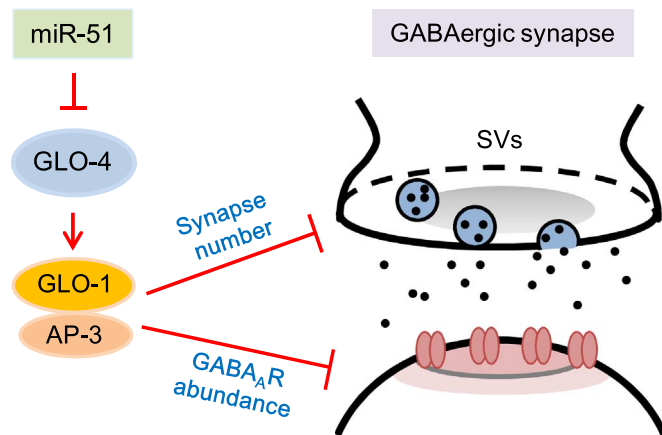


Fig. 6. The schematic picture illustrating that miR-51 regulates GABAergic synapses by targeting Rab GEF GLO-4 and lysosomal trafficking-related GLO/AP-3 pathway in *C. elegans*.

mutants led to the downregulation of GABAergic synapses number and GABA_A receptors abundance, suggesting activation of GLO-4 might repress GABAergic synapses' function. Therefore, we overexpressed GLO-4 driven by *unc-25* promoter in wild-type worms, and found elevating GLO-4 level in GABAergic motor neuron also increased the PTZ and aldicarb hypersensitivities of N2 (Fig. S4). These imply that the balance of GLO-4 expression is important to GABAergic synapses. Either elevation or inhibition of GLO-4 level will results in various defects of GABAergic synapses.

In *C. elegans*, muscles are the postsynaptic terminal where GABA receptors bind to GABA neurotransmitter (Waites et al., 2005). We found that rescue of GLO-4 expression in muscle did not influence the PTZ and aldicarb sensitivity of *mir-51;glo-4* double mutants, and also did not alter their number of GABAergic synapses and abundance of GABA_A receptors. However, these phenotypes were changed by recovery of GLO-4 in GABAergic neurons. As miR-51 is not abundant in muscle (Shaw et al., 2010), these data imply that both miR-51 and GLO-4 might function on presynapses, and then impact postsynaptic UNC-49 receptor distribution in an indirect signal.

GLO/AP-3 pathway is well-known to regulate formation of the lysosome-related organelles (LROs) called gut granules (Hermann et al., 2005). Accumulating evidence shows that lysosomal trafficking is implicated in regulating synaptogenesis and neurotransmitter receptor abundance (Davis et al., 2010; Dermaut et al., 2005). Our results showed that the mutant worms lacking of lysosomal cargo sorting gene *apm-3* had higher density of UNC-49::GFP puncta than that of N2, and miR-51 still influenced this density in the absence of *apm-3*. However, we observed that loss of *mir-51* decreased the UNC-49::GFP puncta's density of N2 in $42.48\% \pm 0.02$ (mean \pm SEM), and reduced that density of *apm-3* mutant in $26.54\% \pm 0.03$ (Fig. S5), suggesting disruption of *apm-3* partially attenuates the effect of miR-51 in UNC-49::GFP puncta's density level. These imply that AP-3 is partially required for miR-51 regulating GABA_A receptor distribution. Moreover, AP-3 might also interact with some factors in other lysosomal trafficking pathways to affect this process.

Taken together, we propose a model that miR-51 regulates GABAergic synapses by repressing GLO-4 expression in GABAergic motor neurons, and then preventing GLO/AP-3 pathway in *C. elegans* (Fig. 6). These findings laid critical foundations for discovering miRNA mimics and inhibitors to cure the human nervous diseases caused by disordered GABA signaling.

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Author contributions

Xiaoxue Li conceived and designed the experiments. Shuai Zhang, Zhibin Fan, Ping Qiao, Yinsuo Zhao Yanan Wang and Da Jiang performed the experiments and analyzed the data. Xiaoxue Li and Shuai Zhang wrote the manuscript. Jun Lu, Xiangming Wang, Xiaojuan Zhu, Yu Zhang and Baiqu Huang revised the manuscript. All the authors discussed the results and commented on the manuscript.

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Conflict of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.02.009.

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