The silkworm glutathione S-transferase gene *noppera-bo* is required for ecdysteroid biosynthesis and larval development

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25 **Abstract**

26 Insect molting and metamorphosis are tightly controlled by ecdysteroids, which are
27 important steroid hormones that are synthesized from dietary sterols in the prothoracic
28 gland. One of the ecdysteroidogenic genes in the fruit fly *Drosophila melanogaster* is
29 *noppera-bo* (*nobo*), also known as *GSTe14*, which encodes a member of the epsilon class
30 of glutathione *S*-transferases. In *D. melanogaster*, *nobo* plays a crucial role in utilizing
31 cholesterol via regulating its transport and/or metabolism in the prothoracic gland.
32 However, it is still not known whether the orthologs of *nobo* from other insects are also
33 involved in ecdysteroid biosynthesis via cholesterol transport and/or metabolism in the
34 prothoracic gland. Here we report genetic evidence showing that the silkworm *Bombyx*
35 *mori* ortholog of *nobo* (*nobo-Bm*; *GSTe7*) is essential for silkworm development.
36 *nobo-Bm* is predominantly expressed in the prothoracic gland. To assess the functional
37 importance of *nobo-Bm*, we generated a *B. mori* genetic mutant of *nobo-Bm* using
38 TALEN-mediated genome editing. We show that loss of *nobo-Bm* function causes larval
39 arrest and a glossy cuticle phenotype, which are rescued by the application of
40 20-hydroxyecdysone. Moreover, the prothoracic gland cells isolated from the *nobo-Bm*
41 mutant exhibit an abnormal accumulation of 7-dehydrocholesterol, a cholesterol
42 metabolite. These results suggest that the *nobo* family of glutathione *S*-transferases is
43 essential for development and for the regulation of sterol utilization in the prothoracic
44 gland in not only the Diptera but also the Lepidoptera. On the other hand, loss of *nobo*
45 function mutants of *D. melanogaster* and *B. mori* abnormally accumulates different
46 sterols, implying that the sterol utilization in the PG is somewhat different between these
47 two insect species.

50 **Keywords (<6)**

51 *Bombyx mori*, ecdysteroids, glutathione *S*-transferase, *noppera-bo*, prothoracic gland,

52 sterol

53

1. Introduction

Ecdysteroids, such as ecdysone and 20-hydroxyecdysone (20E)¹, are steroid hormones that play a central role in the regulation of many developmental and physiological processes in arthropods including insects (Gilbert et al., 2002; Niwa and Niwa, 2014; Spindler et al., 2009). During insect larval development, ecdysteroids are biosynthesized in a specialized endocrine gland, the prothoracic gland (PG). The strict regulation of the ecdysteroid titer throughout the insect's life is critical to its successful development and reproduction. In general, ecdysteroid biosynthetic activity in the PG is considered a major factor in the regulation of ecdysteroid titer (Niwa and Niwa, 2014; Rewitz et al., 2013). Ecdysone is synthesized from dietary cholesterol and/or phytosterols via a series of hydroxylation and oxidation steps (Niwa and Niwa, 2014). Ecdysone is then secreted in the hemolymph and subsequently converted to the more biologically active hormone 20E in the peripheral tissues (Petryk et al., 2003).

A number of genes that are responsible for biosynthetic activity in the PG have been identified and characterized (Niwa and Niwa, 2014). The first step of the ecdysteroid biosynthetic pathway, the 7,8-dehydrogenation of cholesterol to 7-dehydrocholesterol (7dC), is mediated by the [2Fe-2S] Rieske oxygenase Neverland. The next step, the conversion of 7dC to 5 β -ketodiol is known as the 'Black Box', because it has not been precisely characterized. Currently, it is known that the 'Black Box' is mediated by multiple enzymes: the short-chain dehydrogenase/reductase Non-molting glossy (Nm-g)/Shroud and the cytochrome P450 monooxygenases Spook, Spookier and

¹ Abbreviations: 7dC, 7-dehydrocholesterol; 20E, 20-hydroxyecdysone; GST, glutathione *S*-transferase; PG, prothoracic gland; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; s. e. m., standard error of the mean; TALEN, transcription activator-like effector nucleases

CYP6T3. The final hydroxylation steps that convert 5 β -ketodiol to 20E are catalyzed by a series of cytochrome P450s: Phantom, Disembodied, Shadow and Shade. Most of the ecdysteroidogenic enzyme genes described above are highly conserved in not only insects but also in other arthropods, whereas other genes are missing in certain insect species (Niwa and Niwa, 2014).

To date, most *in vivo* functional analyses of these ecdysteroidogenic enzymes have been conducted using the fruit fly *Drosophila melanogaster*. In particular, in *D. melanogaster*, null mutants of *spook*, *shroud*, *phantom*, *disembodied*, *shadow* and *shade*, which are classified as Halloween mutants, exhibit abnormalities in embryonic cuticle differentiation and deficiencies in ecdysteroid levels, resulting in embryonic lethality (Gilbert, 2004; Rewitz et al., 2006). Recently, genetic mutants and double-stranded RNA interference technology have shown the *in vivo* importance of the orthologs of these enzyme genes in other insects, including lepidopteran species such as the silkworm *Bombyx mori* (Niwa et al., 2010) and the rice striped stem borer *Chilo suppressalis* (Shahzad et al., 2014); coleopteran species such as the red flour beetle *Tribolium castaneum* (Hentze et al., 2013) and the Colorado potato beetle *Leptinotarsa decemlineata* (Kong et al., 2014); hemipteran species such as the small brown planthopper *Laodelphax striatellus* (Jia et al., 2013a, 2013b, 2014; Wan et al., 2014b, 2014c) and the white-backed planthopper *Sogatella furcifera* (Jia et al., 2013c; Wan et al., 2014a, 2014b); and orthopteran species such as the desert locust *Schistocerca gregaria* (Marchal et al., 2012, 2011). All of these studies support the idea that the conserved ecdysteroidogenic enzyme genes are crucial for insect development.

Recently, two groups, including ours, identified and characterized the novel Halloween gene *noppera-bo* (*nobo*), also known as *GSTe14*, in *D. melanogaster* (Chanut-Delalande et al., 2014; Enya et al., 2014). *nobo* encodes a glutathione

S-transferase (GST) and is expressed predominantly in the ecdysteroidogenic organs including the PG and the ovary in *D. melanogaster*. Among the known Halloween genes, *nobo* plays a unique role in ecdysteroid biosynthesis; rather than catalyzing the production of ecdysteroid intermediates, *Nobo* appears to regulate the transport and/or metabolism of cholesterol in the PG of *D. melanogaster* (Chanut-Delalande et al., 2014; Enya et al., 2014). Phylogenetic analysis suggests that *GST* genes belonging to the *nobo* family are also found in other dipteran and lepidopteran species; such genes include *GSTe8* in the mosquito *Anopheles gambiae* (Ayres et al., 2011) and *GSTe7* in the silkworm *B. mori* (Yu et al., 2008). To avoid using a confusing numbering system to represent the orthologous *GST* genes of different insect species, we have proposed a unique subfamily name, *noppera-bo* (*nobo*), for these orthologs (Enya et al., 2014). Importantly, the lethality of *D. melanogaster nobo* loss-of-function animals is almost completely rescued by the overexpression of *B. mori GSTe7*, i.e., *nobo-Bm* (Enya et al., 2014). These data strongly suggest that the *nobo* genes of the Diptera and Lepidoptera are functionally equivalent. However, it has not yet been determined whether the *nobo* genes in insect species other than *D. melanogaster* are also essential for ecdysteroid biosynthesis during development.

Here, we report the phenotypic analysis of a *B. mori* genetic mutant of *nobo-Bm*, which was generated by transcription activator-like effector nucleases (TALEN)-mediated genome editing (Daimon et al., 2013). Our results suggest that, similar to *nobo* in *D. melanogaster*, *nobo-Bm* in *B. mori* is essential for development and for sterol utilization in the PG to regulate ecdysteroid biosynthesis. However, there is a substantial difference in the sterol accumulation phenotypes of the PGs from *nobo* loss-of-function *D. melanogaster* and *B. mori* animals.

2. Materials and Methods

2.1. Animal rearing

The nondiapausing strain *w1-pnd* (Takasu et al., 2013) was used as the wild type and to generate the *nobo-Bm* knockout allele. The larvae were reared on an artificial diet (Silk Mate PM, Nosan Corporation, Yokohama, Japan) at 25 °C.

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

Single-strand cDNA synthesis and qRT-PCR analysis were performed as previously described (Shinoda and Itoyama, 2003). The primers for *nobo-Bm* were nobo-Bm-qRTPCR-F1 (5'-TTGCTGTGCTTCCACCATTAGTTC-3') and nobo-Bm-qRTPCR-R1 (5'-ATTTCAATCGCTCTAAGCCACGTC-3'). The primers for *rp49* were rp49-F (5'-CAGGCGGTTCAAGGGTCAATAC-3') and rp49-R (5'-TGCTGGGCTCTTTCCACGA-3'), which have been previously described (Shinoda and Itoyama, 2003). The expression levels were normalized to those of *rp49*.

2.3. TALEN-mediated generation of a gene-targeted *nobo-Bm*^{KO} allele

TALEN expression vectors for generating the gene-targeted *nobo-Bm* knock-out allele were constructed according to a previous study (Takasu et al., 2013). The pBlue-TAL vector was used as the vector backbone. The target sequences were searched using TALEN targeter (Doyle et al., 2012), and the target sequences nobo-TAL1 (5'-CCACCCAATTGATCTCT-3') and nobo-TAL2 (5'-GATTAAAAGCGTCCATT-3') were selected. The DNA recognition sites of the TALEN were constructed using Golden Gate assembly (Cermak et al., 2011). *nobo-Bm*^{Δ85}, the *nobo-Bm* knockout line used for the experiments, was maintained as a heterozygous stock in the parental *w1-pnd*

background without outcrossing to other standard strains.

For *in vitro* TALEN mRNA transcription, a mMACHINE T7 kit (Ambion, Austin, TX, USA) was used according to the manufacturer's instructions. The synthesized mRNA was dissolved in injection buffer (0.5 mM phosphate buffer, pH 7.0) to a final concentration of 200 ng/μl per mRNA. The microinjection of silkworm embryos and the mutant screening were performed as previously described (Takasu et al., 2013).

2.4. Genomic PCR for genotyping of the *nobo-Bm*⁴⁸⁵ allele

Single larvae were squashed in alkaline buffer (50 mM NaOH, 0.2 mM EDTA) and then heated at 95 °C for 5 min. The same volume of 0.2 M Tris-HCl (pH8.0) was added for neutralization. The supernatant was used as the DNA template. KOD FX Neo Polymerase (ToYoBo, Osaka, Japan) was used to amplify the *nobo* genomic region. The genotyping primers for *nobo-Bm*⁴⁸⁵ were nobo-Bm-313-F (5'-CGTTTGATTTGCTGTACGAATGCC-3') and nobo-Bm-313-R (5'-CCCTCGTTGATGTGACTATAAAGTGCC-3').

2.5 Sequencing of *nobo-Bm* transcripts derived from the *nobo-Bm*⁴⁸⁵ allele

Single larvae were squashed in RNAiso Plus (TaKaRa Bio, Shiga, Japan), and then the nucleic acids were extracted and precipitated according to the manufacturer's protocol. In this procedure, both total RNAs and genomic DNAs were simultaneously isolated. After identifying the *nobo-Bm*⁴⁸⁵ homozygous animals via genomic PCR as described above, the total RNA derived from the same animals was used for RT reactions with ReverTra Ace qPCR RT Master Mix (ToYoBo, Osaka, Japan). The RT product was then used as a template for the PCR reactions using KOD FX Neo Polymerase and the primers BmGSTe7-F (5'-CAGTCATATGATGTCCATTGTTTCGGTGTAATATG-3') and

BmGSTe7-R (5'-CTGACTCGAGGTTTGGCTTGTAAGACTCATAAAATA-3').

These primers could amplify the entire coding region of the +^{nobo-Bm} gene. The PCR products were subcloned into pBluescript II SK(-) (Promega, Madison, WI, USA) and sequenced.

2.6. Measurement of the ecdysteroid titer

Either 6 days (+^{nobo-Bm}/^{nobo-Bm} and *nobo-Bm*⁴⁸⁵/^{nobo-Bm}) or 9 days (*nobo-Bm*⁴⁸⁵/*nobo-Bm*⁴⁸⁵) after hatching, 2nd instar larvae were homogenized in methanol. The supernatants were collected and stored at -20 °C until analysis. After methanol extraction, genomic DNA for genotyping PCR was prepared from precipitates. The ecdysteroid titer was measured using an enzyme-linked immunosorbent assay (ELISA) according to a previous study (Shimada-Niwa and Niwa, 2014). Anti-20E rabbit IgG antiserum (20-Hydroxyecdysone EIA Antiserum) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). This antiserum has the same affinity for ecdysone and 20E (Ou et al., 2011; Porcheron et al., 1989). Because 20E (ENZO Life Science, Farmingdale, NY, USA) was used as the standard in this study, the ecdysteroid level was expressed in 20E equivalents.

2.7. Quantification of sterol levels in the PG

Larvae were dissected and the PGs were collected 7 days after hatching. The PGs were stored at -80 °C until analysis. Sterol extraction of sterols and quantification via liquid chromatography-mass spectrometry were performed as previously described (Igarashi et al., 2011)

2.8. Rescue experiments with ecdysteroid intermediates

The offspring of *nobo-Bm*^{Δ85}/+*nobo-Bm* parents were reared on a standard artificial diet at 25 °C. Ten days after hatching, the 2nd instar larvae of *nobo-Bm*^{Δ85} homozygotes, which could be distinguished by their glossy cuticle phenotype, were transferred onto sterol- or steroid-containing foods. After 2 days, the developmental stages were scored. Cholesterol (Sigma), 7-dehydrocholesterol (Sigma) and 5β-ketodiol (provided by Dr. Yoshinori Fujimoto) were dissolved in ethanol. Approximately 9 g of artificial silkworm diet was coated with 100 μl of a 0.2-mg/ml solution of the supplement. Ecdysone and 20E were purchased from Sigma (St. Louis, MO, USA) and ENZO Life Science, respectively. Ecdysone was purified, and its purity was verified by HPLC. Ecdysone and 20E were mixed into the artificial silkworm diet to a final concentration of 100 ppm.

3. Results and Discussion

3.1. The spatial and temporal expression of *nobo-Bm* during larval development

We conducted qRT-PCR experiments to examine the spatiotemporal expression of *nobo-Bm* during the larval development of wild type *B. mori*. Among the 15 different tissues analyzed, *nobo-Bm* was highly expressed in the PG, and weaker *nobo-Bm* expression was observed in several tissues including the corpora allata-corpora cardiaca complex, the brain, the fat body, the epidermis, the Malphigian tubules, the salivary glands and the gonads (Fig. 1A). Temporally, changes in the *nobo-Bm* expression level in the PG closely correlated with changes in the ecdysteroid titer of the hemolymph during 4th and 5th instar larval development (Fig. 1B) (Mizoguchi et al., 2001), which was similar to the expression pattern of other ecdysteroidogenic genes in *B. mori* (Namiki et al., 2005; Niwa et al., 2010, 2005, 2004; Ono et al., 2006; Yoshiyama et al., 2006). These results support our hypothesis that like *D. melanogaster nobo*, *nobo-Bm* is involved in the

regulation of ecdysteroid biosynthesis in the PG in *B. mori*.

3.2. A TALEN-mediated *nobo-Bm* mutant strain exhibits molting defects and a glossy cuticle.

To determine whether the *nobo-Bm* gene plays an essential role in *B. mori* development, we generated a *nobo-Bm* mutant strain using TALEN-mediated gene targeting technology, which has been successfully applied in the generation of a number of *B. mori* mutant strains (Daimon et al., 2013; Takasu et al., 2013). We microinjected mRNAs encoding the TALEN pairs into the embryos (Supplementary Fig. 1 and Materials and Methods 2.3) to induce a double-strand break within the 2nd exon (Fig. 2A). We eventually isolated a *nobo-Bm* mutant strain that lacked 87 bp and had also exogenously added 2 bp in the 2nd exon and 2nd intron of the wild type +^{*nobo-Bm*} locus (Figs. 2A, 2B and Supplementary Fig. 1). We called this deletion allele *nobo-Bm*⁴⁸⁵. The *nobo-Bm*⁴⁸⁵ allele lacks the exon-intron junction between the 2nd exon and the 2nd intron, resulting in an unusual *nobo-Bm* transcript containing part of the 2nd intronic sequence (Fig. 2C and Supplementary Fig. 2). The predicted protein encoded by the *nobo-Bm*⁴⁸⁵ allele lacks half of the GST N-terminal domain and the entire GST C-terminal domain (Fig. 2D), suggesting that the *nobo-Bm*⁴⁸⁵ allele is functionally null.

We found that under regular rearing conditions, *nobo-Bm*⁴⁸⁵ homozygous mutants died at the 2nd instar stage; they never grew into the 3rd instar stage or beyond, while wild type and *nobo-Bm*⁴⁸⁵ heterozygous animals exhibited no developmental phenotypes (Figs. 2E, 2F and Table 1). Interestingly, the cuticle of *nobo-Bm*⁴⁸⁵ homozygous mutants eventually became glossier than that of the control animals (Figs. 2E and 2F). This glossy cuticle phenotype is reminiscent of the classical *B. mori* mutant *non-molting glossy (nm-g)*, which is loss-of-function allele of the ecdysteroidogenic

short-chain dehydrogenase/reductase gene *nm-g/shroud* (Nagata et al., 1992, 1987; Niwa et al., 2010; Tanaka, 1998).

3.3. The larval arrest phenotype of the *nobo-Bm* mutant strain is due to ecdysteroid deficiency.

Because *B. mori nm-g* mutant larvae exhibit a low ecdysteroid titer (Nagata et al., 1987; Niwa et al., 2010; Tanaka, 1998), we expected that the loss of *nobo-Bm* function would also cause a reduction in the ecdysteroid titer. We therefore performed an ELISA to determine whether *nobo-Bm*⁴⁸⁵ homozygous mutants had reduced ecdysteroid titers. We prepared methanol extracts from whole wild type, *nobo-Bm*⁴⁸⁵ heterozygous and homozygous 2nd instar larvae. We found that the ecdysteroid titer in the *nobo-Bm*⁴⁸⁵ homozygous animals was significantly lower than those of the wild type and the heterozygotes (Fig. 3A). These results suggest that *nobo-Bm* is required for ecdysteroid biosynthesis during *B. mori* development.

Furthermore, we conducted a feeding rescue experiment to examine whether the larval arrest phenotype of the *nobo-Bm*⁴⁸⁵ homozygous animals could be rescued by food supplemented with 20E (Table 2). Second instar larvae of *nobo-Bm*⁴⁸⁵ homozygotes that were fed food supplemented with 20E molted and grew into the 3rd instar stage, whereas 2nd instar *nobo-Bm*⁴⁸⁵ homozygous larvae that were fed food supplemented with a vehicle (ethanol) remained arrested. In addition, the rescued 3rd instar *nobo-Bm*⁴⁸⁵ homozygotes did not have the glossy cuticle (Supplementary Fig. 3). These results strongly suggest that *nobo* is required for ecdysteroid biosynthesis during the development of not only dipteran species (*D. melanogaster*) (Chanut-Delalande et al., 2014; Enya et al., 2014) but also of lepidopteran species (*B. mori*).

3.4. The PG cells of the *nobo-Bm* mutant strain abnormal accumulate 7dC.

Previously, we reported that the PG cells of *nobo* loss-of-function *D. melanogaster* larvae show abnormal accumulation of cholesterol (Enya et al., 2014). Therefore, we examined the sterol levels in the PG cells of *nobo-Bm*^{Δ85} homozygotes. Mass spectrometric analysis revealed that the PGs from *nobo-Bm*^{Δ85} homozygotes second instar larvae accumulated significantly higher levels of 7dC, a cholesterol metabolite, than the PGs from control animals (wild type and *nobo-Bm*^{Δ85} heterozygotes) (Fig. 3B). There were no statistically significant differences in the levels of cholesterol and β-sitosterol between the control and *nobo-Bm*^{Δ85} homozygous PG cells (Fig. 3B). Campesterol, desmosterol, ergosterol and stigmasterol were not detected in the PGs of either control or *nobo-Bm*^{Δ85} homozygotes (data not shown). These results suggest that similar to *D. melanogaster nobo*, *nobo-Bm* is essential for development and for the regulation of 7dC utilization in the PGs of *B. mori*.

We also conducted a feeding rescue experiment to determine whether the larval arrest phenotype of the *nobo-Bm*^{Δ85} homozygous animals could be rescued by food supplemented with cholesterol or 7dC. However, neither cholesterol nor 7dC rescued the larval arrest phenotype (Table 2). Given that *nobo-Bm*^{Δ85} appears to be a complete loss-of-function allele, these results suggest that the PG cells that completely lack *nobo-Bm* function cannot utilize sterol(s) for ecdysteroid biosynthesis during larval development. This is also the case for the previously reported knockout allele of *D. melanogaster nobo* that we have previously reported (Enya et al., 2014).

However, it should also be noted that loss of *nobo* function in *D. melanogaster*, but not in *B. mori*, results in the abnormal accumulation of cholesterol (Chanut-Delalande et al., 2014; Enya et al., 2014). In addition, unlike in *B. mori* PGs, 7dC could not be detected by our mass spectrometric analyses even in wild type *D. melanogaster* PGs (Enya et al., 2014). Taken together, these data imply that the storage and utilization of

cholesterol and 7dC in the PG are somewhat different between *D. melanogaster* and *B. mori*. This might reflect the differences of the Nobo-dependent regulatory mechanisms of ecdysteroid biosynthesis between these two species, whereas it remains unclear why such difference occurs between *D. melanogaster* and *B. mori*. To understand the functional differences between *nobo* genes from different insect species, it will be quite important to find specific Nobo substrate(s), which are currently unknown for any insect. Studies aimed at elucidating the enzymatic function of Nobo in several insects are now underway.

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458
 459

Figure legends

Figure 1. The spatiotemporal expression profile of *nobo-Bm* in *B. mori*.

(A) qRT-PCR analysis of *nobo-Bm* mRNA in several tissues from day 2 fourth instar larvae (4th day2). CC-CA; corpus cardiacum-corpus allatum complex. The normalized *nobo-Bm* mRNA level in the PG was set as 1.

(B) The temporal expression profile of *nobo-Bm* in the PG during the fourth instar larval (IV), fifth instar larval (V) and pupal stages. The dashed line is a schematic representation of the developmental changes in the hemolymph ecdysteroid titer based on previously published data (Kiguchi and Agui, 1981; Kiguchi et al., 1985). The normalized *nobo-Bm* mRNA levels in day 0 pupae were set to 1. Each error bar represents the standard error of the mean (s. e. m.) of three biological replicates.

Figure 2. The *nobo-Bm* genomic mutant and its phenotype

(A) The genomic structures of +^{*nobo-Bm*} and the *nobo-Bm*^{*Δ85*} allele. The *nobo-Bm*^{*Δ85*} allele lacks 85 base pairs from the 2nd exon and the 2nd intron. The gray, blue and orange boxes indicate the untranslated regions, the GST N-terminal domain and the GST C-terminal domain, respectively.

(B) Genomic PCR of the +^{*nobo-Bm*} and *nobo-Bm*^{*Δ85*} alleles. Genomic DNA was prepared from wild type, *nobo-Bm*^{*Δ85*} heterozygous and *nobo-Bm*^{*Δ85*} homozygous animals. The PCR primers are shown in A. The PCR products of the +^{*nobo-Bm*} and *nobo-Bm*^{*Δ85*} loci are 313 bp and 228 bp, respectively. In this figure, +^{*nobo-Bm*} and *nobo-Bm*^{*Δ85*} are described as “+” and “*Δ85*,” respectively, for simplicity.

(C) The *nobo-Bm* transcripts derived from the +^{*nobo-Bm*} and *nobo-Bm*^{*Δ85*} alleles. The *nobo-Bm*^{*Δ85*} allele generates a slightly longer transcript than the +^{*nobo-Bm*} allele. The

485 nucleotide sequence of the *nobo-Bm*^{A85}-derived *nobo-Bm* cDNA is described in
 486 Supplementary Fig. 2.

487 (D) The predicted primary structures of the proteins encoded by the +^{*nobo-Bm*} and
 488 *nobo-Bm*^{A85} genes. The *nobo-Bm*^{A85} gene product is thought to contain the first 71 amino
 489 acids of the wild type Nobo-Bm protein and an additional 47 amino-acid extension
 490 encoded in the 2nd intron, which shows no obvious homology to any known proteins.
 491 The *nobo-Bm*^{A85} gene product lacks half of the GST N-terminal domain and the entire
 492 GST C-terminal domain.

493 (E, F) Larvae of *nobo-Bm*^{A85}/*nobo-Bm*^{A85} (top) and control (+^{*nobo-Bm*}/+^{*nobo-Bm*} or
 494 *nobo-Bm*^{A85}/+^{*nobo-Bm*}) animals (bottom) 10 days (E) and 13 days (F) after hatching. The
 495 control animals shown in these photos are 3rd (E) and 4th (F) instar larvae. By contrast,
 496 the *nobo-Bm*^{A85}/*nobo-Bm*^{A85} animals were arrested at the 2nd instar stage even 13 days
 497 after hatching. Note that the *nobo-Bm*^{A85}/*nobo-Bm*^{A85} homozygous larva exhibits a glossy
 498 cuticle phenotype. Scale bar: 0.5 cm.

499

500 **Figure 3.** The ecdysteroid and sterol levels in the *nobo-Bm* genomic mutant

501 In this figure, +^{*nobo-Bm*} is described as “+” for simplicity.

502 (A) The 20E titer in wild type and *nobo-Bm*^{A85} mutant animals. Second instar
 503 +^{*nobo-Bm*}/+^{*nobo-Bm*} and *nobo-Bm*^{A85}/+^{*nobo-Bm*} larvae were collected 6 days after hatching, and
 504 *nobo-Bm*^{A85}/*nobo-Bm*^{A85} animals were collected 9 days after hatching. Each bar
 505 represents the mean ± s. e. m. There were 20 +^{*nobo-Bm*}/+^{*nobo-Bm*} samples, 28
 506 *nobo-Bm*^{A85}/+^{*nobo-Bm*} samples and 11 *nobo-Bm*^{A85}/*nobo-Bm*^{A85} samples. The genotypes
 507 were confirmed by genomic PCR. * *P* < 0.01 by Student's *t*-test.

508 (B) The levels of various sterols in the PG. PGs were collected from 2nd instar larvae 6
 509 days after hatching. Each bar represents the mean ± s. e. m. There were 7 +^{*nobo-Bm*}/+^{*nobo-Bm*}

510 samples, 7 *nobo-Bm*^{Δ85}/*+*^{*nobo-Bm*} samples and 10 *nobo-Bm*^{Δ85}/*nobo-Bm*^{Δ85} samples. *

511 $P < 0.05$ by Student's *t*-test. C, cholesterol; 7dC, 7-dehydrocholesterol; βsito, β-sitosterol.

512

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Figure 1

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Figure 2

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Figure 3

nobo-Bm mutants cause larval arrest
and a glossy cuticle phenotype

nobo-Bm mutants exhibit
a low ecdysteroid titer