Bmmp influences wing shape by regulating anterior-posterior and proximal-distal axis development

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Abstract

Insect wings are subject to strong selective pressure, resulting in the evolution of remarkably diverse wing shapes that largely determine flight capacity. However, the genetic basis and regulatory mechanisms underlying wing shape development are not well understood. The silkworm *Bombyx mori* *micropterous* (*mp*) mutant exhibits shortened wing length and enlarged vein spacings, albeit without changes in total wing area. Thus, the *mp* mutant comprises a valuable genetic resource for studying wing shape development. In this study, we used molecular mapping to identify the gene responsible for the *mp* phenotype and designated it *Bmmp*. Phenotype-causing mutations were identified as indels and single nucleotide polymorphisms in non-coding regions. These mutations resulted in decreased *Bmmp* mRNA levels and changes in transcript isoform composition. *Bmmp* null mutants were generated by CRISPR/Cas9 and exhibited significantly smaller wings. By examining the expression of genes critical to wing development in wildtype and *Bmmp* null mutants, we found that *Bmmp* exerts its function by coordinately modulating anterior-posterior and proximal-distal axis development. We also studied a *Drosophila* *mp* mutant and found that *Bmmp* is
functionally conserved in *Drosophila*. The *Drosophila mp* mutant strain exhibits curly wings of reduced size and a complete loss of flight capacity. Our results increase our understanding of the mechanisms underpinning insect wing development and reveal potential targets for pest control.

Keywords: *Bmmp*; wing shape; silkworm; CRISPR/Cas9
Introduction

Wings endow insects with tremendous adaptive advantages because they enhance survival and fitness by making it possible to change environments rapidly. Insect wings are constantly subject to adaptive evolution and exhibit remarkable diversity in shape. Changes in wing shape result in differences in flight capacity, leading to variations in insect lifestyle [1, 2]. For example, dimorphism in wing shape occurs in a wide range in insects, such as rice planthoppers [3, 4] and aphids [5]. Long-winged morphs can fly, which allows them to escape adverse habitats and track changing resources, whereas short-winged morphs are flightless, but usually possess higher fecundity [1, 2]. In the order Lepidoptera, wing shapes are distinctly different between migratory species and non-migratory species. Typically, migratory moths and butterflies have relatively narrower forewings with straighter costal margins compared to those of non-migratory species [6].

Coordinated regulation of anterior-posterior (A-P) and proximal-distal (P-D) wing axis development plays a crucial role in correct wing shape formation. During this process, wing patterning and proliferation are coordinately modulated by relay signals [7]. For A-P axis development, posterior compartment identity is specified by the *engrailed* gene, which then activates the expression of *hedgehog* [7]. The secreted Hedgehog protein traverses the A-P border and induces expression of *Dpp* and *Wnt1* in anterior cells close to the border [8]. During the process of wing P-D axis development, *apterous* is expressed in the dorsal compartment and activates Notch signaling, which in turn induces *Wnt1* activity at the dorsal-ventral (D-V) border [9, 10]. Wnt1 helps
establish the P-D axis of the wing by activating the *Distal-less* gene, which specifies
the most distal regions of the wing [11, 12]. Reduced levels of Dpp affect both the width
and length of the resulting wing and significantly decrease total wing area [13].
Moderate and uniform amounts of exogenous Wnt1 stimulate proliferative wing growth,
leading to enlargement of the prospective wing [14].

The identification of new factors that influence wing shape will expand our
understanding of the genetic basis of wing diversity. We hypothesized that as-yet
uncharacterized key regulators coordinately regulate both A-P and P-D axis signals
during wing development. To examine this developmental process more closely, we
used the silkworm *Bombyx mori* (Lepidoptera, Bombycidae) *micropterous (mp)* mutant,
which exhibits shortened wing length and enlarged vein spacings. We identified the
gene responsible for the *mp* phenotype and designated it *Bmmp*. We found mutations in
the noncoding regions of *Bmmp* that result in decreased *Bmmp* mRNA levels and
changes in transcript isoform composition. In addition, we generated a *Bmmp* null
mutant and determined that *Bmmp* exerts its effect on wing shape by regulating wing
A-P and P-D axis development.

**Results**

**Characterization of the silkworm *micropterous (mp)* mutant wing phenotype**

To characterize the wing phenotype of the silkworm *mp* mutant, we compared
pupae and moth wing phenotypes of mp and Dazao wildtype (WT) silkworms. Whereas the wings of WT pupae fully cover the third abdominal segment, the wings of mp pupae only cover the second abdominal segment, leaving the third abdominal segment naked (Fig 1A). Further examination demonstrated that the wing length of mp moths was significantly shorter than that of WT moths within each sex, although there was not a significant difference in total wing area (Fig 1B, 1C, 1D). In addition, there was significantly greater spacing between adjacent longitudinal veins in the wings of mp moths compared to those of WT moths within each sex (Fig 1E). These results demonstrate that the mp phenotype is not associated with a specific gender. To reflect the overall changes in wing morphology, we divided the wing length by the sum of longitudinal veins spacings. The resulting value is significantly smaller for mp moths than for WT moths within each sex (Fig 1F).

**Molecular mapping and analysis of candidate genes responsible for the mp phenotype**

To identify candidate gene(s) responsible for the mp phenotype, we performed a genetic linkage analysis using B. mori simple sequence repeat (SSR) markers and newly designed markers polymorphic between WT and mp silkworms. Initially, we roughly mapped the mp phenotype using 456 BC1M individuals and SSR markers on the eleventh linkage group. The results indicate that the gene responsible for the mp phenotype is located within a 12.1-cM region linked to SSR marker S1146 (Fig 2A and 2B). Subsequent fine mapping with 320 BC1M and newly designed primer sets
narrowed the mp locus to an approximately 260-kb region between markers 2810A and 2810C on the nscaf2810 scaffold. The 2810M marker was tightly linked with the mp locus (Fig 2C). Two candidate genes (KWMTBOMO06923 and KWMTBOMO06924) were identified within the 260 kb region, based on annotated sequences obtained from the SilkBase database [15] (Fig 2D).

Because the functions of KWMTBOMO06923 and KWMTBOMO06924 are uncharacterized, we searched for mutations responsible for the mp phenotype by comparing the corresponding genomic sequences from mp silkworms and silkworms with normal wings. Although synonymous single nucleotide polymorphisms (SNPs) mutations were identified in the mp KWMTBOMO06923 and KWMTBOMO06924 genes, no mutations that changed the sequences of the predicted translated proteins were found. We next surveyed all introns within KWMTBOMO06924, as well as putative regulatory regions 2 kb upstream and downstream from the gene. A total of 59 indels and 101 SNPs specific to the mp mutant were identified. (Table S1 and Table S2).

Multiple transcript isoforms of KWMTBOMO06924 are annotated in the B. mori EST database (http://sgp.dna.affrc.go.jp/KAIKObase/). To obtain more detailed isoform information, we generated and sequenced KWMTBOMO06924 cDNA libraries. Sequence alignments revealed that the KWMTBOMO06924 gene is comprised of 10 exons, spanning 36.79 kb of genomic DNA. A total of 28 KWMTBOMO06924 transcript isoforms were identified in WT wing discs. The full-length cDNA sequence contained a 1443-bp open reading frame encoding 481 amino acids, consistent with the
cDNA clone (fwd-02K11) retrieved from the *B. mori* EST database. The protein encoded by the full-length transcript isoform contains three functional domains (BTB, BACK and TLDc) as determined using the SMART online prediction tool. We next examined *KWMTBOMO06924* transcript isoform composition and expression in wing discs from *mp* and WT silkworms. Of the 28 transcript isoforms detected in the WT strain, only 6 were recovered in the *mp* mutant. In addition, one unique transcript was identified in the *mp* silkworms (Fig 3). An intact BTB domain, encoded by exons 1-3, was present in all transcript isoforms identified in both silkworm strains. Quantitative RT-PCR analysis of wing discs from silkworms at the initiation of the wandering stage revealed that total *KWMTBOMO06924* mRNA levels were significantly lower in *mp* vs. WT silkworms during this critical period of wing development (Fig 4).

Together, these results suggest that *KWMTBOMO06924* is responsible for the *mp* phenotype, with causative mutations localized to regulatory regions. Thus, we designated this gene *Bmmp*. The results are consistent with two possible causes for the *mp* phenotype: (1) decreased total *Bmmp* mRNA levels, and (2) reduced variation of *Bmmp* transcript isoforms.

**Null mutation of *Bmmp* results in a significant reduction in wing size**

To elucidate the function of the *Bmmp* gene in wing development and morphology, we utilized the CRISPR/Cas9 system to disrupt *Bmmp*. We selected four genomic targets spanning 130 bp in exon 1 to generate large fragment deletions (Fig 5A). Since
this region is shared across isoforms, any frame-shift mutations would be predicted to
abolish all functional transcripts. sgRNAs were synthesized in vitro for the genomic
targets, mixed with Cas9 protein, and injected into the preblastoderm of Dazao embryos.
In total, 110 injected embryos hatched, and 81 individuals survived to an adult stage.
Out of these 81 silkworms, 67 exhibited markedly smaller wings in pupal and adult
stages, compared to uninjected WT controls (Fig 5B and 5C). To confirm that the
Bmmp deletions caused the decrease in wing size, genomic DNA was extracted from
three moths with small wings. Regions spanning the four sgRNA targets were amplified
by PCR, subcloned, and sequenced. As expected, the three selected moths contained
Bmmp deletions and no wildtype sequences (Fig S1). Notably, five distinct mutations
were identified in moth #11 (Fig S1), demonstrating the presence of mosaicism in
silkworms of the injected generation (generation 0, G0).
To further confirm the function of Bmmp in a uniform genetic background,
homozygous or compound mutant silkworms were obtained by crossing mosaic
knockouts. We randomly surveyed 3 egg batches of generation 1 (G1). All individuals
surveyed were homozygous or compound mutants (Fig 5D), demonstrating that
germline transmission of the mutations was highly efficient. Compared with the WT
control, homozygous or compound mutant silkworms all exhibited significantly smaller
wings (Fig 5E and 5F), consistent with the phenotype observed in G0 mosaics.
It is noteworthy that we obtained homozygous knockout silkworms with an in-
frame 108 bp deletion in the coding region of exon 1 by crossing G1-mp-24 ♀ with G1-
mp-15 ♂ (Fig 5D). Presumably this mutation disrupts the functional BTB domain
without affecting the downstream BACK and TLDc domains (Fig 5D). However, these knockout silkworms were identical in wing phenotype to silkworms harboring frameshift mutations that presumably cause premature termination and functional loss of all three domains. These results suggest that the BTB domain plays an indispensable role in Bmmp gene function.

Taken together, we conclude that Bmmp plays an important role in wing development and the regulation of wing shape. Loss of function of Bmmp results in significantly smaller wings. In addition, we found that the BTB domain is indispensable for Bmmp function. We next sought to identify the mechanism(s) by which Bmmp regulates wing morphology.

**Bmmp regulates genes responsible for wing A-P and P-D axis development**

The decreased wing size of Bmmp biallelic knockout silkworms reflects decreases in wing width and length along the anterior-posterior (A-P) and proximal-distal (P-D) axes, respectively. To detect potential interactions between Bmmp and other genes involved in wing formation, we used qRT-PCR to investigate the expression of key genes responsible for wing A-P and P-D axis development in wing discs from wandering stage Bmmp knockout silkworms. mRNA levels were significantly decreased in Bmmp knockout homozygous and compound heterozygous silkworms for engrailed, hedgehog, dpp, and gbb, which are responsible for wing A-P axis development (Fig 6). Likewise, mRNA levels in knockouts were reduced for apterous
apterous B, vestigial, Wingless (wnt1), and distal-less, which participate in wing P-D axis development (Fig 6). These results suggest that Bmmp directs wing morphology by regulating genes responsible for wing A-P and P-D axis development.

Bmmp gene function is conserved between silkworms and Drosophila

The orthologous Bmmp gene in Drosophila is CG7102. Downregulation of this gene protects Drosophila from hypoxic tissue injury [16]. However, the functional role for CG7102 in Drosophila wing development is not known. To examine whether the function of these two genes is conserved, we first predicted the functional domains of the CG7102 protein product using the SMART online tool. Like Bmmp, CG7102 is predicted to encode BTB, BACK, and TLDc domains. We obtained a Drosophila mp mutant strain that contains an insertion-associated gene mutation in CG7102 from the Bloomington Drosophila Stock Center. Compared to the Drosophila yw control, the wings of the Drosophila mp mutant are curly and significantly smaller in total wing area, although the size difference between the Drosophila strains is not as severe as for Bmmp knockout and WT silkworms (Fig 7A and 7B). We speculate the milder phenotype may be due to genetic differences as the Drosophila mp mutant contains an intronic transposon insertion in the CG7102 gene, whereas the silkworm Bmmp knockouts we generated disrupt exon 1. Our tests show that the Drosophila mp mutant suffers a complete loss of flight capacity (Video S1), while flight is normal in the WT control (yw).
Discussion

The silkworm mp mutant exhibits shortened wing length and enlarged spacing of adjacent longitudinal veins without a decrease in total wing area. In this study, we identified the gene responsible for the mp phenotype and designated it Bmmp. Two possible causes for the mp phenotype are (1) a significant decrease in total Bmmp mRNA levels, and (2) the reduced diversity of Bmmp transcript isoforms in the wing discs. The changes in Bmmp expression in the silkworm mp mutant are likely to be caused by one or more mutations dispersed in non-coding regions of this gene. However, additional experiments would be required to dissect the effects of each of the non-coding mutations. In contrast, frameshift mutations induced by CRISPR/Cas9 mutagenesis into the constitutive exon 1 coding region of Bmmp resulted in significantly decreased wing length, width, and total wing area.

Alternative splicing is a ubiquitous regulatory mechanism of gene expression in eukaryotic organisms. For example, 90% to 95% of human genes are estimated to undergo alternative splicing [17, 18]. Variable mRNA transcript isoforms are translated into different protein isoforms with diverse functions and/or localizations [19]. In our study, we detected 28 distinct Bmmp transcript isoforms in wing discs of WT silkworms. Our findings suggest that Bmmp exerts its effect on wing development, at least in part, by exploiting diversified transcript isoforms, which give rise to different protein products with varying combinations of functional domains. Bmmp proteins can be categorized into three classes, namely, BTB-BACK-TLDc containing proteins, BTB-
BACK containing proteins, and BTB-only proteins. Our results provide insight into the function of the BTB domain. A homozygous knockout silkworm harboring a 108-bp deletion that only disrupts the BTB domain exhibited the same wing phenotype as knockouts harboring frameshift mutations that presumably cause loss of function for all three domains. These results suggest the BTB domain is essential and indispensable for the function of the Bmmp protein. However, further investigation is needed to fully understand the functions of the BTB, BACK, and TLDc domains.

Pest migration, which depends on strong flight performance, is one of the most significant causes of damage to crops and forests [20]. Wing shape has a significant impact on the flight capacity of insects [1, 2]. Therefore, key genes regulating wing shape development are potential targets for pest control [21, 22]. In this study, we demonstrated that the mp orthologous gene CG7102 is functionally conserved in Drosophila. Furthermore, we found flight capacity is completely lost in the Drosophila mp mutant, in which CG7102 contains a transposon insertion. Since mutations responsible for the mp phenotype compromise flight ability, it may be possible to exploit them in future pest control strategies. For example, if mutants can be released to cross into and reduce the fitness of target populations, the use of broad-spectrum pesticides could be reduced or avoided.

Finally, we demonstrated that null mutations of Bmmp decreased mRNA levels for genes involved in wing A-P axis development, including engrailed, hedgehog, Dpp, and Gbb, as well as genes involved in P-D axis development, including apterous A, apterous B, vestigial, Wnt1, and Distal-less. These results indicate that the Bmmp gene
influences wing shape and size by regulating A-P and P-D axis development.

In summary, our results deepen our understanding of wing development in insects and provide a foundation for the development of insect pest control strategies.

289 Materials and Methods

290 Silkworm and Drosophila strains

Silkworm Dazao (wildtype) and mp mutant strains were obtained from the Silkworm Gene Bank at Southwest University (Chongqing, China). Silkworms were reared on fresh mulberry leaves at 25°C.

A Drosophila melanogaster mp mutant was purchased from the Bloomington Drosophila Stock Center (Stock Number: 80643). Drosophila melanogaster strain yw, with the same genetic background as the Drosophila mp mutant, was obtained from SIBCB Drosophila Library (Shanghai, China) and used as wildtype control in all Drosophila experiments. Drosophila strains were maintained at 25°C with standard corn meal medium.

294 Wing morphological measurements

Wings were dissected from silkworms (Dazao, mp mutant, Bmmp knockout mutant) and Drosophila (yw, mp mutant). Wings were imaged using a Leica DVM6 digital microscope. Wing shape parameters (wing area, wing length, adjacent vein spacings) were measured on 30 male and 30 female WT and mp silkworm moths, 23 female WT and 45 female Bmmp knockouts, and 16 male yw and 13 male mp
Drosophila using ImageJ [23]. Experiments were independently repeated three times.

Positional cloning and molecular mapping

Dazao and mp silkworms served as parental strains to produce F₁ progeny. Due to the lack of recombination in female silkworms, 20 progeny from a single-pair backcross between an F₁ female and a mp male (BC₁F) were used for the linkage analysis and 456 progeny from mp female × F₁ male backcrosses (BC₁M) were used for the recombination analysis. Developing embryos were incubated at 25°C in a humidified atmosphere.

We performed preliminary mapping using published SSR markers [24, 25]. SSR markers on chromosome 11 that were polymorphic between the parental strains were used for linkage and recombination analyses. Linkage analysis was conducted with JOINMAP 4.0 using Kosambi’s mapping function [26]. SSR markers were used as anchor points to develop novel markers based on the silkworm genome sequence (International Silkworm Genome Consortium, 2008) for fine mapping with 320 BC₁M.

Identification of mp-specific SNPs and indels

Silkworm mp mutants were analyzed by whole genome sequencing according to a previously published protocol [27]. To identify mp-specific mutations, data were compared to sequences from 127 domestic and wild silkworm strains with normal wing phenotypes from SilkBase [15] and a previous report [27]. Alignments to reference sequences (released in November 2016 by SilkBase [15]) were performed to identify
SNPs and indels in the mp mutant and the 127 silkworm strains, respectively. SNPs and indels were extracted from genomic sequences surrounding KWMTBOMO06923 and KWMTBOMO06924 in the mp strain and the 127 silkworm strains, and then screened for variations specific to mp mutants using an online Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

**Examination of Bmmp transcript isoforms**

To generate Bmmp cDNA libraries, total RNA was extracted from wing discs of mp and WT silkworms. For each strain, wing disc samples were obtained from silkworms from fifth-instar larvae on days 3, 5, and 7 (5L3D, 5L5D, and 5L7D) and from wandering stage silkworms at 0, 24, and 48 hours (W 0 h, W 24 h, and W 48 h). RNA extractions were performed using the MicroElute Total RNA Kit (OMEGA), and reverse transcription was performed using the PrimeScript RT Reagent Kit (Takara). Equal masses (concentration times volume) of the resulting cDNAs from different developmental stages were mixed and used as the templates for PCR amplification. The PCR primers were F: 5’-AAACTAAAACTTTATTTGAGGTTATG-3’, and R: 5’-AATAATCATCGGACTAAATCACCTT-3’. PCR products were subcloned into pEASY-blunt-zero vectors (TransGen) and sequenced.

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted from wing discs of individual silkworms at the wandering stage using the MicroElute Total RNA Kit (OMEGA) and reverse transcription was
performed using the PrimeScript RT reagent Kit (Takara). qRT-PCR experiments were performed using Hieff SYBR Green Master Mix (YEASEN), according to the manufacturer’s recommended procedure. Silkworms were sampled as follows: N=5 or 6 for Dazao and N=10 for Bmmp knockouts at 24 h of the wandering stage; N=5 or 6 for Dazao and N=5 or 8 for Bmmp knockouts at 48 h of the wandering stage. Three independent replicates were performed for all qRT-PCR experiments. Primer sets are listed in Table S3. Eukaryotic translation initiation factor 4A (silkworm microarray probe ID sw22934) was used as the internal control.

**Bmmp knockout generation**

sgRNAs for CRISPR/Cas9 mutagenesis were designed using the CHOP-CHOP online utility (http://chopchop.cbu.uib.no/). sgRNA target sites are shown in Figure 4A. The DNA template for the T7 promoter used to drive *in vitro* transcription was constructed by PCR as described [28]. Briefly, an oligonucleotide containing the T7 promoter and the sgRNA target sequence (N20) was designed as a forward primer with the sequence 5’-TAATACGACTCACTATAGGG(N20)GTTTAGAGCTAGAATAGC.

The T7 promoter sequence is underlined. The reverse primer was 5’-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTA

TTTAACTTGCTATTCTAGCTCTAAAAC-3’. sgRNA synthesis was performed using a T7 RiboMax Large Scale RNA Production System (Promega) following the manufacturer’s instructions.

The bivoltine silkworm strain Dazao was used to generate *Bmmp* knockout
silkworms. To generate non-diapaused eggs, silkworm eggs were incubated at 15°C until hatching, and the larvae were reared on fresh mulberry leaves at 25°C until wandering stage. Adult moths then oviposited non-diapaused eggs, which were used for microinjection. A mixture of sgRNA and Cas9 protein (Thermo Fisher) was incubated at room temperature for 15 min and microinjected into preblastoderm embryos within 5 h of oviposition. Injected embryos were incubated at 25°C and 80% humidity for approximately 10 days until hatching. Larvae were maintained at 25°C and fed fresh mulberry leaves.

**Identification of Bmmp knockout silkworm genotypes**

Genomic DNA was extracted from the wings of Bmmp knockout silkworms at the adult stage using the TIANamp Genomic DNA Kit (TIANGEN). The DNA was used as a PCR template to amplify regions spanning the genomic targets. Two primer sets were used as follows. F1: 5’-TCGGAGCCGCTTATTAAGTGT-3’ and R1: 5’-CAGAAGATGGTTAAGATGACGTT-3’, and F2: 5’-GGTTGCGTTGGTGTTAG-3’ and R2: 5’-TTATCCTGCCCCAGCTGAGAG-3’. PCR products were subcloned into pEASY-blunt-zero vectors (TransGen), and sequenced.

**Statistical analysis**

All values are presented as means ± SEM or means ± SD, as indicated in figure legends. Student’s t test was used to determine p values.
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Competing interests

The authors have declared that no competing interests exist.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

References


Figure 1. Characterization of wing phenotypes of silkworm mp mutant

(A) Representative photograph of male WT (left) and mp (right) silkworm pupae.

Compared to WT pupae, mp pupae exhibit a naked third abdominal segment in the pupal stage, suggestive of a modified wing phenotype. The phenotypically variable
Female *mp* silkworms exhibit the same phenotype. Scale bar, 5 mm. (B) Representative photograph of female WT (top) and *mp* (bottom) silkworm moth wings showing the differences in shape. Male *mp* silkworms exhibited identical phenotypes. Note that scale hairs were removed from the wing to exhibit wing shape characteristics more clearly. Scale bar, 2.5 mm. (C-F) Wings of both male and female WT and *mp* silkworm moths were measured using ImageJ following imaging with a digital microscope. (C) Within each sex, total wing area of WT and *mp* moths do not differ significantly. (D) Wing length was significantly shorter in *mp* moths compared to WT moths. (E) The spacings between adjacent longitudinal veins were significantly larger in *mp* moths compared to WT moths. Filled columns, WT; unfilled columns, *mp*. Column colors correspond to specific vein spacings indicated by lines of the same color overlayed on the photographed wing. (F) Wing length was divided by the sum of spacings between longitudinal veins to reflect overall wing shape. The resulting values were significantly different between *mp* and WT moths. N = 30 for both male and female *mp* and Dazao silkworm moths. ***, P < 0.01; NS, not significant. *DZ*, Dazao, used as wildtype control; *mp*, silkworm *mp* mutant. Error bars represent SD.
Figure 2. Molecular mapping of candidate genes responsible for the mp phenotype

(A) Map of B. mori chromosome 11 with locations of SSR markers used in this study. Five SSR markers and the mp locus are labeled above the map. Map distances are shown in cM. (B) Schematic of scaffolds on chromosome 11. Gray boxes represent the assembled scaffolds; their respective serial numbers are shown below. S1112 mapped to nscaf3031, whereas S1148, S1109, S1147, and S1146 all mapped to nscaf3034. (C) Expanded view of genomic scaffolds used for fine mapping of the mp locus. Newly designed primer sets are shown on the map, and the numbers above them indicate the respective recombination events in 320 BC1M progeny. The mp locus was tightly linked to 2810M, located between markers 2810A and 2810C. (D) Gene annotation in the mp linked region. Two genes were predicted in this region, namely KWMTBOMO06923 and KWMTBOMO06924.
Figure 3. Transcript isoforms in wing discs from \textit{mp} and WT silkworms

Twenty-eight distinct transcript isoforms were identified in wing discs from WT silkworms. Six of the 28 transcript isoforms identified in WT silkworms were also
recovered in wing discs from *mp* silkworms, plus one unique isoform. Dotted lines indicate transcript isoforms identified in both WT and *mp* silkworms. Exon box colors correspond to the encoded domains indicated above each category. Unfilled regions represent exons and portions of exons not included in the specific mature mRNA transcript shown in the figure. Sizes (nucleotides) of truncated exons are shown below the truncations. DZ, Dazao, used as wildtype control; *mp*, silworm *mp* mutant.
Figure 4. Relative Bmmp mRNA levels in wing discs from WT and mp silkworms

Bmmp mRNA levels in wing discs from WT and mp silkworms were quantified by qRT-PCR. Relative Bmmp mRNA levels were significantly higher in WT silkworms compared to mp silkworms at two different developmental stages (5L7D and W0 h). 5L7D, fifth instar at day 7; W0 h, wandering stage at 0 h; ***, P < 0.001; **, P < 0.01. DZ, Dazao, used as wildtype control; mp, silkworm mp mutant. N=3 for both Dazao and mp silkworms at 5L7D and W0 h, respectively. Values are relative to expression of eukaryotic translation initiation factor 4A (defined as 1). Experiment was independently repeated three times. Error bars represent SEM.

DZ

mp
Figure 5. Construction of Bmmp knockout and phenotypic characterization of mutants

(A) Schematic of Bmmp gene structure and nucleotides targeted for mutagenesis by CRISPR/Cas9. Genomic targets (not including PAM) are shown in underlined text, and PAM sequences are shown in red. Black rectangles and broken lines represent exons and introns, respectively, and are not to scale. (B) Bmmp knockout mosaics (mp KO) in
the injected generation (G₀) exhibited naked third abdominal segments in the pupal
stage, suggestive of a changed wing phenotype. (C) Bmmp knockout mosaics (mp KO)
in G₀ exhibited significantly smaller wing areas by visual examination. Scale bar = 10
mm. (D) Mutant alleles detected by sequencing in nine randomly selected G₁ silkworms.
Red, base insertion; bold, base substitution; small deletions are represented with dashes;
for large deletions, the sizes of the deleted regions are shown in parentheses. (E)
Representative photograph of wings of WT and homozygous or compound
heterozygous Bmmp knockout silkworms. Note that scale hairs were removed from the
wings to exhibit the wing shape characteristics more clearly. Scale bar =2.5 mm (F)
Measurement of total wing areas in WT and homozygous or compound heterozygous
Bmmp knockout silkworms. N=23 for WT and N=45 for Bmmp knockouts. WT,
wildtype control, Dazao; mp KO, homozygous or compound heterozygous Bmmp
knockout silkworms. ***, P < 0.001.
Figure 6. Expression of key genes responsible for wing A-P and P-D axis development in wandering stage silkworms

mRNA levels for *engrailed*, *hedgehog*, *wnt1/wingless*, *dpp*, *gbb*, *apterous A*, *apterous B*, *vestigial*, and *distal-less* were measured by qRT-PCR in wing discs from WT and *mp* silkworms at 24 (W24 h) and 48 hours (W48 h) after initiation of the wandering stage.

NS, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; N=5 or 6 for *Dazao* and N=10 for *Bmmp* knockouts at 24 h; N=5 or 6 for *Dazao* and N=5 or 8 for *Bmmp* knockouts at 48 h. Values are relative to expression of eukaryotic translation initiation factor 4A (defined as 1). Experiments were independently repeated three times. WT, wildtype control, *Dazao*, *mp* KO, *Bmmp* knockout homozygous or compound heterozygous silkworms.
Figure 7. Wing phenotype of the *Drosophila mp* mutant

(A) The *Drosophila mp* mutant exhibited curly wings as compared with the WT control (*yw*) by visual examination. Scale bar, 1 mm. (B) Mean total wing area measurements. *Drosophila mp* mutants had significantly smaller wing areas compared with the WT control (*yw*). *, $P < 0.01$. $N = 16$ for *yw* *Drosophila* and $N = 13$ for *mp* mutants. *yw* *Drosophila* and *mp* mutants were both male. Experiments were repeated three times independently. Error bars represent SD.