RNA interference knockdown of *DNA methyltransferase 3* affects gene alternative splicing in the honey bee

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Studies of DNA methylation from fungi, plants, and animals indicate that gene body methylation is ancient and highly conserved in eukaryotic genomes, but its role has not been clearly defined. It has been postulated that regulation of alternative splicing of transcripts was an original function of DNA methylation, but a direct experimental test of the effect of methylation on alternative slicing at the whole genome level has never been performed. To do this, we developed a unique method to administer RNA interference (RNAi) in a high-throughput and noninvasive manner and then used it to knock down the expression of *DNA methyltransferase 3* (*dnmt3*), which is required for de novo DNA methylation. We chose the honey bee (*Apis mellifera*) for this test because it has recently emerged as an important model organism for studying the effects of DNA methylation on development and social behavior, and DNA methylation in honey bees is predominantly on gene bodies. Here we show that *dnmt3* RNAi decreased global genomic methylation level as expected and in addition caused widespread and diverse changes in alternative splicing in fat tissue. Four different types of splicing events were affected by *dnmt3* gene knockdown, and change in two types, exon skipping and intron retention, was directly related to decreased methylation. These results demonstrate that one function of gene body DNA methylation is to regulate alternative splicing.

epigenetics | gene regulation | gene silencing | insect

One of the greatest discoveries of the genomic era is environmental regulation of gene expression; DNA is not just inherited, it is also environmentally responsive. DNA cytosine methylation is an epigenetic mechanism that mediates many environmental influences on gene expression, affecting diverse aspects of organismal function and disease (1), and is one of the best studied mechanisms (2). DNA methylation of CG sites in the promoters of genes in plants and some species of animals acts to repress transcription (3). By contrast, methylation of gene bodies occurs in many species of fungi, plants, and animals (4, 5), but its function has not been clearly elucidated.

We chose to address this issue with the honey bee (*Apis mellifera*) because it has emerged as an important model organism for studying the effects of DNA methylation on development and social behavior (6–9). Moreover, DNA methylation in honey bees is predominantly on gene bodies (7, 8, 10). There are striking differences in methylation between alternative castes that develop from a totipotent female egg—worker and queen (11)—that relate to differences in diet, especially components of royal jelly (12). RNAi silencing of *DNA methyl-transferase 3* (*dnmt3*), which encodes an enzyme involved in de novo methylation (13), alters developmental fate; worker larvae treated with *dnmt3* RNAi show an increased likelihood of developing into queens (11).

Recent studies in both invertebrates and mammals support the idea that methylation is correlated with alternative splicing (AS) of transcripts (7, 14). By comparing brain methylomes of queen and worker bees, a correlation was revealed between DNA methylation and AS (7, 8). An in vitro study of mammalian cells showed that DNA methylation inhibited the binding of transcription factor CCCTC binding factor (CTCF), which affected alternative splicing (14). Regulation of AS is a complicated process that also involves spliceosome assembly (7, 15), chromatin structure (16, 17), siRNA activity (18), and transcriptional elongation (19), in addition to the apparent action of methylation.

To explore the causal relationship of DNA methylation and AS in vivo, we used the honey bee to test for a direct link between DNA methylation and AS. We decreased DNA methylation by *dnmt3* RNA interference (RNAi) and hypothesized that there would be effects on AS.

RNAi has previously been administered to honey bees and other insects for gene knockdown (KD) by injection (20, 21), but this method is time consuming and highly invasive. We developed a unique method to treat large numbers of insects quickly and noninvasively. We coupled small interfering RNA (siRNA) to perfluocarbon-nanoparticles (PFC-NPs) and nebulized the mixture for aerosol application. PFC-NPs have been used in a variety of biomedical applications, including delivery of RNAi to tumor cells (22, 23), but had never before been nebulized.

To test the hypothesis that DNA methylation regulates AS, we used our method to knock down *dnmt3* in honey bee abdominal fat tissue. We chose this tissue because abdominal injections of RNAi have proven especially effective (20, 21), and there are extensive gene expression data from honey bee fat body to compare our results to. Insect fat tissue has analogous functions to both liver and adipose tissue in mammals because they are crucial for both carbohydrate metabolism and lipid storage. We used RNAseq and new AS quantification software (24) to evaluate our hypothesis.

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Data deposition: The sequence reported in this paper has been deposited in the National Center for Biotechnology Information Sequence Read Archive database (accession no. SRP024289).

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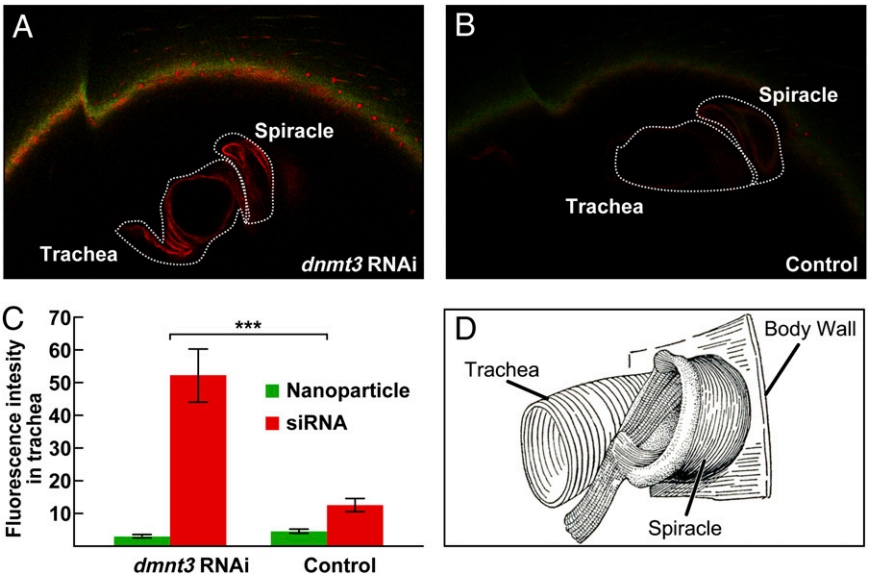

Fig. 1. Noninvasive high-throughput method of delivering RNA interference in vivo. Spraying a nebulized mixture of nanoparticles (PFC-NP) and small interfering RNA (siRNA) on bees allows penetration through the insect tracheal respiratory system. (*A*) Treated: NP (labeled green with Alexa 488) and *dnmt3* siRNA (labeled red with Q-670). (*B*) Control: NP (green) and GFP siRNA (no label). (*C*) Quantitative analysis of fluorescence reveals significantly higher levels of red signal in treated ($n = 5$) comparing to control bees ($n = 6$, $P < 0.001$, two-tailed t test), demonstrating penetration via the trachea. (*D*) Schematic of an inner view of trachea, spiracle atrium, and body wall. Diagram adapted from ref. 25.

Results and Discussion

We first tested the efficacy of our method of administering RNAi. Image analysis indicates that the nebulized nanoparticle–siRNA complex penetrates the spiracles on the thorax and abdomen and travels through the tracheal respiratory system, which functions in insects to deliver oxygen directly to cells via ever-finer branches ending in tracheoles (25). This was shown by spraying caged bees (n = 17–18 bees/cage) with a mixture of PFC-NPs (labeled Alexa 488 in green) and *dnmt3* siRNA (labeled Q-670 in red); control bees were sprayed with PFC-NPs (same label in green) and exogenous GFP siRNA (no label). Confocal microscopy revealed that trachea of *dnmt3* siRNA-treated bees showed significantly higher levels of red fluorescence compared with nonlabeled control bees (Fig. 1). Using the same *dnmt3* RNAi construct (Fig. S1) used previously for a prior honey bee RNAi injection study (11), aerosol application caused an ∼30% knockdown in fat tissue (n = 44 and 42 control and RNAi bees, respectively, across three biological replicates; Fig. 2). We obtained a similar KD by abdominal injection of the PFC-NP–siRNA complex (Fig. S2). By contrast to these results for the abdomen, no consistent KD was detected in the head, so brain analysis was not pursued. This result was unfortunate but not unexpected, given that the penetration points (spiracles) are located on the thorax and abdomen and not the head (25). These results demonstrate that it is possible to effectively exploit the insect tracheal system to deliver siRNA at least to abdominal cells via nebulizing spray in a rapid and noninvasive manner.

dnmt3 siRNA-treated and control bees were used for genome-wide analysis of gene expression, AS, and DNA methylation. We used the TrueSight program to analyze the RNA sequencing

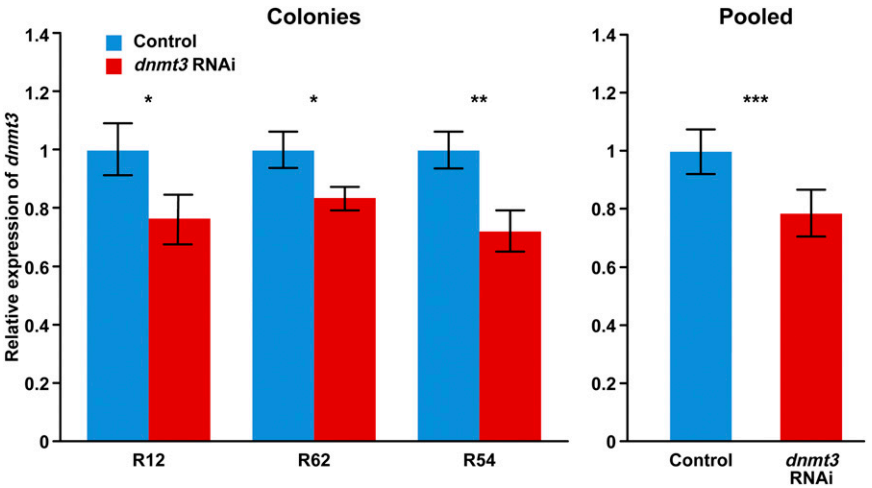

Fig. 2. Effects of spraying a nebulized mixture of nanoparticles and small interfering RNA of *dnmt3* on *dnmt3* expression ± SEM in honey bee abdominal fat body. Results from three biological replicates (bees from unrelated colonies, n = 22 for colony R12; n = 31 for R62, and n = 33 for R54; two-tailed t tests). Analysis of pooled data: mixed-model ANOVA, df = 82, $F = 15.52$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 1. Effects of *dnmt3* knockdown on DSGs, including types of ES, IR, ATE, and AEB on gene alternative splicing (AS)

Types of AS event	No. of DSGs	FDR	Gene example	*P* value of qPCR validation
ES	192	0.01	*GB53079*	0.002
IR	27	0.1	*GB49485*	0.03
ATE	141	0.01	*GB49165*	0.003
AEB	225	0.01	*GB50039*	0.004

Examples are shown in detail in Fig. 3. AEB, alternative exon boundary; ATE, alternative terminal exon; ES, exon skipping; FDR, false discovery rate; IR, intron retention. Gene examples are shown in detail in Fig. 3.

(RNA-seq) data and quantify AS. Detailed description of this application is in ref. 24. Across treated and control samples, RNA-seq yielded robust expression results for 13,008 genes, estimated to comprise 84.9% of the genes predicted from Assembly 4.5 and Official Gene Set 3.2 of the honey bee genome (http://hymenopteragenome.org/beebase/?q=gbrowse_amel). KD of *dnmt3* caused strong effects on gene expression. A total of 2,613 (17.1%) genes showed significant differential expression due to RNAi treatment. Gene Ontology analysis of a subset of these differentially expressed genes (DEGs) that had orthologs in the *Drosophila melanogaster* genome (2,121) revealed that *dnmt3* KD had particularly strong effects on RNA processing, intracellular transport, and protein catabolic processing (Table S1). Approximately 60.3% of the genes were found to be alternatively spliced, which is consistent with findings from *Drosophila* (26), and lower than in mammals (27, 28).

Comparing our results to previous gene expression studies of fat body, our DEG list also was enriched for genes related to honey bee behavioral maturation, which involves a shift from working in the hive when young to foraging when older (29). We compared our DEG list with DEG lists from fat tissue associated with (*i*) hive vs. forager bees; (*ii*) exposure to poor vs. rich diet

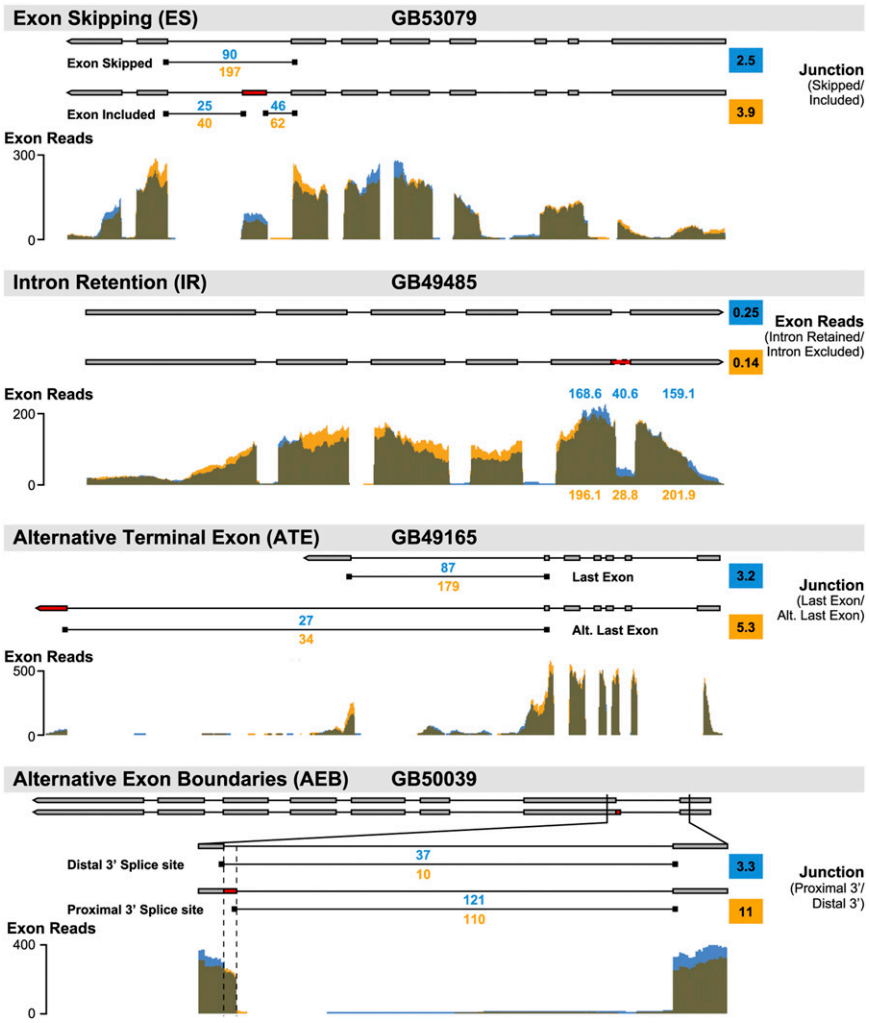

Fig. 3. Effects of *dnmt3* knockdown on differentially spliced genes. (*Upper*) Gene model in both splicing isoforms with the number of RNAseq reads mapped to each splicing junction. (*Lower*) Exon coverage from RNAseq. Blue, control; orange, *dnmt3* knockdown. Derivation of the AS ratios in the colored squares are in *SI Materials and Methods*.

Table 2. Effects of *dnmt3* knockdown (KD) on DNA methylation of DESs and DIRs

Splicing type	Direction	More methylated by *dnmt3* KD	Less methylated by *dnmt3* KD	*P* value
DES	More skipped	13	35	0.019
	Less skipped	14	10	
DIR	Less retained	3	9	0.044
	More retained	3	0	

DES, differentially exon skipped gene; DIR, differentially intron retained gene.

(poor diet accelerates behavioral maturation); and (*iii*) exposure to queen pheromone (which delays behavioral maturation). There were significant overlaps between the *dnmt3* KD DEG list and all three of these DEG lists [298, 368, and 363 genes, respectively; representation factor (RF) = 1.02, 1.37, and 1.37; P = 0.026, 2.36e−12, and 4.34e−12]. There also was a significant overlap of our *dnmt3* KD DEG list and the DEG list from the caste determination study that used *dnmt3*-silenced larvae (11) (RF = 2.62, P = 5.77e−5). These results suggest that nutritional regulation of behavioral maturation involves differential methylation of genes in the honey bee fat body.

KD of *dnmt3* caused strong effects on AS. A total of 524 genes showed significant differences in AS due to treatment [false discovery rate (FDR) < 0.1]. Moreover, there were significant differences for four different types of AS events: exon skipping (ES), alternative terminal exons (ATE), alternative exon boundaries (AEB), and intron retention (IR) (Table 1). This result revealed a broader effect of *dnmt3* KD on AS than expected. Results with quantitative PCR (qPCR) for selected genes (Fig. 3; Fig. S3) confirmed the effects of *dnmt3* KD on AS.

To examine the possibility that *dnmt3* KD exerted its effects by altering the expression of genes known to act as AS factors, we investigated the RNA-seq results for all known AS factors or AS factor-like genes in honey bees (by orthology to *Drosophila*). Of 72 AS factor or AS factor-like genes, 16 were differentially expressed (Table S2). There was no correlation between DNA methylation changes in gene bodies or promoter regions and the expression of these 16 genes (r = 0.25 and −0.12, P = 0.35 and 0.66, respectively). We cannot rule out the possibility that changes in expression of AS factor genes caused all of the above-reported change in AS, but we consider this unlikely, given the close association between the RNAi KD–induced changes in methylation and AS that we detected, as we report in the following paragraphs.

To address the issue of whether the effects of *dnmt3* KD on AS were due to decreases in DNA methylation, *dnmt3* siRNA-treated and control bees were used for genome-wide analysis of DNA methylation by bisulfite sequencing (BS-seq). Using pooled samples for a single analysis of treated and control bees, *dnmt3* KD caused a 21% decrease in DNA methylation, similar to the level of KD of *dnmt3* itself (~30%). Sequencing coverage was comprehensive, covering 86.5% of all cytosine-phosphate-guanines (CpGs) with at least one read in the control and 80.1% in the *dnmt3* KD sample (Fig. S4).

BS-seq results support the contention that the effects of *dnmt3* KD on AS are at least partly due to decreases in DNA methylation. There was a significant relationship between *dnmt3* KD, DNA methylation, and exon skipping: decreased DNA methylation (due to *dnmt3* KD) was associated with increased ES (Table 2; P = 0.019, two-tailed Fisher's exact test, n = 72 differential exon skipping events; Fig. 4). A galactokinase-like gene *GB46657* is represented in Fig. 4 as an example of DNA methylation affecting ES. These genome-wide findings are consistent with results from honey bees for a single gene (8). A study in human cell lines (14) reported that DNA methylation prevented CTCF from binding to the alternative exon, leading to an increase in ES. The specific effect of DNA methylation on AS may vary in different genomic landscapes and in different species (30). Our results provide experimental evidence at the whole-genome level for a direct link between alternative splicing and DNA methylation in a *dnmt3* KD background.

BS-seq also revealed a significant association with decreased DNA methylation and less IR (P = 0.044, two-tailed Fisher's exact test; Table 2). We did not test for a relationship between DNA methylation levels and AEB or ATE because no practical method has been developed yet: alternative exon boundaries are very short in cases of AEB, and ATE is often close to the regulatory regions. Other mechanisms in addition to DNA methylation are thus likely involved in the regulation of different forms of AS. Our results with BS-seq data demonstrate that DNA methylation of gene bodies affects alternative splicing on a genome-wide scale, specifically for ES and IR.

Alternative splicing of mRNA transcripts is an important mechanism for increasing the diversity and complexity of phenotypes that are generated from a genome (31) and can show

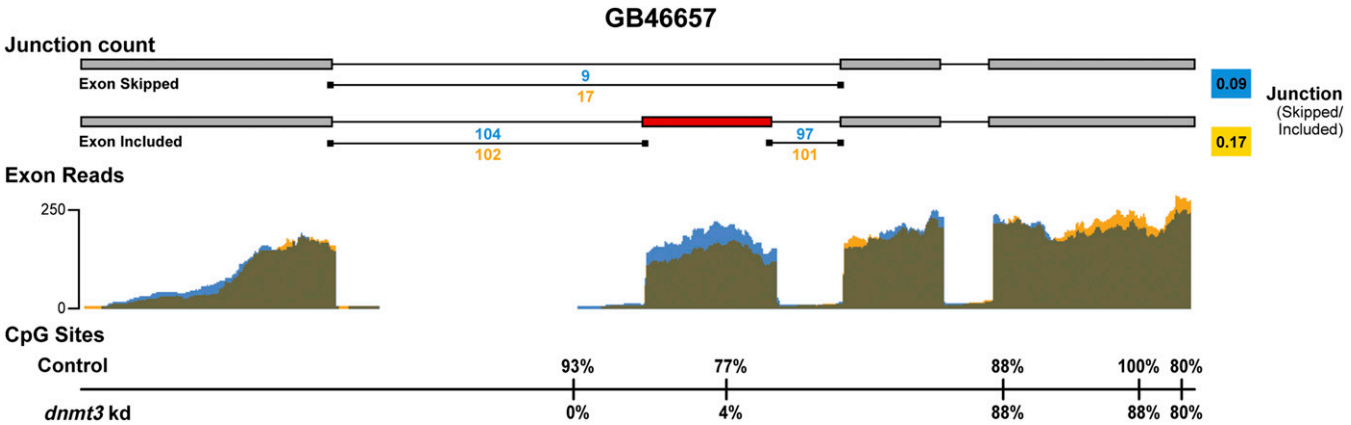

Fig. 4. Effects of *dnmt3* knockdown on exon skipping and methylation in gene GB46657 (galactokinase-like). (*Top* and *Middle*) Same information as in Fig. 3. (*Bottom*) mCpG sites with vertical lines, together with percent differences between treatments.

strong species-specific differences (32, 33). AS has been shown to play fundamental roles in sex determination, development, behavior, and disease, and in worker honey bees, sterility, which is a hallmark of eusociality (1, 34, 35). Recently, DNA methylation has been detected in the genomes of other important social insects including several ants and termites (36, 37) and has been implicated in caste differentiation and behavioral maturation (8, 38). Further studies of DNA methylation and AS will help understand the mechanisms and evolution of phenotypic plasticity.

Materials and Methods

Bees. Honey bees were a mix of European races typical of Illinois, predominantly *Apis mellifera ligustica*. They were maintained according to standard practices at the University of Illinois Bee Research Facility. We used worker bees derived from single-drone inseminated queens for RNA-seq analysis to reduce interindividual genetic variability and worker bees from a naturally mated queen colony for BS-seq analysis. Treated and control bees for each biological replicate were from the same colony.

RNA Interference Treatment. We administered a nebulized aerosol mixture of PFC-NPs made in the Wickline laboratory and small siRNA for *dnmt3* (Sigma). The control was siRNA for EGFP-S1 (IDT). siRNA oligo sequences are given in Table S3. The doses of PFC-NP and siRNA were 200 pM and 1 μM, respectively, in a total volume of 1,368 μL (mixture of PFC-NP, siRNA, and molecular graded water). A higher ratio of *dnmt3* siRNA/NP (200 pM/2 μM) also was tested, but did not appear as efficacious. The reason may be that the number of siRNA molecules per nanoparticle was increased at a higher dose and the charge of the transfection complex changed, so the delivery of the transfection complex was not efficient. This treatment was administered to groups of 17–18 bees for 5 min. After treatment, the bees were placed in a standard Plexiglas cage and held in a dark incubator at 32 °C, 40% relative humidity, for 96 h. They were fed pollen paste [50% (wt/vol) honey/50% (wt/vol) pollen] and sugar syrup [50% sucrose (wt/vol) in water], which was replaced daily. Bees were collected 96 h after spray and frozen in liquid nitrogen, and then kept at −80 °C for analysis. Abdominal fat bodies were stored in RNAlater-ICE (catalog no. AM7030; Ambion by Life Technologies) overnight and then dissected and stored at −80 °C. For injection experiments, PFC-NP:siRNA (10 pM:10 nM) was administered in 400 nL of insect saline solution and administered to 1-d-old bees abdominally. Holding conditions were as for the sprayed bees.

Image Processing to Determine Mode of Action of Nanoparticle–RNAi Spray. To determine whether the nanoparticle-RNAi spray acts by penetrating the insect tracheal respiratory system, 1-d-old bees ($n = 17–18$) were sprayed with both 1 μM siRNA of GFP control (no labeling) and 200 pM Alexa 488 fluorescently labeled nanoparticles (green) or with 1 μM Q-670 siRNA of *dnmt3* (red) and nanoparticles (green) with the above dose. Abdominal spiracles and trachea were dissected in insect saline solution 24 h after spraying. Samples were dehydrated in solutions of 25%, 50%, 75%, 100%, and 100% methanol for 20 min, respectively, and maintained in 100% methyl salicylate until imaging. The images were captured by a Zeiss LSM 700 confocal microscope at 20×.

RNA-seq. We selected individuals showing typical KD for RNA sequencing (6 treated and 6 control). Libraries were made with the TruSeq RNA sample preparation kit per the manufacturer's instructions (Illumina). One library was made per individual and barcoded. The 12 libraries were pooled and quantitated by qPCR, and the pool was sequenced on two lanes for 100 cycles, in paired-end mode, on an Illumina HiSeq2000 machine (Illumina). We used the TruSeq SBS sequencing kit (version 3) and analyzed the results with Casava1.8 (pipeline 1.9). Library construction and RNA-seq sequencing were performed at the University of Illinois W.M. Keck Center for Comparative and Functional Genomics. The number of sequencing reads was ∼56–72 M/sample, and the average cDNA length was 220 bp. Reads from each sample were aligned onto the *A. mellifera* genome assembly v4.5 using TrueSight (24), allowing up to two mismatches for both exonic and junction spanning reads. These reads were mapped to 14,124 genes of 15,314 annotated genes in the Official Gene Set v3.2. TrueSight assigns a probability score to each of the predicted splice junctions for each gene, which indicates their reliability based on coding potentials and RNA-seq mapping quality. We used all splice junctions with TrueSight score greater than 0.5 (on a scale from 0 to 1). BEDTools (39) was used to calculate the read coverage of the nonredundant exon models based on TrueSight mapping results (in binary alignment/map format). Complete methods and further analyses are provided in *SI Materials and Methods*.

BS-seq Analysis. Genomic DNA (1 μg/group) extracted from pooled abdominal fat body samples (18 bees per group) was used to generate BS-seq libraries using the premethylated adapter method based on a previously published protocol (40). The single-end libraries were sequenced on an Illumina HiSeq2000, following the manufacturer's manual. Data processing, aligning Bisulfite-converted reads, and methylation assessments are described in ref. 40. Identical reads were collapsed into single reads. The methylation level was calculated as the ratio of methylated reads over all reads covering each CpG site, using a threshold of 20% methylation and read coverage ≥4 in either control or treated samples. We used this threshold because it resulted in a comparable number of methylated cytosine-phosphate-guanine (mCpG) sites (123,023) to previous studies (7). At this threshold there was no methylated cytosine signal from the mitochondrial genome. *Dnmt3* KD caused an overall decrease in CpG methylation levels (an average of −0.045 per mCpG).

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