UNC-64 and RIC-4, the plasma membrane-associated SNAREs syntaxin and SNAP-25, regulate fat storage in nematode *Caenorhabditis elegans*

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Abstract: Objective To investigate whether genes required for synaptogenesis and synaptic function are also involved in fat storage control in *Caenorhabditis elegans*. **Methods** Fat storage was examined in mutants of genes affecting the synaptogenesis and synaptic function. In addition, the genetic interactions of SNAREs *syntaxin/unc-64* and *SNAP-25/ric-4* with *daf-2*, *daf-7*, *nhr-49*, *sbp-1* and *mdt-15* in regulating fat storage were further investigated. The tissue-specific activities of *unc-64* and *ric-4* were investigated to study the roles of *unc-64* and *ric-4* in regulating fat storage in the nervous system and/or the intestine. **Results** Mutations of genes required for the formation of presynaptic neurotransmission site did not obviously influence fat storage. However, among the genes required for synaptic function, the plasma membrane-associated SNAREs *syntaxin/unc-64* and *SNAP-25/ric-4* genes were involved in the fat storage control. Fat storage in the intestinal cells was dramatically increased in *unc-64* and *ric-4* mutants as revealed by Sudan Black and Nile Red stainings, although the fat droplet size was not significantly changed. Moreover, in both the nervous system and the intestine, expression of *unc-64* significantly inhibited the increase in fat storage observed in *unc-64* mutant. And expression of *ric-4* in the nervous system completely restored fat storage in *ric-4* mutant. Genetic interaction assay further indicated that both *unc-64* and *ric-4* regulated fat storage independently of *daf-2* [encoding an insulin-like growth factor-I (IGF-I) receptor], *daf-7* [encoding a transforming growth factor- β (TGF- β) ligand], and *nhr-49* (encoding a nuclear hormone receptor). Besides, mutation of *daf-16* did not obviously affect the phenotype of increased fat storage in *unc-64* or *ric-4* mutant. Furthermore, *unc-64* and *ric-4* regulated fat storage probably through the *ARC105/mdt-15*- and *SREBP/sbp-1*-mediated signaling pathways. In addition, fat storage in *unc-64*; *ric-4* was higher than that in either *unc-64* or *ric-4* single mutant nematodes, suggesting that *unc-64* functions in parallel with *ric-4* in regulating fat storage. **Conclusion** The plasma membrane-associated SNAREs *syntaxin/unc-64* and *SNAP-25/ric-4* function in parallel in regulating fat storage in *C. elegans*, probably through the *ARC105/mdt-15*- and *SREBP/sbp-1*-mediated signaling pathways.

Keywords: fat storage; synaptic function; UNC-64; RIC-4; *Caenorhabditis elegans*

1 Introduction

Regulation of fat storage is a fundamental process, and abnormalities in fat storage would induce important human diseases^[1]. Currently, it has been proven that the nematode *Caenorhabditis elegans* (*C. elegans*) is an excellent model for analyzing the mechanisms of fat storage^[2]. The worms have homologs of many mammalian lipogenic and lipolytic

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enzymes, as well as genes encoding a wide range of components involved in mammalian fat regulation cascade^[2,3]. In addition, insulin signaling is also a primary mechanism for controlling metabolic physiology in nematodes^[4]. Down-regulating the transforming growth factor-beta (TGF- β) pathway promotes fat accumulation in adults^[5]. Moreover, NHR-49, MDT-15 and SBP-1 mainly constitute the components of metabolic sensors and coordinate regulation of metabolic pathway to function in the metabolic sensing mechanisms in *C. elegans*^[6,7].

Furthermore, TUB-1, a protein expressed in ciliated sensory neurons, may regulate fat storage either by modulating transport and sensing, or by responding to signals in ciliated neurons^[8]. The *bbs-1* gene, which could synergistically increase fat storage in a *kat-1* mutant, has been identified by genetic screen. This gene acts in 15 ciliated neurons, and regulates fat storage in the same pathway as that of *tub-1*^[9]. In addition, studies have revealed that the classical neurotransmitters have dramatic effects on fat regulation in nematodes^[10,11]. Mutation of tryptophan hydroxylase gene *tph-1* (a critical factor for serotonin biosynthesis) results in a noticeable increase of fat storage^[10]. In addition, inactivation of specific dopaminergic and glutamergic receptors also alters the fat deposits, but with no adverse effect on growth rate or viability^[11]. Thus, it is assumed that a close relation between fat storage and neurotransmission or presynaptic assembly may exist in nematodes.

Previous studies have suggested the importance of synaptic transmission to the lifespan control machinery in animals^[12,13]. Mutations of genes encoding homologs of syntaxin and a Ca^{2+} -dependent activator protein for secretion can extend lifespan in a DAF-16-dependent fashion^[12]. Shen *et al.* further systematically screened for genes involved in aging regulation from genetic loci encoding synaptic proteins, by lifespan assay and accumulation of lipofuscin autofluorescence^[13]. Nevertheless, it is still largely unclear whether the genes required for synaptogenesis and synaptic function are also involved in fat storage control in *C. elegans*.

Thus, the present study was aimed mainly to investigate the possible involvement of these synaptogenesis- and synaptic function-required genes in fat storage control. The

possible genetic interactions of these genes with known important signaling pathways in regulating fat storage in *C. elegans* were further analyzed.

2 Materials and methods

2.1 Strains and culture conditions A total number of 16 genetic loci were selected in the present study, including *unc-13*, *unc-10*, *syd-2*, *rpm-1*, *dlk-1*, *mkk-4*, *pmk-3*, *scd-2*, *fsn-1*, *snb-1*, *ric-4*, *rab-3*, *snt-1*, *unc-18*, *unc-64*, and *hbl-1*. All these genes encode synaptic proteins and are required for synaptogenesis or synaptic functions (Table 1).

Nematodes used in the present study included wild-type N2, *daf-2(e1370)*, *unc-13(e323)*, *unc-10(md1117)*, *syd-2(ju37)*, *rpm-1(ju41)*, *dlk-1(ju476)*, *mkk-4(ju91)*, *pmk-3(ok169)*, *scd-2(ok565)*, *fsn-1(gk429)*, *snb-1(md247)*, *ric-4(md1088)*, *rab-3(y250)*, *snt-1(md290)*, *unc-18(md299)*, *unc-64(md130)*, *hbl-1(ok725)*, *daf-2(e1370)*, *daf-16(mu86)*, *daf-7(m62)*, *nhr-49(gk405)*, *sbp-1(ep176)* and *mdt-15(tm2182)* mutants, originally obtained from the Caenorhabditis Genetics Center (funded by the NIH National

Table 1. Genes required for synaptogenesis and synaptic functions in *C. elegans*

Genes	Encoded proteins
<i>unc-13</i>	Neurotransmitter release regulator
<i>unc-10</i>	Rab3 effector RIM1
<i>syd-2</i>	LAR-interacting protein
<i>rpm-1</i>	Inhibitor of type V adenylyl cyclases
<i>dlk-1</i>	Serine/threonine protein kinase
<i>mkk-4</i>	Mitogen-activated protein kinase (MAPK) kinase MKK4
<i>pmk-3</i>	P38 MAP kinase
<i>scd-2</i>	Protein tyrosine kinase
<i>fsn-1</i>	SOCS box protein SSB-1
<i>snb-1</i>	Synaptobrevin/VAMP-like protein
<i>ric-4</i>	SNAP-25 (synaptosome-associated protein) component of SNARE complex
<i>rab-3</i>	GTPase Rab3
<i>snt-1</i>	Ca^{2+} -dependent phospholipid-binding protein Synaptotagmin
<i>unc-18</i>	Vesicle trafficking protein Sec1
<i>unc-64</i>	SNARE protein Syntaxin 1
<i>hbl-1</i>	LAR transmembrane tyrosine phosphatase-interacting protein liprin

Center for Research Resource, USA). *daf-2(e1370)* mutant served as a positive control. The strains were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* (*E. coli*) OP50 at 20 °C as described previously^[14]. Gravid nematodes were washed off from the plates into centrifuge tubes, and were lysed with the bleaching mixture (0.45 N NaOH, 2% HOCl). Age synchronous populations of nematodes were obtained as described previously^[15].

2.2 Sudan Black staining As described previously^[2], well-fed young adult nematodes were washed in M9 buffer and fixed in 1% paraformaldehyde in M9 buffer. The nematodes were then subject to 3 freeze-thaw cycles and dehydrated through an ethanol series. The nematodes were stained overnight in a 50% saturated solution of Sudan Black in 70% ethanol. Then they were rehydrated and photographed.

2.3 Nile Red staining Nile Red staining was performed as described^[11]. Briefly, Nile Red (5H-benzo[α]phenoxazine-5-one, 9-diethylamino) powder (N-1142 Molecular Probes) was dissolved in acetone at the concentration of 500 μ g/mL. Then, it was diluted in phosphate buffered saline (PBS) to a final concentration of 0.05 μ g/mL and added on top of NGM plates already seeded with OP50 or RNAi bacteria. Nematodes at the egg stage were then grown on plates with Nile Red, and photographed with epifluorescence microscopy (rhodamine channel). The staining phenotypes were assessed prior to starvation at the young adult stage. Fat content was monitored and evaluated using ImageJ Software (NIH Image) by determining the average intensity in each animal's intestine. More than 30 animals were counted for statistical analysis.

2.4 DNA construction The nervous system specific fragment of *unc-14* promoter [*Punc-14* (Hind III/Pst I)], and the intestine specific fragment of *ges-1* promoter [*Pges-1* (Xba I/Bam HI)] were cloned into the pPD95_75 vector, respectively. Then the cDNA fragments of *ric-4* (Bam HI), *unc-64* (Bam HI), *mdt-15* (Kpn I), and *sbp-1* (Kpn I) were further subcloned into the pPD95_75 vector behind the *Punc-14* (Hind III/Pst I) fragment. Meanwhile, the cDNA fragments of *unc-64* (Kpn I), *mdt-15* (Kpn I), and *sbp-1* (Kpn I) were further subcloned into the pPD95_75 vector behind the *Pges-1* (Xba I/Bam HI) fragment. Transgenic worms were then generated as previously described^[16]. The marker vector *Pdop-1::rfp* and the

constructs were coinjected into gonads of hermaphrodite animals.

2.5 Statistical analysis Data were expressed as mean $\pm$ SD, and analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett's *t*-test. Graphs were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). *P* < 0.05 was considered as statistically significant.

3 Results

3.1 Fat storage evaluation by Sudan Black staining and Nile Red staining Fat storage in wild-type N2 and mutants were examined by using Sudan Black staining. As shown in Fig. 1, compared with wild-type N2, mutations of *unc-64* and *ric-4* both resulted in a remarkable increase of fat storage in nematodes. However, no obvious changes in fat storage were observed in *unc-13(e323)*, *unc-10(md1117)*, *syd-2(ju37)*, *rpm-1(ju41)*, *dlk-1(ju476)*, *mkk-4(ju91)*, *scd-2(ok565)*, *fsn-1(gk429)*, *snb-1(md247)*, *rab-3(y250)*, *snt-1(md290)*, *unc-18(md299)*, *h1b-1(ok725)*, or *pmk-3(ok169)* mutant. In *C. elegans*, the fat droplets in intestinal cells and hypodermal cells can be directly visualized after Sudan Black staining^[4]. The numbers of detectable fat droplets in intestinal cells in *unc-64(md130)* and *ric-4(md1088)* mutants were approximately 1.26- and 1.23 -folds to that in wild-type N2. However, the numbers of detectable fat droplets in hypodermal cells in *unc-64(md130)* and *ric-4(md1088)* mutants were similar to that in wild-type N2. In addition, no noticeable changes in fat droplet size were observed in any examined synaptic mutant, compared with that in wild-type N2 (data not shown). As a positive control, mutation of *daf-2* also induced a sharp increase of fat storage in *C. elegans*, consistent with the previous report^[4]. Moreover, the levels of fat storage in *unc-64(md130)* and *ric-4(md1088)* mutants were still lower than that in *daf-2(e1370)* mutant nematodes.

In worms, the intestine (an endodermal derivative) is the major site of energy store and fat storage. Adding Nile Red to *E. coli* OP50, the laboratory diet of *C. elegans*, could cause uptake and incorporation of the dye Nile Red into lipid droplets in intestinal cells^[11]. By this manner, the fat droplets were visualized in intestinal cells in living nematodes. As shown in Fig. 2, among the examined mutants, only the mu-

Fig. 1 Fat storage in wild-type N2 and mutant nematodes as monitored by Sudan Black staining. *daf-2(e1370)* mutant was used as a positive control. The left arrowheads indicate the position of the second pharyngeal bulb, and the right arrowheads indicate the position of vulva.

tants of *unc-64(md130)* and *ric-4(md1088)* exhibited significant ($P < 0.01$) increases in fat storage, compared with the wild-type N2, although these increases were not so dramatic as that observed in *daf-2(e1370)* mutant. The intensities of Nile Red fluorescence in *unc-64(md130)* and *ric-4(md1088)* mutants increased by 191% and 180%, respectively, compared with that of wild-type. Similarly, no obvious changes in Nile Red fluorescent puncta size were observed in any examined synaptic mutant compared with that of wild-type. Therefore, the fat deposition revealed by Nile Red staining was consistent with that observed by Sudan Black staining.

3.2 Tissue-specific activities of UNC-64 and RIC-4 in regulating fat storage It has been clear that *unc-64* is expressed in both the nervous system and the intestine, and *ric-4* is expressed only in the nervous system. The tissue-specific activities of UNC-64 and RIC-4 in regulating fat storage were then investigated in nematodes. As shown in Fig. 3A, expression of *unc-64* in both the nervous system (*Punc-14-*

unc-64) and the intestine (*Pges-1-unc-64*) could significantly ($P < 0.01$) inhibit, but not completely restore, fat storage in *unc-64* mutant nematodes. Moreover, expression of *ric-4* in the nervous system (*Punc-14-ric-4*) could completely restore fat storage in *ric-4* mutant nematodes.

3.3 Genetic interactions of *unc-64* and *ric-4* with insulin signaling pathway in regulating fat storage Since Nile Red staining does not influence growth rate, brood size, feeding or lifespan^[11], the possible genetic interactions of *unc-64* and *ric-4* with insulin signaling pathway were further investigated by visualizing the nematode fat storage using Nile Red staining. In *C. elegans*, *daf-16* encodes 3 members of the fork head family of transcription factors^[17], and *daf-2* encodes the insulin-like growth factor-I (IGF-I) receptor^[4]. As shown in Fig. 4A, *daf-2(e1370)* mutants showed an increase in fat storage. Fat storage in *unc-64(md130);daf-2(e1370)* was significantly higher ($P < 0.01$) than that in either *unc-64(md130)* or *daf-2(e1370)* single mutant nematodes.

Fig. 2 Fat storage in wild-type N2 and mutant nematodes as monitored by Nile Red staining. **A:** Pictures of Nile Red staining. The left arrowheads indicated the position of the second pharyngeal bulb, and the right arrowheads indicated the position of vulva. **B:** The relative intensities of Nile Red fluorescence in wild-type N2 and mutants. Data were expressed as mean $\pm$ SD. ** $P < 0.01$ vs N2.

Fig. 3 Tissue-specific activities of UNC-64 and RIC-4 in regulating fat storage. **A:** Tissue-specific activities of UNC-64 in regulating fat storage as monitored by the quantification of Nile Red fluorescence. **B:** Tissue-specific activities of RIC-4 in regulating fat storage as monitored by the quantification of Nile Red fluorescence. Data were expressed as mean $\pm$ SD. NS, no significant difference. ****** $P < 0.01$.

Fig. 4 Genetic interactions of *unc-64* and *ric-4* with insulin signaling pathway in regulating fat storage. **A:** Genetic interactions of *unc-64* and *ric-4* with *daf-2* in regulating fat storage. **B:** Genetic interactions of *unc-64* and *ric-4* with *daf-16* in regulating fat storage. Data were expressed as mean $\pm$ SD. ****** $P < 0.01$.

Similarly, fat storage in *daf-2(e1370); ric-4(md1088)* was significantly higher ($P < 0.01$) than that in either *ric-4(md1088)* or *daf-2(e1370)* single mutant nematodes. These data suggest that *unc-64* and *ric-4* may act independently of *daf-2* in regulating fat storage in *C. elegans*.

Moreover, in *C. elegans*, mutation of *daf-16* alone did not influence the nematode fat storage. As shown in Fig. 4B, fat storage in *daf-16(mu86); unc-64(md130)* mutant was similar to that in *unc-64(md130)* mutant, and fat storage in *daf-16(mu86); ric-4(md1008)* was similar to that in *ric-4*

(*md1088*) mutant. Thus, mutation of *daf-16* did not obviously affect the increased fat storage observed in *unc-64* (*md130*) or *ric-4*(*md1088*) mutant nematodes.

3.4 Genetic interactions of *unc-64* and *ric-4* with TGF- β pathway in regulating fat storage TGF- β signaling pathway functions in parallel with insulin-like signaling pathway to control whether *C. elegans* larvae develop into fast metabolizing or fat storing adults, and *daf-7* encodes a key component in the TGF- β signaling pathway^[5]. As shown in Fig. 5, mutation of *daf-7* caused a sharp increase of fat storage in nematodes. Moreover, fat storage in *daf-7*(*m62*); *unc-64* (*md130*) was significantly higher ($P < 0.01$) than that in either *unc-64*(*md130*) or *daf-7*(*m62*) single mutant nematodes. Similarly, fat storage in *daf-7*(*m62*); *ric-4*(*md1088*) was higher ($P < 0.01$) than that in either *ric-4*(*md1088*) or *daf-7*(*m62*) single mutant nematodes. These data also suggest that *unc-64* and *ric-4* act independently of *daf-7* in regulating fat storage in *C. elegans*.

3.5 Genetic interactions of *unc-64* and *ric-4* with metabolic sensors and coordinated regulation of metabolic pathway in regulating fat storage In *C. elegans*, mutation of *mdt-15* or *sbp-1* will suppress fat storage^[7], whereas mutation of *nhr-49* will increase fat storage^[6]. The possible role of genetic interactions of *unc-64* and *ric-4* with metabolic sensors and coordinated regulation of metabolic pathway in regulating fat storage was further investigated. As shown in Fig. 6A, levels of fat storage in both *mdt-15*(*tm2182*); *unc-64*(*md130*) and *mdt-15*(*tm2182*); *ric-4*(*md1088*) were significantly lower than that in either *unc-64*(*md130*) or *ric-4*(*md1088*) single

mutant nematodes, and similar to that in *mdt-15*(*tm2182*) single mutant nematodes. In addition, as shown in Fig. 6B, fat storage in both *sbp-1*(*ep176*); *unc-64*(*md130*) and *sbp-1*(*ep176*); *ric-4*(*md1088*) was also significantly lower than that in either *unc-64*(*md130*) or *ric-4*(*md1088*) single mutant nematodes, and similar to that in *sbp-1*(*ep176*) single mutant nematodes. These data suggest that *unc-64* and *ric-4* may regulate fat storage through *mdt-15*- and *sbp-1*-mediated signaling pathways in *C. elegans*.

Moreover, as shown in Fig. 6C, fat storage in *nhr-49* (*gk405*); *unc-64*(*md130*) was higher ($P < 0.01$) than that in either *unc-64*(*md130*) or *nhr-49*(*gk405*) single mutant nematodes. Similarly, fat storage in *nhr-49*(*gk405*); *ric-4* (*md1088*) was higher ($P < 0.01$) than that in either *ric-4* (*md1088*) or *nhr-49*(*gk405*) single mutant nematodes. Therefore, *unc-64* and *ric-4* may act independently of *nhr-49* in regulating fat storage in *C. elegans*.

3.6 Tissue-specific activities of MDT-15 and SBP-1 in regulating fat storage *mdt-15* and *sbp-1* genes are expressed in both the nervous system and the intestine in nematodes. The tissue-specific activities of MDT-15 and SBP-1 in regulating fat storage in nematodes were also studied. As shown in Fig. 7A, expression of *mdt-15* in both the nervous system (*Punc-14- mdt-15*) and the intestine (*Pges-1- mdt-15*) could significantly ($P < 0.01$) increase fat storage in *mdt-15* mutant nematodes. Similarly, as shown in Fig. 7B, expression of *sbp-1* in both the nervous system (*Punc-14- sbp-1*) and the intestine (*Pges-1- sbp-1*) could significantly ($P < 0.01$) increase fat storage in *sbp-1* mutant nematodes. Therefore, expression in

Fig. 5 Genetic interactions of *unc-64* and *ric-4* with TGF- β pathway in regulating fat storage. Data were expressed as mean $\pm$ SD. ** $P < 0.01$.

Fig. 6 Genetic interactions of *unc-64* and *ric-4* with metabolic sensors and coordinated regulation of metabolic pathway in regulating fat storage. **A:** Genetic interactions of *unc-64* and *ric-4* with *mdt-15* in regulating fat storage. **B:** Genetic interactions of *unc-64* and *ric-4* with *sbp-1* in regulating fat storage. **C:** Genetic interactions of *unc-64* and *ric-4* with *nhr-49* in regulating fat storage. Data were expressed as mean $\pm$ SD. ** $P < 0.01$.

Fig. 7 Tissue-specific activities of MDT-15 (A) and SBP-1 (B) in regulating fat storage, as monitored by the quantification of Nile Red fluorescence. Data were expressed as mean $\pm$ SD. ** $P < 0.01$.

the nervous system and the intestine both accounted for the functions of *mdt-15* and *sbp-1* in regulating fat storage in nematodes.

3.7 Genetic interaction of *unc-64* with *ric-4* in regulating fat storage The genetic interaction between *unc-64* and *ric-4* in regulating fat storage in nematodes was also examined. As shown in Fig. 8, fat storage in *unc-64(md130); ric-4(md1088)* was higher ($P < 0.01$) than that in either *unc-64(md130)* or *ric-4(md1088)* single mutant nematodes. Therefore, *unc-64* might function in parallel with *ric-4* in regulating fat storage in *C. elegans*.

Fig. 8 Genetic interaction of *unc-64* with *ric-4* in regulating fat storage. Data were expressed as mean $\pm$ SD. ** $P < 0.01$.

4 Discussion

Synaptogenesis is a process involving the formation of a neurotransmitter release site at a presynaptic neuron and a receptive field at the postsynaptic neurons, and the precise alignment of pre- and post-synaptic specialization. In nematodes, active zones with electron-dense structures at the presynaptic termini provide the structural basis for exocytosis and endocytosis, and mediate the Ca^{2+} -triggered neurotransmitter release^[18]. As a member of the liprin family, SYD-2 is mainly localized at the active zone, and regulates the differentiation of presynaptic termini, particularly the formation of the active zone^[19]. Regulator of Presynaptic Morphogenesis (RPM-1) is a periactional zone protein and may

restrict the development or promote the maturation of different synapses, since mutation of *rpm-1* causes enlargement in some GABAergic neuromuscular junctions (NMJs) with an excessive number of active zones, while other GABAergic NMJs disappear^[20,21]. F-box Synaptic protein (FSN-1) is physically associated with RPM-1 and the homologues of SKP1 and Cullins to form a new type of SCF complex (Skp-1, Cullin and an F-box protein) at presynaptic periactional zones. And this neuron-specific, SCF-like complex provides a localized signal to attenuate presynaptic differentiation^[22]. Suppressor of Constitutive Dauer formation (SCD-2) may be a target or a downstream effector by which FSN-1 stabilizes synaptic formation^[22]. Furthermore, RPM-1 negatively regulates a p38 MAP kinase pathway composed of the dual leucine zipper-bearing MAPKKK DLK-1, the MAPKK MKK-4, and the p38 MAP kinase PMK-3^[23]. In the present study, mutations of these important genes required for the formation of a neurotransmitter release site in the presynaptic neurons did not obviously influence fat storage, at least suggesting that there is no direct association between presynaptic neurotransmission site formation and fat storage control in *C. elegans*. Nevertheless, it is still largely unclear whether regulation of the postsynaptic neurotransmission site formation and the precise alignment of pre- and postsynaptic specialization will be involved in the fat storage control in nematodes.

In animals, chemical synapses are important in providing the information flow between presynaptic and postsynaptic neurons^[24]. Synaptic vesicles should be tethered to the sites of release (docking) and then become a component to be fused with the plasma membrane (priming)^[25]. UNC-18 is a Sec1-related protein required for synaptic vesicle exocytosis^[26]. In addition, mutation of *unc-18* results in reductions in the sizes of the readily releasable pool and docked vesicles at the active zone, suggesting that UNC-18 functions as a facilitator of vesicle docking, directly or indirectly^[26]. Besides, electropharyngeogram (EPG) recording shows that mutation of *rab-3* would cause vesicle dispersal at synapses and subtle synaptic defects, suggesting that RAB-3 may be involved in vesicle localization or docking^[27]. Mutation in *snt-1* has not been demonstrated to induce a vesicle docking defect, al-

though it is clear that this mutation causes defects in synaptic function^[28]. The priming process involves the formation of trans-SNARE complexes, in which the synaptic vesicle protein synaptobrevin (also called VAMP) forms a helical bundle with the plasma membrane proteins syntaxin and SNAP-25^[29]. It is previously considered that the formation of SNARE complexes is an essential priming reaction, as evidenced by the correlation between the size of the readily releasable pool and the level of SNARE complex assembly^[30]. In *C. elegans*, the vesicle-associated synaptobrevin is encoded by *snb-1*, and the plasma membrane-associated SNAREs syntaxin and SNAP-25 are encoded by *unc-64* and *ric-4*, respectively. UNC-13 is also required for priming synaptic vesicles for exocytosis and synaptic transmission^[31]. Mutations of UNC-10/RIMs, the putative RAb-3A effectors, could regulate the synaptic vesicle exocytosis and neurotransmitter release^[32]. HLB-1 positively regulates synaptic transmission and is involved in the regulation of both presynaptic and postsynaptic differentiation^[33]. In the present study, the roles of these synaptic function-involving genes in fat storage control were examined, and mutations of only *unc-64* and *ric-4* showed significant effects on fat storage. In *C. elegans*, it has been proven that the open form of syntaxin UNC-64 is required for both docking and priming of synaptic vesicles^[34,35]. Therefore, our data suggest a close association between regulation of docking and priming of synaptic vesicles and fat storage control in nematodes.

Our findings on the tissue-specific activities of UNC-64 and RIC-4 in regulating fat storage further suggest that expression of *unc-64* in the nervous system could largely rescue the fat storage defect in *unc-64* mutant, and expression of *ric-4* in the nervous system could completely rescue the fat storage defect in *ric-4* mutant. These data strongly imply that the transportation of synaptic vesicles, especially the docking and priming of synaptic vesicles, may play an important role in the control of fat storage of animals.

In *C. elegans*, genes can be inactivated by RNAi initiated with double-stranded RNA expressed in *E. coli* food^[36]. By using RNAi to specifically disrupt the gene expression in *C. elegans*, Ashrafi *et al.* have identified 305 genes whose inactivation could cause a reduction in body fat storage and

112 genes whose inactivation results in an increase in fat storage^[11]. However, the genes *unc-64* and *ric-4* are not found in the list of these identified genes. One of the main reasons is that only about 12% of the genes that function in the nervous system can be inactivated by the clones in this double-stranded RNAi bacterial library used by Ashrafi *et al.*^[37]. Another reason is that the current RNAi library only covers about 86% of the known and predicted genes, and certain classes of genes may be missing or underrepresented^[11].

In *C. elegans*, reconstitution of the insulin receptor in neurons but not in muscle can partially restore the increased fat content in insulin receptor knockout nematodes^[38]. *daf-7* which encodes a TGF- β ligand, is expressed in one pair of ASI sensory neurons, and its transcription is modulated by daumone, a constitutively secreted pheromone that *C. elegans* use to assess population density. This suggests that the TGF- β signaling pathway responds to environmental conditions and functions as a central regulator for homeostasis^[5,39]. Here our results demonstrate that *unc-64* and *ric-4* function in parallel with *daf-2* or *daf-7* in regulating fat storage in nematodes, and mutation of *daf-16* did not obviously influence the phenotype of increased fat storage in *unc-64* or *ric-4* mutant. These data imply that, at least in the neurons, regulation of fat storage by UNC-64 and RIC-4 is not through the insulin or the TGF- β signaling pathway in *C. elegans*.

Studies in mammals have indicated that some of the cellular metabolic sensors can also regulate the metabolic homeostasis and behavioral responses in the nervous system^[40,41], such as the *sbp-1* signaling pathway, the *mdt-15* signaling pathway, and the *nhr-49* signaling pathway in *C. elegans*^[6,7]. NHR-49 is a nuclear hormone receptor and serves as a key regulator of fat saturation by modulating pathways that control fat consumption and fatty acid saturation, and regulate metabolic gene expression^[6]. However, our data suggest that *unc-64* and *ric-4* also act independently of *nhr-49* in regulating the nematode fat storage in *C. elegans*. SBP-1, a sterol regulatory element binding protein (SREBP), promotes fatty acid homeostasis by regulating the expressions of lipogenic enzymes. And RNAi inhibition and loss of function mutation in *sbp-1* cause dramatic reductions in fat content and bio-

genesis of intestinal lipid droplets^[2,7]. MDT-15, a transcription co-activator, is required for fatty acid homeostasis, and for the control of the transcriptions of genes governing desaturation of stearic acid to oleic acid^[7]. Moreover, in both mammalian cells and *C. elegans*, SREBP function requires the physical interaction with a transcription co-activator, ARC105/MDT-15^[7]. Our data further show that fat storage in both the *mdt-15*; *unc-64* double mutant and the *mdt-15*; *ric-4* double mutant was similar to that in *mdt-15* mutant, and fat storage in both the *sbp-1*; *unc-64* double mutant and the *sbp-1*; *ric-4* double mutant was similar to that in *sbp-1* mutant, suggesting that in neurons, *unc-64* and *ric-4* regulate fat storage may at least partially through the *mdt-15/sbp-1* complex-mediated signaling pathway in *C. elegans*.

The *unc-64*, *mdt-15*, and *sbp-1* genes have a similar expression pattern, and are expressed in both the nervous system and the intestine. The tissue-specific activities of UNC-64 demonstrate that the expression of *unc-64* in the intestine could also largely rescue fat storage in *unc-64* mutant. Moreover, expressions of *mdt-15* or *sbp-1* in both the nervous system and the intestine could significantly increase fat storage in *mdt-15* or *sbp-1* mutant nematodes. These data suggest that expressions in both the nervous system and the intestine will account for the functions of *unc-64*, *mdt-15*, and *sbp-1* in regulating fat storage in nematodes. In addition, in the nervous system, *unc-64* functions in parallel with *ric-4* in regulating fat storage in nematodes.

Based on these results, a model was raised to explain the functions of *unc-64* and *ric-4* in regulating fat storage (Fig. 9). In the nervous system, *unc-64* and *ric-4* function in parallel in regulating fat storage through the *mdt-15/sbp-1* complex-mediated signaling pathway, while in the intestine, only *unc-64* regulates fat storage through the *mdt-15/sbp-1* complex-mediated signaling pathway. That is, besides the intestine, the nervous system also plays a key role in regulating the process of fat storage. In addition, the nervous system and the intestine may employ 2 different mechanisms to regulate fat storage. In the nervous system, fat storage may be closely associated with synaptic vesicle transportation. However, further studies are still needed to clarify whether

Fig. 9 A model established to explain the functions of *unc-64* and *ric-4* in regulating fat storage in nematodes.

the regulation of docking and priming of synaptic vesicles is important or even critical for fat storage control in animals. One of the possible strategies is identifying the neurons required for fat storage control by laser ablation, and then elucidating the roles of *unc-64* and *ric-4* genes in these neurons in fat storage control in *C. elegans*. In the intestine, the vesicle transportation may also serve as an important cellular basis for the fat storage process, and *unc-64* may regulate this process through the *mdt-15/sbp-1* complex-mediated signaling pathway. Moreover, it is still unknown concerning the correlations between the functions of the nervous system and the functions of intestine during the fat storage process in animals, and whether *unc-64*, *mdt-15*, and *sbp-1* are involved in such correlation also needs to be investigated.

In conclusion, by observing the phenotypes of *unc-64* and *ric-4* mutants, our present study strongly imply the possible close relationship between the regulation of docking and priming of synaptic vesicles and fat storage control, and this close association may be further regulated by the *mdt-15/sbp-1* complex-mediated signaling pathway.

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质膜相关的 SNAREs syntaxin/UNC-64 与 SNAP-25/RIC-4 蛋白调控秀丽线虫的脂肪积累

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摘要: **目的** 研究神经突触组装与功能调控相关基因是否参与秀丽线虫的脂肪积累调节。**方法** 分析神经突触组装与功能调控相关基因突变体的脂肪积累变化, 进而观察 SNAREs *syntaxin/unc-64* 和 *SNAP-25/ric-4* 基因与 *daf-2*、*daf-7*、*nhr-49*、*sbp-1* 及 *mdt-15* 所介导的信号通路在调控脂肪积累上的遗传关系。对 *unc-64* 与 *ric-4* 基因进行组织特异性活性分析, 以确定它们在神经系统与肠道内对脂肪积累的影响。**结果** 突触前为神经突触组装所必需的基因的突变并未明显影响脂肪积累。对调控神经突触功能的基因进行分析的结果显示, SNAREs *syntaxin/unc-64* 与 *SNAP-25/ric-4* 基因均参与了脂肪积累的调节。利用苏丹黑染色与尼罗红标记法观察到 *unc-64* 与 *ric-4* 突变体肠道中脂肪积累显著增加, 而 *unc-64* 与 *ric-4* 突变体中积累的脂肪颗粒并未出现尺寸的显著变化。在神经系统与肠道中, *unc-64* 基因的表达均能显著降低 *unc-64* 突变体动物的脂肪积累, 而基因 *ric-4* 在神经系统的表达则可以完全恢复 *ric-4* 突变体动物的脂肪积累。遗传分析表明, *unc-64* 与 *ric-4* 对脂肪积累的调控独立于 *daf-2* (IGF-I 受体基因)、*daf-7* (TGF- β 配体基因) 和 *nhr-49* (核激素受体基因), 且不受 *daf-16* 基因突变的影响。进一步的研究结果显示, *unc-64* 与 *ric-4* 可能经由 *ARC105/mdt-15* 与 *SREBP/sbp-1* 介导的信号通路来调节动物的脂肪积累进程。此外, *unc-64*; *ric-4* 双突变体的脂肪积累水平要显著高于 *unc-64* 或 *ric-4* 单突变体的脂肪积累水平, 提示这两个基因通过平行的遗传通路调控脂肪积累。**结论** 质膜相关的 SNAREs *syntaxin/unc-64* 和 *SNAP-25/ric-4* 基因通过平行的遗传通路并经由 *ARC105/mdt-15* 与 *SREBP/sbp-1* 介导的信号通路来调控秀丽线虫的脂肪积累。

关键词: 脂肪积累; 神经突触功能; UNC-64; RIC-4; 秀丽线虫