Non-molting glossy/shroud encodes a short-chain dehydrogenase/reductase that functions in the ‘Black Box’ of the ecdysteroid biosynthesis pathway

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SUMMARY
In insects, the precise timing of molting and metamorphosis is strictly guided by a principal steroid hormone, ecdysone. Among the multiple conversion steps for synthesizing ecdysone from dietary cholesterol, the conversion of 7-dehydrocholesterol to 5β-ketodiol, the so-called ‘Black Box’, is thought to be the important rate-limiting step. Although a number of genes essential for ecdysone synthesis have recently been revealed, much less is known about the genes that are crucial for functioning in the Black Box. Here we report on a novel ecdysteroidogenic gene, non-molting glossy (nm-g)/shroud (sro), which encodes a short-chain dehydrogenase/reductase. This gene was first isolated by positional cloning of the nm-g mutant of the silkworm Bombyx mori, which exhibits a low ecdysteroid titer and consequently causes a larval arrest phenotype. In the fruit fly, Drosophila melanogaster, the closest gene to nm-g is encoded by the sro locus, one of the Halloween mutant members that are characterized by embryonic ecdysone deficiency. The lethality of the sro mutant is rescued by the overexpression of either sro or nm-g genes, indicating that these two genes are orthologous. Both the nm-g and the sro genes are predominantly expressed in tissues producing ecdysone, such as the prothoracic glands and the ovaries. Furthermore, the phenotypes caused by the loss of function of these genes are restored by the application of ecdysteroids and their precursor 5β-ketodiol, but not by cholesterol or 7-dehydrocholesterol. Altogether, we conclude that the Nm-g/Sro family protein is an essential enzyme for ecdysteroidogenesis working in the Black Box.

KEY WORDS: Black Box, Bombyx mori, Drosophila melanogaster, Ecdysone, Halloween mutant, Prothoracic gland, Short-chain dehydrogenase/reductase

INTRODUCTION
In insects and other arthropods, steroid hormones designated as ecdysteroids, such as ecdysone and its derivative, 20-hydroxyecdysone (20E), control the precise temporal progression of development (Thummel, 2001; Gilbert et al., 2002; Mirth and Riddiford, 2007; Spindler et al., 2009). Ecdysone is synthesized via a series of hydroxylation and oxidation steps in the prothoracic gland (PG) during postembryonic development and in the ovary in adults (Gilbert et al., 2002). Ecdysone is subsequently converted to 20E by 20-hydroxylase present in the peripheral tissues (Gilbert et al., 2002).

In the past decade, molecular genetic studies using the fruit fly Drosophila melanogaster have successfully identified several genes crucial for the conversion of intermediates in ecdysone biosynthesis. The dehydrogenation of cholesterol to 7-dehydrocholesterol (7dC), the first step for synthesizing ecdysone, is mediated by the Rieske-domain protein Neverland (Nvd) (Yoshiyama et al., 2006) (Fig. 1). The terminal hydroxylation steps in ecdysteroid biosynthesis in the PG, namely 5β-ketodiol to ecdysone, are catalyzed by three cytochrome P450 mono-oxygenases: Phantom (Phm; CYP306A1), Disembodied (Dib; CYP302A1) and Shadow (Sad; CYP315A1) (Chávez et al., 2000; Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004; Niwa et al., 2005) (Fig. 1). The conversion of ecdysone to 20E is also mediated by a P450 mono-oxygenase, Shade (Shd; CYP314A1), in the peripheral tissues (Petryk et al., 2003). All of these genes encoding P450 enzymes have been identified from embryonic lethal mutants called Halloween mutants that exhibit ecdysone deficiency in embryos (Chávez et al., 2000; Gilbert and Warren, 2005).

Although the first and final biochemical steps of ecdysteroid biosynthesis are relatively well-characterized at the molecular level, little is known about the enzymes involved in the intervening conversion processes from 7dC to 5β-ketodiol. These uncharacterized reactions are commonly referred to as the ‘Black Box’ (Fig. 1), for which no stable intermediate has been identified. It is believed that the Black Box contains the rate-limiting step in the production of ecdysone (Warren and Hetru, 1990; Gilbert et al.,

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prothoracicotropic hormone (PTTH) and a subsequent intracellular ecdysteroid biosynthesis. However, it is unclear whether play a role in the Black Box (Namiki et al., 2005; Ono et al., 2006).

The identification and functional characterization of genes involved in ecdysteroidogenesis are essential to understand the mechanisms by which ecdysteroidogenesis is precisely controlled in insects. These modifications include the oxidation of β-alkanol to its ketone form, the oxidation of carbon-6 with concomitant loss of the 4- and 6-hydrogens to form the 6-keto group, and 14α-hydroxylation (Warren and Hetru, 1990; Gilbert et al., 2002; Lafont et al., 2005). Therefore, the identification and functional characterization of genes involved in the Black Box are essential to understand the mechanisms by which ecdysteroidogenesis is precisely controlled in insects.

To uncover an unidentified gene responsible for ecdysteroid biosynthesis, we focused on a genetic mutant designated non-molting glossy (nm-g) of the silkworm Bombyx mori, another classical model insect utilized for the study of endocrinology. Previous studies have revealed that the nm-g mutant causes a reduced ecdysteroid titer and larval arrest mainly at the first instar stage (Nagata et al., 1987; Tanaka, 1998). Here, we report that the gene responsible for nm-g mutants encodes a short-chain dehydrogenase/reductase (SDR). We also show that the shroud (sro) mutant, one of the Halloween mutants in D. melanogaster, is caused through the loss of function of the nm-g ortholog. Finally, we demonstrate that the Nm-g/Sro family acts as an indispensable player for a specific conversion step called the Black Box in ecdysteroid biosynthesis (Fig. 1).

MATERIALS AND METHODS

Animal strains and culture

Wild-type strains of the silkworm B. mori were C108 (The University of Tokyo) and the KINSHU × SHOWA F1 hybrid. The nm-g mutant strain (Nagata et al., 1987) was a46 (Kyushu University). Silkworm larvae were reared on mulberry leaves or on an artificial diet (Silkkmate, Nihon Nosan Kogyo) at 25°C.

D. melanogaster flies were reared on standard agar-cornmeal medium at 25°C under a 12-hour light, 12-hour dark photoperiod. yw was used as the wild-type. sro1 (Jürgens et al., 1984) was obtained from the Drosophila Genetic Resource Center. Other strains were kind gifts as follows: sro754 and sro34-105 (Giesen et al., 2003) from C. Klämbt (University of Münster, Germany); kay1 (Zeitlinger et al., 1997) from D. Bohmann (University of Rochester, NY, USA); 2-286-GAL4, in which GAL4 is expressed during embryogenesis (see Fig. S1 in the supplementary material) and strongly expressed in the larval PG (Timmons et al., 1997), from C. S. Thummel (University of Utah, USA); and pth neuron-ablated flies (pth-GAL4;UAS-grim) (McBrayer et al., 2007), pnm-GAL4 and the TM3[Actin5c-GFP] balancer from M. B. O’Connor (University of Minnesota, USA). sro RNAi was performed using three independent UAS-sro-inverted repeat (IR) transgenic lines that targeted different CG12068 (sro) open reading frame (ORF) regions (see Fig. S2A in the supplementary material). UAS-sro-IR1 was established as described below. UAS-sro-IR2 and UAS-sro-IR3 were obtained from the Vienna Drosophila RNAi Center.

Genetic mapping and molecular cloning of nm-g

For linkage and recombination analysis, a single-pair cross between a female (C108) and a male (a46) produced the F1 offspring. Then, the cross (a46 × C108) × (a46 × C108) produced F2 progeny. Primer sets were designed from the single nucleotide polymorphism (SNP) linkage map (Yamamoto et al., 2006; Yamamoto et al., 2008) and the B. mori genome sequence (Mita et al., 2004; Xia et al., 2008) (see Table S1A in the supplementary material). PCR and SNP markers were generated at each position on linkage group 17 and the markers that showed polymorphisms between C108 and a46 were used for the genetic analysis of 839 F2 individuals with the nm-g phenotype (see Table S2 in the supplementary material). Total RNA for each individual genotyped was isolated using the RNeasy Mini Kit (Qiagen). Synthesis of single-stranded cDNA and qRT-PCR were performed as previously described above.

Quantitative RT-PCR (qRT-PCR)

For quantifying the sro transcript level in sro754 homozygous and control embryos, individual embryos from sro754/TM3[Actin5c-GFP] parents were collected 2-6 hours after egg laying (AEL). Extraction of both genomic DNA and total RNA from individual embryos was performed as essentially described (Ghanim and White, 2006). sro754 homozygote and other progenies were distinguished by PCR amplification of the GFP gene region on the TM3 balancer with genome DNA and primers (see Table S1D in the supplementary material). Total RNA for each individual transgenic embryo was isolated by RNeasy Mini Kit (Qiagen). Synthesis of single-stranded cDNA and qRT-PCR were performed as previously described above.

Fig. 1. A schematic representation of the roles of Nm-g/Sro in ecdysteroid biosynthesis. Nm-g/Sro plays a crucial role in the conversion step(s) between 7-dehydrocholesterol and 5β-ketodiol, the so-called Black Box.

2002) and is ultimately under the regulation of the neuropeptide prothoracicotrophic hormone (PTTH) and a subsequent intracellular signaling pathway (Gilbert et al., 2002; Rewitz et al., 2009). A recent study reported that the Halloween gene spook (spo) and its paralog spookier (spok), which also encode cytochrome P450s, play a role in the Black Box (Namiki et al., 2005; Ono et al., 2006). However, it is unclear whether spo and spok are only a single genetic component of the Black Box because the conversion of 7dC to 5β-ketodiol must be achieved through modifications at multiple carbon positions. These modifications include the oxidation of β-alkanol to its ketone form, the oxidation of carbon-6 with concomitant loss of the 4β- and 6-hydrogens to form the 6-keto group, and 14α-hydroxylation (Warren and Hetru, 1990; Gilbert et al., 2002; Lafont et al., 2005). Therefore, the identification and functional characterization of genes involved in the Black Box are essential to understand the mechanisms by which ecdysteroidogenesis is precisely controlled in insects.
In situ RNA hybridization
To generate a template for synthesizing sense and antisense sro RNA probes, the ORF region of sro was amplified by PCR using primers (see Table S1H in the supplementary material) and subcloned to pGEM-T (Promega). Synthesis of DIG-labeled RNA probes and in situ hybridization were performed as previously described (Niwa et al., 2004).

Rescue experiments with ecdysonoids intermediates
Cholesterol, 7αCholesterol, 7α,20E, ecyanosce and 20E were purchased from Sigma. 5β-ketodiol (3β,14α-dihydroxy-5β-cholesten-7-en-6-one) was prepared as previously described (Niwa et al., 2004). For rescue experiments for D. melanogaster embryos, sro/+/sroKI trans-heterozygous embryos were collected 6–9 hours AEL, dechorionated and incubated with or without 100 mM 20E for 3 hours as previously described (Ono et al., 2006). After incubation, the embryos were washed and allowed to develop further on agar-apple juice plates with yeast paste containing 1 mg/ml or no 2E in 3.3% ethanol at a final concentration. For the feeding rescue experiment for B. mori nm-g mutants, we collected the nm-g mutant larvae at day 5 of first instar. At this stage, wild-type animals entered into the molting stage, whereas the nm-g mutants did not. Because the nm-g mutant larvae were smaller than the control larvae, the nm-g/nm-g homozygotes were distinguished from the nm-g/+;nm-g and +/+;nm-g/+;nm-g bodies by size. Ten selected nm-g mutant larvae were reared on 100 mg ml−1 of 0.2 mg/ml of each intermediate dissolved in 100% ethanol. Two days later, developmental stages were scored. The percentage of nm-g mutants that entered into the molting stage was calculated. This assay was performed at least five times for each intermediate. To assess the phenotype of D. melanogaster sro RNAi animals, the 24–36 hours AEL first instar larvae of yw;UAS-sro-IR1/+;2-286-GAL4/+ were collected. Progeny carrying either UAS or GAL4 transgenes alone were used as controls. Feeding rescue experiments for D. melanogaster larvae were performed as previously described (Yoshiyama et al., 2006).

Ecdysteroid titer measurements
Day 5 first instar nm-g mutants were transferred to mulberry leaves supplemented with ecdysteroids intermediates. Then, silkworms were collected and squashed 1 day after the transfer, which was 1 day before the day before the wild-type animals entered into the molting stage under our culture conditions. In experiments using D. melanogaster control and sro RNAi larvae were collected 54-66 hours AEL when a large amount of 20E is produced in wild type (Sullivan and Thummel, 2003). Sample preparation and a radioimmunoassay for ecdysteroid titer measurement were performed as described (Takeda et al., 2004). Cholesterol, 7α-dihydroxy-5β-cholesten-3-one (α-chol) and 7α-dihydroxy-5β-cholesten-3-one (β-chol) were purchased from Sigma.

RESULTS
Positional cloning of nm-g in B. mori
To specify a candidate region for a responsible gene of nm-g mutant (Fig. 2A), we performed genetic linkage analysis using the SNP linkage map and the B. mori genome sequence. First, we roughly mapped and narrowed the nm-g mutation region on linkage group 17 (Fig. 2B; see also Fig. S1A and Table S2 in the supplementary material). Two scaffolds (Bm scaf131 and Bm scaf21) corresponding to this region were found in the B. mori genome database (see Fig. S1B in the supplementary material). We then designed primer sets based on the sequences of these scaffolds (see Table S1A in the supplementary material) and further delimited the nm-g locus to an ~330 kb-long position on Bm scaf21 that contained seven predicted genes (Fig. 2B; see Fig. S3C in the supplementary material). RT-PCR experiments revealed that the expression of one of the seven genes, BGIBMGA007047, was detected in the wild-type but not in the nm-g homozygous larvae (Fig. 2C), whereas the expression of the other genes examined did not exhibit any obvious differences between the

UAS vectors, overexpression of genes and generation of transgenic strains
Overexpression of genes in flies was performed using the GAL4/UAS system (Brand and Perrimon, 1993). To generate UAS constructs to overexpress nm-g and sro, specific primers (see Table S1F in the supplementary material) were used for PCR to add a BglII site and a NotI site at the 5′ and the 3′ ends, respectively, of each of the cDNA fragments corresponding to the ORFs. Each ORF region was digested with BglII and NotI and the fragment was ligated into a pUAST vector. For transgenic RNAi (Kennerdell and Carthew, 2000), we generated a UAS-sro-IR1 that was a genomic-cDNA fusion construct as represented in Fig. S2B in the supplementary material. The primers for constructing the UAS-sro-IR1 are described in Table S1G in the supplementary material. The D. melanogaster transinvertase transformants were established using standard protocols.

Identification of point mutations in EMS-induced sro mutants
Genomic DNA was extracted from the balanced sro/+/sroKI trans-heterozygous adults. DNA fragments covering the entire sro gene region and the first exon of kayakIDSfoxBG31956 were amplified by PCR using genomic DNA with primers (see Table S1H in the supplementary material) and then sequenced.

Fig. 2. The identification and characterization of the B. mori nm-g locus. A) nm-g homozygous mutants (left) and nm-g heterozygous or wild-type larvae (right) photographed 10 days after hatching. Scale bar: 1 cm. B) Mapping of nm-g. The nm-g locus was in an ~330 kb region between 0.45 M and 0.78 M nucleotides on Bm_scaf21, located around 24.9 CM on linkage group 17. The ~330 kb region contains seven putative transcripts predicted by the Chinese genome consortium. The BGIBMGA007047 (nm-g) gene is marked in red. See Fig. S2A-C in the supplementary material for more details. C) RT-PCR analysis with wild-type (WT) and nm-g mutants using the primers indicated in D. 18S ribosome was used as an internal control. D) The genomic structure of the nm-g gene in wild type (C108) and nm-g mutant strains (a46). White boxes, gray boxes and black bars indicate untranslated regions, the ORF and intronic regions, respectively. An insertion on the nm-g mutant chromosome is marked in red.
wild-type and the nm-g homozygous larvae (see Fig. S3D in the supplementary material). Moreover, the nm-g mutant chromosome had a 5.2 kb insertion sequence in the third exon of BGIBMGA007047 (Fig. 2D; see Fig. S4 in the supplementary material). Because the insertion disrupted the entire BGIBMGA007047 ORF, we concluded that BGIBMGA007047 was the gene responsible for nm-g mutants (\(+^{nm-g}\)). Hereafter, we describe the BGIBMGA007047 or \(+^{nm-g}\) gene as nm-g for simplicity.

**The D. melanogaster Halloween mutant shroud causes loss of function of the nm-g ortholog**

A BLAST search revealed that the B. mori nm-g gene is the most closely related to CG12068 of all of the predicted genes of *D. melanogaster* (Fig. 3A,B). CG12068 is located at the 99C1 cytological position on the third chromosome. Curiously, this position is in the vicinity of the Halloween mutant shroud (sro) that has been mapped to the 99A-100A interval (Jürgens et al., 1984). sro is known to exhibit the typical Halloween-class phenotype (Jürgens et al., 1984; Chávez et al., 2000), epitomized by an undifferentiated cuticular structure in embryos (Fig. 4A,B). Conversely, a previous study has reported that sro1, indeed located next to CG12068 on the third chromosome (Fig. 4C). A basis of this conclusion is that two independent EMS-induced lethal alleles of sro, sro1 and sro\(^{O4-105}\), were not complemented with sro\(^{P54}\) (Fig. 4D), in which a P-element is inserted in the 5\(^\prime\) upstream region of kay (Giesen et al., 2003) (Fig. 4C). Paradoxically, the strongest kay mutation, kay\(^{I}\), results in embryonic lethality but not the typical Halloween-class morphological defects (Jürgens et al., 1984; Zeitlinger et al., 1997; Giesen et al., 2003). We therefore examined whether CG12068, but not kay, was the true gene responsible for sro mutants.

First, the EMS-induced kay\(^{I}\) and sro\(^{O4-105}\) alleles were complementary to the kay\(^{I}\) allele (Fig. 4D), suggesting that the gene responsible for the EMS-induced sro mutants must be different from the kay gene. This result was consistent with another recent report (Hudson and Goldstein, 2008). Second, qRT-PCR analysis revealed that the sro\(^{P54}\) homozygous mutant embryos exhibited a large reduction in the CG12068 transcript level compared with the control embryos (Fig. 4E), indicating that sro\(^{P54}\) causes loss of function not only in kay but also in CG12068. Third, both the sro\(^{I}\) and the sro\(^{O4-105}\) chromosomes had C-to-T nucleotide substitutions (see Fig. S5 in the supplementary material), which led to nonsense mutations that disrupted the catalytic centers of the deduced CG12068 protein structures (Fig. 3A; see Fig. S5 in the supplementary material). Finally, the lethality of the sro\(^{I}\) mutant was rescued by the overexpression of either *D. melanogaster* CG12068 or *B. mori* nm-g (Table 1). These results strongly support our hypothesis that CG12068 is the gene responsible for the sro mutants and is the functional ortholog of nm-g. Hereafter, we call the CG12068 gene *sro*.

**The embryonic lethality of the D. melanogaster sro mutant is rescued by delivering 20E**

As the Halloween mutants have defects in ecdysteroid biosynthesis (Chávez et al., 2000), we next determined whether the lethality of the sro mutants was rescued by delivering 20E midway through embryogenesis. With control ethanol treatment, no sro\(^{I}\)/sro\(^{O4-105}\) embryos (n=134) developed into first instar larvae. By contrast, by the 20E application, approximately 52% of those embryos (n=259) hatched into first instar larvae, whereas the rescued animals died at the first instar larva on a normal diet (n=59). This larval lethality was also due to ecdysone deficiency, as approximately 45% of the rescued sro\(^{I}\)/sro\(^{O4-105}\) first instar larvae (n=58) grew into the second larval stage on a diet supplemented with 20E. These results suggest that the sro is required for ecdysone production in both embryonic and larval development in *D. melanogaster*.
**nm-g/sro encode a short-chain dehydrogenase/reductase**

The predicted full ORF of *B. mori nm-g/* *D. melanogaster sro* encodes a protein that belongs to short-chain dehydrogenase/reductases (SDRs), which constitute a large family of enzymes that catalyze NAD(P)(H)-dependent oxidation and/or reduction reactions (Kalberg et al., 2002; Kavanagh et al., 2008). The primary structure of Nm-g/Sro contains several motifs that are typical of SDR proteins, such as a NAD/NADP binding domain, a catalytic center (Fig. 3A; see Fig. S5 in the supplementary material) and other characteristic secondary structures (see Fig. S5 in the supplementary material). A phylogenetic analysis demonstrates that putative *nm-g/sro* orthologs are conserved among insect species as well as the crustacean *Daphnia pulex* (Fig. 3B). Whereas the Nm-g/Sro proteins are substantially similar to some vertebrate steroidogenic SDR proteins, such as 11β- and 17β-hydroxysteroid dehydrogenases (Wu et al., 2007) (Fig. 3B; see Fig. S5 in the supplementary material), apparent *nm-g/sro* orthologs cannot be found outside the arthropods (Fig. 3B). These results suggest that the occurrence of *nm-g/sro* genes is limited to animals that produce ecdysteroids, similar to the Halloween P450 genes (Rewitz and Gilbert, 2008).

**nm-g/sro is predominantly expressed in the PG and ovaries**

Spatial expression of *nm-g* was restricted to the PG and was weak in the ovary, both of which synthesize ecdysone (Fig. 5A). A change in the *nm-g* expression level in the PG also correlated well with the change in the hemolymph ecdysteroid titer during development (Fig. 5B), which was similar to other ecdysteroidogenic genes, such as *B. mori spo* and *phm* (see Fig. S6 in the supplementary material).

In *D. melanogaster*, the early embryonic expression of the Halloween P450 genes is essential for embryonic ecdysteroid production prior to the formation of the PG (Gilbert and Warren, 2005; Namiki et al., 2005; Ono et al., 2006). The embryonic expression pattern of *sro* also correlated with a change in the embryonic ecdysteroid titer in *D. melanogaster*. Although no or little *sro* mRNA was maternally loaded (Fig. 5C; see Fig. S7A in the supplementary material), the *sro* transcript level drastically increased around stage 3, mainly in the epithelial cells (see Fig. S7B in the supplementary material). The maximal level of embryonic *sro* expression was observed at 2–4 hours AEL (Fig. 5C), which roughly corresponds to embryonic stages 5–9 (see Fig. S7C–E in the supplementary material). Then, *sro* expression was gradually decreased in later embryogenesis (Fig. 5C; see Fig. S7F,G in the supplementary material). These results suggest that *sro* mRNA accumulates prior to the maximal ecdysteroid titer during *D. melanogaster* embryogenesis (Maróy et al., 1988).

During the larval stage, *sro* was expressed predominantly in the ring gland, which contains the PG cells of the first instar larval stage and later stages (Fig. 5D,E; see Fig. S7I in the supplementary material). In the ring gland, the *sro* transcript was exclusively observed in the PG (Fig. 5F) but not in the corpus allatum or corpus cardiacum, which are components of the ring gland (Fig. 5F). In the developing egg chambers of adult females, *sro* was expressed in the nurse cells (Fig. 5G; see Fig. S7H in the supplementary material), in which some genes involved in ecdysone production are known to be expressed (see Discussion). Furthermore, similar to the *nev* and *Halloween* P450 genes (McBrayer et al., 2007), *sro* expression was significantly reduced in the third instar larvae in which the *pith* gene-expressing neurons were ablated (Fig. 5H). All of these results demonstrate that *D. melanogaster sro* expression is also spatiotemporally correlated with ecdysone production.

**The larval arrest phenotype of the loss of nm-g/sro function is rescued by feeding of 20E in both B. mori and D. melanogaster**

To assess the importance of *sro* in the PG during *D. melanogaster* larval development, we also examined the phenotypes of either the overexpression or the knockdown of the *sro* gene in the PG of developing flies using the GAL4/UAS system. In a wild-type background, the overexpression of *sro* using the PG-expressing GAL4 lines 2-286-GAL4 and *phm-GAL4* had no visible effect on development (data not shown). To knock down *sro*, we performed transgenic RNA interference (RNAi) experiments (Kennerdell and
Carthew, 2000) using a transgenic line carrying an inverted repeat construct corresponding to the sro mRNA under the control of the UAS promoter (UAS-sro-IR1; see Fig. S2A,B in the supplementary material). Hereafter, we refer to the animals in which the sro RNAi was driven by 2-286-GAL4 and UAS-sro-IR1 as ‘sro RNAi animals’ for simplicity. In the sro RNAi animals, an ~90% reduction in the level of the sro mRNA was achieved at the first and second instar larvae (see Fig. S2C,D in the supplementary material). sro RNAi animals completed embryogenesis, hatched normally and showed no apparent morphological or behavioral defects until the second instar larvae. After 72 hours AEL, the control animals possessed large mouth hooks with numerous small teeth and everted anterior spiracular papillae of the trachea, which is typical for third instar larvae. However, even after 72 hours AEL, sro RNAi animals retained the second instar larval-type morphologies for both the mouth hook and the anterior spiracular insertion (Fig. 6A,C). All sro RNAi animals died prior to 144 hours AEL at the second instar larval stage. The identical larval arrest phenotype was also observed when using the sro RNAi driven by the other PG-expressing GAL4 driver, phm-GAL4. In addition, two other sro RNAi transgenic lines that targeted different regions of the sro gene (see Fig. S2A in the supplementary material) also caused lethality when driven by 2-286-GAL4 or phm-GAL4 (see Table S3 in the supplementary material), suggesting that the effect of the RNAi was specific to sro and was not an off-target effect.

We also found that sro RNAi animals fed yeast paste containing 20E just after hatching grew to the third instar larval stage, as judged by the mouth hook and anterior spiracular morphologies (Table 2; Fig. 6B,D). Similarly, consistently with a previous report (Nagata et al., 1987; Tanaka, 1998), the larval arrest phenotype of the B. mori nm-g mutant was rescued by feeding the larvae edysone (Table 2; see Fig. S8 in the supplementary material). These results demonstrate that nm-g and sro are essential for larval development via the regulation of edysone production.

**A measurement of ecdysteroid titer in sro RNAi animals using radioimmunoassay**

Because the B. mori nm-g mutant larvae are known to exhibit a low ecdysteroid titer (Nagata et al., 1987; Tanaka, 1998), it was expected that the loss of sro function would also cause a reduction of the ecdysteroid titer in D. melanogaster. We therefore performed a radioimmunoassay to examine whether the sro RNAi caused a reduction in ecdysteroid titers. Ethanolic extracts from the control and the sro RNAi second instar larvae were prepared 54-66 hours AEL, which is the time when a large amount of 20E is produced in the wild type (Sullivan and Thummel, 2003). However, the sro RNAi animals exhibited a normal level of ecdysteroids when compared with the control animals (see Fig. S9 in the supplementary material). This result was in fact consistent with the previous study demonstrating that sro mutant embryos do not show the reduced ecdysteroid titer (Chávez et al., 2000). This point is argued in the Discussion below.

**nm-g/sro plays a crucial role in the Black Box**

To determine which ecdysteroid conversion step is affected by the loss of nm-g/sro function, we performed a feeding experiment with various precursors of edysone biosynthesis. We expected that an exogenously applied intermediate downstream of the conversion step by Nm-g/Sro would overcome the larval arrest phenotype observed in the loss of nm-g/sro function animals. When 5β-ketodiol was added to the food, ~90% of the B. mori nm-g mutant animals were rescued and entered the molting stage (Table 2; see Fig. S8 in the supplementary material). Similarly, in D. melanogaster, ~70% of the sro RNAi animals on 5β-ketodiol-supplemented food molted into the third larval instar (Table 2). By contrast, food containing cholesterol and 7dC did not rescue the
larval growth arrest in the loss of nm-g/sro function larvae of both species (Table 2; see Fig. S8B,C in the supplementary material). In B. mori nm-g mutants, the application of 5β-ketodiol, but not cholesterol or 7dC, also rescued the low ecdysteroid titer and exhibited a higher titer than the wild-type ecdysteroid level (Fig. 7). The failure of feeding rescue by 7dC was not due to the instability and accidental degradation of 7dC under our experimental conditions because the larval growth arrest of day 3 wild-type and day 6 nm-g mutant first instar larvae were collected and squashed for their measurements (also see Materials and methods). The results are depicted as picograms (pg) of 20E equivalents/mg wet body weight on the y-axis. Each bar represents the mean ± s.e.m. from two independent samples for each feeding condition. Control animals (con.) were reared without intermediates. **, P<0.01 by Student’s t-test.

### DISCUSSION

In this study we identified the non-molting glossy (nm-g)/shroud (sro) genes that belong to a family of short-chain dehydrogenase/reductase (SDR) proteins. Some SDRs are involved in steroid biosynthesis in vertebrates (Wu et al., 2007). Although the 3-dehydroecdysone 3α-reductase, which contributes to the inactivation of ecdysteroids, belongs to the SDR family in the cotton leafworm Spodoptera littoralis (Takeuchi et al., 2000), no SDRs have been identified as ecdysteroidogenic enzymes so far. All of our results indicate that Nm-g/Sro plays a crucial role in ecdysteroid biosynthesis in insects.

A previous study argued that D. melanogaster sro and kay belong to the same complementation group (Giesen et al., 2003). However, we consider it unlikely that the gene responsible for the sro mutation is kay/D-fos for the following reasons. First, all EMS-induced sro mutants are complemented with the validated kay mutant, which is also consistent with a recent report (Hudson and Goldstein, 2008). Second, both of the independent EMS-induced sro alleles have nonsense mutations that disrupt the catalytic centers of the SDR proteins. Finally, lethality of the EMS-induced sro mutant is rescued by transgenes of D. melanogaster CG12068 and B. mori nm-g. We have assumed that the atypical characteristics of the P-element insertion allele sro1 leading to the conclusion of a previous study (Giesen et al., 2003) that showed non-complementation with both sro and kay (Fig. 4D). Because another previous study argues that the sro1 mutant has a point mutation in the first exon of kay (Hudson and Goldstein, 2008), we also re-examined their finding. We found three synonymous nucleotide substitutions, but not any nonsynonymous changes, in the ORF region in the first exon of kay (see Fig. S10 in the supplementary material). Therefore, the sro1 mutant chromosome at least does not cause any amino acid changes in Kay proteins.

B. mori nm-g mutants show a lower ecdysteroid titer during their larval development (Nagata et al., 1987) (Fig. 7). Conversely, radioimmunoassays performed by this study and the previous study (Chávez et al., 2000) demonstrate that sro RNAi animals exhibit the normal ecdysteroid titer in D. melanogaster. We also measured...

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**Table 2. Rescue experiments with ecdysteroid intermediates**

| Intermediate          | % of rescued nm-g | % of rescued sro RNAi |
|-----------------------|-------------------|-----------------------|
| ETOH (control)        | 0 (30)            | 0 (71)                |
| Cholesterol           | 0 (30)            | 0 (107)               |
| 7-dehydrocholesterol  | 0 (30)            | 0 (89)                |
| 5β-ketodiol           | 90 (30)           | 74 (147)              |
| Ecdysone              | 93 (30)           | n.d.                  |
| 20-hydroxyecdysone    | n.d.              | 43 (118)              |

The percentage of nm-g silkworms that entered into the moulting stage and sro RNAi flies growing into the third instar stage was examined. Each number in parentheses refers to the total number of animals in each experiment. n.d., not determined.

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**Fig. 6. D. melanogaster sro RNAi animals are rescued by the feeding of 5β-ketodiol.** (A-D) Whole bodies (A, B) and dissected mouth hooks (C, D) of sro RNAi animals at 120 hours AEL. The animals were reared on food supplemented with control ethanol (A, C) or 5β-ketodiol (B, D). Yellow arrows indicate the teeth of mouth hooks. sro RNAi animals raised on a regular diet died in the second instar larval stage, exhibiting singular insertions of anterior tracheal pits (A, arrows and inset) and 2-5 teeth on mouth hooks (C). 5β-ketodiol-fed sro RNAi animals molted in the third instar larval stage as judged by the branched morphology of the anterior tracheal pits (B, arrowheads and inset) and numerous small teeth on the mouth hook (D), which are typical features of third instar larvae. Scale bars: 500 μm in A, B; 10 μm in C, D.

**Fig. 7. Ecdysteroid titers in the B. mori wild type and the nm-g mutants on food supplemented with intermediates.** Day 3 wild-type and day 6 nm-g mutant first instar larvae were collected and squashed for their measurements (also see Materials and methods). The results are depicted as picograms (pg) of 20E equivalents/mg wet body weight on the y-axis. Each bar represents the mean ± s.e.m. from two independent samples for each feeding condition. Control animals (con.) were reared without intermediates. ***, P<0.01 by Student’s t-test.
the ecdysteroid titer in sro RNAi animals supplemented with cholesterol, 7dC, 5β-ketodiol and 20E, but no obvious difference was detected between the control and the sro RNAi animals (see Fig. S9 in the supplementary material). The sro mutants have been classified as Halloween mutants, many of which exhibit ecdysteroid deficiencies in embryos (Chávez et al., 2000). In addition, the phenotypes caused by the loss of sro function are clearly rescued by the application of 20E or 5β-ketodiol, suggesting that D. melanogaster sro is essential for ecdysone production as well as B. mori nm-g. We assume that the loss of sro function might lead to the accumulation of the substrate of Sro in the Black Box. The accumulated substrate might cause a production of an unknown byproduct that can be recognized by an anti-ecdysone antibody used for the radioimmunostay, as discussed previously (Chávez et al., 2000). The data also suggest an unknown difference in the synthesis and/or metabolism of ecdysteroids between B. mori and D. melanogaster.

Like the Halloween P450 genes, the spatiotemporal expression pattern of nm-g/sro correlates with in vivo ecdysone production. Conversely, there are also some differences between sro and other Halloween genes. For example, sro is not expressed in the primordial PG in the late embryonic stage of D. melanogaster (see Fig. S7G in the supplementary material). The embryonic PG is thought to be less crucial for the production of embryonic ecdysteroid, as the ecdysteroid titers continuously and gradually decrease after the primordial ring gland forms by stage 14-15 during embryogenesis (Fig. 5C). Therefore, the embryonic PG without sro expression would not affect embryonic ecdysone production. Meanwhile, the difference in expression between sro and other Halloween genes suggests that the transcriptional regulation of nm-g/sro is possibly different from that of the other Halloween genes. The other difference is that the sro transcript is not observed in the follicle cells, which are thought to be a source of ecdysone (Riddiford, 1993; Gilbert et al., 2002), but it is seen in the nurse cells (Fig. 5G). Previous studies have shown that all the Halloween genes are expressed mainly in the follicle cells (Chávez et al., 2000; Warren et al., 2002; Petryk et al., 2003; Niwa et al., 2004; Warren et al., 2004; Namiki et al., 2005; Ono et al., 2006). However, it should be noted that another ecdysteroidogenic gene, neverland, is strongly expressed in nurse cells (Yoshiyama et al., 2006). In conjunction with our observation, an interesting possibility is that some conversion steps in ecdysteroid biosynthesis take place in the nurse cells and the other steps occur in the follicle cells. If this scenario is correct, certain intermediates should be shuttled between the nurse cells and the follicle cells. Further analysis is needed for understanding of ovarian ecdysteroid production.

The SDR family has great intra- and interspecies functional diversity (Kallberg et al., 2002; Kavanagh et al., 2008). Some mammalian SDRs catalyze the oxidoreduction of hydroxyl and oxo functions at distinct positions in the steroid hormones (Wu et al., 2007), raising the possibility that the Nm-g/Sro proteins could catalyze oxidation or reduction reactions in ecdysteroid biosynthesis. In addition to Halloween P450 Spook and its closest orthologs (Ono et al., 2006), our data suggest that Nm-g/Sro is involved in conversion of 7dC to 5β-ketodiol, the so-called Black Box (Fig. 1), which is considered the rate-limiting step in ecdysteroid biosynthesis (Gilbert et al., 2002). Because overexpression of nm-g and sro does not affect development, the transcriptional regulation of nm-g/sro might not be rate-limiting. Thus far, we have not assigned a specific enzymatic activity to any substrate for Nm-g/Sro, as no intermediates between 7dC and 5β-ketodiol have been precisely identified in insects (Warren and Hetru, 1990; Gilbert et al., 2002). One possibility is that Nm-g/Sro catalyzes the 3β-dehydrogenation of 7dC to 3-oxo-7dC (cholesta-5,7-diene-3-one), which is hypothesized to be involved in the initial step in the Black Box (Dauphin-Villemant et al., 1997; Warren et al., 2009). An alternative possibility is that Nm-g/Sro might act as an ecdysteroid 5β-reductase converting Δ^4-diketol to diketol. This is the putative last step in the Black Box proposed from studies using the Y-organs of the crab Carcinus maenas (Blais et al., 1996), although Δ4-diketol has neither been isolated nor shown to be a product in the ecdysteroid biosynthesis pathway in insects (Gilbert et al., 2002). Further studies to identify the exact step in which Nm-g/Sro is involved would shed light on the molecular mechanisms controlling developmental timing in insects. Studies on the elucidation of the enzymatic function of Nm-g/Sro are now underway.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Strain: 2-286-GAL4

*in situ* probe: GAL4 gene

| Stage  | Antisense | Sense |
|--------|-----------|-------|
| 1      | ![Image](image1.png) | ![Image](image2.png) |
| 5      | ![Image](image3.png) | ![Image](image4.png) |
| 9      | ![Image](image5.png) | ![Image](image6.png) |
| 13     | ![Image](image7.png) | ![Image](image8.png) |
Fig. S10
Niwa et al.
Fig. S2
Niwa et al.

A

116 172
CG12068 gene region
1063
UAS-sro-IR1
172 773
UAS-sro-IR2
662 987
UAS-sro-IR3
228 533

B

DmCG12068RNAi1-2
DmCG12068RNAi2-1

BglII
Inverted CG12068_{171-172}
NotI
CG12068_{172-773}
XbaI

DmCG12068RNAi1-1
DmCG12068RNAi2-2

C

The 1st instar larval stage

D

The 2nd instar larval stage

sro/rp49 (%)

| 2-286-GAL4 or UAS-sro-IR1 | 2-286-GAL4 >UAS-sro-IR1 |
|---------------------------|--------------------------|
| 100                       | *                        |

sro/rp49 (%)

| 2-286-GAL4 or UAS-sro-IR1 | 2-286-GAL4 >UAS-sro-IR1 |
|---------------------------|--------------------------|
| 100                       | **                       |
Fig. S5
Niwa et al.
Fig. S6
Niwa et al.

A

B

C

fourth instar larvae  fifth instar larvae  pupae
pg 20E equivalent / mg wet weight

control
sro RNAi

control cholesterol 7dC 5β-ketodiol

P = 0.25
P = 0.46
P = 0.33
P = 0.10

P = 0.47

* P = 0.20

20E
Table S1. The primer sets used in this study

(A) Linkage analysis in Table S2

| Name              | Sequence (5'-3')                        | Scaffold  | Position      |
|-------------------|-----------------------------------------|-----------|---------------|
| nscaf2874-1F      | TCCCTTATTTTCAATAACGCAACCA              | Bm_scaf131| 411132-411157 |
| nscaf2874-1R      | TATTCTCTCGATATTTGCCCTGTCGTA            | Bm_scaf131| 412015-412040 |
| nscaf2865-29F     | TAATTTCTCAACGCAGCGCTGTAAC              | Bm_scaf21 | 321784-321808 |
| nscaf2865-29R     | GGGATTTCTAAATGAGGCCCTGAAAGA            | Bm_scaf21 | 322741-322765 |
| nscaf2865-30F     | ATTTCTACTTGTTAAGTCGCTGGA               | Bm_scaf21 | 426441-426466 |
| nscaf2865-30R     | AAATCTGCTGATACGGCAGCGCTGGA             | Bm_scaf21 | 427407-427431 |
| prgy0385-F        | TGTCGACTGCGGTAATGCTGTAAGC              | Bm_scaf21 | 455338-455365 |
| prgy0385-R        | GCAGTTGGAATTCTGTTTTA                   | Bm_scaf21 | 454454-454478 |
| nscaf2865-35F     | TCTCGGAAAGGATTGATGATGCA                | Bm_scaf21 | 650876-650900 |
| nscaf2865-35R     | TTGTTTTATGGCCCTCTGATTAC               | Bm_scaf21 | 777509-777533 |
| nscaf2865-32F     | ACCGGTCCTTACACTTACCTCAGA               | Bm_scaf21 | 778399-778423 |
| nscaf2865-16F     | TAGCCGGGGGACTACTGCTGTA                | Bm_scaf21 | 834000-834024 |
| nscaf2865-16R     | GGCCCAAGCACTTGCATTCGTTTTA             | Bm_scaf21 | 834882-834906 |
| nscaf2865-6F      | TCTCGGGAAGGATTGATGATGCA                | Bm_scaf21 | 1683479-1683503|
| nscaf2865-6R      | TTGACCTTIAAGTGAAGCAGCGAGG              | Bm_scaf21 | 1684319-1684343|

(B) PCR for amplifying the 7 nm-g candidate shown in Fig. 2C and Fig. S3D

| Primer-F          | Primer-R                        |
|-------------------|---------------------------------|
| BGIBMGA007045-F   | AATAATGGAAGGCGATGCTATTGTTG      |
| BGIBMGA007045-R   | TTAGGGTTTCTGATAGCAGGCCAAAT      |
| BGIBMGA007046-F   | ATGAAACACAGGCTTGGTTCACTAA       |
| BGIBMGA007046-R   | CGTTAATACAAATGCCAGGCTAGAGG      |
| BGIBMGA007047-F   | GTCCGTTAGCCGCTTCTGTAGCTGA       |
| BGIBMGA007047-R   | TGAATGAAATAGGCAAGGCAACTATC      |
| BGIBMGA007048-F   | ATGTTTTTATTGCTGCTTCTGATG        |
| BGIBMGA007048-R   | TACAATACACCAAGGCGTTTCATTG       |
| BGIBMGA007049-F   | TTTGAATCTTTACTCCTACCCCAACC      |
| BGIBMGA007049-R   | GAAACATGAGCCTTGGCCTGTGTG        |
| BGIBMGA007050-F   | TTGGACCACACACCAACTTACCGA        |
| BGIBMGA007050-R   | TGGCAATTCTCTCGTTGTTGATGAGT      |
| BGIBMGA007060-F   | CAGGCAACACAGAAGAAGAGGCAACG      |
| BGIBMGA007060-R   | CAATAAAACAGATTACCCGGAAAGGT      |
| 18S-F             | TTGGACGGAAAGGGCCACCCACCC        |
| 18S-R             | TTGGACGGAAAGGGCCACCCACCC        |

(C) Genomic PCR in Fig. 2D and Fig. S4

| Primer-F          | Primer-R                        |
|-------------------|---------------------------------|
| GTCCGTTAGCCGCTTCTGTAGCTGA | TGAATGAAATAGGCAAGGCAACTATC |

(D) Genotyping sroP54 homozygous embryos

| Primer-F          | Primer-R                        |
|-------------------|---------------------------------|
| GAACTACAAAGACAGCGTCGTA | CGATAGGGCGAGTATGGTGGA |

(E) qRT-PCR

| Primer-F          | Primer-R                        |
|-------------------|---------------------------------|
| TGTGATAGTGACTGGCTGGTGGC | GGCTTTCCGCTGCTCGTTTCC |
| GCATGAGGCTATGCGGAGGCA | ACCAGGCGCGGAATCCGG |
| CAGGCAACACAGAAGAGGCAACG | TGGACGGAAAGGGCCACCCACCC |
| CGATAGGGCGAGTATGGTGGA | TGGACGGAAAGGGCCACCCACCC |
| CAGGCAACACAGAAGAGGCAACG | TGGACGGAAAGGGCCACCCACCC |

Note: Genomic structures around the nm-g locus of the wild-type and the nm-g mutant were determined by sequencing genomic fragments amplified with the primers BGIBMGA007047-F and BGIBMGA007047-R.

Reference

| Reference |
|-----------|
| Shinoda and Itoyama, 2003 |
| Shinoda and Itoyama, 2003 |
| McBrayer et al., 2007 |
| McBrayer et al., 2007 |
### F) Constructing overexpression vectors

| CG12068-Bm-F   | AGATCTCCACCATGGTACGTTACGTCCTTG |
| CG12068-Bm-R   | GCGGCGGCTACTTGGTATCGAGGGAGC    |
| CG12068-Dm-F   | AGATCTCCACCATGACGAGTCAGTTCTTC |
| CG12068-Dm-R   | GCGGCGGCTAAATCTCTTCTGCTGTTCCG |

### G) Constructing the UAS-sro-IR vector

| DmCG12068RNAi1-1 | GCGGCGGCACTCCTGACGTTGTCCTGAG |
| DmCG12068RNAi1-2 | AGATCTCACTGAAAGCCCTCAGTCATC |
| DmCG12068RNAi2-1 | GCGGCGGCTTCTTGCAGAGCCGGAGC |
| DmCG12068RNAi2-2 | TCTAGACACTTGAAGCCCTGCTGCACTC |

### H) Genomic PCR for the CG12068 (sro) locus and the 1st exon of the kayak/D-fos/CG33956 region

| CG12068-genome-F | GGATCTCTTCTGTTTACTTCTGAG |
| CG12068-genome-R | CAAGTATTCTTAAATGTCCTACAATG |
| kayak-1stExon-F  | CGTAAAGTGCTGCAATTAGCAC |
| kayak-1stExon-R  | ACCTCGAATTATGAGGATACCTTC |

### I) Constructing a plasmid for in situ hybridization

| sro-F          | ATGAGCGGCGACTCACTTCTTTCG |
| sro-R          | CTAATCTTCTCCGTGGATGTTCAGTCAG |
| GAL4-F         | CTGTCTTCTGAGAACCAAGCATTCG |
| GAL4-R         | AGCATCCTGGCATAAAATCCAC |
Table S2. Linkage analysis of F2 segregants

| Primer     | Method | C108 | a46  | F₁   | F₁,*   |
|------------|--------|------|------|------|--------|
| nscaf2874-1F/1R  | PCR    | A    | B    | A/B  | B      | A/B (28) |
| nscaf2865-29F/29R | PCR    | A    | B    | A/B  | B      | A/B (4)  |
| nscaf2865-30F/30R | seq    | A    | B    | A/B  | B      | A/B (1)  |
| prgy0385-F/R    | PCR    | A    | B    | A/B  | n.d.   | A/B (1)  |
| nscaf2865-35F/35R | PCR    | A    | B    | A/B  | B      | B        |
| nscaf2865-32F/32R | seq    | A    | B    | A/B  | A/B (1)| B        |
| nscaf2865-16F/16R | PCR    | A    | B    | A/B  | A/B (4)| B        |
| nscaf2865-6F/6R  | PCR    | A    | B    | A/B  | A/B (20)| B        |

*A* indicates the C108 (WT) homozygous genotype, 'B' indicates the a46 (nm-g mutant) homozygous genotype and 'A/B' indicates the heterozygous genotype. Heterozygous genotypes are indicated by the shaded sections. This region indicates the unlinked region to nm-g. The bold letters indicate the primer sets designed at the closest positions to the nm-g linked region. *In linkage analysis, 839 F₂ individuals with the nm-g phenotype were used. The numbers shown in parentheses indicate the number of heterozygous genotypes (A/B). The nm-g linked region is between prgy0385-F/R and nscaf2865-32F/32R. n.d., not determined.
Table S3. *sro* RNAi experiments using three independent transgenic RNAi lines

| Genotype                      | Number of adults |
|-------------------------------|------------------|
| UAS-sro-IR1/+; 2-286-GAL4/+   | 0 (74)           |
| UAS-sro-IR2; 2-286-GAL4       | 0 (170)          |
| UAS-sro-IR3/+; 2-286-GAL4/+   | 0 (106)          |
| UAS-sro-IR1/+; phm-GAL4/+     | 0 (139)          |
| UAS-sro-IR2; phm-GAL4         | 0 (126)          |
| UAS-sro-IR3/+; phm-GAL4/+     | 0 (101)          |

The numbers of viable adults were scored. Numbers in parentheses indicate the number of viable progeny with the presence of balancer markers from the parental strains.
Table S4. List of the proteins represented in Fig. 3B

| Organism                      | Protein Name                  | Accession Number          |
|-------------------------------|-------------------------------|----------------------------|
| Anopheles gambiae (Aga)       | AGAP000882 [XP_316858]        |                            |
|                               | AGAP002521 [XP_312416]        |                            |
|                               | AGAP003984 [XP_318434]        |                            |
|                               | AGAP005532 [XP_315532]        |                            |
|                               | AGAP011811 [XP_001238364]     |                            |
| Apis mellifera (Ame)          | CG1386 [XP_624540]            |                            |
|                               | CG7601 [XP_394428]            |                            |
|                               | CG8888 [XP_392612]            |                            |
|                               | *CG12068 [XP_0011120169]      |                            |
|                               | CG31937 [XP_395330]           |                            |
| Bombyx mori (Bmo)             | BGIBMGA000511 [NM_001046959]  |                            |
|                               | *BGIBMGA007047/Nm-g [A8361434]|                            |
|                               | BGIBMGA008095 [NM_001046690]  |                            |
|                               | [http://silkworm.genomics.org.cn/cgi-bin/gbrowse_details/silkdb?name=BGIBMGA008096-TA] | BGIBMGA008096 |
|                               | [http://silkworm.genomics.org.cn/cgi-bin/gbrowse_details/silkdb?name=BGIBMGA008889-TA] | BGIBMGA008889 |
| Caenorhabditis elegans (Cel)  | DHS-2 [NP_871815]             |                            |
|                               | DHS-16 [NP_504554]            |                            |
|                               | DHS-20 [NP_505941]            |                            |
|                               | F20G2.1 [NP_506406]           |                            |
|                               | F20G2.2 [NP_506407]           |                            |
| Culex quinquefasciatus (Cqu)  | *CPIJ011409 [XP_001861765]    |                            |
| Drosophila melanogaster (Dme) | CG3699 [NP_569875]            |                            |
|                               | CG8888 [NP_610724]            |                            |
|                               | CG10425 [NP_651363]           |                            |
|                               | *CG12068 [A8361435]           |                            |
|                               | CG31559 [NP_731045]           |                            |
| Daphnia pulex (Dpu)           | *GNO_96774 [http://wfleabase.org/genepage/daphnia/NCBGNO_96774] | GNO_96774 |
|                               | GNO_300483 [http://wfleabase.org/genepage/daphnia/NCBGNO_300483] | GNO_300483 |
|                               | GNO_598054 [http://wfleabase.org/genepage/daphnia/NCBGNO_598054] | GNO_598054 |
|                               | GNO_660094 [http://wfleabase.org/genepage/daphnia/NCBGNO_660094] | GNO_660094 |
|                               | GNO_662094 [http://wfleabase.org/genepage/daphnia/NCBGNO_662094] | GNO_662094 |
| Homo sapiens (Hsa)            | 3-hydroxybutyrate dehydrogenase type 1 (BDH1) [NP_004042] | 3-hydroxybutyrate dehydrogenase type 1 (BDH1) |
|                               | Short chain dehydrogenase/reductase family 9C member 7 (SDR9C7) [NP_683695] | Short chain dehydrogenase/reductase family 9C member 7 (SDR9C7) |
|                               | 11β-hydroxysteroid dehydrogenase 2 (HSD11β2) [NP_000187] | 11β-hydroxysteroid dehydrogenase 2 (HSD11β2) |
| Nasonia vitripennis (Nvi)     | *CG12068 [XP_001600976]       |                            |
|                               | ENSANGP00000021187 [XP_001599364] | ENSANGP00000021187 |
|                               | MGC81046 [XP_001604749]       |                            |
|                               | RH09070p [XP_001601503]       |                            |
| Pediculus humanus corporis    | *PHUM419870 [XP_002429087]    |                            |
| Tribolium castaneum (Tca)     | TcasGA2_TCO000474 [E2Z98067]  |                            |
|                               | LOC656669 [XP_968272]         |                            |
|                               | LOC660823 [XP_972119]         |                            |
|                               | *LOC661894 [XP_973118]        |                            |

The GenBank Accession number or the public web resource for each protein is represented in square brackets. Asterisks indicate the proteins that belong to the Nm-g/Sro family.