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The Expression and Role of Caveolin-1 in Different Development Periods of *Drosophila melanogaster*

Yejun Zhang,[1,2] Ying Wang,[1] Changqing Qu,[1] Hui Huang,[1] Hongyan Li,[1,2] Yan Li,[1] Yuying Cui[1] and Wei Zou[1,2]*

[1]School of Life Sciences, Liaoning Normal University, Dalian, 116081, China
[2]Liaoning Key Lab of Biotechnology and Molecular Medicine Development, Dalian, 116081, China

Yejun Zhang and Ying Wang contributed equally to this work.

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Authors' Contribution
YZ and YW conceived and designed the study, analyzed the data and wrote the article. CQ executed histological studies, HH performed RT-PCR, while HL performed blotting. YL and YC were responsible for *Drosophila* culture. WZ supervised the work.

Key words
Caveolin-1; *Drosophila melanogaster*; expression.

A B S T R A C T

Caveolin-1 is an iconic structural molecule of Caveolae. Because of its pivotal role in the process of cell proliferation, apoptosis and other related signaling pathways of human tumors, caveolin-1 has been extensively studied. It has been found that caveolin-1 expresses in various types of invertebrates and vertebrates and is highly conserved in evolution. However, there has been no report about the caveolin-1 of insect so far. In the present study, caveolin-1 was amplified in the larvae and adults of *Drosophila melanogaster* to observe their expression levels. The *in vivo* distributions of caveolin-1 were observed in larvae, pupae and adults through immunofluorescence and confocal technology. Furthermore, the expression of caveolin-1 was detected in larvae, pupae and adults through Western blotting. The results showed that caveolin-1 exists in *D. melanogaster*, and its expression and distribution varied in different developmental stages. The *in vivo* distribution of caveolin-1 was mainly in the brain, muscles and gut wall in adult, a little in the brain and fat bodies in pupa and a little in the fat bodies in larva. The expression levels of caveolin-1 were significantly higher than in adults in the larvae and pupae. The expression of the protein was significantly higher in the female adult than in the adult male.

INTRODUCTION

Lipid raft was a micro region containing special lipid and protein in membrane lipid bilayer (Gajate and Mollinedo, 2015). Caveolae, which is a special lipid raft structure formed from cell membrane invagination, was enriched in cholesterol and sphingomyelin, and primarily played the important role in membrane transport, signal transduction, substrate transport and endocytosis (Martin, 2013). Caveolins and flotillins are two families of the membrane proteins correlating with Caveolae of the mammals (Bickel *et al*., 1997). Caveolins are integral membrane proteins with molecular weight of 21 - 24 kDa, and as the scaffold structure, they recruit a lot of signal molecules**.** So far, 3 members of the caveolin family have been found in mammals: caveolin-1, caveolin-2 and caveolin-3. Among these, caveolin-1 had two subtypes - alpha and beta (Williams and Lisanti, 2004). Co-expression of caveolin-1 and caveolin-2 is widely distributed in various types of tissues and cells, such as epithelial cells, endothelial cells, adipocytes, fibroblasts, central nervous system and the placental tissues. It is generally believed that caveolin-2 forms heterooligomeric complex with caveolin-1 involved in the transport process of the later (Chen and Che, 2014). Caveolin-3 is only specifically expressed in skeletal muscle cells, myocardial cells and smooth muscle cells, and more so in the first two muscle cells (Marx, 2001; Park *et al.*, 2002).

Caveolin-1 is the most important protein in the caveolin family (Frank *et al.*, 2008). It is involved in the formation of caveolae, endocytosis, signal transduction, angiogenesis and other physiological process (Chidlow and Sessa, 2010). It also plays an important role in cholesterol homeostasis and vesicular transport, but could also combine with a variety of signaling molecules which would activate or inhibit different intracellular signaling pathways, and regulate the expression of related genes (Fridolfsson *et al.*, 2014; Liu *et al.*, 2012). At present, caveolin-1 has been studied as key molecule in the signaling pathways of cell proliferation and apoptosis in the process of human tumorigenesis (Shatz and Liscovitch, 2008; Ubaldo *et al.*, 2010).

Caveolin-1 which is highly conserved in evolution has been found to express in many invertebrate such as nematode pole worm, hookworm, animalcule, sea urchins and vertebrate species. Parker *et al.* (2007) reported that the caveolin-1 of *Caenorhabditis elegans* was homologous with caveolin-3 of human, and is expressed in nerve cells and muscle of body wall. Sato *et al.* (2006) and Scheel *et al.* (1999) reported that it could

* Corresponding author: weizou60@hotmail.com.
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dynamically regulate the maturation of the germ cell line of the androgynous adult. Hernández-Bello *et al.* (2008) cloned the caveolin-1 gene of *Trichinella spiralis* and used fragment specific cDNA probes, and reported its sex-specific expression in the oocyte maturation and early embryo development process. Caveolin-1 monoclonal antibodies have been prepared for the experimental study of nematode, which laid the foundation for the study of the caveolin-1 of model species (Hadwiger *et al.*, 2010).

The caveolin-1 gene of *Artemia sinica* an arthropoda has been cloned. Zhang *et al.* (2011) have shown that the caveolin-1 protein of *Artemia sinica* has the same physiological function as in vertebrate. Li *et al.* (2014) cloned the full-length sequence of the caveolin-1 gene of *Artemia sinica*, and obtained the nucleotide sequence of 675bp. There is no report on the caveolin-1 of insect.

In this study the expression levels of caveolin-1 in different developmental stages of *D. melanogaster* were observed by RT-PCR, immunofluorescence and Western blot. It is being reported for the first time that caveolin-1 exists in *D. melanogaster* and the expression of this protein in *D. melanogaster* is highly variable in different developmental stages, which lays foundation for further study on caveolin-1 of *D. melanogaster* and other insects.

MATERIALS AND METHODS

Materials and reagents

The wild type *D. melanogaster* was supplied by the Genetics Laboratory of Liaoning Normal University. DEPC was purchased from Amresco Co. (America); M-MLV reverse transcriptase, 5×M-MLV buffer, dNTP mixture, random primer-hexadeoxyribonucleotide mixture [pd(N)6] were all purchased from TaKaRa (Dalian, China). Taq enzyme, dNTP mixture and 10×PCR buffer were all purchased from Tiangen Biotech (Beijing) Co., Ltd. Caveolin-1 Mouse IgG_{2b} 7C8 was purchased from Santa Cruz Biotechnology Co. (America). Hoechst 33342 and Anti-Mouse IgG-TRITC were purchased from Sigma Co. (America). Anti-fluorescence quencher was purchased from Shanghai biyotime Co. Goat anti rabbit IgG with horseradish enzyme and Goat anti mouse IgG with horseradish enzyme were purchased from Beijing Jinqiao Bio Technology Co., Ltd. ECL™ Chemiluminescence Kit was purchased from Thermo Scientific Co. (America). Caveolin-1 monoclonal anti rabbit antibody was purchased from Cell signaling Co. (America). The Artemia anti rabbit antibody was prepared by the Laboratory of Lin Hou. Other reagents such as bromine phenol blue, xylene, ethanol, formaldehyde, acetic acid, picric acid, orchid agent, bleaching agent, hematoxylin, eosin and ultra pure water were domestic and analytical pure.

Culture of D. melanogaster

The specimens of *D. melanogaster* were cultured in the maize medium at room temperature, and the culture medium was prepared by agar (10g/L), sugar (100g/L), maize (90g/L) and propionate (7mL/L).

RT-PCR of caveolin-1

The Caveolin-1 sequences of Zebrafish, *Xenopus* and Nematoda were searched from the NCBI database, and the PCR primers were synthesized in high homology. The upstream primer was
"5'-GGACGGTATCACGGCAAGA-3'",
the downstream primer was
"5'-TACCACTCGTCAGCCTCA-3'".
The total RNA of the larvae and adults of the *D. melanogaster* was extracted by TRIzol reagent, and the integrity of RNA was detected by agarose gel electrophoresis. This RNA was used for RT-PCR with random primers. The reaction conditions were as follows: denaturation at 35°C for 10min, annealing at 42°C for 1h and elongation at 70°C for 15 min, followed by 1 cycle. Amplificated the target gene fragment with the above synthetic primers, the reaction conditions were as follows: initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 56°C for 30s and elongation at 72°C for 30s, with a final extension at 72°C for 10min. Observed through agarose gel electrophoresis and gel imaging system.

Preparation of paraffin section

Larva, pupa and adult *D. melanogaster* were put into Bouin's liquid and fixed for several hours, followed by moving directly into the 70% alcohol for rinse, until the materials are not yellow anymore. The samples were dehydrated, embedded in paraffin, and 4 μm thick sections were cut and stored at room temperature. The paraffin sections were stained with hematoxylin and eosin.

Thirty eight paraffin sections of larva of *D. melanogaster* were prepared, out of which No. 5, 16 and 27 were stained with H and E. Twenty paraffin sections of pupa of *D. melanogaster* were prepared. Out of which No. 4, 8 and 12 were stained with H and E. Thirty one paraffin sections of adult of *D. melanogaster* were prepared. Out of which No. 7, 15 and 23 were stained with H and E.

Immunofluorescence and confocal technology

The paraffin sections of *D. melanogaster* were dewaxed and dewatered in xylene and ethanol concentration gradient solutions; washed and blocked with 1% BSA at 37°C for 30min avoid light and incubated with caveolin-1 antibody at 4°C for 48h; washed and incubated for 1.5h with IgG-TRITC secondary antibody at room temperature; then washed and incubated with Hoechst 33342 in dark for 5min at room temperature, washed with PBS and double distilled water; added anti fluorescence quencher and mounted the sections; observed the sections with laser confocal microscope.

Western blotting

Total proteins were extracted respectively from larva, pupa and adult samples of *D. melanogaster* with radio immunoprecipitation assay (RIPA) lysis buffer and quantified using the Bradford method. Each protein sample was fractionated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes, blocked with 5% evaporated skimmed milk for 1h at room temperature. According to the best titer of different antibodies, PBST was used to dilute the primary antibodies and then the blocked PVDF membranes were incubated overnight at 4°C. Next day, the membranes were incubated with HRP-conjugated secondary antibody for 90 min at room temperature and exposed in the darkroom. The western blots were photographed and the images were analyzed with Image J software. Image gray scale analysis method in the Image J documentation was used to convert the data to column charts. The expression intensities of caveolin-1 specific bands were normalized against the Gapdh-specific bands.

Statistical analysis

All experiments were repeated more than three times independently, data were analyzed using t-test and analysis of variance by SPSS 14.0 software. The data were expressed with mean ± SD, $^*P<0.05$ referred to significant difference, and $^{**}P<0.01$ referred to extremely significant difference.

RESULTS

Expression of caveolin-1 gene in larvae and adults

The total RNA extracted from the larvae and adult of *D. melanogaster* (Fig. 1A) was used as templates, the cDNA was obtained through reverse transcription and amplified by PCR with forward and reverse primers of caveolin-1, the PCR products were detected by agarose gel electrophoresis. The results showed that caveolin-1 exists in *D. melanogaster*, and the expression levels of it varied in different developmental stages, the expression level of adult was significantly higher than that of the larva (Fig. 1B).

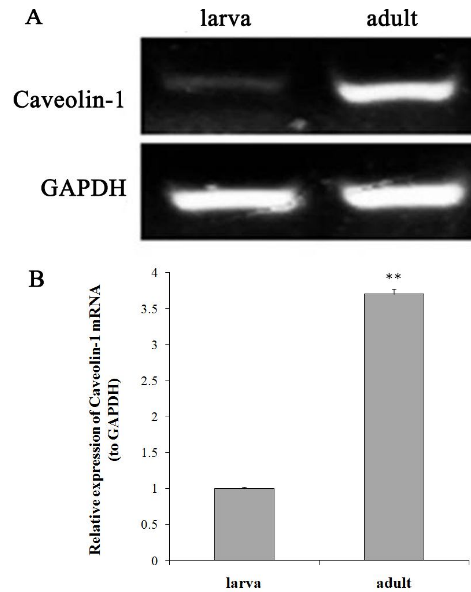

Fig. 1. The expression of caveolin-1 mRNA of the larva and adult of *D. melanogaster.*
A, The band intensities for caveolin-1 mRNA were normalized against that of the GAPDH; B, Quantification of caveolin-1 mRNA shown in A. n=3, $^{**}P<0.01$.)

Histological structure of different developmental stages

Figures 2A, 2B and 2C show structure of salivary glands, alimentary tract, fat bodies, muscles and Malpighian tubules. Figures 2D, 2E and 2F, show structure of brain, eyes, muscles, salivary glands, alimentary tract, fat bodies and wings. Figure 2G, 2H and 2I show structures such as brain, eyes, mouthparts, muscles, heart, salivary glands, alimentary tract, wings, gonads, malpighian tubules and fat bodies.

Expression of caveolin-1 in different developmental stages by immunofluorescence

Paraffin sections of different developmental stages of *D. melanogaster* were performed immunofluorescence experiments and the expression and distribution of caveolin-1 were observed through the confocal microscope (the protein of caveolin-1 displayed red and the cell nuclei which showed blue was stained by Hoechst 33342). The results showed that the expression and distribution of caveolin-1 varied in different developmental stages of *D. melanogaster*. The distribution of caveolin-1 was very little only in the fat

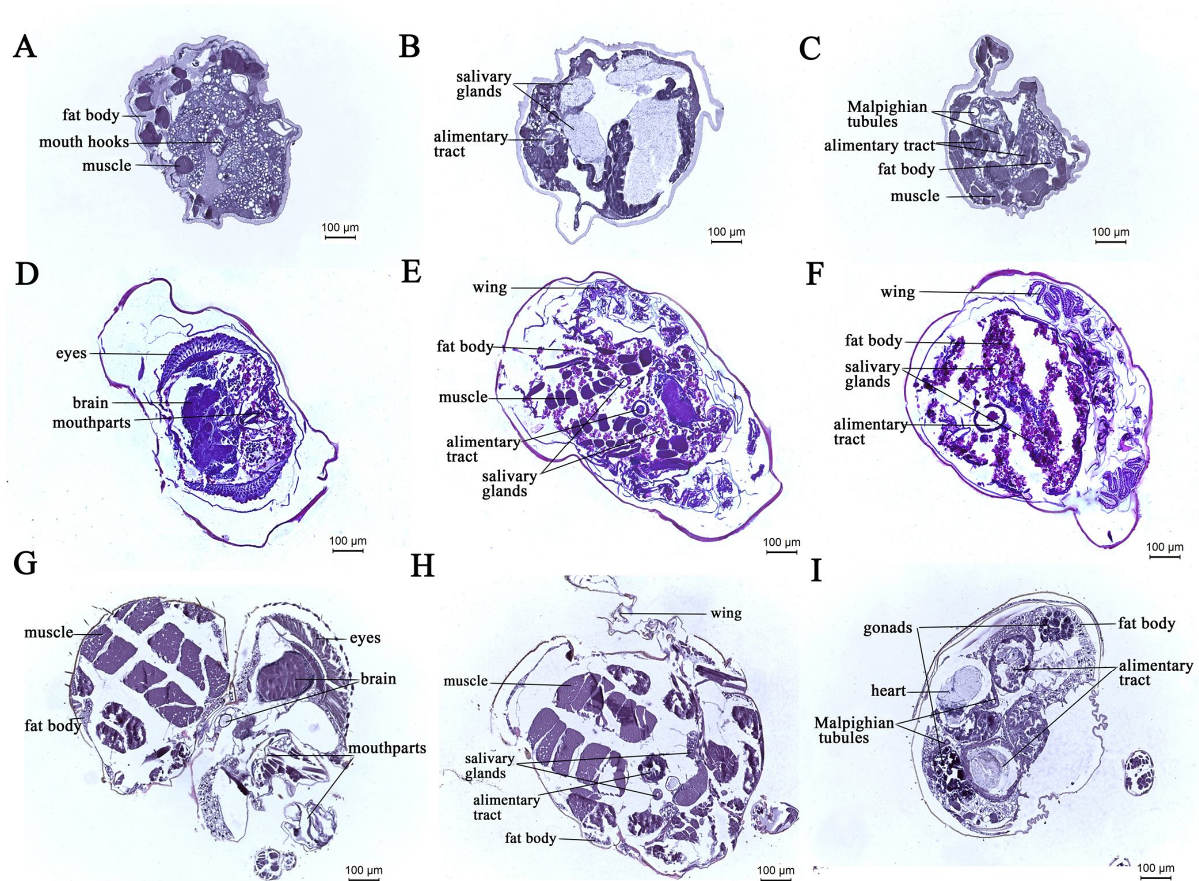

Fig. 2. The transverse section of different developmental stages of *D. melanogaster*; A, B, C belong to the larva, A, No. 5 transverse section; B, No. 16 transverse section; C, No. 7 transverse section; D, E, F belong to the pupa, D, No. 4 transverse section; E, No. 8 transverse section; F, No. 12 transverse section; G, H, I belong to the adult, G, No. 7 transverse section; H, No. 15 transverse section; I, No. 23 transverse section..

bodies in the larva (Fig. 3A). In pupa it was mainly observed in the brain and a little in the fat bodies (Fig 3B); whereas in adults it was mainly found in the brain, muscles and gut wall, and a little in the fat bodies (Fig.3C).

Expression of caveolin-1 in different developmental stages by Western blot

Western blot was used to detect the expression of caveolin-1 in the larva, pupa and adult of *D. melanogaster*. The results showed that caveolin-1 was mainly expressed in the adults of *D. melanogaster* (Fig. 4A) and the expression level of the female was significantly higher than that of the male (Fig. 4A, 4B).

DISCUSSION

Caveolin-1 protein in mammals is composed of 178 amino acid residues, including 3 regions of N terminal (1-101), transmembrane region (102-134) and C terminal (135-178) (Zhang *et al.*, 2014). N and C terminals were special membrane attachment domains (MADS), both exposed to the cytosol, the middle transmembrane part which was made of hydrophobic amino acid residues formed a hairpin structural and inserted into the membrane, thereby the protein chain was divided into two cytosol regions. Two structural domains at the N terminal played the major role in the structure and function: one was the 61-101 amino acid residues near the transmembrane region, which was known as oligomerization domain, it was the oligomer part formed from the interaction between caveolins monomers; another was the 82-101 amino acid residues included in the oligomerization domain, called caveolins scaffolding domain (CSD), it was the region of interaction between caveolin-1 and intracellular other signal molecules

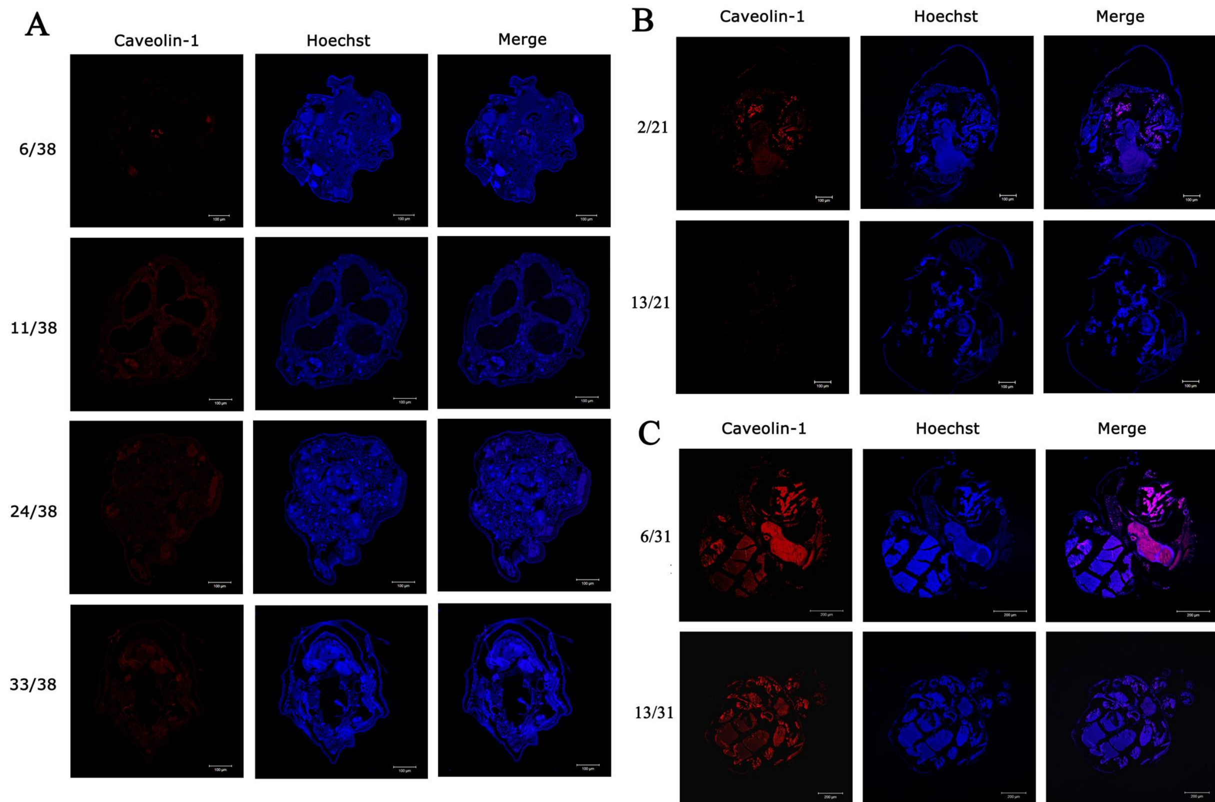

Fig. 3. The distribution of caveolin-1 in different stages of *D. melanogaster*; A, larva; B, pupa; C, adult).

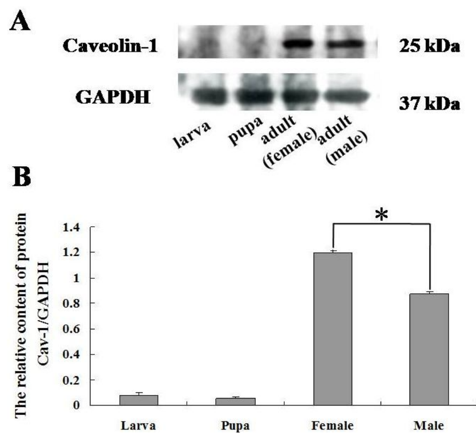

Fig. 4. Western blotting analysis of the expression of caveolin-1 protein at different development stages in *D. melanogaster*.
A, The band intensities for caveolin-1 protein were normalized against that of the GAPDH protein. B, Values were expressed as arbitrary units of relative value. Significant differences of different sex of adult (n=3, $*P<0.05$, $**P<0.01$) were analyzed by one-way analysis of variance (ANOVA).)

(Hernández-Bello *et al.*, 2008; Parker *et al.*, 2007; Zou *et al.*, 2003), was also a highly conserved skeleton region in caveolin-1.

Caveolin-1 expressed in the larval and adult stages of *D. melanogaster*, whereas the expression was significantly higher in adult than that in the larvae.

Caveolin-1 has been found to express in many species of vertebrates and invertebrates. Caveolin-1 is highly conserved in evolution. According to the high conservation of the caveolin-1, we selected the caveolin-1 antibody from mouse and *Artemia sinica* as the primary antibody of *D. melanogaster* in immunofluorescence and western-blot experiments.

We observed the *in vivo* distribution of caveolin-1 in different stages of *D. melanogaster* through immuno-fluorescence and confocal technology. The results showed that the distribution of caveolin-1 varied in different developmental stages of *D. melanogaster*. The *in vivo* distribution of caveolin-1 is very little in the fat bodies of larvae, mainly in the brain and a little in the fat bodies in pupa, mainly in the brain, muscles and gut wall, a little in the fat bodies of adults. In general, the expression of caveolin-1 in the adult was significantly

greater than those of the pupa and larva.

The results of western blot also showed that the caveolin-1 expressed mainly in adult of *D. melanogaster.* Moreover, the expression levels of female was significantly higher than that of the male, suggesting that caveolin-1 might be related to the gender development in *D. melanogaster*.

Since the expression of caveolin-1 was mainly evident in the adult stage of *D. melanogaster* compared to larval and the pupal stages it was speculated that caveolin-1 mainly affected the growth and physiological functions of the adult. The previous studies of our lab have indicated that caveolin-1 was related to the physiological and pathological changes of mammalian brain function, and played important role in the neuron development and synaptic plasticity (Wang *et al.*, 2007; Zou *et al.*, 2006). In this experiment, we observed obvious expression of caveolin-1 in the brain of *D. melanogaster* which suggests that caveolin-1 might have important role in the development of brain of insects. In addition, caveolin-1 also showed strong expression in the muscles of thorax, suggesting that caveolin-1 was also very closely related to the development and function of the muscles in *D. melanogaster*.

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CONCLUSIONS

The expression level and distribution of caveolin-1 varied in *Drosophila melanogaster*. The *in vivo* distribution of Caveolin-1 was mainly found in the brain, muscles and gut wall in adult. The expression level of caveolin-1 in adults were significantly higher in adults than in the larvae and pupae. Moreover, the expression was significantly higher in the female adult than in the male adult.

Statement of conflict of interest

The authors have declared no conflict of interest.

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