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Insights into the repression of fibroin modulator binding protein-1 on the transcription of *fibroin H-chain* during molting in *Bombyx mori*

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ABSTRACT

Fibroin modulator binding protein-1 (FMBP-1) is a novel DNA-binding protein containing a conserved score and three amino acid peptide repeat (STPR) domain. The roles of factors containing STPR domain are less known. Although multiple transcription factors are involved in the transcriptional regulation of silk protein genes during the development of silkworm, the mechanism of transcriptional repression of silk protein genes during molting remains unclear. Here, we found that FMBP-1 expression was contrary to that of *fibroin heavy chain* (*fib-H*) during the fourth molting period of *Bombyx mori*. FMBP-1 repressed *fib-H* promoter activity by directly binding to the −130 element in the *fib-H* promoter region. We also identified two proteins, Bmsage and Bmdimm, that interacted with FMBP-1 in the posterior silk gland of silkworm larvae, and further verified these interactions by far western blotting and microscale thermophoresis *in vitro*, as well as co-immunoprecipitation and bimolecular fluorescence complementation at the cellular level. The luciferase reporter assay showed that the interaction between FMBP-1 and Bmdimm antagonized the activation of Bmdimm on *fib-H* transcription, but did not affect FMBP-1-mediated transcriptional repression on *fib-H* gene. Therefore, we proposed the following mechanism of *fib-H* transcriptional repression by FMBP-1 during the molting of silkworm larvae: 1) FMBP-1 directly binds to the −130 element in the *fib-H* promoter to repress *fib-H* transcription; 2) FMBP-1 interacts with Bmdimm to antagonize the activation of Bmdimm on *fib-H* transcription. Our findings promote a better understanding of *fib-H* transcriptional regulation and provide novel insights into the transcriptional repression of *fib-H* by FMBP-1 and basic helix-loop-helix factors Bmdimm during the molting of silkworm larvae. Our study also provides valuable information regarding the biological function of factors containing STPR domain.

1. Introduction

Most of the interaction patterns between protein and DNA are mediated by base recognition. Recently, fibroin modulator binding protein-1 (FMBP-1) from *Bombyx mori* is highlighted as a novel DNA-binding protein which interacts with its target sequence by DNA shape recognition (Yu et al., 2016). FMBP-1 has a novel DNA-binding domain containing a score motif and three amino acid peptide repeats (STPR). Each repeat of the STPR domain forms a short α-helix maintained by an N-cap and salt bridge, and has different effects on the interaction of STPR with DNA (Saito et al., 2008). STPR interacts with a deformed B-DNA in a zig-zag structure extending along the major groove of DNA (Saito et al., 2007; Takiya et al., 2009; Yu et al., 2016). The binding of STPR to specific DNA induces a conformational change in FMBP-1 that increases its overall α-helix content, and this binding could be stimulated by Y-box protein of *Bombyx mori* (Takiya et al., 2004). STPR domain is highly conserved in *Caenorhabditis elegans*, *Drosophila*, mouse, and human with more than 80% sequence identity (Nonaka et al., 2010; Takiya et al., 2005).

Bombyx mori is a domestic insect with huge economic importance

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and a good model of *Lepidoptera* insects due to its potential applications in science, agriculture, industry and medicine (Hino et al., 2006; Ogawa et al., 2007; Tomita et al.; 2003, 2007). Silkworms spin a mass of silk protein to wrap themselves into a cocoon at the end of the final instar. Silk yield is closely dependent on the developmental stage of silkworm and the synthesis of silk proteins (Xia et al., 2014). The silk gland of *Bombyx mori* is divided into anterior, middle and posterior regions. As the main component of silk protein, fibroin comprises three components, fibroin heavy chain (fib-H), fibroin light chain (fib-L) and P25, which are synthesized and secreted in the posterior silk gland (PSG). The expression of silk protein genes is strictly regulated with territorial and developmental specificities (Suzuki, 1994). *Fibroin* gene is repetitively switched on and off during development (Maekawa and Suzuki, 1980), with tissue-specific expression regulated at the transcriptional or post-transcriptional level (Hui and Suzuki, 1989; Maekawa and Suzuki, 1980). A large number of transcription factors, such as silk gland factor BmFkh/SGF-1 (Mach et al., 1995), SGF-2 (Ohno et al., 2013; Tsuda and Suzuki, 1983), POU-M1/SGF-3 (Hui et al., 1990; Kimoto et al., 2012; Xu et al., 1994), SGF-4 (Hui et al., 1990), FBF-A1 (Hui et al., 1990), FMBP-1 (Takiya et al., 1997), BmFTZ-F1 (Zhou et al., 2016), Bmsage (Zhao et al., 2014), and Bmdimm (Zhao et al., 2015), are shown to be correlated with *fib-H* transcription. The gene expression profiles of these factors in silk glands have been characterized individually (Kimoto et al., 2010; Kokubo et al., 1996). In *Bombyx mori*, FMBP-1 is a ubiquitous transcription factor that binds to AT-rich elements upstream of the promoter and intron regions of *fib-H* (Takiya et al., 1997). FMBP-1 expression is inhibited by Bmo-miR-2758, a microRNA (Wang et al., 2016). Bmsage and Bmdimm are basic helix-loop-helix (bHLH) family transcription factors, which are homologs of *Drosophila* sage and dimm and expressed specifically in the posterior silk gland of silkworm larvae (Jones, 2004). Bmdimm binds to a consensus hexanucleotide sequence (E-box), and interacts with SGF-1 (Zhao et al., 2014) and Bmsage (Zhao et al., 2015) to promote *fib-H* transcription at the end of the fifth instar, whereas Bmsage does not bind to the E-box element. Additionally, Bmsage regulates silk gland development during early embryonic stage (Fox et al., 2013; Xin et al., 2015).

The structure and properties of STPR domain have been extensively studied, while the biological roles of proteins containing STPR domain are still less known. In the present study, we found that FMBP-1 repressed *fib-H* transcription during the fourth molting of silkworm larvae. Furthermore, we revealed FMBP-1 repressed *fib-H* transcription *via* direct binding of FMBP-1 to the −130 element upstream of *fib-H* promoter and interacts with Bmdimm and Bmsage. Our results not only provide novel insights into the transcriptional repression of *fib-H* gene by FMBP-1 during molting, but also have important significance for the functional research of other factors including STPR domain.

2. Materials and methods

2.1. Biological materials and reagents

Silkworm strain Dazao (P50) was from our laboratory (Southwest University, Chongqing, China) and reared as previously described (Liu et al., 2016a). *Bombyx mori* embryo cells (BmE cells) were cultured at 27 °C in Grace's medium containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA). 4′6-diamidino-2-phenylindole (DAPI) was purchased from Sigma (St. Louis, MO, USA). Polyvinylidene fluoride (PVDF) membrane was from Roche (Basel, Switzerland).

2.2. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was prepared using TRIzol reagent (Life Technologies). Reverse transcription was performed with a Takara PrimeScript RT reagent Kit (Shiga, Japan). mRNA was quantified by qRT-PCR on an ABI7500 real-time PCR system (Life Technologies) using SYBR Premix Ex Taq II (Takara). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference. The primers used for qRT-PCR are listed in Table S1. Each of amplification was performed in triplicate. The reaction system (20 μL) was comprised of 0.8 μL forward or reverse primer, 0.4 μL Rox reference dye, and 10 μL SYBR Primer Ex Taq II. The reaction conditions were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s with a melting stage at 60 °C for 6 s. Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method.

2.3. Western blotting

Different tissues were collected from silkworm larvae on day 3 of the fifth instar. The posterior silk gland was dissected from day 0 of the fourth instar to day 0 of the fifth instar of silkworm larvae, and then immediately frozen in liquid nitrogen. These tissues were crushed in a mortar under liquid nitrogen and homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Beijing, China) containing a mixture of protease inhibitors (Beyotime) for 30 min. The supernatant was collected by centrifugation at 4 °C for 15 min. Protein concentration was determined by BCA protein assay kit (Beyotime). Total protein (100 μg) was denatured at 95 °C for 10 min, separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto PVDF membrane at 100 V for 60 min. The PVDF membrane was blocked in 5% (v/v) skim milk at 37 °C for 1 h and incubated with mouse anti-FMBP-1 (1:10,000 dilution) at 4 °C overnight. After five rinses with Tris-buffered saline containing Tween-20 (TBST, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.1% Tween-20), the membrane was incubated with goat anti-mouse IgG conjugated with horseradish peroxidase at 37 °C for 1 h. The signals were detected with an enhanced chemiluminescence kit (Life Technologies).

2.4. Dual luciferase assay

The firefly luciferase reporter plasmid FHP-Exon was constructed by inserting the promoter sequence of *fib-H* (−924 to +66) into pGL3-basic vector (Promega, Madison, WI, USA) using specific primers (Table S2). *Ie1* and *ie2* are essential genes for DNA replication of *Bombyx mori* nucleopolyhedrovirus. These two genes have strong promoters which are commonly used to express foreign genes in cell lines derived from insect species (Imai et al., 2005; Jarvis et al., 1990; Shotkoski et al., 1996). Here, the −130/E-box element was inserted into *ie2* promoter using specific primers (Table S2) to yield −130-*ie2*-luc and E-box-*ie2*-luc vectors, respectively. BmE cells were cultured in 24-well plates and transfected with the firefly luciferase reporter plasmid and renilla luciferase reporter plasmid (pRL-*ie1*-SV40) at a ratio of 10:1. The overexpression plasmids, FMBP-1-1180, Bmsage-1180, Bmdimm-1180 or Red-1180 and FHP-Exon/−130-*ie2*-luc were co-transfected into BmE cells using X-treme GENE HP DNA transfection reagent (Roche), respectively. After 48 h, the cells were washed with phosphate-buffered saline (PBS) and collected to measure promoter activity with the Dual-Glo Luciferase assay kit (Promega). In addition, FMBP-1-1180 and Bmsage-1180 were co-transfected with pRL-*ie1*-SV40 and FHP-Exon or −130-*ie2*-luc or E-box-*ie2*-luc into BmE cells for luciferase assay, respectively. Similarly, FMBP-1-1180 and Bmdimm-1180, or FMBP-1-1180, Bmsage-1180 and Bmdimm-1180 were co-transfected with pRL-*ie1*-SV40 and FHP-Exon or −130-*ie2*-luc or E-box-*ie2*-luc into BmE cells for luciferase assay, respectively. Red-1180 was used as a negative control. All assays were conducted in triplicate. Statistical significance was analyzed using Student's *t*-test.

2.5. Electrophoretic mobility shift assay (EMSA)

The oligonucleotides labelled with biotin at the 5′ end were incubated at 95 °C for 10 min, and annealed to generate the probe. Wild-type and unlabeled probes were used as competitors. The sequences of

the competitors were: −130 probe (−152 to −125): 5′-Biotin-GTAT TGTTATGTTAA ATAAAAAGATTAA-3'; cold probe: 5′-TATTGTTATGT TAAATAAAAAGATTAA-3'. FMBP-1 was expressed in *Escherichia coli* (*E. coli*) and purified by Ni-NTA affinity chromatography. The fused poly-histidine tag was cleaved by Prescission protease (GE Healthcare, IL, USA) and removed by gel filtration. EMSA was performed at 25 °C for 20 min in 20 μL solution containing FMBP-1, 50 ng poly(dI-dC), 2.5% glycerol, 0.05% NP-40, 50 mM KCl, 5 mM $MgCl_2$, 4 mM EDTA and DNA probe. For the binding assay, the amounts of FMBP-1 were 0.5, 1.0, or 2.0 μg. For the competition assay, the competitor concentrations were 250 nm (1X), 6.25 mM (25X) and 12.5 mM (50X). For the super-shift assay, 0.5, 1.0, and 2.0 μg anti-FMBP-1 antibody was individually added and incubated at 25 °C for 10 min. The samples were transferred onto a positively charged nylon membrane (GE Healthcare) and run at 100 V for 1.5 h on a 6% polyacrylamide gel at 4 °C, and then cross-linked under irradiation of 254 nm ultraviolet light for 1 min. The membrane was incubated with blocking buffer at 25 °C. After 15 min, the blocking buffer was replaced with fresh blocking buffer containing 66.7 μL streptavidin-horseradish peroxidase. The signal was measured with a light-shift chemiluminescence kit (Life Technologies).

2.6. Immunoprecipitation (IP) and Co-immunoprecipitation (Co-IP)

To identify which proteins interact with FMBP-1 in the posterior silk gland, IP was performed. The nuclear fractions were isolated from the posterior silk gland of silkworm larvae at the fourth molting stage with a NE-PER nuclear and cytoplasmic extraction kit as the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). Protein G beads (50 μL) were washed with PBS once, and then incubated with 5% bovine serum albumin (BSA) at 25 °C while rotating for 30 min. Next, 5 μg anti-FMBP-1 antibody was added and incubated with the beads at 25 °C for 1 h. IgG was used as a control. FMBP-1 (20 μg) was added after washing the bead-antibody complexes three times with PBS. The complexes were collected by a DynaMag-2 magnet (Invitrogen) and washed three times with PBS. The nuclear fractions (5 mg) were subsequently added and incubated while rotating at 4 °C for 4 h. The precipitates were washed with PBS four times, and then detected with a fast silver staining kit (Beyotime).

Co-IP was performed using protein agarose A/G beads (Santa Cruz Biotechnology, Dallas, TX, USA) to further validate the interaction of FMBP-1 with Bmsage and Bmdimm. The nuclear fractions were isolated from BmE cells at 48 h post-transfection as described above. Highly pure plasmids were prepared using Qiagen plasmid midi kit (Hilden, Germany). The overexpression plasmids FMBP-1-1180 and Bmsage-1180, or FMBP-1-1180 and Bmdimm-1180 were co-transfected into BmE cells, respectively. After 48 h, anti-FMBP-1 antibody was added to 50 μL BSA-blocked Dynabeads (5%, w/v; Santa Cruz Biotechnology), and incubated at 25 °C while rotating for 30 min. The Dynabeads-antibody complexes were collected by centrifugation and washed twice with PBS. The nuclear fractions (5 mg) were subsequently added and incubated while rotating at 4 °C for 4 h. After centrifugation, the precipitates were washed with PBS four times and analyzed by western blotting.

2.7. Far western blotting

Far western blotting was performed as previously described (Liu et al., 2016b; Wu et al., 2007). Bmsage and Bmdimm were provided by Shuguang Wei and Peng Zhao, respectively. Recombinant Bmsage and Bmdimm were expressed with His and sumo tags in *E. coli* and purified by Ni-NTA affinity chromatography, after which the fused tags were cleaved by sumo protease and separated from Bmsage and Bmdimm with a Ni-NTA column. Bmsage and Bmdimm (1 μg/each) were separated on 12.5% SDS-PAGE and transferred to PVDF membranes. BSA was used as a negative control. Denatured BSA, Bmsage, and Bmdimm were renatured on the PVDF membranes by gradient dilution of guanidine hydrochloride solution. The PVDF membrane was blocked and then incubated with FMBP-1, followed by a standard western blotting procedure. Similarly, different truncated FMBP-1 variants were separated by 15% SDS-PAGE and analyzed using the same procedures described here.

2.8. Microscale thermophoresis (MST)

MST was carried out on a Monolith NT.115 (NanoTemper, Munich, Germany). Bmsage and Bmdimm was labelled with fluorescein isothiocyanate (FITC) to form highly stable fluorescent dye-protein conjugate in PBS buffer (Jerabek-Willemsen et al., 2011; van den Bogaart et al., 2012; Wienken et al., 2010), and then exchanged into TBST buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween-20) to a final concentration of 10 μM. FMBP-1 (1 mg/mL) was prepared through a 16 step dilution into TBST buffer, and then mixed with FITC labelled Bmsage or Bmdimm (10 μL, 100 nM) at a volume ratio of 1:1. The mixture (20 μL) was loaded into NT hydrophobic capillaries to start MST measurement. The LED power and MST power were set as 80% and 40%, respectively. The default settings including time for initial fluorescence, thermophoresis recording, and after-thermophoresis fluorescence were used without modification. The dissociation constant (K_D) was calculated using the NTAnalysis program (NanoTemper). Each set of experiments was repeated in triplicate.

2.9. Subcellular localization and bimolecular fluorescence complementation (BiFC)

cDNA extracted from silkworm larvae on day 2 of the fourth instar was used as a template for PCR amplification of *FMBP-1*. *FMBP-1* was inserted into A4-RFP-1180 vector for overexpression. Immunofluorescence was performed as previously described (Liu et al., 2016a). BmE cells were cultured on glass coverslips with medium. The overexpression plasmid FMBP-1-RFP was transfected into cells using X-treme GENE HP DNA transfection reagent (Roche). After 48 h, the cells were washed with PBS, and then fixed in 4% (w/v) paraformaldehyde at 25 °C for 15 min, followed by treatment with 0.02% Triton-X 100 at 25 °C for 10 min and washed with PBS three times. Next, DAPI was added to stain the cell nucleus. After 15 min, the cells were washed with PBS three times. The coverslips were mounted on glass slides and sealed with anti-fluorescence quenching reagent (Beyotime). Fluorescent signals were recorded on an Olympus confocal fluorescence microscopy Fv1000 (Tokyo, Japan).

BiFC was performed as previously described (Kerppola, 2006). *FMBP-1*, *Bmsage*, and *Bmdimm* were inserted into pENTR11 vector (Life Technologies), respectively. All plasmids were verified by sequencing. Gateway reactions were then performed between the resulting plasmids and destination vectors (pie2cVW and pie2nVW) (Mitsunobu et al., 2012; Zhou et al., 2016) to yield the plasmids FMBP-1-pie2cVW, FMBP-1-pie2nVW, Bmsage-pie2cVW, Bmsage-pie2nVW, Bmdimm-pie2cVW and Bmdimm-pie2nVW. BiFC assay was performed by co-transfecting FMBP-1-pie2nVW, Bmsage-pie2cVW or FMBP-1-pie2nVW and Bmdimm-pie2cVW into BmE cells. FMBP-1-pie2nVW and pie2cVW were co-transfected into BmE cells as a control. Cell staining and fluorescence observation were conducted as described above.

3. Results

3.1. Expression profiles of FMBP-1 and fib-H

qRT-PCR showed that FMBP-1 was ubiquitously expressed in different tissues of silkworm larvae on day 3 of the fifth instar (Fig. 1A). The expression level of FMBP-1 in the posterior silk gland was higher than that in the anterior and middle silk gland, indicating that this protein is related to silk protein synthesis. Western blotting further confirmed the qRT-PCR results (Fig. 1D). In the posterior silk gland,

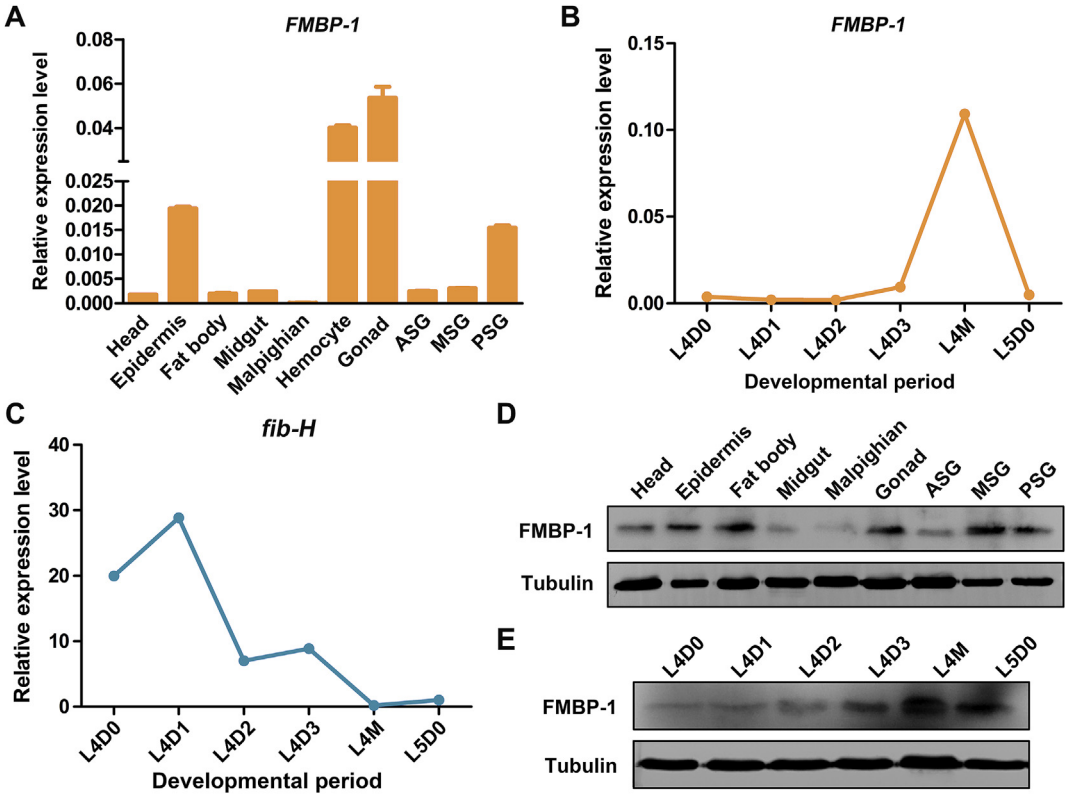

Fig. 1. FMBP-1 expression profile in *Bombyx mori*. A. The relative expression level of *FMBP-1* in different tissues of silkworm larvae on day 3 of the fifth instar by qRT-PCR. B-C. The relative expression level of *FMBP-1* (B) and *fib-H* (C) in the posterior silk gland of silkworm larvae from day 0 of the fourth instar to day 0 of the fifth instar by qRT-PCR. D. The expression level of *FMBP-1* in different tissues of silkworm larvae on day 3 of the fifth larval instar by western blotting. E. The expression level of *FMBP-1* in the posterior silk gland of silkworm larvae from day 0 of the fourth instar to day 0 of the fifth instar by western blotting. *GAPDH* was as used as a control in A, B, and C. Tubulin was used as a control in D and E. L, Larval instar; D, day; M, molting stage.

from day 0 of the fourth instar to day 0 of the fifth instar, qRT-PCR showed that the expression of *FMBP-1* increased slowly, reached the maximum on the fourth molting, and then decreased sharply to a very low level on day 1 of the fifth instar (Fig. 1B). Western blotting showed a similar tendency at protein level as those of *FMBP*-1 mRNA, although with a slight delay (Fig. 1E). However, the expression of *fib-H* showed nearly the opposite trend as *FMBP-1*. *Fib-H* transcription reached a maximum on day 1 of the fourth instar and then decreased gradually. At the fourth molting, *fib-H* transcription reached a minimum (Fig. 1C). Our results imply that FMBP-1 may repress *fib-H* transcription during the fourth molting of silkworm larvae.

3.2. Cellular localization of FMBP-1

To evaluate FMBP-1-mediated regulation on *fib-H* transcription, we constructed an overexpression vector of FMBP-1 fused with red fluorescence protein (RFP) at its C-terminus, and then transfected it into BmE cells. After 48 h, cell fluorescence was observed on an Olympus Fv1000 confocal fluorescence microscope. The results showed red fluorescence was concentrated in the cells nuclei, though some leakage into the cytoplasm of the cell (Fig. 2A), suggesting that FMBP-1 functions as a transcription factor in the cell nucleus.

3.3. FMBP-1 repressed fib-H promoter activity

To evaluate the regulation of FMBP-1 on *fib-H* transcription, we overexpressed FMBP-1 in BmE cells, and then analyzed its effect on *fib-H* promoter activity. Compared to luciferase activity when RFP was overexpressed as a control, FMBP-1 overexpression significantly reduced the luciferase activity under the control of *fib-H* promoter (Fig. 2B), suggesting that FMBP-1 repressed *fib-H* promoter activity.

3.4. FMBP-1 directly bound to the negative regulatory −130 element in fib-H promoter

A previous study reported the binding of FMBP-1 to the −130 element upstream of *fib-H* promoter (Takiya et al., 1997). To verify FMBP-1-mediated repression on *fib-H* transcription, we performed an EMSA experiment using FMBP-1 and the synthesized −130 probes. The results showed that in the absence of FMBP-1, only the synthesized DNA probe was observed (Fig. 2C, lane 1). As FMBP-1 increased, lagging strips appeared and became increasingly obvious (Fig. 2C, lanes 2–4), indicating the direct binding of FMBP-1 and the −130 element. In the presence of increasing competitive cold probe, the lagging strips gradually weakened (Fig. 2C, lanes 5–7), suggesting that binding of FMBP-1 and the −130 element could be competitively suppressed by adding unlabeled cold probe. Furthermore, the addition of increasing anti-FMBP-1 antibody resulted in the disappearance of the previous lagging strip and the appearance of a super-shift lagging strip (Fig. 2C, lanes 8–10). These results suggest FMBP-1 directly binds to the −130 element upstream of *fib-H* promoter.

To reveal the role of −130 element on *fib-H* promoter activity, we ligated the −130 element to *ie2* promoter and co-transfected this sequence along with FMBP-1 into BmE cells. The results showed that the relative luciferase activity of −130-*ie2*-luc was significantly lower than that of *ie2*-luc (Fig. 2D), indicating the −130 element is a negative regulatory element.

3.5. Identification of the interaction of FMBP-1 with Bmsage and Bmdimm

Bmdimm activates *fib-H* transcription by binding to the E-box element (−432 ~ −407) of *fib-H* promoter and interacting with Bmsage. The −130 element (−152 ~ −125) is downstream of the E-box

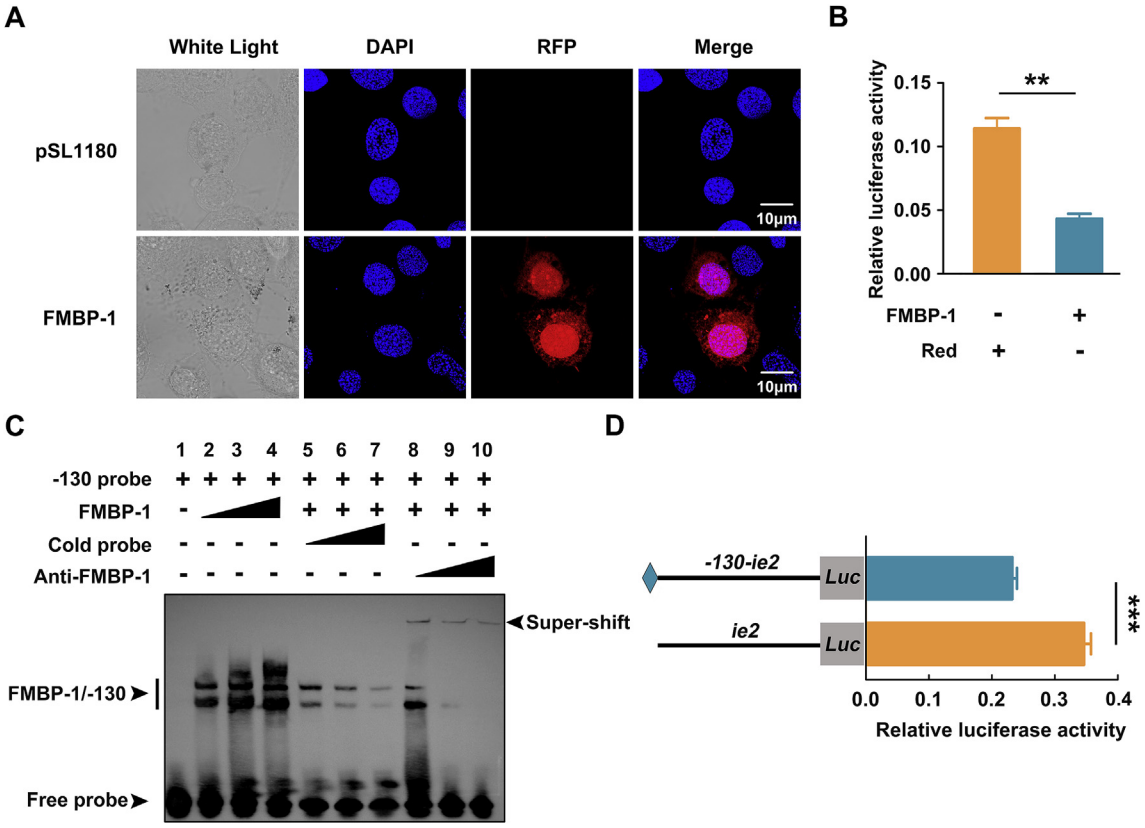

Fig. 2. FMBP-1 repressed *fib-H* promoter activity by binding to the -130 element. A. Cellular localization of FMBP-1 in BmE cells. The cells were fixed in the medium containing DAPI at 48 h post transfection and photographed on a Fv1000 confocal microscope. B. A luciferase assay using a co-transfection of three recombinant plasmids into BmE cells. The *fib-H* and *ie1* promoters were inserted into the firefly luciferase reporter plasmid, which was named as FHP-Exon and pRL-*ie1*-SV40, respectively. FHP-Exon (0.5 μg), pRL-*ie1*-SV40 (0.01 μg) and FMBP-1180 or Red-1180 (0.5 μg) were co-transfected into BmE cells. Red-1180 was used as an irrelevant complementary plasmid for control. C. EMSA of FMBP-1 with −130 element of *fib-H* promoter. The amounts of FMBP-1 in lanes 2–4 were 0.5 μg, 1 μg and 2 μg, respectively. The amounts of cold probe in lanes 5–7 were 250 nm (1X), 6.25 mM (25X) and 12.5 mM (50X), respectively. The amounts of anti-FMBP-1 in lanes 8–10 were 0.5 μg, 1 μg and 2 μg, respectively. D. Effect of FMBP1 on the activity of −130-ie2 promoter. Luciferase assay was performed at 48 h post-transfection of FMBP-1180, pRL-*ie1*-SV40 and *-130-ie2* or *ie2* into BmE cells. Red-1180 plasmid was used as a control. The relative luciferase activity is presented as a ratio of firefly luciferase activity to renilla luciferase activity. Data from three independent experiments are shown as the mean ± S.E. **p < 0.01; ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

element (Fig. 3A). Therefore, we investigated the possible interaction between FMBP-1, Bmdimm, and Bmsage. We prepared nuclear extracts of the posterior silk gland from silkworm larvae at the fourth molting stage, and then captured FMBP-1 and its interacting partner from the nuclear extracts using anti-FMBP-1 antibody. SDS-PAGE showed two bands with molecular sizes comparable to those of Bmsage and Bmdimm (Fig. 3B). Further, we identified the hypothetical strips on SDS-PAGE with specific Bmsage and Bmdimm antibodies (Fig. 3C), which demonstrated that the proteins captured by anti-FMBP-1 antibody contained Bmsage and Bmdimm.

We then investigated the expression profiles of *Bmsage* and *Bmdimm* in the posterior silk gland of silkworm larvae from day 0 of the fourth instar to day 0 of the fifth instar. The expression profile of *Bmsage* was consistent with that of *FMBP-1* (Fig. 3D), whereas the expression profile of *Bmdimm* contrasted that of *FMBP-1* (Fig. 3E). These results indicate the interactions between FMBP-1, Bmsage and Bmdimm in the posterior silk gland of silkworm larvae during the fourth molting period.

To confirm the interaction of FMBP-1, Bmsage and Bmdimm, far western blotting was performed. Two obvious bands in the lanes of Bmsage and Bmdimm were detected by anti-FMBP-1 antibody, whereas no band appeared in the control lane of BSA (Fig. 4A), indicating the specific interaction of FMBP-1 with Bmsage and Bmdimm. To determine the dissociation constant (K_D) of FMBP-1 for Bmsage and Bmdimm, we performed microscale thermophoresis (MST) experiments. The fluorescence of FITC-labeled Bmsage/Bmdimm increased with increasing concentrations of FMBP-1 (Fig. 4B and C), indicating that FMBP-1 binds to Bmsage and Bmdimm directly in solution, which is consistent with the results of far western blotting and co-IP. The K_D values of FMBP-1/Bmsage and FMBP-1/Bmdimm were calculated to be 6930 ± 347 nM and 1730 ± 170 nM, respectively.

3.6. Essential domains for interaction of FMBP-1with Bmsage and Bmdimm

FMBP-1 contains an AP2 domain at the N-terminus and STPR domain at the C-terminus (Fig. 5A). To determine the essential domain of FMBP-1 for the interactions with Bmsage and Bmdimm, we expressed and purified different truncated variants of FMBP-1 from *E. coli* (Fig. 5B), and then investigated the interactions of these variants with Bmsage and Bmdimm by far western blotting. The result showed Bmsage and Bmdimm bound to multiple variants of FMBP-1, except the STPR domain (Fig. 5C and D), suggesting that the sequences at both ends of STPR domain are essential for the interaction of FMBP-1 with Bmsage and Bmdimm.

3.7. FMBP-1 interacted with Bmsage and Bmdimm in BmE cells

To further determine whether FMBP-1 binds to Bmsage and Bmdimm in cells, we constructed overexpression vectors of Flag-FMBP-1, Myc-Bmsage and Myc-Bmdimm, co-transfected these vectors into BmE cells, and then measured the interactions of these factors by Co-IP.

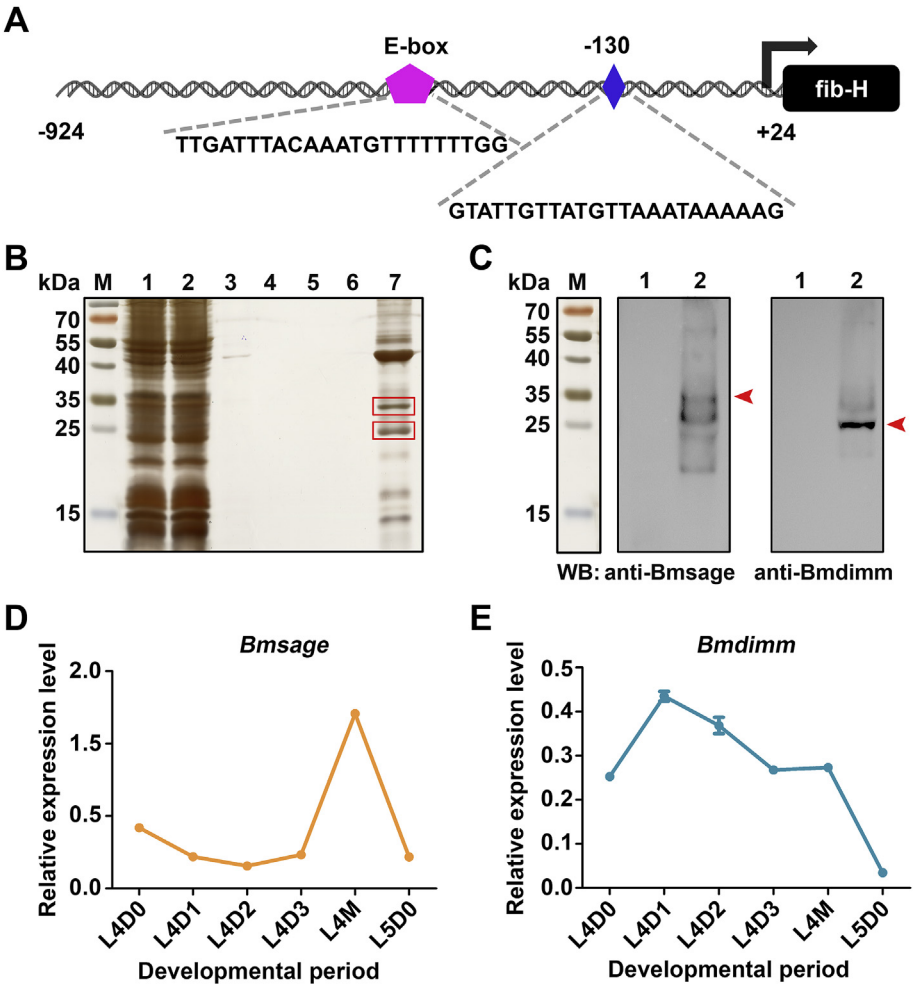

Fig. 3. Identification of the interacting proteins of FMBP-1 from the nuclear extracts of posterior silk gland cells from silkworm larvae on day 3 of the fourth instar. A. The cis-regulatory elements Ebox and −130 associated with FMBP-1 and Bmdimm in the *fib-H* promoter region, respectively. B. Silver staining of co-precipitated products by SDS-PAGE. Lane 1, the nuclear extracts of posterior silk gland cells from silkworm larvae on day 3 of the fourth instar; lane 2, the supernatant of previous nuclear extracts after precipitation by anti-FMBP-1 antibody; lanes 3–6, PBS eluent from protein agarose A/G beads; lane 7, the co-precipitated products by anti-FMBP-1 antibody. C. Western blotting of the co-precipitated products. Lane 1, IgG; Lane 2, the co-precipitated products by anti-FMBP-1 antibody. D-E. The expression profiles of *Bmsage* (D) and *Bmdimm* (E) in the posterior silk gland of silkworm larvae from day 0 of the fourth instar to day 0 of the fifth instar by qRT-PCR. *GAPDH* was used as a control.

The results showed that Bmsage was present in the anti-FMBP-1 precipitates (Fig. 6A–a), while FMBP-1 was detected in the anti-Bmsage precipitates (Fig. 6A and b). Similarly, Bmdimm was present in the anti-FMBP-1 precipitates (Fig. 6B–a) and FMBP-1 was present in the anti-Bmdimm precipitates (Fig. 6B–b).

We also conducted BiFC assay to better visualize the interactions of FMBP-1 with Bmsage and Bmdimm in cells. The results showed that BmE cells emitted green fluorescence when FMBP-1 and Bmdimm were co-expressed in the cells, while no fluorescence was observed in the control. We also observed green fluorescence in the cells co-expressing FMBP-1 and Bmsage or Bmsage and Bmdimm, respectively (Fig. 6C). These results demonstrate that FMBP-1 interacted with Bmsage and Bmdimm in the cell nucleus.

3.8. *FMBP-1 repressed fib-H transcription by interacting with Bmdimm and Bmsage*

Bmdimm promotes *fib-H* transcription by binding to the E-box element in *fib-H* promoter and interacting with Bmsage (Zhao et al., 2015). Our results showed that FMBP-1 repressed *fib-H* transcription by

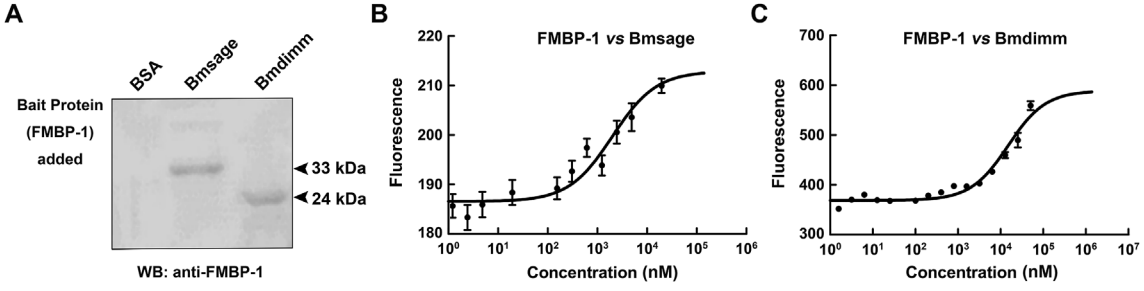

Fig. 4. Interaction analysis of FMBP-1 with Bmsage and Bmdimm *in vitro*. A. Far western blotting. Bmsage and Bmdimm were separated by SDS-PAGE, transferred onto PVDF membranes, denatured, and then incubated with FMBP-1 after renaturation. Western blotting was performed with anti-FMBP-1 antibody. BSA served as a negative control. B-C. Interaction analysis of FMBP-1 with Bmsage (B) and Bmdimm (C) by MST. Bmsage and Bmdimm were labelled with FITC, respectively, and exchanged into TBST buffer to a final concentration of 10 μM. FMBP-1 (1 mg/mL, 40 μM) was prepared through a 16 step dilution into TBST buffer, and then mixed with the labelled Bmsage or Bmdimm (10 μL, 100 nM). The mixture (20 μL) was loaded into NT hydrophobic capillaries for MST measurement. The curve was fitted by the NTAnalysis program. In this experiment, the concentrations of Bmsage and Bmdimm were fixed (50 nM), the initial FMBP-1 concentration was 10 μM, and FMBP-1 was diluted in 16 steps by gradient. Each experiment was repeated three times.

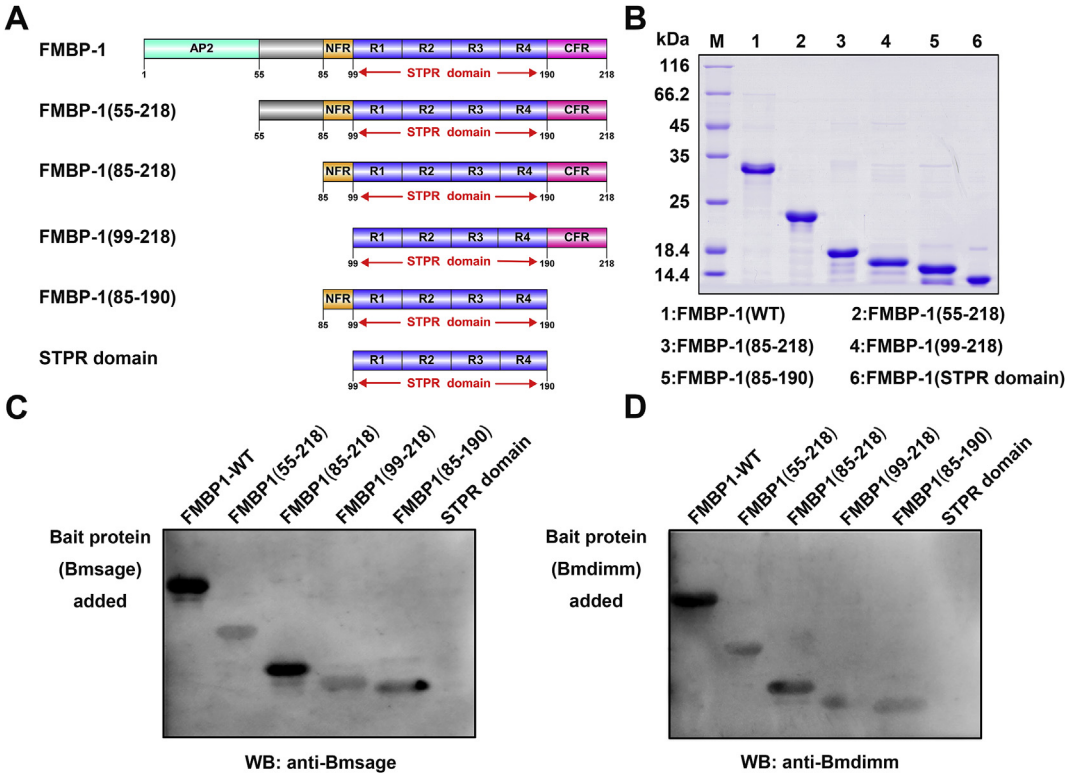

Fig. 5. Essential domains for FMBP-1 binding with Bmsage and Bmdimm. A. Schematic diagram of different truncated variants of FMBP-1. B. SDS-PAGE of different FMBP-1 variants. Far western blotting analysis of the interactions between FMBP-1 variants and (C) Bmsage and (D) Bmdimm. FMBP-1 variants were separated by SDS-PAGE, transferred onto PVDF membranes, and incubated with Bmsage and Bmdimm after denaturation and renaturation. Western blotting was performed with anti-Bmsage (C) and anti-Bmdimm (D) antibody.

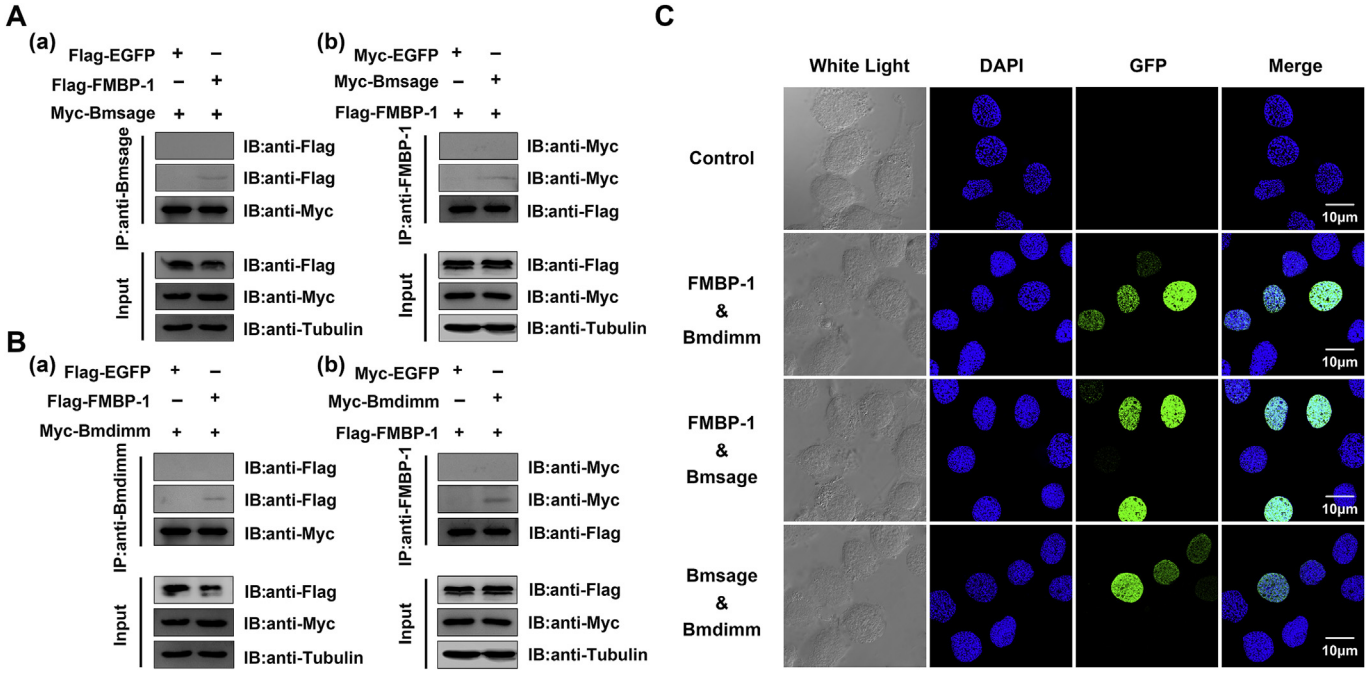

Fig. 6. Interaction analysis of FMBP-1 with Bmsage and Bmdimm at the cellular level. A-B. Co-IP assay of the interactions of FMBP-1 and Bmsage (A), Bmdimm (B). Tubulin was used as a control. The cellular nucleus extractions were obtained from BmE cells at 48 h post-transfection by NE-PER nuclear and cytoplasmic extraction reagent kit. The interactions of FMBP-1 and Bmsage or Bmdimm were detected by bidirectional co-immunoprecipitation using anti-Flag and immunoblotting with the reciprocal anti-Myc antibody or anti-Myc antibody and immunoblotting with the reciprocal anti-Flag antibody. C. BiFC assay of the interactions of FMBP-1, Bmsage, and Bmdimm. BiFC assay was performed by co-transfecting FMBP-1-pie2nVW, Bmsage-pie2cVW, or FMBP-1-pie2nVW and Bmdimm-pie2cVW into BmE cells. FMBP-1-pie2nVW and pie2cVW were co-transfected as a control.

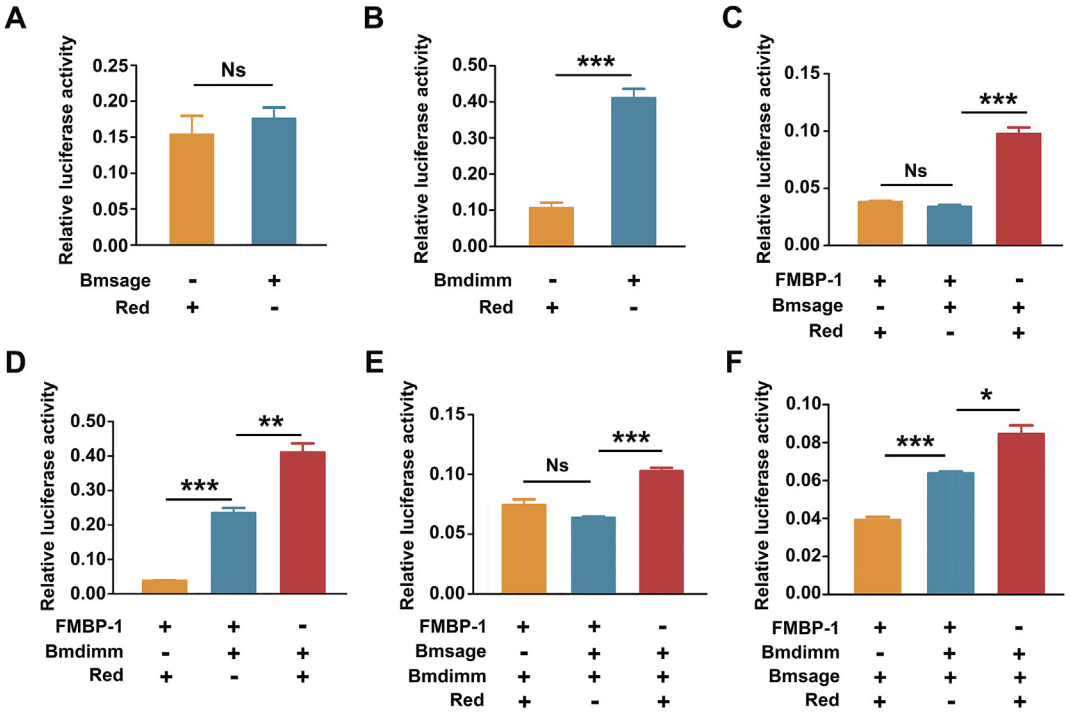

Fig. 7. Luciferase reporter assay of the interactions of FMBP-1, Bmsage, and Bmdimm on *fib-H* promoter activity in BmE cells. The relative luciferase activity of *fib-H* promoter under overexpression of Bmsage (A), Bmdimm (B), co-expression of FMBP-1 and Bmsage (C), and co-expression of FMBP-1 and Bmdimm (D), co-expression of FMBP-1, Bmsage and Bmdimm (E–F). Overexpression of FMBP-1, Bmsage, and Bmdimm was detected by western blotting. Tubulin was used as a control. All experiments were performed in three biological replicates. The relative luciferase activity is represented as the mean ± S.E. *p < 0.05; **p < 0.01; ***p < 0.001; Ns, no significant difference.

binding to the negative regulatory −130 element and interacted with Bmsage and Bmdimm. To reveal how FMBP-1, Bmsage, and Bmdimm regulate *fib-H* transcription during the fourth molting of silkworm larvae, we performed dual luciferase reporter assays. Bmsage alone did not affect *fib-H* promoter activity (Fig. 7A), whereas Bmdimm significantly activated *fib-H* promoter activity (Fig. 7B), which is consistent with the previous report (Zhao et al., 2015). FMBP-1 not only significantly suppressed the activity of *fib-H* promoter in BmE cells (Fig. 7C), but also suppressed Bmdimm-mediated activation on *fib-H* promoter activity (Fig. 7D). Bmsage did not affect the repression of FMBP1 on *fib-H* promoter activity in both cases (Fig. 7E and F).

To determine whether the interactions of FMBP-1, Bmsage and Bmdimm affected their effects on the corresponding regulatory elements in *fib-H* promoter, we ligated E-box and −130 elements to *ie2* promoter, and then co-transfected the cells with FMBP-1, Bmsage, and Bmdimm to measure the luciferase activity of the constructed promoters. The results showed Bmdimm activated the promoter activity of E-box-*ie2*, and FMBP-1 repressed the activation of Bmdimm on E-box-*ie2* promoter activity (Fig. 8A). Bmsage did not affect the repression of FMBP-1 on Bmdimm activation of E-box-*ie2* promoter (Fig. 8B). Similarly, FMBP-1 repressed the promoter activity of −130-*ie2*, and Bmdimm and Bmsage did not affect the repression of FMBP-1 on −130-*ie2* promoter (Fig. 8C and D).

4. Discussion

The genes encoding silk proteins are expressed during intermolting, and repressed during molting of *Bombyx mori* (Ishikawa and Suzuki, 1985; Maekawa and Suzuki, 1980; Suzuki and Suzuki, 1974; Tripoulas and Samols, 1986), with *fibroin* genes specifically expressed in the posterior silk gland of silkworm larvae (Maekawa and Suzuki, 1980; Suzuki and Brown, 1972; Suzuki and Giza, 1976; Suzuki and Suzuki, 1974; Takiya et al., 1990). *Fib-H* expression is selectively switched on and off by transcription factors during silkworm development. The genes responsible for *fib-H* transcription are switched on and off at specific stages (Maekawa and Suzuki, 1980). Multiple transcription factors are involved in activating *fib-H* transcription during intermolting (Kimoto et al., 2010; Liu et al., 2016a; Zhao et al., 2014). However, the precise mechanism associated with *fib-H* transcriptional repression during molting remains unclear. In this study, we revealed the mechanism of *fib-H* transcriptional repression by FMBP-1 during the fourth molting of silkworm larvae. Unlike FMBP-1, BmFTZ-F1 represses *fib-H* transcription during the early stage of fifth instar larvae (Zhou et al., 2016). Thus, studying *fib-H* transcriptional repression during molting will provide more information on the mechanism associated with silk protein synthesis.

Compared to other transcription factors regulating silk protein genes transcription, FMBP-1 is a ubiquitous factor (Fig. 1) and contains a unique DNA-binding domain STPR at its C-terminus (Saito et al., 2007; Takiya et al., 2009). The difference between FMBP-1 and other DNA-binding factors is the interacting patterns of DNA and protein. The STPR domain binds to DNA in a specific conformation. So far, little is known about the biological function of the factors including STPR domain from *Caenorhabditis elegans* to humans. Although the structure of STPR and its specific interaction with DNA have been documented (Nonaka et al., 2010; Saito et al., 2008; Takiya et al.; 2005, 2009; Yu et al., 2016), the role of FMBP-1 on *fib-H* transcription remains unclear. Here, we found that FMBP-1, a well-known transcriptional factor containing STPR domain, repressed *fib-H* transcription during the fourth molting of silkworm larvae, and further revealed the mechanism of FMBP-1 on *fib-H* transcriptional repression.

The expression profile of *FMBP-1* was opposite to that of *fib-H*, particularly during the fourth molting stage of *Bombyx mori* (Fig. 1). FMBP-1 was found in the cell nucleus and specifically bound to the −130 element to reduce *fib-H* promoter activity (Fig. 2). The results indicate that FMBP-1 represses *fib-H* transcription during molting. In eukaryotes, gene transcription is a delicate and complex process. Multiple factors, including BmFkh/SGF-1, SGF-2, POU-M1/SGF-3, SGF-4,

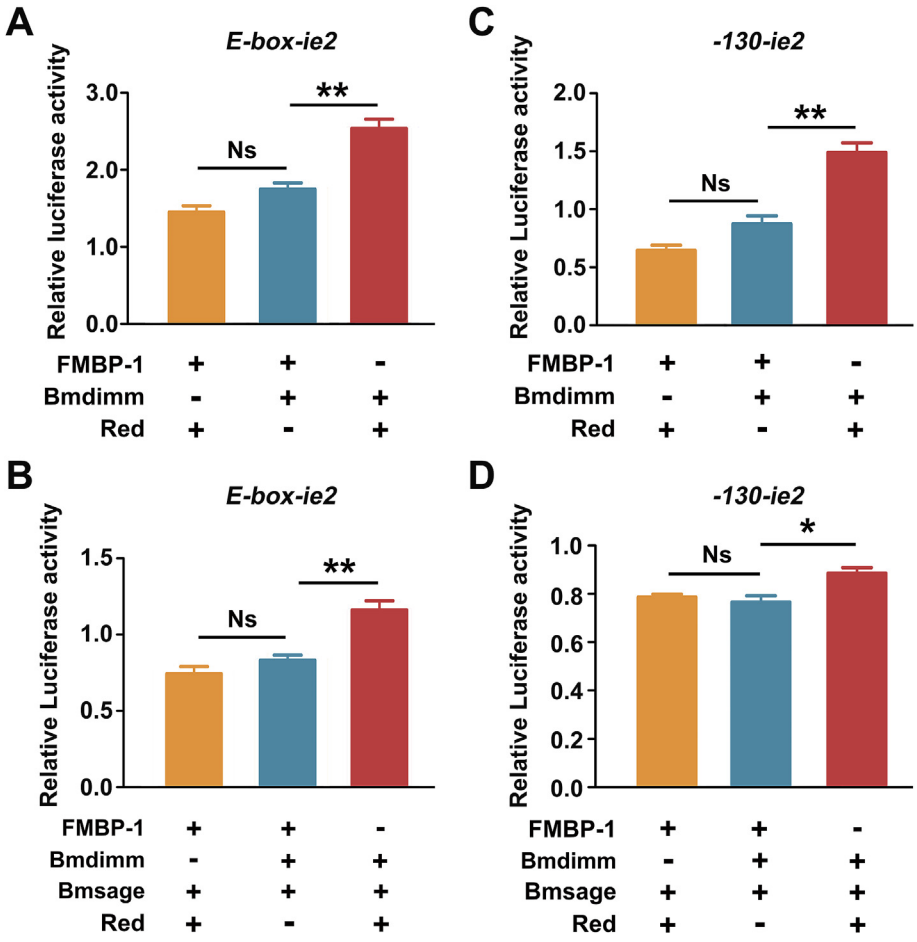

Fig. 8. Luciferase reporter assay of the interactions of FMBP-1, Bmsage, and Bmdimm on the effect of E-box and -130 elements in BmE cells, respectively. The relative luciferase activity of *E-box-ie2* promoter under co-expression of FMBP-1 and Bmdimm (A), or co-expression of FMBP-1, Bmsage and Bmdimm (B). The relative luciferase activity of *-130-ie2* promoter under co-expression of FMBP-1 and Bmdimm (C), or co-expression of FMBP-1, Bmsage and Bmdimm (D). Overexpression of FMBP-1, Bmsage, and Bmdimm were detected by western blotting. Tubulin served as a control. All experiments were performed in triplicate. The relative luciferase activity is shown as the mean ± S.E. *p < 0.05; **p < 0.01; Ns, no significant difference.

FBF-A1, FMBP-1, BmFTZ-F1, Bmsage, and Bmdimm (Hui et al., 1990; Kimoto et al., 2012; Mach et al., 1995; Ohno et al., 2013; Takiya et al., 1997; Tsuda and Suzuki, 1983; Xu et al., 1994; Zhao et al., 2014, 2015; Zhou et al., 2016), are involved in *fib-H* transcriptional regulation at specific stages (Maekawa and Suzuki, 1980). Therefore, studying silk protein genes transcriptional regulation is important for understanding the transcriptional regulation of eukaryotic genes. Some transcription factors require the formation of functional dimers prior to binding DNA (Amoutzias et al., 2008; Smale, 2012). Dimer formation occurs either before or after DNA binding (Kohler and Schepartz, 2001). bHLH transcription factors play important roles in eukaryote development, such as sex determination, cardiogenesis, myogenesis, neurogenesis, and hematopoiesis (Abrams et al., 2006; Fox et al., 2013; Ohtsuka et al., 2001; Park et al., 2008; Qian et al., 2000), and mediate DNA binding and dimerization *via* the highly conserved HLH domain (Amoutzias et al., 2008). Bmsage and Bmdimm are members of the bHLH family, and specifically expressed in the silk gland of silkworm. Bmdimm binds to the E-box element to activate *fib-H* transcription during intermolting, whereas Bmsage does not. In this study, we found that FMBP-1 binds to Bmsage and Bmdimm in the posterior silk gland of silkworm larvae (Figs. 3–6). Further, we confirmed the combinatorial effect of FMBP-1, Bmsage, and Bmdimm on *fib-H* transcription in a luciferase reporter assay (Fig. 7). These results suggest that the interaction of FMBP-1, Bmsage, and Bmdimm regulates *fib-H* transcription during the fourth molting stage of silkworm larvae.

Co-expression of Bmsage and FMBP-1 did not affect *fib-H* promoter activity compared to FMBP-1 alone (Fig. 7C), indicating that the interaction between Bmsage and FMBP-1 did not affect FMBP-1 regulation of *fib-H* transcription. However, co-expression of Bmdimm and FMBP-1 reduced the activating effect of Bmdimm alone on the *fib-H* promoter (Fig. 7D), indicating the interaction of Bmdimm and FMBP-1 affected both FMBP-1 and Bmdimm regulation on *fib-H* transcription. Although Bmsage bound to Bmdimm and FMBP-1, it did not affect *fib-H* promoter activity in the presence of Bmdimm and FMBP-1 (Fig. 7E). In the presence of Bmsage, the interaction of FMBP-1 and Bmdimm antagonized each other to affect *fib-H* promoter activity. Additionally, the interaction of Bmsage and Bmdimm activated *fib-H* promoter activity in the presence of FMBP-1 (Fig. 7F), which is consistent with the results of a previous report (Zhao et al., 2015). We found that E-box-*ie2* promoter activity in the presence of FMBP-1 and Bmdimm was significantly lower than that in the presence of Bmdimm alone (Fig. 8A). Similarly, co-expression of FMBP-1, Bmsage, and Bmdimm reduced the promoter activity of E-box-*ie2* compared to that in the presence of Bmsage and Bmdimm (Fig. 8B). These results suggest that the interaction of FMBP-1 and Bmdimm interfered the activation of Bmdimm on E-box-*ie2* promoter activity by binding to the E-box element, resulting in reduced E-box-*ie2* promoter activity. The promoter activity of −130-*ie2* in the presence of FMBP-1 or FMBP-1/Bmsage was not affected regardless of the presence of Bmdimm (Fig. 8C and D), indicating that the interaction of FMBP-1 and Bmdimm did not interfere the effect of FMBP-1 on −130-*ie2* promoter activity.

In summary, we propose the following regulatory mechanisms associated with FMBP-1-mediated repression on *fib-H* transcription during molting of silkworm larvae (Fig. 9): 1) FMBP-1 directly binds to the −130 element in *fib-H* promoter to repress *fib-H* transcription; 2) FMBP-1 interacts with Bmdimm to antagonize the activation of Bmdimm on *fib-H* transcription. To further elucidate the transcriptional regulation of *fib-H* mediated by multiple factors, additional studies are still needed. Notably, we only focused on the repression of FMBP-1 on *fib-H* transcription during the fourth molting of silkworm larvae. During

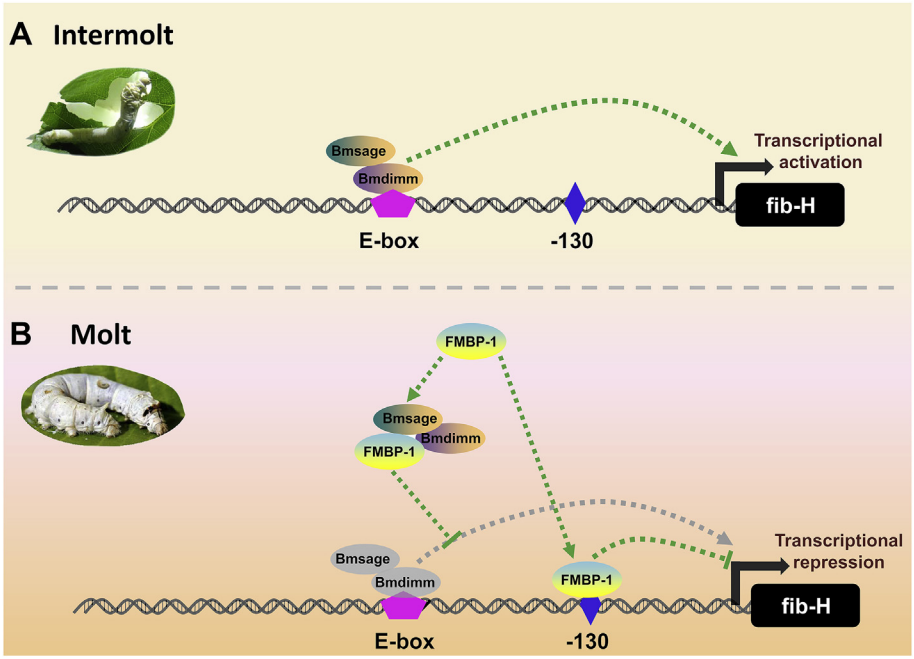

Fig. 9. Schematic diagram of the transcriptional repression of *fib-H* mediated by FMBP-1 during molting of *Bombyx mori*. (A) Bmdimm binds to the E-box element in *fib-H* promoter and interacts with Bmsage to activate the transcription of *fib-H* during the fourth intermolting period of silkworm larvae; (B) FMBP-1 binds to the −130 element and interacts with Bmsage and Bmdimm to repress *fib-H* transcription during the fourth molting period of silkworm larvae. Intermolt and molt represent the fourth feeding and molting period, respectively.

other developmental stage, the transcriptional regulation of *fib-H* and its relationship with FMBP-1 require further analysis. Our results promote a better understanding of *fib-H* transcriptional regulation and provide novel insight into the transcriptional repression of *fib-H* mediated by FMBP-1 and bHLH factors during molting. Our study will also be beneficial for functional exploration of factors containing STPR domain.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.12.004.

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