

RESEARCH REPORT

Identification of *BmBrat* gene in silkworm, *Bombyx mori*XX Ke^{1#}, H Gao^{1#}, K Chen², L Zhang¹, S Chen¹, X Wang¹, Z Wei¹, L Yang¹, H Cui^{1*}

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Abstract

NHL protein family is evolutionarily conserved in either *vertebrates* or *invertebrates*. It has been extensively studied in mammals for the function of regulating cell proliferation and differentiation. *Brat*, known as the homologue of *TRIM* in insects, may have the same function. In this paper, we identified and characterized *BmBrat* in silkworm. The gene expression profile indicated that *BmBrat* was expressed in all tissues and the expression was especially high in ovary and head at larval stage. The expression profile was also detected at different stages of embryo development, and reached a peak at 4th and 5th days of the embryonic period. Subcellular localization of *BmBrat* in hemocytes revealed that it was specifically expressed in cytoplasm. Over-expression of *BmBrat* in silkworm significantly reduced the rate of cell proliferation by BrdU staining. This finding suggested that *BmBrat* played an important role in cell processes.

Key Words: silkworm, *BmBrat*, gene expression profile, subcellular localization**Introduction**

The name NHL originates from three members, which were earlier discovered: NCL-1, HT2A and LIN-41. These three factors have relationship with the RNA metabolism, and may be involved in post-transcriptional regulation of gene expression (Loedige *et al.*, 2009). Researches showed that family members of the NHL contain three conserved motifs: a RING, one or two B-box and a coiled-coil domain (Petrera *et al.*, 2012; Tocchini *et al.*, 2015). NHL domain plays a vital role in regulating the protein activity of Brat (Brain tumor) (Marchetti *et al.*, 2014). *Brat* itself as a member of the NHL protein family is a kind of tumor suppressor genes, but it is also a candidate gene controlling cell proliferation (Loedige *et al.*, 2014). In the *Drosophila* nervous system, the expression of *Brat* largely limits the peripheral nervous system, ventral nerve cord and brain development during embryonic stage (Bello *et al.*, 2006). In stem cells development system, *Brat*, *Prospero* and *Numb* work as cell fate determination factors, promoting the changes of cell fate (Lee *et al.*,

2006). *Numb* is a membrane-bound protein involved in the regulation of Notch signaling pathway (Cotton *et al.*, 2013). During neural precursor cell division, *Numb* gathered to one end of the cell to combine with the membrane, distributed into the ganglion mother cells through asymmetric cell division (Hutterer *et al.*, 2005). Then *Numb* interacts with *Sanpodo*, which is α -ubiquitin mediated protein activity regulating factor, to inhibit the Notch protein (Berdnik *et al.*, 2002). The inhibition of Notch signaling pathway, which plays a crucial role in proliferation and differentiation of neuroblastoma cells, would result in a cell fate transformation, which means self-renewing cells can gradually begin to differentiate (Wang *et al.*, 2011). *Prospero* is a transcriptional regulatory protein, which exists both in the embryo and larvae neuroblast, and was continuously expressed in the differentiated glial cells (Choksi *et al.*, 2006; Reichert, 2011). *Prospero* functions as a downstream regulator of *Brat*, and plays a key role in cell fate determination and cell proliferation regulation. Thus studying *Brat* in cell differentiation and proliferation can provide theoretical basis for differentiation of stem cells. The homolog of *Brat* in human and mammalian is the TRIM protein family, and they all play a role in the development, differentiation, host cell antiviral defense and cancer. TRIM family is a subgroup of the NHL domain, such as TRIM2, TRIM3, TRIM32

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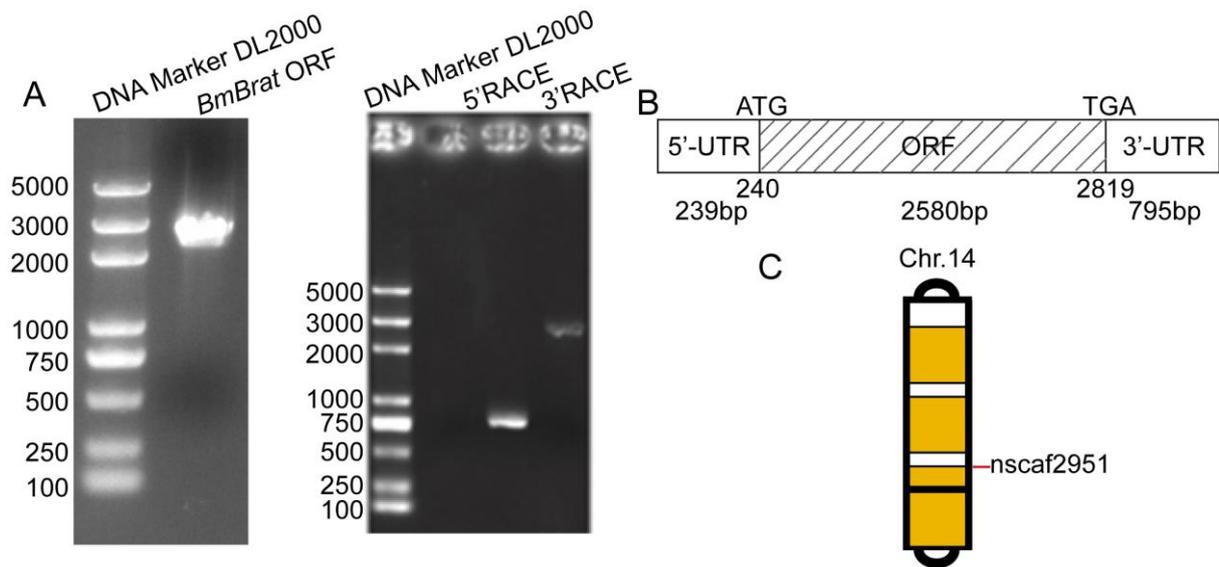


Fig. 1 Complete cloning of silkworm *BmBrat*. (A) PCR of *BmBrat*. (B) The diagram of the complete *BmBrat* cDNA. (C) The location of *BmBrat* on chromosome 14

and TRIM71 in mammalian, Brat, Mei-p26, Abba and Wech in *Drosophila*, and Ncl-1, Nhl-1, Nhl-1, Nhl-3, Lin41 in *Caenorhabditis elegans* (Nisole *et al.*, 2005; Neumuller *et al.*, 2008; Sardiello *et al.*, 2008). Among these, *TRIM3*, known as a tumor suppressor, can inhibit cancer development by binding to *CDK* inhibitor *p21^{WAF1/CIP1}* to prevent the accumulation of Cyclin D1-*CDK4* (Boulay *et al.*, 2009; Liu *et al.*, 2014). Reducing *TRIM3* expression in mice can increase and accelerate the platelet-derived growth factor (PDGF) induced glioma incidence (Boulay *et al.*, 2009; Chen *et al.*, 2014). However, there is almost no report on the *BmBrat* gene in silkworm, *Bombyx mori* at present, and the exact function of *BmBrat* gene remains unknown. In this study, we reported the identification, characterization, expression analysis and functional study of the *BmBrat* gene in silkworm, *B. mori*, which is not only an important economical insect for silk production, but also an excellent research model of *invertebrate*.

Materials and Methods

Biological materials

Bombyx mori strain Dazao (P50) used in our study is provided by the Gene Resource Library of Domesticated Silkworm of Southwest University. The larvae was fed with fresh mulberry leaves under standard conditions. Different tissues were extracted in 1× DEPC-treated PBS, flash-frozen and stored at -80 °C until needed. The cultured silkworm embryo-derived cell line was established by our laboratory, and it was cultured in Grace' medium supplemented with 10% (V/V) FBS.

RNA extraction and cDNA synthesis

Total RNA was extracted from the head, midgut, epidermis, silk gland, fat body, malpighian tubules, testis, ovary, haemocytes and embryo with TRIzol

reagent referring to the protocol provided by the manufacturer. After being treated with RNase-free DNase I, the first-strand cDNA was synthesized with M-MLV reverse transcriptase, and the products were stored at -20 °C.

Bioinformatic and phylogeny analysis

The ORF was verified with the ORF Finder software (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The *BmBrat* protein domain was predicted using SMART (<http://smart.embl-heidelberg.de/>). The deduced amino acid sequences of *BmBrat* was aligned using the Clustal X program, and a phylogenetic tree was constructed by the neighbor-joining method (Tamura *et al.*, 2007). The sequences used in this study were downloaded from GeneBank (<http://www.ncbi.nlm.nih.gov/>).

Quantitative real-time PCR (qRT-PCR)

For qRT-PCR analysis, a StepOne Plus™ Real-Time PCR system (Applied Biosystems, USA) and the SYBR Master Mix (Promega, USA) were used under the following conditions: 95 °C for 10 min, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The housekeeping gene *BmGAPDH* was used as an internal control. All reactions were performed in triplicates in a total volume of 20 μL. Sequences of qRT-PCR primers were designed as follows:

BmBrat-F, 5'-CGTGCCTTACTTGTCTGT-3'; *BmBrat*-R, 5'-GCAGTTGGGGCAGAGGT-3'; *GAPDH*-F, 5'-CATTCCGCGTCCCTGTTGCTAAT-3'; *GAPDH*-R, 5'-GCTGCCTCCTTGACCTTTTGC-3'.

Relative mRNA expression levels were calculated by using the standard $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001). The online t-test software GraphPad Software (<http://www.graphpad.com/quickcalcs/ttest1.cfm>) was used to evaluate the statistical significance ($P < 0.05$).

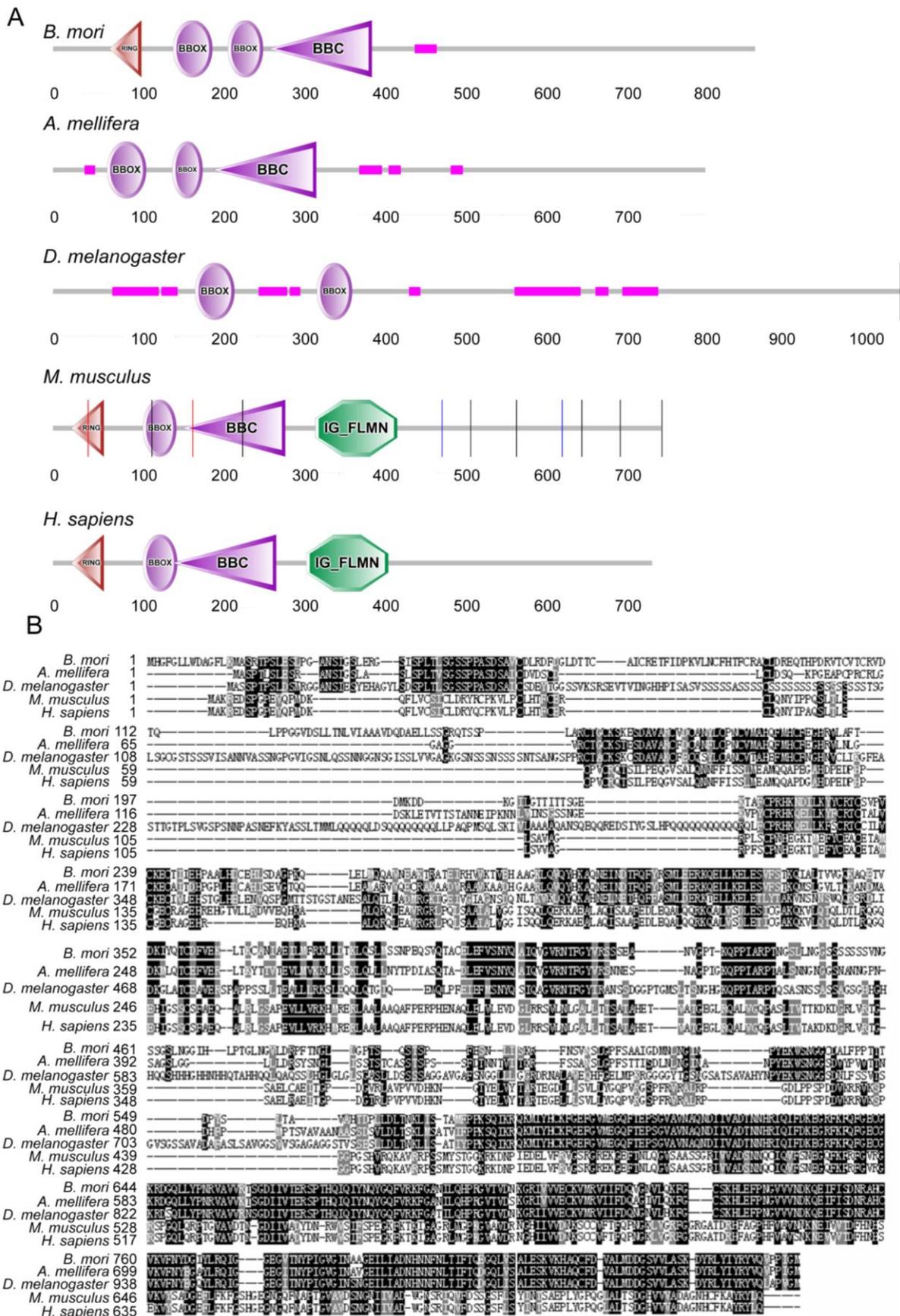


Fig. 2 Bioinformatic analysis of *BmBrat*. (A) Multiple protein domain alignment of *BmBrat* in several species. *BmBrat* protein has a conserved RING domain, two B-BOX domain and a BBC domain. (B) Multiple sequence alignment of *BmBrat* in several species, and *BmBrat* gene has high homology in these species

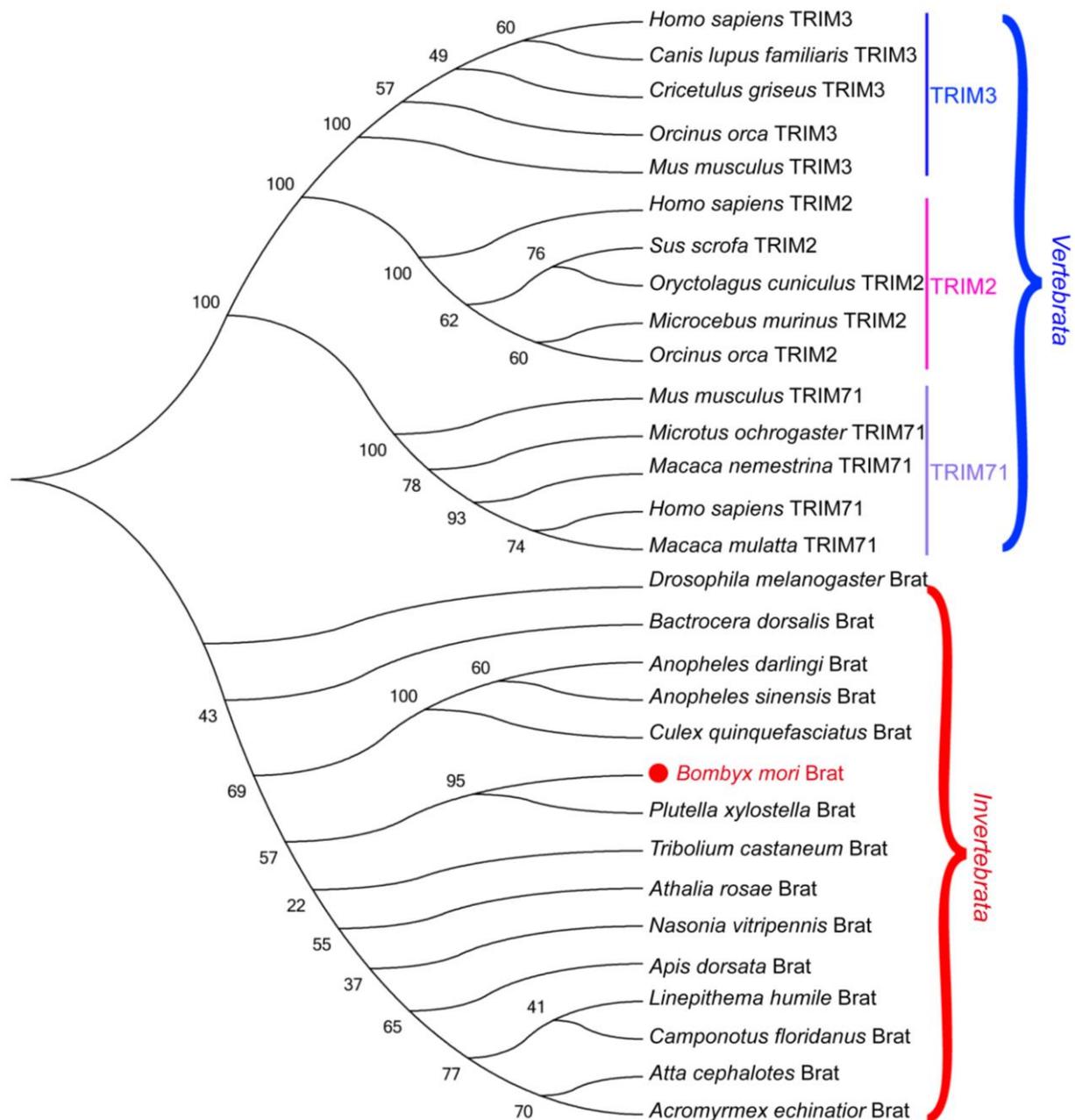


Fig. 3 The phylogenetic tree of *BmBrat*. The phylogenetic tree of the *BmBrat* based on the full-length amino acid by the neighbor-joining method. The number closed to individual branches represents the percentage of 1000 bootstrap iterations supporting the branch, and values below 60% were omitted. The silkworm *BmBrat* are labeled with circles

Cell transfection and *BmBrat* overexpression

The *BmBrat-Flag* fragment for overexpression was generated by PCR using Dazao (P50) head cDNA template. Then it was ligated into the pSL1180-A4-DsRed plasmid. 2×10^5 BmE cells were seeded into 24-well plates for 24 h, and transiently transfected with pSL1180-A4-DsRed-*BmBrat-Flag* vector with X-tremeGENE HP DNA Transfection Reagent (Roche). After 72h transfection, cells were processed for western blot and immunofluorescence assay. Observation of the fluorescent signal was performed by using the confocal fluorescence

microscope (Olympus FV1000, Olympus Corporation, Tokyo, Japan).

BrdU staining

After transfection with the overexpression vector, cells were grown on coverslips, and incubated with 10 μ g/ml BrdU (Sigma) for 3 h, then washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 20 min. Subsequently, cells were pre-treated with 1 mol/L HCl, and blocked with 10% goat serum for 1 h, followed by a monoclonal rat primary antibody against BrdU

(1:200, ab6326, Abcam, Cambridge, MA, USA) for 1 h and Alexa Fluor® 488 goat anti-rat IgG secondary antibody, (H+L; Invitrogen). DAPI (300 nM) was used for nuclear staining; the percentage of BrdU was calculated at least from 10 microscopic fields (Nikon 80i, Nikon Corporation, Tokyo, Japan).

Flow cytometry

For cell cycle analysis, flow cytometry was performed in BmE cells overexpressing *BmBrat*. 1×10^6 cells were harvested and fixed with 70% ethanol, stained with propidium iodide (PI) (BD Biosciences), then incubated with RNaseA for 30 min at room temperature. Finally, the cell samples were analyzed by Flow Cytometry using a FACS C6 (BD BioSciences), and the data was analyzed with CellQuest Pro software.

Results

Cloning and identification of *BmBrat* in silkworm

BmBrat full-length sequence was obtained and identified (Fig. 1 A and B), which was located on chromosome 14 (Fig. 1 C), containing 859 amino acid residues. There is a conserved RING domain, two B-BOX domain and a BBC domain in *Brat* protein sequence of silkworm (Fig. 2 A). The homology of *Brat* from silkworm and other species was explored via multiple sequence alignment using clustal X (Fig. 2 B). Results of multiple protein domain alignment (Fig. 2 A) showed some differences between silkworm and other species in the genetic structure, especially in the sequence similarity among species. Although a relatively conserved NHL domain was found in the C-terminal region in all analyzed species, there were some differences between *invertebrates* and mammals. It was found that the sequence conservation of each domain was rather low, especially the conservation of the RING domain. However, the homology of the NHL domains in *invertebrates* and mammals, respectively, was very high. In addition, the cysteine in the BBOX domain was highly conserved, the hydrophobic amino acid was relatively conserved in the coiled-coil domain. Furthermore, phylogenetic analysis of *BmBrat* from several species using MEGA6 software generated a phylogenetic tree (Fig. 3). And results showed that, in evolution, *BmBrat* had far relationship with *vertebrate* homologous gene family *TRIM*. However, *Danaua plexippus* had the closest relationship with silkworm.

BmBrat is highly expressed in ovary and head

The mRNA expression levels of *BmBrat* over the embryo period from day 1 to day 9 and in various tissues at day 3 of 5th instar larvae (L5D3) were analyzed by qRT-PCR, the results of which were shown in Fig. 4. We found that the *BmBrat* was highly expressed at day 4 and day 5, especially in day 5, and the expression level went down rapidly after day 5. We collected and compared most of organs in silkworm, including testis, ovary, epidermis, silk gland, malpighian tubule, midgut, head, fat body, and hemocytes. The data showed that *BmBrat* was expressed in various organizations, including the highest in the ovary, followed by the head.

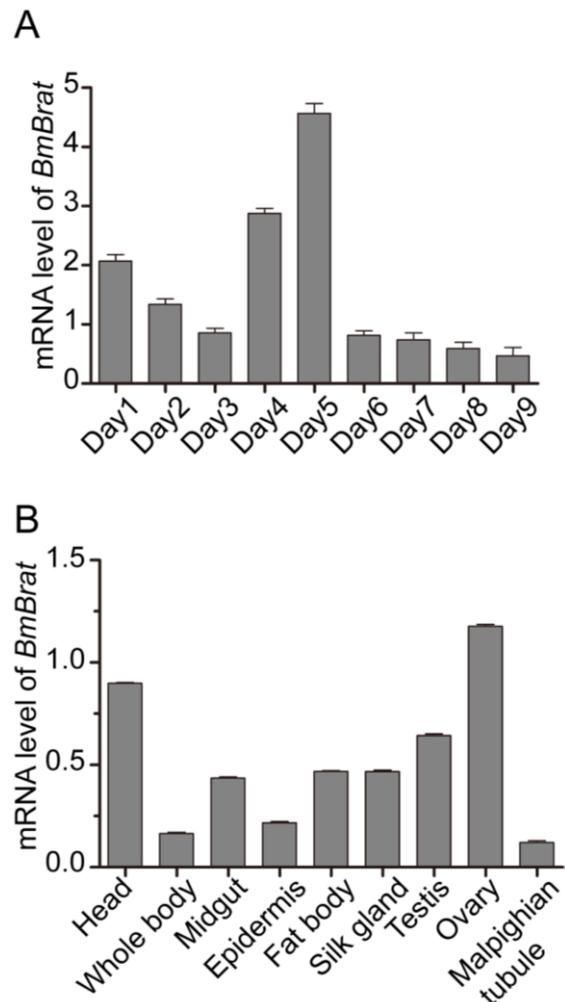


Fig. 4 The expression profile of *BmBrat*. (A) The temporal and spatial expression profiles of *BmBrat* in silkworm embryos. cDNAs were obtained from embryos at different stages. (B) Tissue-specific expression of *BmBrat* in silkworm larvae. cDNAs were obtained from the head, whole body, midgut, epidermis, fat body, silk gland, testis, ovary, malpighian tubule of L5D3 larvae. qRT-PCR was used to analyse *BmBrat* mRNA expression levels, and *BmGAPDH* was used as an internal control. All reactions were performed in triplicate

BmBrat is specifically located in cytoplasm

For further analysis of *BmBrat* in expression characteristics silkworm, full length expression vector pSL1180-A4-*BmBrat*-FLAG-Dsred was constructed (Fig. 5A). Then we overexpressed *BmBrat* in BmE cell line, pSL1180-A4-Dsred empty vector was used as control. Results showed that in the control group, the red signal of Dsred evenly spread out in cytoplasm and nucleus. On the other hand in the experimental group, the *BmBrat* signal only existed in cytoplasm but not in nucleus. In addition, we observed that the position of *BmBrat* signal is different in the prophase, metaphase, early telophase, and late telophase (Fig. 5C). This result suggested that the *BmBrat* was contributed in different positions in different period of cell division.

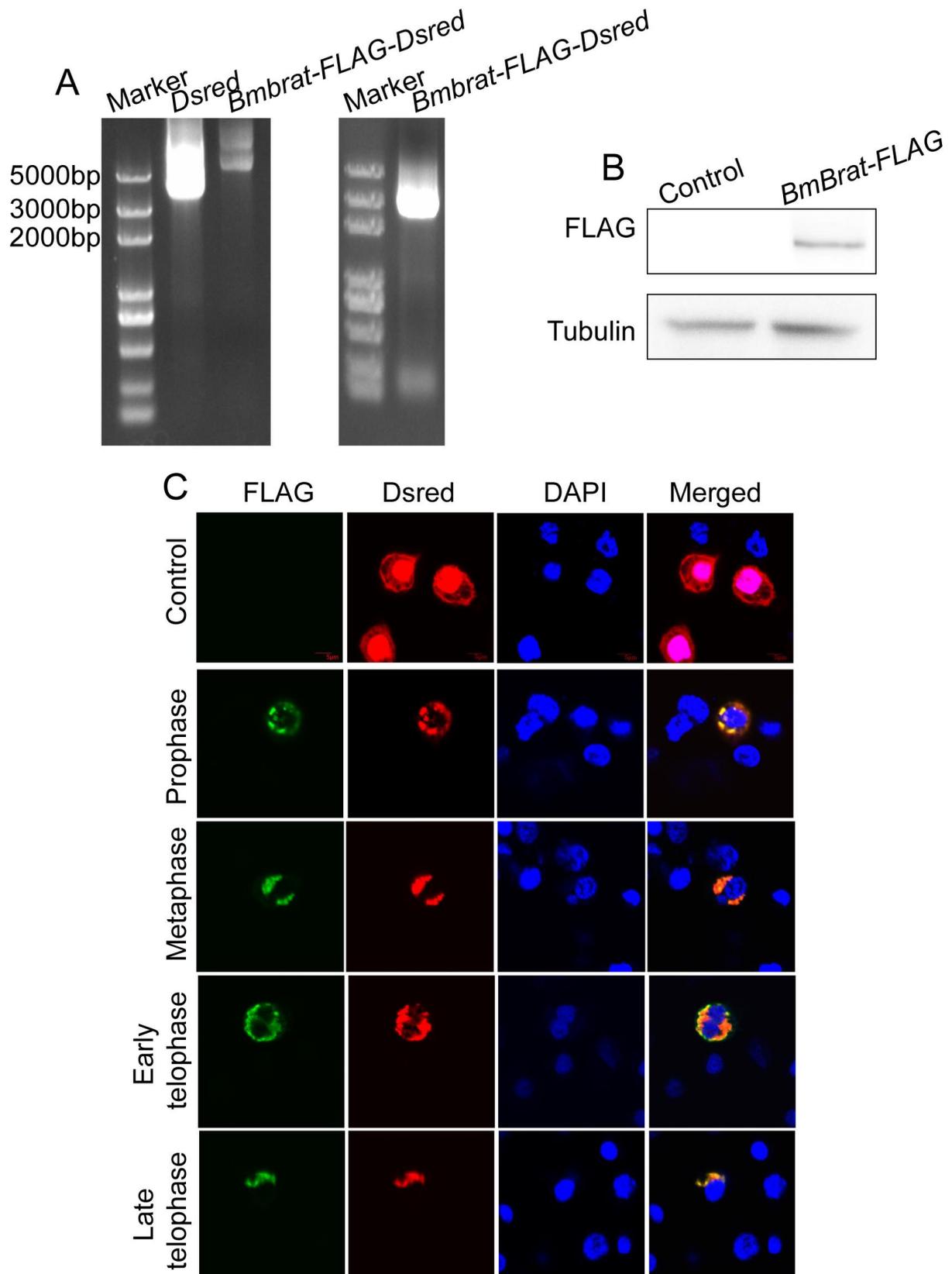


Fig. 5 The over-expression and localisation of *BmBrat* in silkworm BmE cells. (A) Construction of the pSL1180-A4-*BmBrat*-Dsred vector. *BmBrat* cloned into pSL1180-A4-Dsred vector with FLAG tag. (B) Western blot analyses of over-expression of *BmBrat* gene in BmE cells. Over-expression of *BmBrat* gene in BmE cells was obtained by transfection of *BmBrat*-FLAG-Dsred plasmid. (C) Localisation of *BmBrat* protein in BmE cells by immunofluorescence assay. FLAG (green) showed the distribution of *BmBrat*-FLAG; Dsred (red) showed the distribution of *BmBrat*-Dsred; DAPI (blue) showed the cell nuclei; Scale bar: 5 μ m

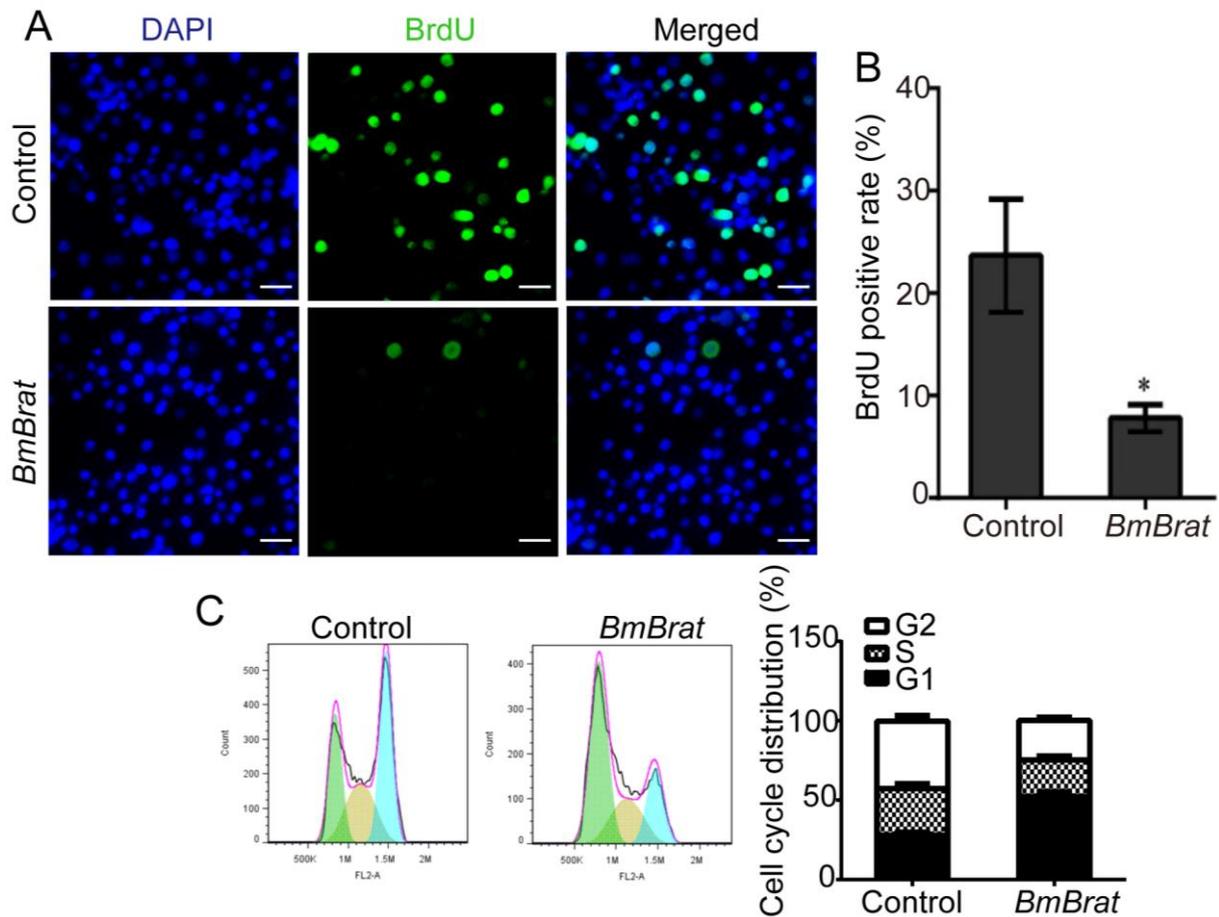


Fig. 6 Over-expression of *BmBrat* inhibits cell proliferation in silkworm BmE cells. (A) BrdU staining was used to investigate cell proliferation in *BmBrat* up-regulation cells. Immunofluorescence detected by Anti-BrdU antibody. Scale bar: 20 μ m. (B) Statistical analysis of BrdU positive cells ratio. Statistical analysis were performed using two-tailed student's t-test, * $p < 0.01$. (C) FACS analysis were performed to investigate *BmBrat* overexpression influence cell cycle of BmE cells

Over-expression of *BmBrat* inhibits the cell proliferation in BmE cells

In order to figure out the role of *BmBrat* in cell process, BrdU staining was utilized after it was up-regulated in BmE cell line. First we use anti-Flag antibody to make sure that the *BmBrat* was successfully overexpressed (Fig. 5B). The BrdU staining result revealed that the BrdU positive cells decreased significantly (Fig. 6 A and B), which means the cell proliferation was suppressed after overexpression of *BmBrat*. Moreover, FACS analysis was performed to check the changes of cell cycle in BmE cells which were overexpressed of *BmBrat*. And the result shown in Figure 6 C clarified that the cell cycle of BmE cells was arrested in G1 phase, and the S phase was significantly decreased, when overexpression of *BmBrat* was performed. It is suggested that the function of *BmBrat* may be to inhibit cell proliferation.

Discussion

NHL, particularly *TRIM* family, has been commonly studied in mammals for the function in

cell proliferation especially in cancer development (Petrera *et al.*, 2012). Bioinformatic analysis of *BmBrat* showed the gene encoding protein has four relatively conservative structure domain from the N terminal to C terminal, respectively a RING, two B-Box and a BBC structure domain, which were typical NHL structure domains. indicating it might function in the cell proliferation and differentiation (Tocchini *et al.*, 2015).

Expression spectrum analysis of *BmBrat* in silkworm embryonic showed that the amount of gene expression reached the peak at 4th and 5th day and went down after that. Silkworm eggs need about 10 days to incubate, and this period can be divided into divers stages according to the embryonic development process. The earlier 6 days of the incubation period is time for organ formation, and 4th and 5th day is the very summit of that, and the gene was highly expressed at this time. The organ development completed after the earlier 6 days, and the gene expression was downregulated significantly at the same time. This suggested that *BmBrat* might be associated with silkworm organ formation process in the embryonic development. L5D3 larvae

expression spectrum analysis illustrated that the gene was almost expressed in all tissues, especially in ovary and head, which means that the *BmBrat* was a tissue-specific gene and might have some certain functions that are still unknown.

Results of localization analysis showed that the gene expression in embryonic cells *BmBrat* exists in cytoplasm, as part of the cells to start dividing *BmBrat* protein in the cells due to the role of cell polarity factors together to the bottom, asymmetrical distribution in the daughter cells. Symmetrical division of cells, the protein will average distribution into the daughter cells. To sum up, *BmBrat* genes in silkworm growth process plays a very important role. Speculation *BmBrat* genes involved in cell division, and is associated with the cell cycle, and the inhibition of cell proliferation.

It has been reported the *Brat* has a variety of functions in *Drosophila* differentiating germ cells. It is known the maintenance of germline stem cell is regulated by the Dpp signaling pathway (Xie *et al.*, 1998; Chen *et al.*, 2003) and translation inhibitory factor *Nanos* (*Nos*)/*Pumilio* (*Pum*) (Forbes *et al.*, 1998). In the ovary, *Pum-Nos* adjust the mRNA level of the *Brat*, limiting its expression in the cystoblasts (Muraro *et al.*, 2008). Up-regulation of Dpp signal can promote the inhibition on *Brat* mRNA generation by *Pum-Nos* (Li *et al.*, 2009). When cells divide, Dpp signal come into silence, the loss of Dpp signal downregulated *Nos*, which accelerate expression of the *Brat*. In return, *Brat* formed new compounds with *Pum* to inhibit mRNA levels of *Mad* and *dMyc* which works in Dpp signaling pathway (Ohlstein *et al.*, 1997), reducing the response capacity of cystoblasts to Dpp signal, promoting cell differentiation (Harris *et al.*, 2011). Therefore, whether *BmBrat* has the similar function in silkworm remains to be further studied.

Competing interests

The authors disclose no potential conflicts of interest.

Acknowledgement

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