Article

RNAi KNOCKDOWN OF *Bm*Rab3 LED TO LARVA AND PUPA LETHALITY IN SILKWORM *Bombyx mori* L.

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Rab3 GTPases are known to play key a role in vesicular trafficking, and express highest in brain and endocrine tissues. In mammals, Rab3 GTPases are paralogs unlike in insect. In this study, we cloned Rab3 from the silk gland tissue of silkworm Bombyx mori, and identified it as BmRab3. Our in silico analysis indicated that BmRab3 is an isoform with a theoretical isoelectric point and molecular weight of 5.52 and 24.3 kDa, respectively. Further, BmRab3 showed the C-terminal hypervariability for GGT2 site but having two other putative guanine nucleotide exchange factor/GDP dissociation inhibitor interaction sites. Multiple alignment sequence indicated high similarities of BmRab3 with Rab3 isoforms of other species. The phylogeny tree showed BmRab3 clustered between the species of Tribolium castaneum and Aedes aegypti. Meanwhile, the expression analysis of BmRab3 showed the highest expression in middle silk glands (MSGs) than all other tissues in the third day of fifth-instar larva. Simultaneously, we showed the differential expression of BmRab3 in the early instar larva development, followed by higher expression in male than female pupae. In vivo dsRNA interference of BmRab3 reduced the expression of BmRab3 by 75% compared to the control in the MSGs in the

Grant sponsor: National Basic Research Program of China; Grant number: 2012CB114601; Grant sponsor: the National Natural Science Foundation of China; Grant number: NSFC:31372374/C1703; Grant sponsor: the Science and Technology Innovation Team of Zhejiang Province; Grant number: 2010R50031; Grant sponsor: Chinese Universities Scientific Fund.

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ARCHIVES OF INSECT BIOCHEMISTRY AND PHYSIOLOGY, Vol. 00, No. 0, 1–13 (2015)
Published online in Wiley Online Library (wileyonlinelibrary.com).
 DOI: 10.1002/arch.21228

first day. But as the worm grew to the third day, the difference of BmRab3 between knockdown and control was only about 10%. The knockdown later witnessed underdevelopment of the larvae and pharate pupae lethality in the overall development of silkworm B. mori L. © *2015 Wiley Periodicals, Inc.*

Keywords: silkworm *Bombyx mori* (*B. mori*); *Bm*Rab3; middle silk glands (MSG); dsRNA interference

INTRODUCTION

The first clone of *Rab3* gene including Rab1, Rab2, and Rab4 (in mammalian and homologous to the previously identified yeast SEC4/Ypt) was achieved using oligonucleotide strategy in 1987 (Touchot et al., 1987). Since then, several Rab proteins have been identified from eukaryotes. From simplest eukaryotes (10 Rabs) to human (over 70 Rabs), eukaryotic genomes encode several Rab family members (Zhang et al., 2007; Pereira-Leal, 2008; Diekmann et al., 2011).

Rab3 is one of the major constituents of synaptic vesicles and is involved in wider aspects of cycle such as docking, priming, and fusion (Sudhof, 2004; Ng and Tang, 2008). The superfamily of Rab3 GTP-binding has four isoforms (Rab3A–Rab3D) (Schlüter et al., 2004). These isoforms have highest expression in brain and endocrine tissues besides their regulatory role in the exocytosis of hormones as well as neurotrasmitters (Coppola et al., 2002).

Several studies in other species have indicated that Rab3 interacts with Rab3 GTPase cycle involving proteins such as Rab3 guanine nucleotide exchange factor (GEF) (Wada et al., 1997), Rab3-GTPase-activating protein (Fukui et al., 1997), and GDP dissociation inhibitor (GDI) (Araki et al., 1990), including Rab3 putative effectors Sec 15 (Wu et al., 2005), Rabphilin (Shirataki et al., 1993), and Rab3-interacting molecule (Rim) (Wang et al., 1997). Rab3 including all other GTPases undergo posttranslational modification at the C-terminal sites. Rab GTPases C-terminal hypervariability is likely for specific localization (Chavrier et al., 1991) and posttranslational addition of geranylgeranyl residues at the C-terminal site is known for membrane-associated stabilization of Rab proteins (Musha et al., 1992).

In silkworm *Bombyx mori*, small GTP-binding proteins in the brain or brain-corpus cardiacum-corpus allatum complex (BC-CC-CA) are involved in secreting prothoracicotropic hormone (PTTH) hormone (Shirai et al., 1998). Rab7 immunohistochemical reactivity was restricted to the pars intercerebralis and dorsolateral protocerebrum neurons in the brain of *B. mori* (Uno et al., 2010a). Rab1 and Rab14 were shown to have GTPase and ATPase activities in *B. mori* (Uno et al., 1998, 2010b).

Interestingly, PTTH is a neuropeptide that stimulates insect's postembryonic development. Prothoracic glands are stimulated by PTTH for synthesizing and releasing ecdysone (Agui et al., 1979; Gilbert et al., 2000). Finally, ecdysone and 20-hydroecdysone acts on targeted tissues. Besides, earlier investigations on *B. mori* silk protein synthesis had also suggested that ecdysteroids, juvenile hormones (Daillie, 1979; Couble et al., 1983; Tripoulas and Samols, 1986), and brain factor (Sehnal and Michalik, 1984) regulate silk glands action. Importantly, silk gland constitutes anterior silk gland (ASG), middle silk gland (MSG), and posterior silk gland (PSG) that are all morphologically and functionally diversified. MSG produces sericins for

nurturing sticky coating to fibroin protein synthesized from PSG. Finally, they together protrude through ASG as cocoon thread. Recently, it was also suggested that neuropeptides
discharge into hemolymph may be the action of clock neuron through direct electrical coupling, synaptic transmission (or secretion) (Vafopoulou et al., 2007). Therefore, it is likely that vesicular trafficking pulses have something to do with neuropeptides secretion.

Therefore, herein, we report for the first time the highest expression of *BmRab3* in MSG compared to PSG and the whole of silk gland tissues or other tissues in general. In the early instars development, *BmRab3* shows differential expression followed by higher expression in male pupa than that in female pupa. Silencing of *BmRab3* resulted in underdevelopment of larva with pharate pupa fatality.

MATERIALS AND METHODS

Insect

Silkworm *B. mori* L (strain P50) was reared on mulberry leaves under common temperature and humidity of 26±1°C and 75–85% with a photoperiod of 12:12 LD.

Total RNA Isolation and RT-PCR

Total RNA was separately isolated from samples using RNAiso PLus, according to the manufacturer's instructions (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China). For RNA samples of the developmental stages (third day of first, second, third, and fourth-instar larva, and third day of male and female pupae). For tissue samples, the whole insect bodies were dissected and separated the head, hemolymph, skin, ventral nerve, fat body, gut, MSG, PSG, and whole SG from first and third day of fifth instar and wandering stages of fifth-instar larva.

RNA sample for cloning was prepared from whole silk gland tissue, and used as template for the first strand of cDNA synthesis. Quality of total RNAs were determined with 260/280 absorbance using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). PCR was performed on the resulting cDNAs with the designed primers (Fig. 1). Thermal cycling conditions with initial denature at 94°C for 5 min followed by 30 cycles of 30 sec at 94°C, 30 sec annealing at 58°C, 3 min extension at 72°C, and a final extension at 72°C for 4 min. Amplified PCR product was analyzed on 1.0% agarose gel with the ethidium bromide staining. The amplified PCR product was recovered, cloned into pMD18-T vector, and verified by sequencing (Sangon Biotech Co., Ltd., Shanghai, China).

Expression Analysis of BmRab3 During Developmental Stages or Tissues

Isolated total RNA was reverse transcribed using PrimeSkript® RT Reagent kit with gDNA Eraser (TAKARA) as per the manufacturer's instruction. Then a sample of 2 μl cDNA was used in 20 μl volume for real-time qRT-PCR. Real time was performed using the SYBR. The qRT-PCR primers for *BmRab3* and *Actin3* as internal control primers are listed in Table 1. RT-PCR was performed using an ABI7300 System (Applied Biosystems, Foster, CA) and SYBR®Premix Ex TaqTM (TAKARA). The two steps amplification consisted of a 30 sec at 95°C followed by target amplification of 40 cycles at 95°C for 5 sec and 60°C for 31 sec. The absence of unwanted by-products was confirmed by automated melting

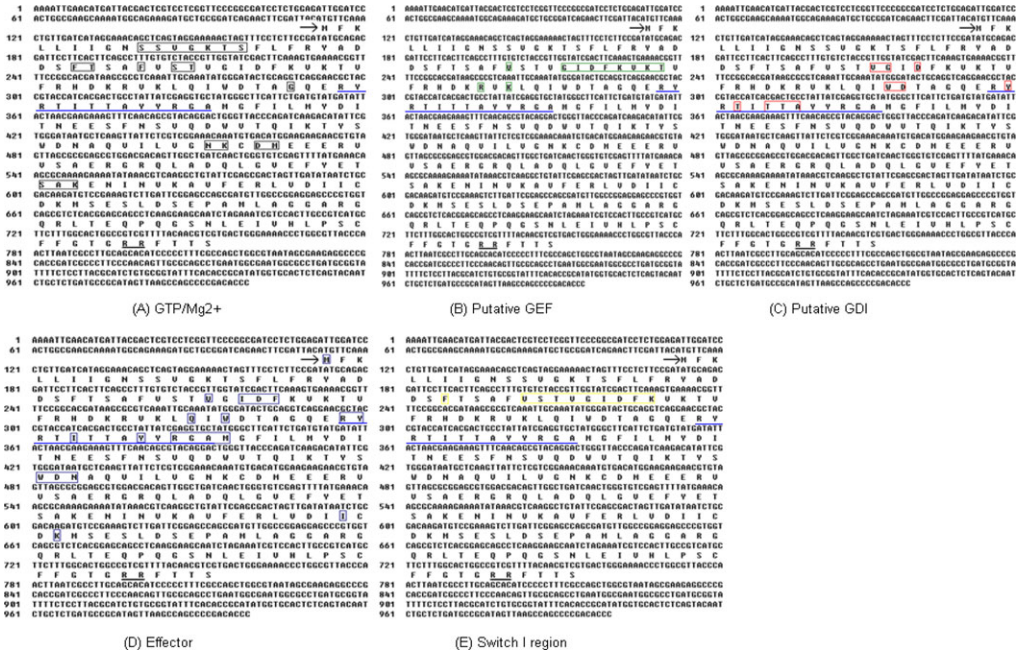


Figure 1. Sequence analysis of *Bm*Rab3 comparing the deduced nucleotide and amino acid showing conserved protein domain: (A) GTP/, black box; (B) putative GEF-binding site, green box; (C) putative GDI-binding site, red box; (D) effector, blue box; (E) switch I region, yellow box; light-blue line, REP recognition motif; arrow head, methionine; and underlined black bold line, isoprenylation sites at the C-terminal sites.

Table 1. Primers Used in Cloning, qRT-PCR, and dsRNAi of BmRab3 of Silkworm B. mori L

Primers	*Sequence (5′ -3′)*
(A) For PCR	
*Bm*Rab3(F)	**ggatcc** ACTGGCGAAGCAAAATGG
*Bm*Rab3(R)	**tctaga** TTGCTTCCTTGAGGCTGC
(B) For qRT-PCR	
*Bm*Rab3(F)	GACGATTCCTTCACTTCAGCC
*Bm*Rab3(R)	TCCGACGAGAATAACTTGAGC
For actin3 control	
Actin3 (F)	GCGCGGCTACTCGTTCACTACC
Actin3 (R)	GGATGTCCACGTCGCACTTCA
(C) For dsRNA synthesis	
*Bm*Rab3(F)	GGATCCTAATACGACTCACTATAGGACTGGCGAAGCAAAATGG
*Bm*Rab3(R)	GGATCCTAATACGACTCACTATAGGTTGCTTCCTTGAGGCTGC

The bolds of ggatcc and tctaga are the restriction enzyme cut sites of *Apo*I and *Xba*I respectively.

curve analysis after the PCR stage. The ectopic transcripts of each gene were calculated based on crossing the point analysis with standard curves generated from standard cDNA. Reactions were performed all in triplicate.

Synthesis and Injection of dsRNA

dsRNAs template synthesis corresponding to *BmRab3* using gene-specific primers consisting T7 polymerase sites for sense and antisense are shown in Table 1. T7 promoter

region corresponding to nucleotide sequences are underlined: initial denature at 94°C for 5 min followed by 34 cycles of 30 sec at 94°C, 30 sec annealing at 63°C, 3 min extension at 72°C, and a final extension at 72°C for 8 min. Amplified PCR product was analyzed on 1.0% agarose gel with the ethidium bromide staining. The templates were used to generate dsRNAs using T7 RiboMAX Express RNAi system according to the manufacturer's instructions (Promega Corperation, Madison, WI). Upon synthesis, dsRNAs were diluted with diethyl pyrocarbonate treated water and OD value measured (260/280), and the products were analyzed by gel electrophoresis to further confirm the annealing. The diluted samples were brought to final concentration and first-day fifth-instar larvae were injected with 5 μl each of ds*Bm*Rab3 interference using a 10 μl microsyringe (Hamilton). Control larvae were also injected with 5 μl each of ds-EGFP. After injection, larvae were maintained under normal conditions and samples were collected every 24 hr for 3 days consecutively, and the remaining worms were reared for physical or morphological changes analysis until the adult stage.

Statistical Analysis

All experiments were performed in three independent biological replication and reactions of each sample were carried out in triplicate. All data were determined by one-way ANOVA analysis.

RESULTS

cDNA Cloning and Sequence Analysis of BmRab3

Using GenBank databases (http://www.ncbi.nlm.nih.gov/) EST (Accession: NM_001044155.1) and SilkDB CDS (http://silkworm.genomics.org.cn/) (Gene ID: BGI*BM*GA000295-TA), the gene of *BmRab3* was blast searched and designed the degenerated primers (Table 1). Later, using DNA star software, we deduced 214 amino acid residues from the resulted 999 bp length cDNA obtained from PCR amplification and sequencing results of the gene. The comparative cDNA sequence and amino acid is shown (Fig. 1).

Further, *BmRab3* consists of 645 bp open reading frame (ORF) followed by a molecular weight and isoelectric point of 24.55/5.52 Da, 25 strongly basic amino acids, 28 strongly acidic amino acids, 74 hydrophobic amino acids, and 61 polar amino acids. Isoprenylation site including homologues for evolution (evolutionary motif conservation) was searched with the deduced amino acid using http://mendel.imp.ac.at/PrePS/index.html(Maurer-Stroh and Eisenhaber, 2005) and it hit 21 clusters in 2,166 sequences. Rab-small GTPases scored 1,314 maximum hits (ClusID: 46229098). Best hit *E*-value was 1×10^{-105} scoring "+" for Rab-small GTPases and Evo value was 633.613. It had shown absence of "CaaX" box for FT and GGT1 bindings but missing one cysteine within the last five positions (Fig. 1) of the sequence binding sites for geranyl-geranyltransferase 2 (GGT2). Hitting sequences also had shown 1,289 GGT2 when FT and GGT1 hit values were 167 and 45 only. In the taxonomic kingdom, Eukaryota scored 1,303 while the synthetic and the viruses scored 10 and 1, respectively. HMMER search for GGT2's resulted HMM1 (Rab GTPase fold *E*-value): 3.3×10^{-101} (= significant) and HMM2 (REP-interaction score): 23.1 (= good). Predicted REP recognition motif was "RYRTITTAYYRGA" (between 82 and 94 amino acids) (Figs. 1 and 2). Using this amino acid, a protein homology search by

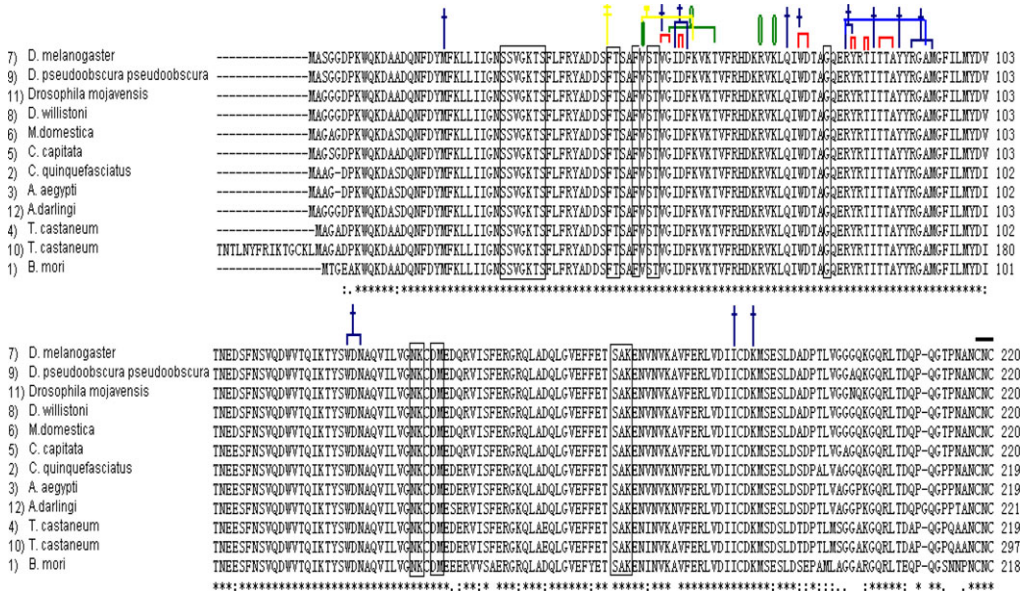


Figure 2. Multiple sequence alignment of *Bm*Rab3 with others organisms using ClustalW2. Indicated are black box, site for GTP/; green, putative GEF-binding site; red, putative GDI-binding site; blue, effector; yellow, switch I region; light-blue line, REP recognition motif. Isoprenylation site in C-terminal is shown by black color bold line overhead. Reference NCBI sequence number and maximum amino acid identity percentage are given in the results. Consensus symbols identity: *, positions having single or fully conserved residue; :, strongly similar conserved groups properties; and ., weakly similar conserved groups properties, respectively.

http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins confirmed that it is 97% highly homologous to the ras-related GTP-binding protein Rab3 of *B. mori* (Accession number: NP_001037620.1). Therefore, we established this sequence as Rab3 of *B. mori* and named *BmRab3*. Additionally, the translated amino acid sequence shared significant similarity with already reported Rab3 GTPases, which belong to the superfamily of P-loop_NTPase superfamilies.

Other organisms that shared high homologous to *BmRab3* with their NCBI accession numbers were: *Culex quinquefasciatus* gi|170049010|/*Cq*Rab-3D, *Aedes aegypti* gi|157112793|/*Aa*Rab-3C, *Tribolium* castaneum gi|91093088|/*Tc*Rab-3C, *Ceratitis capitata* gi|498997133|/*Cc*Rab3, *Musca domestica* gi|557759178|/*Md*Rab3, *Drosophila melanogaster* gi|17737457|/*Dm*Rab3, *D. willistoni* gi|195429579|/*Dw*Rab3, *D. pseudoobscura* gi|125811091|/*Dpp*Rab3, *T. castaneum* gi|270013048|/*Tc*Rab3, *D. mojavensis* gi|195120790|/*Dm*Rab3, and *Anopheles darlingi* gi|568249997|/*Ad*Rab-3D, and produced high homologous similarities in order of: 93, 93, 92, 91, 91, 91, 91, 90, 91, 90, and 93%, respectively. Therefore, the highest homologous scored to *Bm*Rab3 were *C. quinquefasciatus, A. aegypti, and A. darlingi*; and the lowest scored were *D. pseudoobscura, and D. mojavensis*, respectively. Multiple sequence alignment of *Bm*Rab3 with other organisms was compared using ClustalW2 (Fig. 2).

Amino acid residues for GTP/Mg2+, effectors, putative guanine nucleotide-exchange factors (GEFs) and guanine nucleotide-dissociation inhibitors (GDIs), and Switch I region are gathered from http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi site. Alignment indicated that BmRab3 sequences are highly conserved in all organisms, particularly in relation to residues for GTP/Mg2+ binding (Fig. 2), as it also implies in

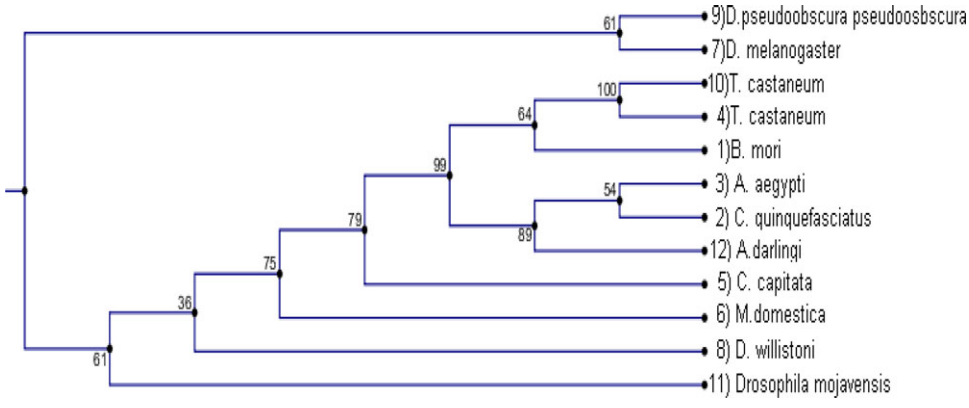


Figure 3. Phylogeny tree analysis using amino acid sequences of BmRab3 with other organisms. The NJ (neighboring-joining) tree was prepared using Free CLC sequence viewer Version 6.8.1 (Copyright 2010 CLC Bio A/S, Aarhus, Denmark). NCBI sequence references are shown in the results. A bootstrap value is indicated at each node, and the value is a measure of confidence in the branch. Bootstrap analysis is adjusted with 100 replicates.

other Rab GTPases members. Additionally, some area of the sequence shows comparative residue diversity. Isoprenylation sites at the C-terminal were found to be diversified. Phylogeny tree branch of BmRab3 was clustered between the species of *T. castaneum* and *A. aegypti* (Fig. 3), and is consistent according to the eukaryotic evolutionary extant.

Expression Analysis of BmRab3 During Development Stages in Silkworm B. mori

We examined the expression of *BmRab3* during development stages in silkworm *B. mori*, and the expression for the tissue specific on third day of fifth-instar larval. The results showed that the transcript expression of *BmRab3* in MSG was much higher than the PSG or the whole SG or all other analyzed tissues followed by its lowest expression in the head tissue (Fig. 4B). Further, we showed expression of *BmRab3* in MSG on third day of fifth-instar larvae reached a peak, which consisted the highly active synthesis of sericins in MSG in this stage (Fig. 4C). The higher expression of *BmRab3* was screened in male pupae than that of female pupae in the third day of pupation (Fig. 4D).

Effects of BmRab3 Knockdown in MSG Tissue and on Larval and Pupa Morphology

To further understand the function of *BmRab3* in *B. mori*, we performed an in vivo knockdown of *BmRab3*. The relative transcriptional expression of *BmRab3* in MSG was significantly reduced compared to control when the larva was treated with *BmRab3*-dsRNA. (Fig. 5A). dsRNA interference of *BmRab3* reduced the expression of *BmRab3* by 75% compared to the control in the MSGs in the first day. But as the worm grew to the third day, the difference of *BmRab3* between knockdown and control was only about 10%.

The knockdown of *BmRab3* in the MSG tissue resulted in abnormality in physiology and morphology of silkworm *B. mori*. During the course of development, it brought a total fatality as indicated in both larvae and pupae (Fig. 5B, C). Therefore, the changes resulted from mRNA transcript lead to RNA reduction and simultaneously brought a

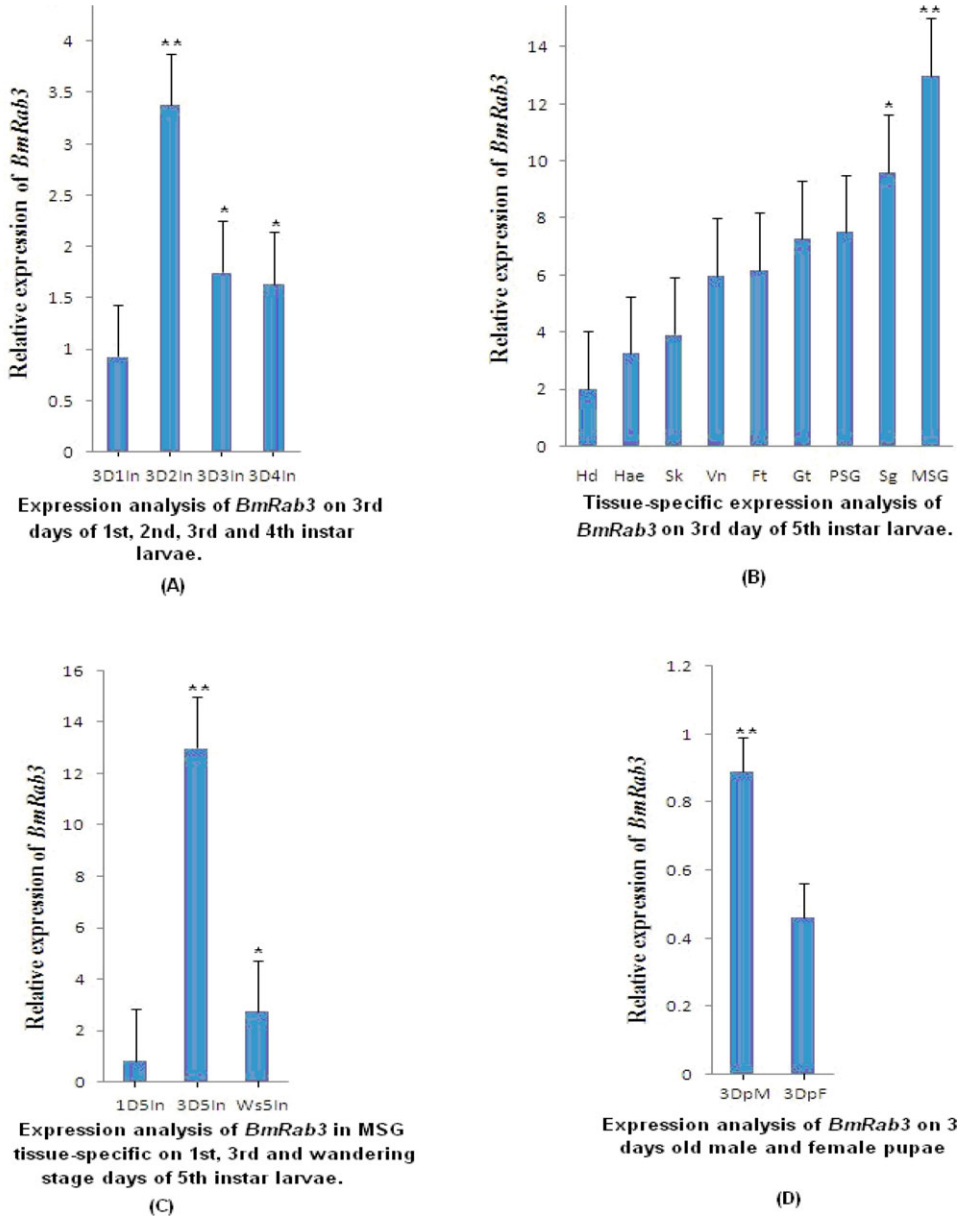
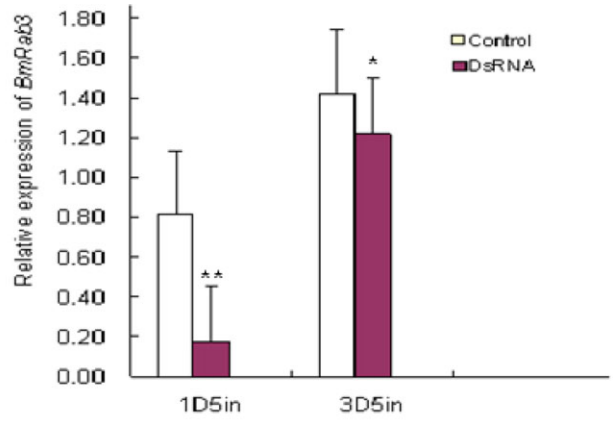
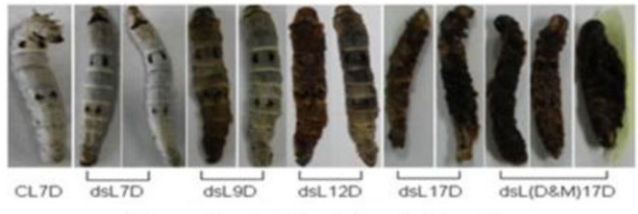


Figure 4. Analysis of transcript level of *BmRab3* during development stages in silkworm, *B. mori*. (A) *BmRab3* expression analysis in third-day-old early instar larva development (3D1In, third-day first-instar larva; 3D2In, third-day second instar; 3D3In, third-day third instar; and 4Din, fourth-day fourth instar); (B) tissue-specific expression analysis on third day of fifth-instar larva (Hd, head; Hae, hemolymph; Sk, skin; Vn, ventral nerve; Ft, fat; Gt, gut; Fib, posterior silk gland; Sg, silk gland; and Ser, middle silk gland); (C) *BmRab3*-specific MSG expression profile in fifth-instar larva (1D5In, first-day fifth instar; 3D5In, third-day fifth instar; and Ws5In, wandering stage fifth instar; (D) *BmRab3* expression comparison in male and female pupae (3DpM, third-day male pupae and 3DpF, third-day female pupae), respectively. *BmActin 3* was as the normalization gene. All experiments were performed in three independent biological replication and reactions of each sample were carried out in triplicate. Data are represented as mean ± SE; $*P < 0.05$ and $**P < 0.01$.



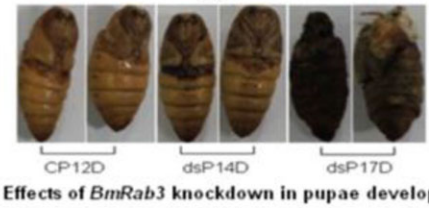
RNAi knockdown of BmRab 3 on 1D and 3D of 5th instar larvae tissue-specific MSG

(A)



Effects of *BmRab3* knockdown in *B.mori* larvae

(B)



Effects of *BmRab3* knockdown in pupae development

(C)

Figure 5. Effects of *BmRab3* knockdown in silkworm. (A) *BmRab3* transcript level in MSG tissue after *BmRab3*-dsRNA treatment. 1D5in, first day of fifth instar; 3D5in, third day of fifth instar; (B) in vivo effects of *BmRab3* knockdown on morphological changes in *B. mori* larva (CL7D, 7 days old ds-EGFP-injected control larv; dsL7D, dsL9D, dsL12D, dsL17D, 7, 9, 12, 17 days old ds*BmRab3*-injected larva; and dsL(D&M) 17D, ds*Bm*Rab3-injected larva (death and melted within cocoon); (C) knockdown effects in pupae (CP12D, 12 days old ds-EGFP; dsP14D and dsP17D, 14 and 17 days old ds-*Bm*Rab3-injected pupae, respectively. ds-EGFP of 5 μl (1 μg/μl dissolved in diethyl pyrocarbonate water) was injected as control. All experiments were performed in three independent biological replication and reactions of each sample were carried out in triplicate. Data are represented as mean ± SE; $*P < 0.05$ and $**P < 0.01$.

significant morphological change in the knockdown larvae quite earlier (Fig. 5B, dsL7D) as compared to control larvae (Fig. 5B, CL7D).

Further, the knockdown larvae brought a significant change and ultimately dead and dried (Fig. 5B, dsL9D, dsL12, and dsL17D), followed by failure to transform into pupa stage timely and melted within the cocoons (Fig. 5B, dsL(D&M)17D). In the meantime, we

also noticed fatality in the knockdown pupae (Fig. 5C, dsP14D and dsP17D) as compared to its control (Fig. 5C, CP12D).

DISCUSSION

Recently, it has been immerged that Rab GTPases play a central role in regulating membrane carrier motility. Rab3 belongs to the wider superfamily of Ras GTP-binding protein, which belongs to the wider family of Rab GTPases, controls the identity of membrane and eukaryotic cells trafficking (Stenmark, 2009). We first reported in this study the identification of *BmRab3* cloned from silk gland tissue, and the highest expression of *BmRab3* in MSG than the PSG or the whole of SG tissues and other tissues studied in the silkworm *B. mori*. Knockdown of *BmRab3* in MSG shows larvae and pupae fatality in the organism development.

We reported that *Bm*Rab3 showed a C-terminal hypervariability for GGT2 site, with a homologous similarity of 97% to the ras-related GTP-binding protein Rab3 of silkworm *B. mori* (Accession number: NP_001037620.1), and having a superfamily of Ras-like_GTPase.

Our in silico data also further supported the conservation within the various GTPase families. As indicted by our result in Figs. 2 and 3, orthologous even from the distantly related groups share 65–85% sequence identity. The strong conservation and its universality show that even the latest common ancestor of eukaryotes already possessed small GTPases that are involved in such cellular activities equal to the one involved by the present-day family members. Overall, this implies that the majority of the functional diversification of the eukaryote-specific GTPase families takes place after getting separated from prokaryotic ancestors but prior to diversification of the current-day eukaryotic groups. Truly, the phylogeny of small GTPases in fact indicates the evolutionary history of central components of the eukaryotic cell.

HMMER analysis for GGT2 indicated that HMM1 (Rab GTPase) and HMM2 (REP) are both significantly conserved (Figs. 1 and 2). Complex of Rab and REP are recognized by the lipid attaching GGT2. Isoprenylation sites at the C-terminal were found to be diversified (requiring at least one cysteine in C-terminus within the last five terminus) (Fig. 1), and this confirmed the binding sites for GGT2. This is quite significant because in human also a very specific carboxyl-terminal motif is nonessential for GGT2 (Alexandrov et al., 1999), although several cysteines -CC, -CXC, -CCX, -CCXX, -CCXXX or single -CXXX are available in close proximity to carboxyl terminus. Interestingly the superfamily of *Bm*Rab3, with others *Bm*Ras1 and *Bm*Ras2 from silkworm *B. mori* were also reported to be neither farnesylated nor palmitoylated but rather geranylgeranylated (Moriya et al., 2010).

We hypothesized whether BmRab3 is highly upregulated on any of the given silk-producing domains such as MSG, PSG, or whole SG, in particular. The results showed that the highest in vivo expression of BmRab3 was presented in MSG than PSG or whole of SG on the third day of fifth-instar larvae. In fact, Rab proteins are known for its regulation of several processes including transport of neurotransmitter and neuropeptide, and silk-producing domains serve as the streamline for secretory network function in silkworm B. mori. The MSG of silkworm is an organ for synthesis and secretion of large number of sericin proteins, especially in the mid-fifth instar. So the highest expression of *Bm*Rab3 in this period (Fig. 4B, C) is deemed as consistent with the silk protein synthesis and secretion.

To understand the function, we knockdowned *BmRab3* in vivo by injecting dsRNA into the fifth-instar larva of silkworm *B. mori*. RNAi knockdown of *BmRab3* reduced the expression of *BmRab3* by 75% compared to the control in the MSGs in the first day. But as the worm grew to the third day, the difference of *BmRab3* between knockdown and control was only about 10%. This suggested that RNA interference gradually reduce over time.

The knockdown of *Bm*Rab3 in MSG tissue in the larva showed downregulated 24 and 72 h post dsRNA injection and resulted in severe underdevelopment in terms of its morphology and physiology of larva and subsequent larva or pharate pupa fatality compared to control. Therefore, it is likely that knockdown of *BmRab3* results in disruption of neuronal trafficking leading to the loss of homeostatic control of neural function and ultimately death to the organism.

In conclusion, the identification of *BmRab3* in silkworm *B. mori* and its functional studies are very important. Our in silico analysis of *Bm*Rab3 showed the C-terminal hypervariability for GGT2 sites. Interestingly, our tissue expression analysis of *BmRab3* showed the highest expression in MSG tissue than other tissues including the PSG and whole SG. Generally, the tissue-specific expression underscored strong evidence to the corresponding tissues and its physiological functions. Though the function of Rab3 is still vague, our study provided a fundamental clue to the significant function of *BmRab3* in the MSG tissue of *B. mori*. Significantly, we found that suppression of *BmRab3* in vivo MSG leads to larvae and pupae fatality in the present study. With several unknown regulatory system in silk protein biosynthesis, *Bm*Rab3 might offer an opportunity to understand the MSG regulatory protein network in silkworm biology.

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