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Dusky-like is required for epidermal pigmentation and metamorphosis in *Tribolium castaneum*

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Dusky-like (Dyl) is associated with the morphogenesis of embryonic denticle, adult sensory bristle and wing hair in *Drosophila melanogaster*. And whether Dyl involved in insect post-embryonic development and its signal transduction are poorly understood. Here, phylogenetic analysis revealed that *dyl* displayed one-to-one orthologous relationship among insects. In *Tribolium castaneum*, *dyl* is abundantly expressed at the late embryonic stage. Tissue-specific expression analysis at the late adult stage illustrated high expression of *dyl* in the fat body and ovary. Knockdown of *dyl* resulted in the defects in larval epidermal pigmentation and completely blocked the transitions from larval to pupal and pupal to adult stages of *T. castaneum*. We further discovered that *dyl* RNAi phenotypes were phenocopied by *blimp-1* or *shavenbaby* (*svb*) silencing, and *dyl* was positively regulated by *blimp-1* through *svb* in *T. castaneum*. These results suggest that Dyl functions downstream of Blimp-1 through Svb for larval epidermal pigmentation and metamorphosis. Moreover, *ftz-f1* was down-regulated after RNA interference (RNAi) suppressing any of those three genes, indicating that Ftz-f1 works downstream of Dyl to mediate the effects of Blimp-1, Svb and Dyl on metamorphosis in *T. castaneum*. This study provides valuable insights into functions and signaling pathway of insect Dyl.

Dusky (Dy), Dusky-like (Dyl) and Miniature were identified as the transmembrane proteins containing a zona pellucid (ZP) domain in *Drosophila melanogaster*[1]. Phylogenetic analysis indicated that they have a common ancestor in *D. melanogaster*[2]. Sequence comparison illustrated that Dy has higher amino acid sequence identity with Dyl (70% amino acid sequence identity) than Miniature (only 45% amino acid sequence identity with Dy) at the ZP domains[3].

RNA *in situ* hybridization and immunostaining showed that *dy* and *miniature* are expressed in pupal wings by 28 hours after puparium formation (APF), but the expression of *dyl* could not be detected in pupal wings at the same stage[3]. Consistent with their expressions, Dy and Miniature share similar roles for wing morphogenesis in *D. melanogaster*, either of which mutation reduced the size of the whole wings by decreasing the size of individual wing epidermal cell[3]. Different from Dy and Miniature, *dyl* mutant embryos were characterized by very small, unhooked denticles, often with a split extremely[2]. Knocking down *dyl* in adults resulted in stub bristles with pigmentation abnormalities[4]. Recently, it is found that *dyl* mutation caused split, thinned, multiple and often very short hairs and a unique planar cell polarity phenotype of adult wing in *D. melanogaster*[5]. These studies of Dyl only focused on embryonic denticles, adult bristles and wing hairs of *D. melanogaster*, which provides limited information about *dyl* functions in the insect. On the other hand, a confused problem has emerged among the current studies that *dy* is evolutionarily more close to *dyl* than *miniature*, whereas *dy* shows similar expressions and functions with *miniature*. Thus, investigation of the functions of Dyl in the insect will help to clarify the functional relationship among Dyl, Dy and Miniature.

At the transcriptional level, the epidermal expression of *dyl* was abolished in *shavenbaby* (*svb*) mutant embryos of *D. melanogaster*[2], suggesting that Svb is upstream of Dyl. *Svb* is a key selector gene that integrates Wingless and DER pathways to control epidermis differentiation[6], and it controls the expressions of *miniature*, *singed* and *forked* in embryonic denticles of *D. melanogaster*[7]. But the functional relationship between *svb* and *dyl* in insects is unclear up to now. Moreover, additional upstream genes and downstream genes of Dyl should be identified in the insect.

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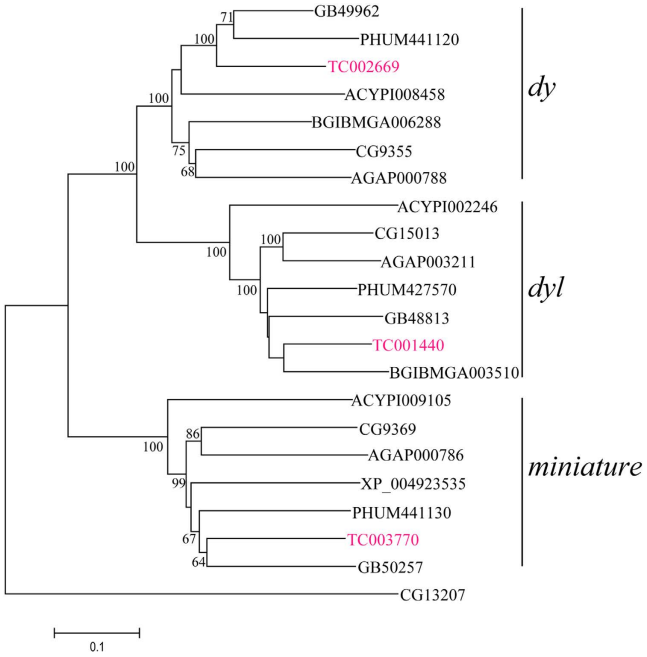

Figure 1. Phylogenetic tree of insect *dy* and *dyl* by Neighbor-joining method. TC NO. indicates the *T. castaneum* gene; GB NO. indicates the *A. mellifera* gene; PHUM NO. indicates the *P. h. humanus* gene; ACYPI NO. indicates the *A. pisum* gene; BGIBMGA NO. and XP_004923535 indicate *B. mori* genes; AGAP NO. indicates *A. gambiae* gene; CG NO. indicates the *D. melanogaster* gene. The tree is rooted by *D. melanogaster* no mechanoreceptor potential A (nompA) (CG13207). The bootstrap value below 60% was removed from phylogenetic tree.

Recently, we discovered that not only *dyl* but also *blimp-1* and *ftz-f1* transcripts were down-regulated after RNA interference (RNAi) suppressing the G protein-coupled receptor gene, *methuselah-like1* (*Tcmthl1*) (Accession number: HQ188199, orthologous to *Drosophila mthl5*) of *Tribolium castaneum*[8], which is involved in lifespan, development, stress resistance and reproduction[9], suggesting that there might be certain relationship among *dyl*, *blimp-1* and *ftz-f1*. In *D. melanogaster*, *blimp-1* RNAi caused lethality at the pupal stages in most of the observed animals[10], and elevated expressions of *ftz-f1* from 6 h[11] to 8 h APF but reduced the expression level of *ftz-f1* by 10 h APF[10]. While, Ftz-f1 was required for cholesterol converted into 20-hydroxyecdysone to control the metamorphosis in *D. melanogaster*[12] and *Leptinotarsa decemlineata* (Say)[13]. Based on these clues, it is hypothesized that *dyl* might be associated with *blimp-1* and *ftz-f1* and involved in the metamorphosis of insect.

To address these issues, we investigated the function of Dyl in *T. castaneum* by RNAi in this study. Furthermore, we explored how *dyl* relates to Blimp-1, Svb and Ftz-f1. These Results indicate that Dyl, Svb and Blimp-1 are essential for larval epidermal pigmentation and metamorphosis in *T. castaneum*.

Results

Dyl is widely distributed and conserved in insects. Using *Drosophila dyl* as a reference, 20 homolog genes were obtained from the genome database of *D. melanogaster*, *T. castaneum*, *Anopheles gambiae*, *Bombyx mori*, *Apis mellifera*, *Acyrthosiphon pisum* and *Pediculus humanus humanus*. Phylogenetic analysis revealed that *dyl* has the orthologue in each insect and shows one-to-one orthologous relationship among insects. In addition, its two paralogues, *dy* and *miniature*, also have this orthologous relationship in insects, respectively. And *dyl* is evolutionarily close to *dy* but far away from *miniature* (Figs 1 and S1). Sequence alignment of the proteins encoded by these three genes from *D. melanogaster* and *T. castaneum* also demonstrated that Dyl shares more sequence identity with Dy than Miniature. Structurally, these three genes are coding for the transmembrane proteins containing a ZP domain, respectively (Fig. S2).

Developmental and tissue expressions of *dyl* in *T. castaneum*. Before functional analysis of *dyl*, we measured mRNA levels of *dyl* at different stages and tissues by quantitative real-time PCR (qRT-PCR) analysis. In *T. castaneum*, *dyl* reached peak expression in the late embryonic stage, while its pupal and adult expression levels were very low (Fig. 2a). Tissue-specific expression analysis at the late adult stage illustrated *dyl* has high expressions in the fat body and ovary, moderate expressions in the epidermis and accessory gland but low expressions in the gut (Fig. 2b).

***dyl* is essential for larval epidermal pigmentation and metamorphosis of *T. castaneum*.** To clarify the function of *dyl* (Accession number: *TC001440*), dsRNAs of *dyl* were injected into larvae and pupae of *T. castaneum*. The qRT-PCR analysis indicated that the mRNA levels of *dyl* were significantly reduced after dsRNA injection (Fig. 3a). It is observed that all *dyl* dsRNA-treated insects arrested at the larval stage and failed to molt into pupae. The epidermis of these larvae has pigmentation abnormalities. Injection of *dyl* dsRNA into early

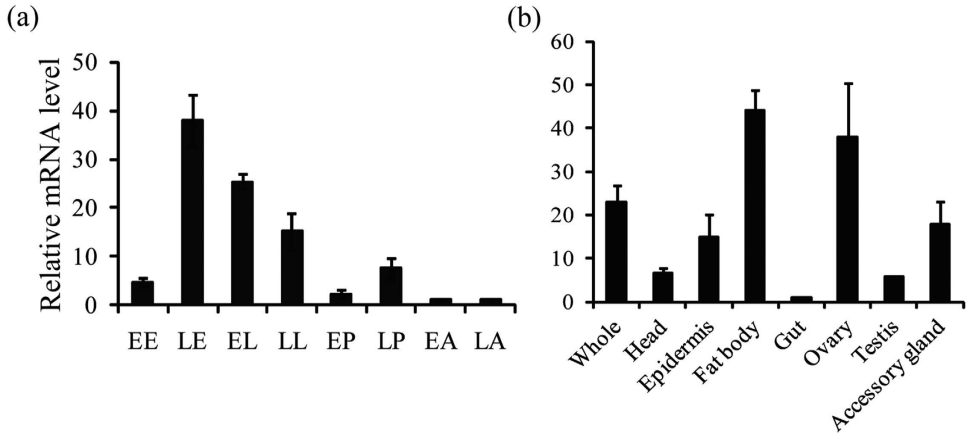

Figure 2. Developmental (**a**) and tissue (**b**) expression patterns of *dyl* in *T. castaneum* by qRT-PCR. EE, early eggs; LE, late eggs; EL, early larvae; LL, last-instar larvae; EP, early pupae; LP, late pupae; EA, early adults; LA, late adults. Tissues were isolated from adults (10-day old). One μg of total RNA from each sample was converted into cDNA for qRT-PCR.

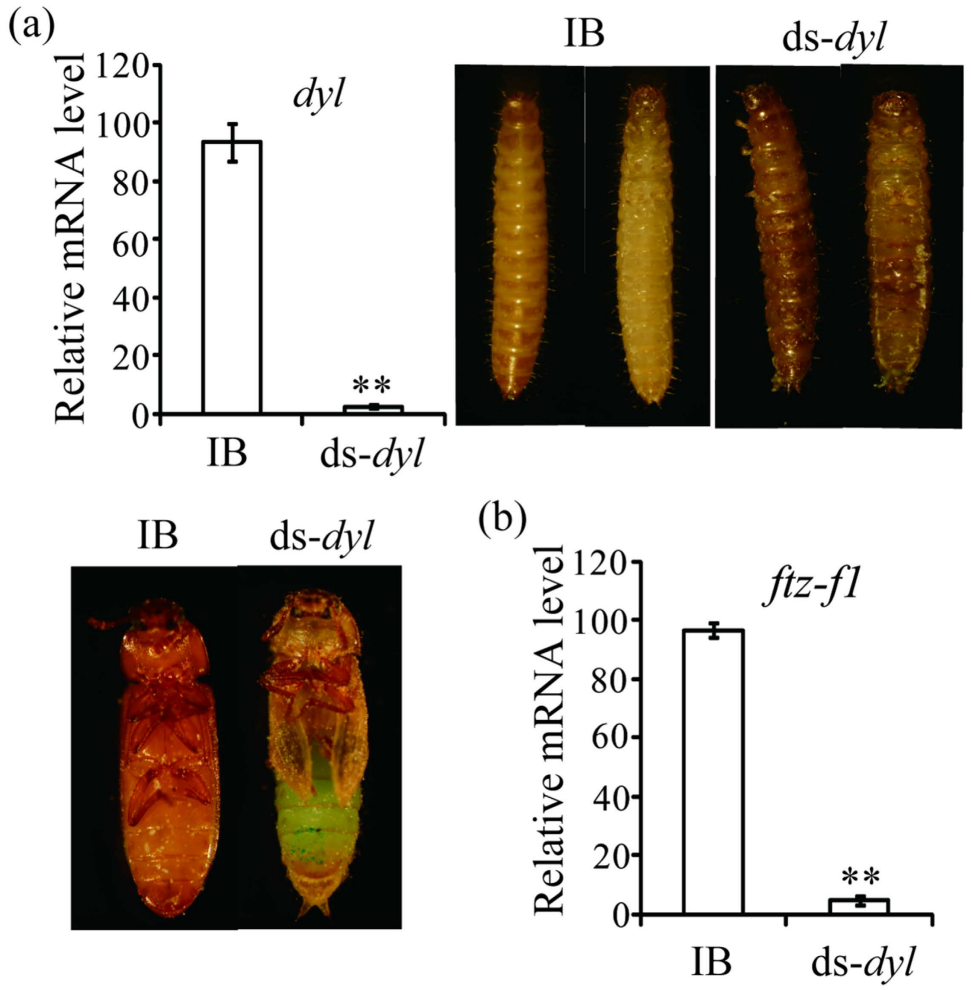

Figure 3. Knockdown of *dyl* affected *T. castaneum* pupation, eclosion (**a**) and reduced *ftz-f1* level (**b**). IB, beetles injected with physiological buffer; ds-*dyl*, beetles injected with *dyl* dsRNA. Three individuals of each group were used to extract total RNA for qRT-PCR analysis.

pupae resulted in developmental arrest prior to eclosion in *T. castaneum* (Fig. 3a). Thus, *dyl* is essential for larval epidermal pigmentation and metamorphosis of *T. castaneum*.

Dyl positively regulates *ftz-f1* expression in *T. castaneum*. It is shown that reduction of *dyl* strongly inhibited the metamorphosis in *T. castaneum*, and it is likely that there is cross-talk between Dyl and 20-hydroxyecdysone pathways. Therefore, we decided to check the expression of *ftz-f1* that is required for 20-hydroxyecdysone synthesis. As expected, we found that the mRNA level of *ftz-f1* (Accession number: *TC002550*) was down-regulated by nearly 89% compared with IB after *dyl* knockdown (Fig. 3b), suggesting that *dyl* positively regulates *ftz-f1* expression in *T. castaneum*. The result illustrates that Dyl is implicated in 20-hydroxyecdysone pathway.

Svb plays vital roles in larval epidermal pigmentation, metamorphosis and promotes expressions of *dyl*, *miniature*, *singed* and *forked* in *T. castaneum*. By RNAi, *svb* (Accession number: *TC008099*) was silenced at larval and pupal stages in *T. castaneum*, respectively. Knock-down of *svb* resulted in arrested development at the larval stage of *T. castaneum*. These larvae have abnormally pigmented epidermis, could not molt into pupae, and then died at larval stage. Pupal RNAi of *svb* also led to the failure to initiate the eclosion of *T. castaneum* (Fig. 4a). In *svb* dsRNA-treated insects, approximately 85% of *dyl* mRNA level was eliminated. It is also observed that more than 50% expressions of *miniature* (Accession number: *TC003770*), *singed* (Accession number: *TC006673*) and *forked* (Accession number: *TC005627*) were declined by *svb* RNAi (Fig. 4b). Thus, *dyl*, *miniature*, *singed* and *forked* are indeed the targets of Svb in *T. castaneum*. Furthermore, *svb* knockdown abolished 89% transcript of *ftz-f1* (Fig. 4c). Taken together, these results indicate that Svb is required for larval epidermal pigmentation and metamorphosis, and promotes the expressions of *dyl*, *miniature*, *singed* and *forked* in *T. castaneum*.

Blimp-1 is required for metamorphosis, epidermal pigmentation, and positively regulates *svb* and its targets and *ftz-f1* expressions in *T. castaneum*. Injections of dsRNAs for *blimp-1* (Accession number: *TC014741*) into larvae or pupae depleted about 90% mRNA of itself in *T. castaneum*. Reduced *blimp-1* levels caused developmental arrest at the larval stage of *T. castaneum*. The treated larvae with abnormally pigmented epidermis could not molt into pupae. Injection of *blimp-1* dsRNAs into pupae led to the failure to start the eclosion (Fig. 5a). By qRT-PCR, we demonstrated that *blimp-1* knockdown significantly reduced mRNA levels of *svb* and its targets, and *ftz-f1* in *T. castaneum* (Fig. 5b,c). Overall, Blimp-1 is also essential for larval epidermal pigmentation, metamorphosis, and positively modulates expressions of *svb* and its targets in *T. castaneum*.

Discussion

In this study, we reveal that *dyl*, *svb* and *blimp-1* are required for larval epidermal pigmentation and metamorphosis of *T. castaneum* (Figs 3a, 4a and 5a). At the transcriptional level, *dyl* positively regulated *ftz-f1* expression but was modulated by *blimp-1* and *svb* (Figs 3–5).

In *D. melanogaster*, *dyl* was detected in trichome cells of embryo epidermis but not pupal wings[2,3]. While, *dyl* mutation or knockdown resulted in very small and unhooked denticles of embryo[2], stub bristles with pigmentation abnormalities[4], split, thinned, multiple and often very short hairs and a unique planar cell polarity phenotype of adult wing[5] in *D. melanogaster*. In this study, *dyl* also showed high expressions in the late embryonic stage and low expressions in the early pupal stage of *T. castaneum* (Fig. 2a). However, RNAi knocking down *dyl* at the late larval and early pupal stages caused the defects in larval epidermal pigmentation and metamorphosis of *T. castaneum* (Fig. 3). Moreover, parental RNAi of *dyl* led to significant embryonic lethal effect in *T. castaneum*, indicating that *dyl* also played some key roles in embryo development of *T. castaneum,* which may be much more critical than that of it in *D. melanogaster*. Tissue-specific analysis revealed that *dyl* is highly expressed in the fat body and ovary of adult in *T. castaneum* (Fig. 2b), supporting the embryonic lethal effect induced by parental RNAi of *dyl*. Thus, it seems that *dyl* shares some common functions between *D. melanogaster* and *T. castaneum*, but has occurred functional shift between them.

In *D. melanogaster*, the epidermal expression of *dyl* was abolished or reduced in *svb* mutant embryos[2], indicating that *dyl* is positively regulated by *svb*. In addition to *dyl*, the expressions of *miniature*, *singed* and *forked* were also controlled by *svb* in *D. melanogaster*[7]. In *T. castaneum*, *svb* knockdown reduced *dyl*, *miniature*, *singed* and *forked* (Fig. 4b), whereas *dyl* silencing showed no effects on *svb* and *forked* expressions (Fig. S3). RNAi of *dyl* or *svb* led to the defects in larval epidermal pigmentation and metamorphosis in *T. castaneum* (Figs 3a and 4a). These results confirm that Svb is upstream of Dyl for larval epidermal pigmentation and metamorphosis.

In *D. melanogaster*, *blimp-1* knockdown showed lethality at pupal stages in most of the observed animals. Many of them eclosed but died shortly thereafter or died during eclosion[10]. Interestingly, silence of *blimp-1* also resulted in the defects of larval epidermal pigmentation and metamorphosis of *T. castaneum* (Fig. 5a), which is similar to *dyl* or *svb* RNAi phenotypes (Figs 3a and 4a). At the transcriptional level, *blimp-1* RNAi reduced the levels of *svb*, *dyl*, *miniature*, *singed* and *forked* in *T. castaneum* (Fig. 5b). Therefore, Dyl is downstream of Blimp-1 through Svb for larval epidermal pigmentation and metamorphosis in *T. castaneum*.

In *blimp-1* knockdown flies, high-level expressions of *ftz-f1* was detected from 6h[11] to 8h APF, but the expression level of *ftz-f1* was greatly reduced by 10 h APF, demonstrating that Blimp-1 works as a repressor for premature expression of *ftz-f1* but a activator for latish expression of *ftz-f1*[10]. By qRT-PCR analysis, it is shown that *blimp-1* knockdown down-regulated *ftz-f1* expression at the last-instar larvae of *T. castaneum* (Fig. 5c), suggesting that the expression of *ftz-f1* is positively regulated by *blimp-1* at the last-instar larvae. Likewise, *svb* or *dyl* RNAi also reduced *ftz-f1* level in *T. castaneum* (Figs 3b and 4c). In *T. castaneum*, high expression levels of *dyl* (Fig. 2b) and *ftz-f1*[14] were detected in the adult fat body and ovary, demonstrating that *dyl* and *ftz-f1* possessed the similar tissue-specific expression pattern. These results indicated that Ftz-f1 works downstream of Dyl, Blimp-1 and Svb

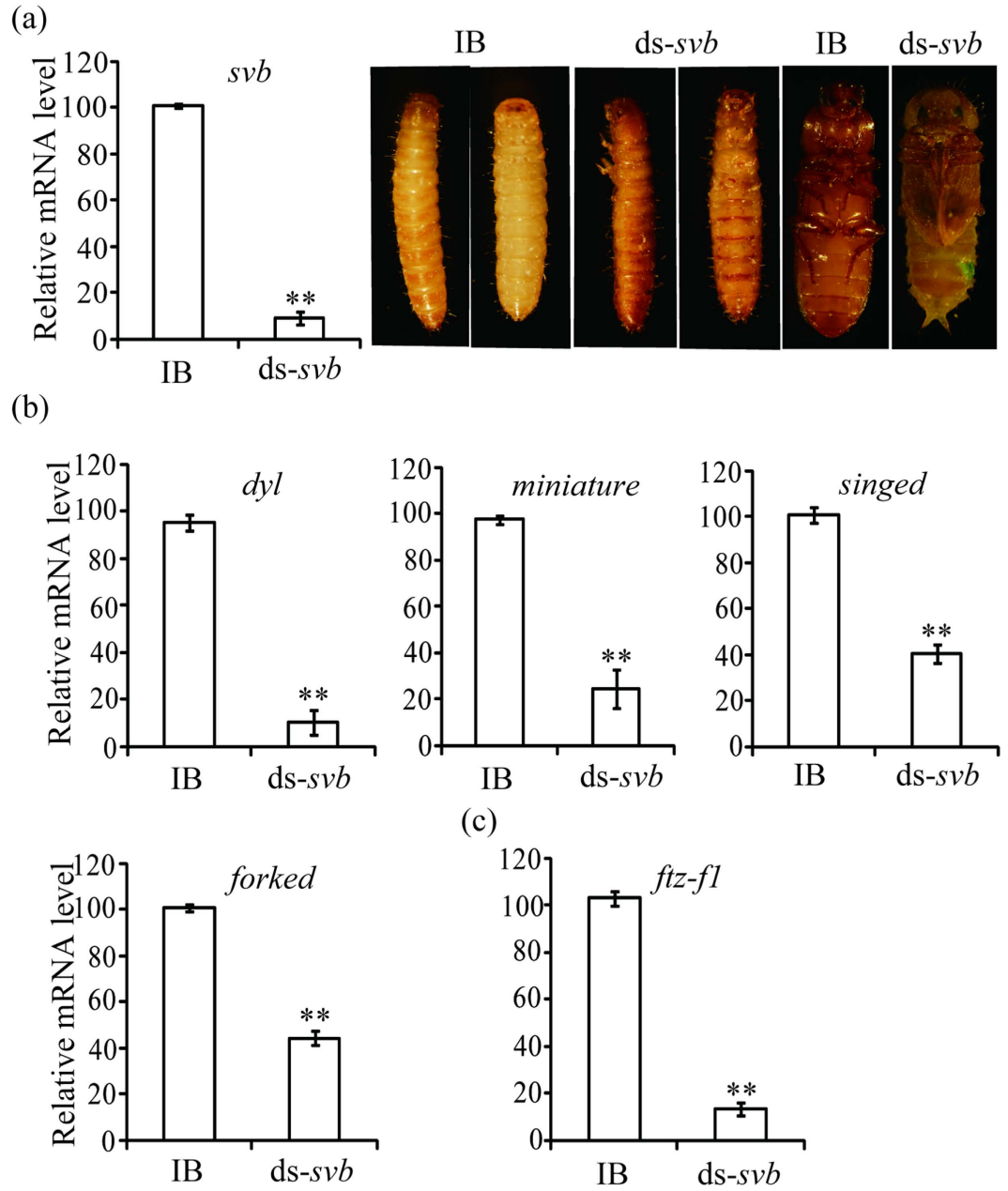

Figure 4. *Svb* RNAi affected pupation and eclosion (**a**) and reduced the expressions of *dyl*, *miniature*, *singed*, *forked* (**b**) and *ftz-f1* (**c**) in *T. castaneum*. IB, beetles injected with physiological buffer; ds-*svb*, beetles injected with *svb* dsRNA. Three larvae or pupae of each group were used to extract total RNA for qRT-PCR analysis.

in *T. castaneum*. As reported previously, Ftz-f1 was required for cholesterol converted into 20-hydroxyecdysone in *D. melanogaster*[12] and *L. decemlineata* (Say)[13]. Mutation of *ftz-f1* resulted in arrested development at embryonic, larval and pupal stages in *D. melanogaster*[15]. Reduced levels of *ftz-f1* by RNAi also caused arrested development at larval stage[12]. These studies demonstrate that *ftz-f1* is essential for metamorphosis. Therefore, the effects of Dyl, Svb and Blimp-1 on metamorphosis were modulated by Ftz-f1 in *T. castaneum*. It is concluded that Dyl functions downstream of Blimp-1 through Svb but upstream of Ftz-f1 for metamorphosis (Fig. 6).

Developmental study showed that the presence/absence of *svb* expression ultimately determined the pattern of denticles and dorsal hairs[16]. It was further found that Svb controls the expression of cuticle proteins[17] and enzymes that increase trichome pigmentation and hardness[7]. These studies reveal the vital roles of Svb for epidermis differentiation. In *T. castaneum*, an obvious defect in epidermal pigmentation was observed in *svb* knockdown larvae (Fig. 4a), confirming the involvement of Svb in epidermal pigmentation. As the target of Svb, *dyl* was only expressed in embryonic tissues that will secrete cuticle, including epidermis, trachea and foregut[2,3]. Knocking down *dyl* function disrupted cuticle formation in bristles[4]. As is the case in bristles, hairs lacking *dyl* function also showed abnormality in chitin deposition[5]. In *T. castaneum*, *dyl* silencing caused defects in larval epidermal pigmentation (Fig. 3a). In *D. melanogaster*, Chitinase 6 was identified as a potential candidate of Dyl[5].

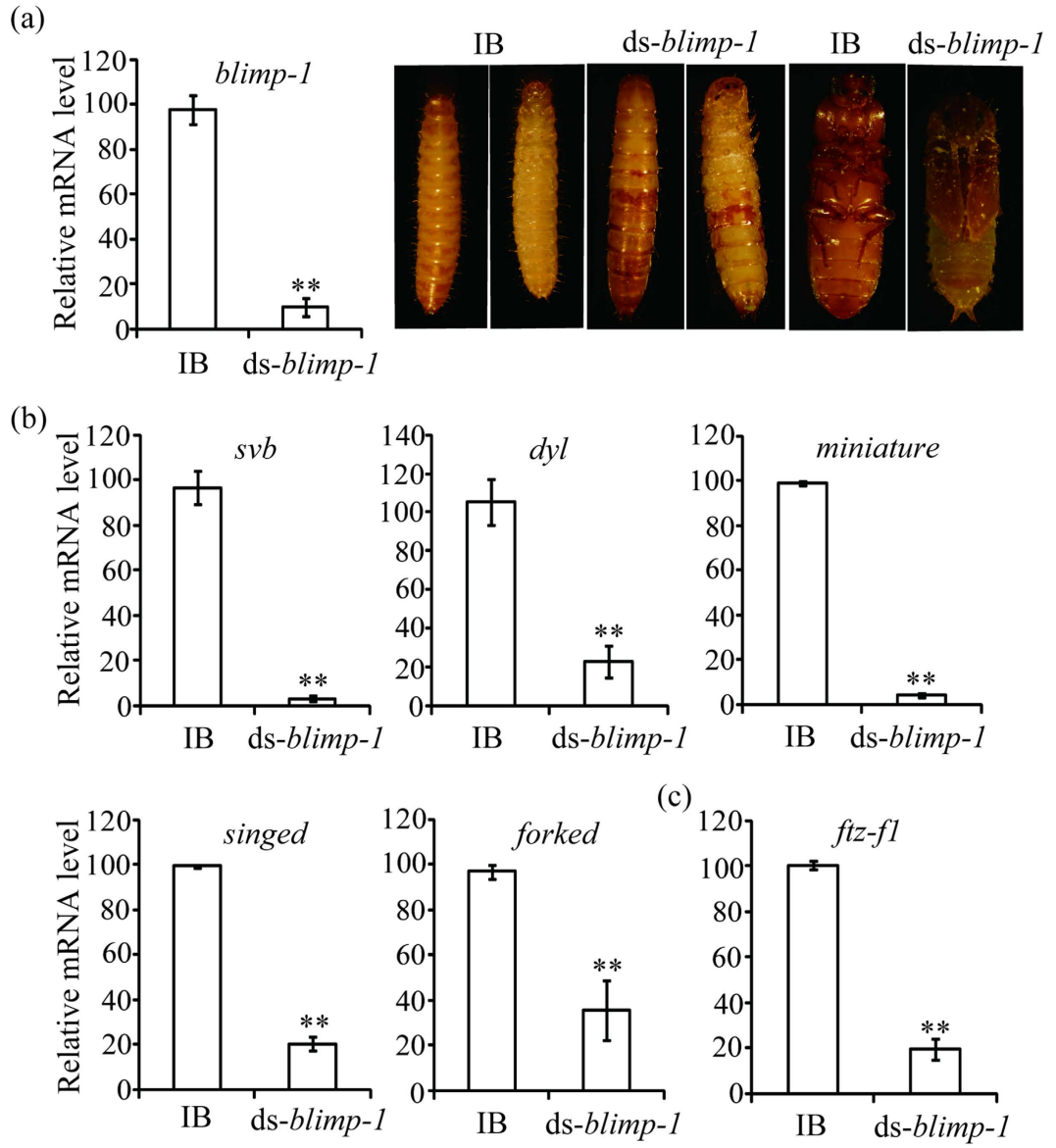

Figure 5. *Blimp-1* RNAi affected pupation and eclosion (**a**) and down-regulated the mRNA levels of *svb* and its targeted genes (**b**) and *ftz-f1* (**c**) in *T. castaneum*. IB, beetles injected with physiological buffer; ds-*blimp-1*, beetles injected with *blimp-1* dsRNA. Three larvae or pupae of each group were used to extract total RNA for qRT-PCR analysis.

It is likely that epidermal pigmentation defects arose from *dyl*, *svb* or *blimp-1* knockdown are associated with chitin deposition. However, how Dyl, Svb and Blimp-1 modulate larval epidermal pigmentation is unclear at present and need to be studied in the future.

Though *dyl*, *dy* and *miniature* of *D. melanogaster* were originated from one ancestral gene[2], sequence comparison illustrated that Dyl showed more sequence similarities with Dy than Miniature at ZP domains[3]. Based on seven insect genome sequences, our study shows that insect *dyl*, *dy* and *miniature* were divided into three separate clusters of the phylogenetic tree (Figs 1 and S1), providing clues to the functional relationship among these three genes. In *D. melanogaster*, *dy* and *miniature* were detected in pupal wings by 28 hours APF, while *dyl* expression was not detected in pupal wings of the same stage[3]. Functionally, both Dy and Miniature are required for cytoskeletal reorganisation during wing morphogenesis[3], while *dyl* shows significant effects on embryonic denticles formation[2], adult bristle cuticle formation[4] and adult hair integrity and planar cell polarity in *D. melanogaster*[5]. In *T. castaneum*, *dyl* is shown to be required for larval epidermal pigmentation and metamorphosis (Fig. 3a). These results suggest that Dyl is functionally divergent with Dy and Miniature in the insect.

Methods

Insect strains. The *Tribolium castaneum* GA-1 strain was used for all experiments. Insects were reared in whole wheat flour containing 5% brewer's yeast at 30 °C under standard conditions as described previously[18,19].

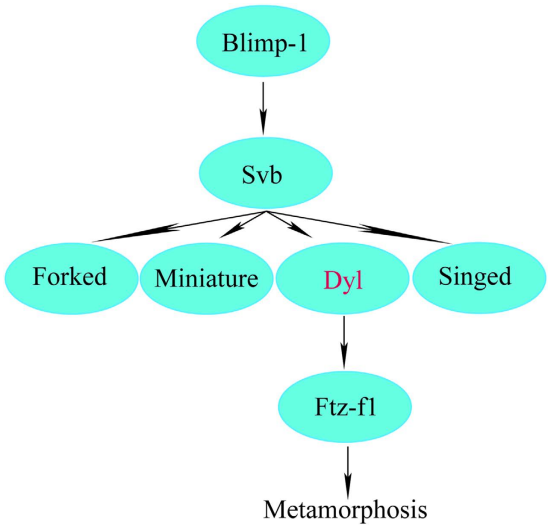

Figure 6. Preliminary regulation model of Dyl in *T. castaneum*.

Gene	Sense primer (5′-3′)	Anti-sense primer (5′-3′)	Utility
dyl	TAATACGACTCACTATAGGGGTACGGCCAAAGATCATGAG	TAATACGACTCACTATAGGGGCCGACTGAATGACCACA	dsRNA synthesis
	TCACCTTTGGGATATTAACCG	CGCATGTGAGTCTTTTCGCAC	PCR
ftz-f1	TAGACAAAACGCAAAGGAAAAGG	GTTTGTACATCGGACCGAATTT	PCR
svb	TAATACGACTCACTATAGGGGAAAATACCCCCAAACCACA	TAATACGACTCACTATAGGGTGTCCAGTATCACAATCCATGT	dsRNA synthesis
	GAGGCACACGAGGACGCATA	CTTCAGACAGTGCGATTCCAAC	PCR
miniature	TACGAGAAAACTGCCTCCAAAC	CCTCCTCCATCAGACGCGA	PCR
singed	TGCTCAAAACGCGGTCTAATAC	TCGTTTCGTGCTCCAATCG	PCR
forked	CAGATGTTGAGAGAGGCGGA	GCACTCGTGTGTTTCTTGGATTT	PCR
blimp-1	TAATACGACTCACTATAGGGCACGACAACTACCCTAACCA	TAATACGACTCACTATAGGGGCTTCTTACAAATGCCACAC	dsRNA synthesis
	TCTATGCCAAAGATGCGGTAC	TCATTTTGTATTGACAGGCGATTAA	PCR
rps3	TCAAATTGATCGGAGGTTTG	GTCCCACGGCAACATAATCT	PCR

Table 1. List of primers used.

RNA extraction and cDNA synthesis. Using RNAiso^{TM}Plus (TaKaRa), total RNA was isolated from the eggs, larvae, pupae and adults of *T. castaneum*. And 1 μg of total RNA was converted to cDNA by Moloney Murine Leukemia Virus reverse transcriptase (TaKaRa) and an Oligo(dT)$_{18}$ primer (TaKaRa).

Phylogenetic analysis. With the sequences of *D. melanogaster dy*, *dyl* and *miniature* (http://flybase.org/), we searched the genome databases of these insects, including *T. castaneum* (http://beetlebase.org/), *A. gambiae* (https://www.vectorbase.org/index.php), *B. mori* (http://silkworm.genomics.org.cn/), *A. mellifera* (http://hymenopteragenome.org/beebase/), *A. pisum* (http://www.aphidbase.com/aphidbase/), *P. h. humanus* (https://www.vectorbase.org/index.php) and National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/guide/). These sequences were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Then the Neighbour-joining tree was reconstructed by MEGA 5 using the bootstrap method with 1000 replications (Fig. 1). To test the topology of Neighbor-joining tree, we reconstructed the Maximum likelihood tree by MEGA 5 (Fig. S1).

qRT-PCR. To check the gene expression profiles, qRT-PCR was performed with FastStart Universal SYBR Green Master (ROX) (Roche). The data are expressed here as the relative mRNA levels normalized to a control gene, *T. castaneum ribosomal protein S3* (*rps3*)[20], using the ΔΔCT method[21]. Three batches of samples were used for qRT-PCR. The primers were listed in Table 1.

RNAi. RNAi was performed as previously[9,20]. Negative controls consisted of injections of either *vermillion* (*ver*) dsRNA or an equal volume of buffer only (IB). Knock-down levels of the target genes were examined by qRT-PCR at the fifth day after injection. These experiments were performed for three biological replications.

Statistical analysis. The mean values of the RNAi-treated insects versus the mean values of the control insects were compared using the one-way ANOVA program of SPSS version 13.0. All the data are presented as the mean ± standard error. "*" indicates $p < 0.05$, and "**" indicates $p < 0.001$.

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Author Contributions

Conceived and designed the experiments: C.J.L. and B.L. Performed the experiments: C.J.L. and X.P.Y. Analyzed the data: C.J.L. and B.L. Contributed reagents/materials/analysis tools: C.J.L. and X.P.Y. Wrote the paper: C.J.L. and B.L. All authors gave final approval for publication.

Additional Information

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