Nutritional Control of Reproductive Status in Honeybees via DNA Methylation

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Fertile queens and sterile workers are alternative forms of the adult female honeybee that develop from genetically identical larvae following differential feeding with royal jelly. We show that silencing the expression of DNA methyltransferase Dnmt3, a key driver of epigenetic global reprogramming, in newly hatched larvae led to a royal jelly–like effect on the larval developmental trajectory; the majority of Dnmt3 small interfering RNA–treated individuals emerged as queens with fully developed ovaries. Our results suggest that DNA methylation in *Apis* is used for storing epigenetic information, that the use of that information can be differentially altered by nutritional input, and that the flexibility of epigenetic modifications underpins, profound shifts in developmental fates, with massive implications for reproductive and behavioral status.

Many organisms respond to environmental conditions by displaying phenotypic plasticity, that is, producing different phenotypes from the same DNA genome (*1*, *2*). In social insects, the production of contrasting adult morphologies as well as different reproductive and behavioral systems is critical to their social organization and division of labor (*3*–*6*). These differences are likely to arise at the most basic level from differential somatic imprinting during development on the same genome. Honeybees (*Apis mellifera*) differentially feed genetically identical female larvae to create mainly workers and, when required, a few queens (*7*–*11*). Young nurse bees in the hive produce and feed a largely biochemically uncharacterized substance named royal jelly to larvae destined to become queens, whereas the other larvae are fed with less-sophisticated food (*8*–*11*). Despite their identical clonal nature at the DNA level, workers and queens differ markedly in morphological and physiological features and have contrasting reproductive capabilities, strikingly diverse life spans, and very different behavioral repertoires. It is not understood, however, how differential nutrition is linked to gene expression and how alterations in diet alter pathways that modify the developmental trajectory of an organism.

Studies in mammals suggest that environmental stimuli such as diet can alter the epigenetic state of the genome and affect gene expression by modifying DNA methylation and histone acetylation patterns (*12*, *13*). In addition, strong epidemiological data reveal that cardiovascular and diabetes mortality in children can be influenced by the nutritional status of their parents and grandparents (*14*). In experimental mammalian contexts such as the *agouti* mouse, a number of contrasting phenotypes, such as yellow and obese or brown and slim, can be controlled by varying the mother's diet before, during, and after pregnancy (*15*). *Agouti* gene expression can be silenced by DNA methylation, and its quantities are variable in genetically identical individuals because of epigenetic modifications established during early development (*16*). Differential maternal behavior in the rat also alters the methylation status of the promoter of the glucocorticoid receptor of her pups (*17*).

Epigenetic regulation thus facilitates the integration of intrinsic signals and environmental signals by using highly conserved enzymatic machinery

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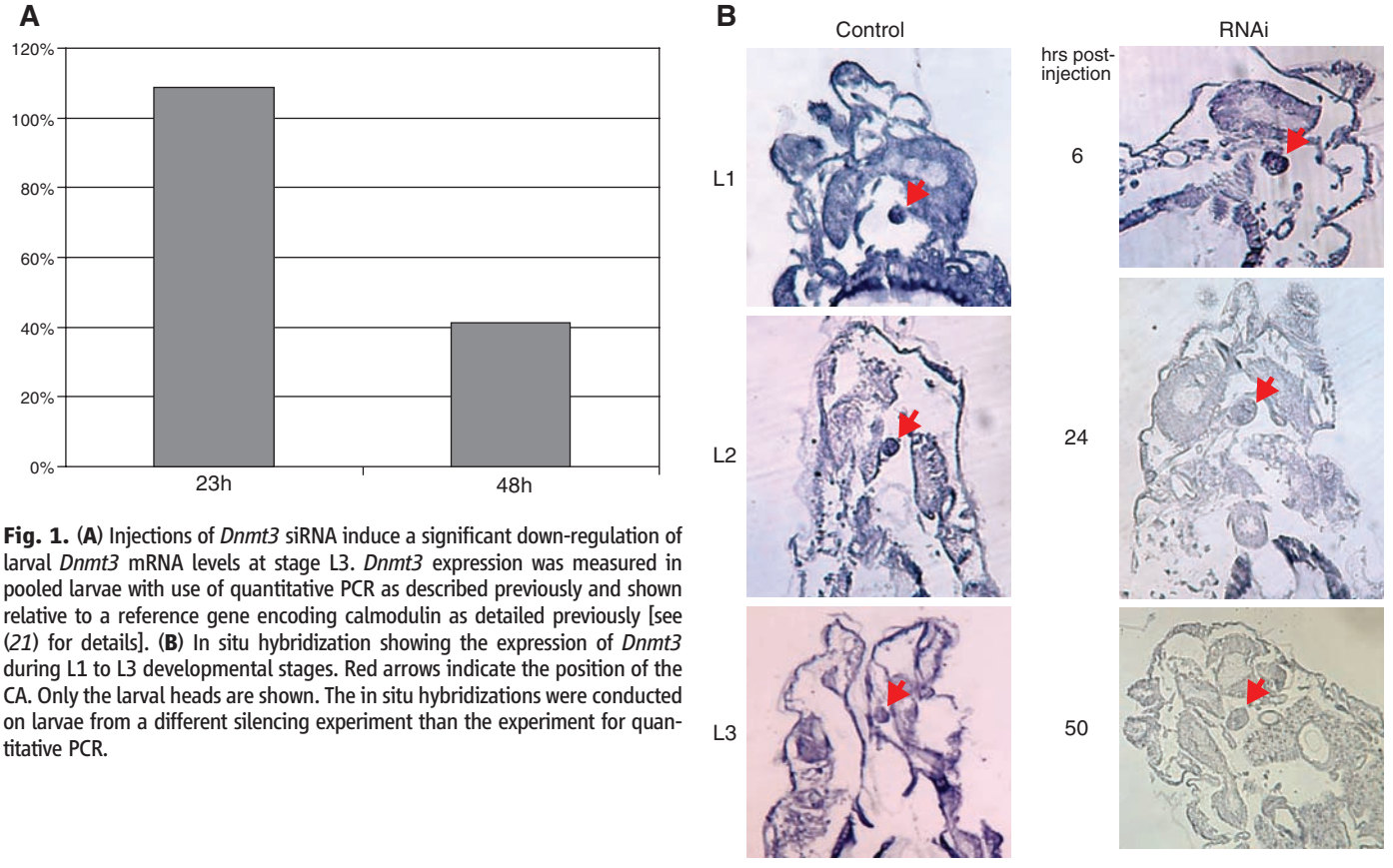

Fig. 1. (**A**) Injections of *Dnmt3* siRNA induce a significant down-regulation of larval *Dnmt3* mRNA levels at stage L3. *Dnmt3* expression was measured in pooled larvae with use of quantitative PCR as described previously and shown relative to a reference gene encoding calmodulin as detailed previously [see (*21*) for details]. (**B**) In situ hybridization showing the expression of *Dnmt3* during L1 to L3 developmental stages. Red arrows indicate the position of the CA. Only the larval heads are shown. The in situ hybridizations were conducted on larvae from a different silencing experiment than the experiment for quantitative PCR.

that includes DNA cytosine-5-methyltransferases (Dnmts), histone deacetylases (HDAs), and methyl-binding proteins (MBPs) (*18*). Although Dnmts appear to be widely conserved in evolution, evidence for a fully functional DNA methylation system in insects has not been forthcoming. Recently, we reported that the honeybee has a full complement of all three functional Dnmts with in vivo properties similar to those of the CpG methylation system in vertebrates (*19*). *Apis* has two orthologs of Dnmt1, one ortholog of Dnmt2, and one of Dnmt3 (*19*, *20*). However, dipterans, such as *Drosophila* and mosquitoes, lack some members of this family and possess only a Dnmt2 ortholog. The *Apis* genome also encodes conserved MBPs including components of the nucleosome remodeling and HDA complex. Because all three Dnmt enzymes are shared by humans and honeybees but not by other commonly used model invertebrates, such findings establish the honeybee as a model to not only study the function of DNA methylation in invertebrates, but also for examining any fundamental overlaps that may help in understanding the nutritional basis of epigenetic reprogramming in humans. Although the global methylation landscape of the *Apis* genome remains ill defined, several methylated genes have already been identified, revealing that CpG methylation occurs preferentially within the coding exons but not at the 5′ and 3′ regions of the transcription units (*19*).

Accordingly, we examined how this highly conserved molecular machinery in *Apis* could be used to elucidate the contrasting anatomical, physiological, and behavioral characteristics of honeybee castes that are brought about by differences in their early life environment.

We used RNA interference (RNAi) technology to silence the expression of Dnmt3 in newly hatched L1 larvae and in embryos because this de novo methylase has been implicated in establishing DNA methylation patterns during development (*18*, *20*). Injections of Dnmt3 small interfering RNA (siRNA) into newly hatched larvae were well tolerated and led to a transient but significant decrease in the amount of Dnmt3 message, whereas this treatment was lethal for embryos (*21*). Both quantitative polymerase chain reaction (PCR) and in situ hybridization show that the amount of Dnmt3 mRNA is lower in siRNA-treated larvae than in control individuals (Fig. 1), with the strongest silencing occurring 48 to 50 hours post-injection, a time that coincides with the L2-to-L3 larval transition, a critical "decision-making" period in larval development (*7*–*11*). In contrast, injections of a "nonlarval" gene, *uth* (*22*), had no detectable effects on the amount of Dnmt3 mRNA.

This interference with Dnmt3 expression triggers profound developmental changes leading to contrasting adult outcomes (Fig. 2; see fig. S1 for data from individual experiments). In the Dnmt3 siRNA-treated larvae, the majority of emerging adults (72%) were queens with fully developed ovaries, and the remaining 28% were typical workers with rudimentary ovaries (Fig. 2). In contrast, in the larval group injected with a control gene siRNA (*uth*), 77% of adults emerged as workers, whereas the remaining 23% exhibited queenlike morphological features. However, these queenlike individuals had grossly underdeveloped ovaries with no more than 50 to 80 ovarioles per ovary compared with at least 120 to 190 ovarioles in those queens emerging in the Dnmt3 siRNA experiment (Fig. 2). The ovaries of siRNA-induced queens are practically indistinguishable from the ovaries of a virgin queen reared in the hive on pure royal jelly. Workers have only rudimentary ovaries with two to six ovarioles.

Do these phenotypic effects correlate with methylation changes in larval DNA? Because global methylation in the honeybee genome is low, we examined the methylation status of cytosines in a single gene, *dynactin p62*, that we had previously shown to be differentially methylated during development [figure S3 in (*19*)]. Dietary changes lead to differential expression of this gene in *Drosophila*, further underscoring its role in growth and feeding-related processes (*23*, *24*).

As with all genes so far examined for CpG methylation in *Apis*, *dynactin p62* is methylated exclusively within the coding exonic sequences. Within the exonic landscape, we chose exons 5, 6, and 7 for bisulfite conversion because they contain a total of 10 CpG dinucleotides (Fig. 3), a high concentration compared with other genes we have examined. With this 0.5-kb bisulfite-converted and PCR-amplified fragment, we first mapped differences in *dynactin p62* methylation by using DNAs isolated from hive-reared whole larvae (late L3) destined to become either queens or workers. As shown in Fig. 3A, there is a detectable decrease in the overall amount of methylation of *dynactin p62* in the queen larvae (48%) versus the worker larvae (58%), suggesting that the methylation state of certain genes may correlate with the larva's developmental trajectory. However, because there are virtually no cell divisions during larval growth and larval tissues are both highly polypoid and heterogeneous with regard to their ploidy level (see examples in fig. S2), varying DNA dosages at any locus introduces technical limitations on methylation measurements in whole larvae. Therefore, to better evaluate the effectiveness of Dnmt3 RNAi silencing, we used only DNAs extracted from larval heads (late L3). Although the larval head contains several cell types (*25*),

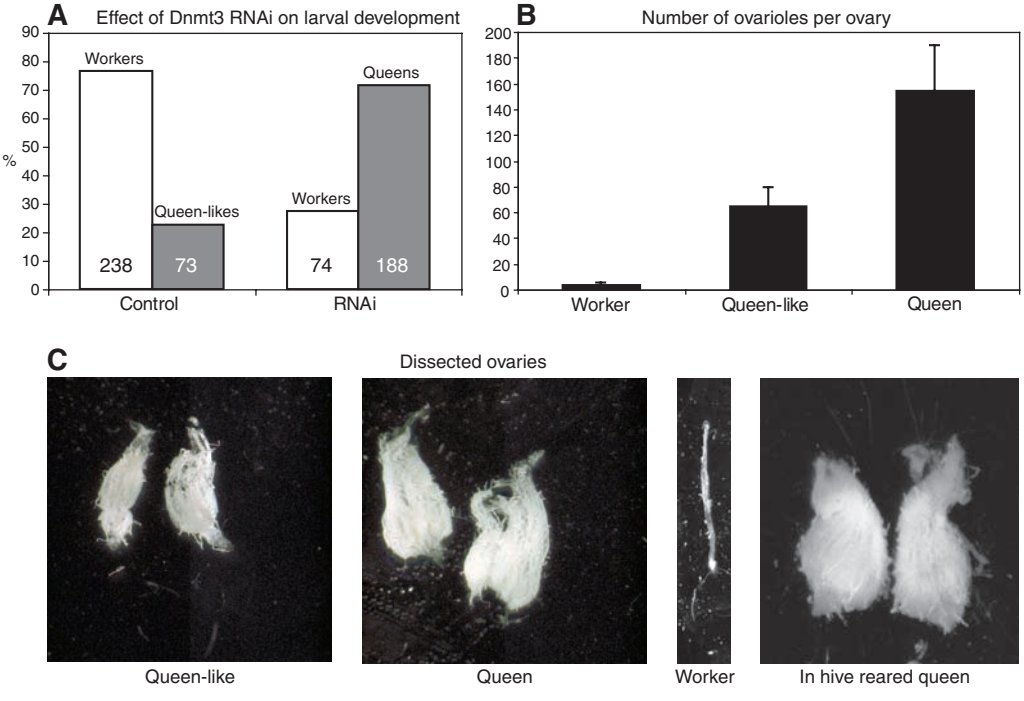

Fig. 2. Effect of Dnmt3 silencing on caste development in honeybees. Newly emerged larvae were injected either with a nonlarval control gene, *uth*, siRNA or with *Dnmt3* siRNA and allowed to develop until adulthood in a climate-controlled incubator. In both groups, the larvae developed normally, but the emerging adults displayed contrasting phenotypes. (**A**) The number of adults in each phenotypic category (workers, queens, and queenlikes). (**B**) The number of ovarioles per single ovary in each phenotypic class. Range error bars encompass the lowest and highest values. (**C**) Examples of ovaries dissected from each category and, for comparison, from a virgin queen reared in the hive on royal jelly. Queenlikes have queen morphological features but fewer ovarioles per ovary than queens [see (B)]. Workers have only rudimentary ovaries with two to six ovarioles. The figure is a compilation of four independent experiments. See (*21*), table S2, and fig. S1 for more details and results from individual experiments.

the tissues most relevant to developmental processes are brain neurosecretory cells and corpora allata (CA), the gland producing juvenile hormone implicated in the control of development and caste determination (*7*). The CA undergo several phases of endomitosis that lead to a high degree of its polyploidization in both queens and workers, but the rate of growth of the CA in queens is significantly accelerated, and at the completion of larval development (L5) the queen CA are twice the size of the worker CA (*26*). The high amount of Dnmt3 expression in CA shown in Fig. 1B may well be indicative of a link between the gland's function and its methylation status. Furthermore, the highly polyploid nature of the CA at stages L3 to L5 (fig. S2) must correlate with massive DNA replication that provides an opportunity for either adding or removing the methyl tags to target loci.

The results shown in Fig. 3 are consistent with this expectation. As illustrated in Fig. 3B, the 10% decrease in *dynactin p62* methylation in the heads of queen larvae is basically the same as the decrease found in the whole bodies, but the overall amount of CpG methylation in clones sequenced from both worker and queen larval heads is significantly higher than those analyzed from the whole bodies. The heads of worker larvae show 73% methylation across the *dynactin* fragment compared with only 58% in clones extracted from the workers' whole bodies, and for the queen larvae the head clones show 63% methylation versus 48% in the whole bodies (Fig. 3, A and B, right). This finding suggests that during larval development a high amount of CpG methylation and/or demethylation is associated with selected tissues, most likely with those that undergo massive DNA replication, such as cells of the CA and neurosecretory cells.

The analysis of the Dnmt3 RNAi silencing experiments in laboratory-reared larvae reveals a profile that is remarkably similar to that seen in the hive-reared individuals (Fig. 3C). The heads of worker larvae in the control group (worker-destined) show 79% methylation across the *dynactin p62* fragment, whereas the clones extracted from the Dnmt3 siRNA-injected larvae (queen-destined) show 63% methylation (Fig. 3C).

Interestingly, individual CpG sites reveal greater differences between the castes than those illustrated by the average methylation amounts across the entire *dynactin* fragment. For CpG sites 2 and 4, the decrease in cytosine methylation between the control and siRNA-treated clones is more than 30%, and for CpG site 10 the difference is 25% (Fig. 3C). These CpGs also show more than the average differential methylation in the heads of hive-reared larvae (Fig. 3B). This finding suggests that certain CpG sites might be preferentially methylated, but further studies are required to determine whether CpG methylation in *Apis* is used for transcriptional silencing of individual genes or is part of a global mechanism controlling transcriptional domains across the whole genome.

To identify members of networks regulated by methylation during larval growth and to gain some understanding of the epigenetic hierarchy that leads to alternative developmental paths we used the honeybee genomic oligonucleotide microarray to compare global gene expression between the control and Dnmt3-silenced lar-

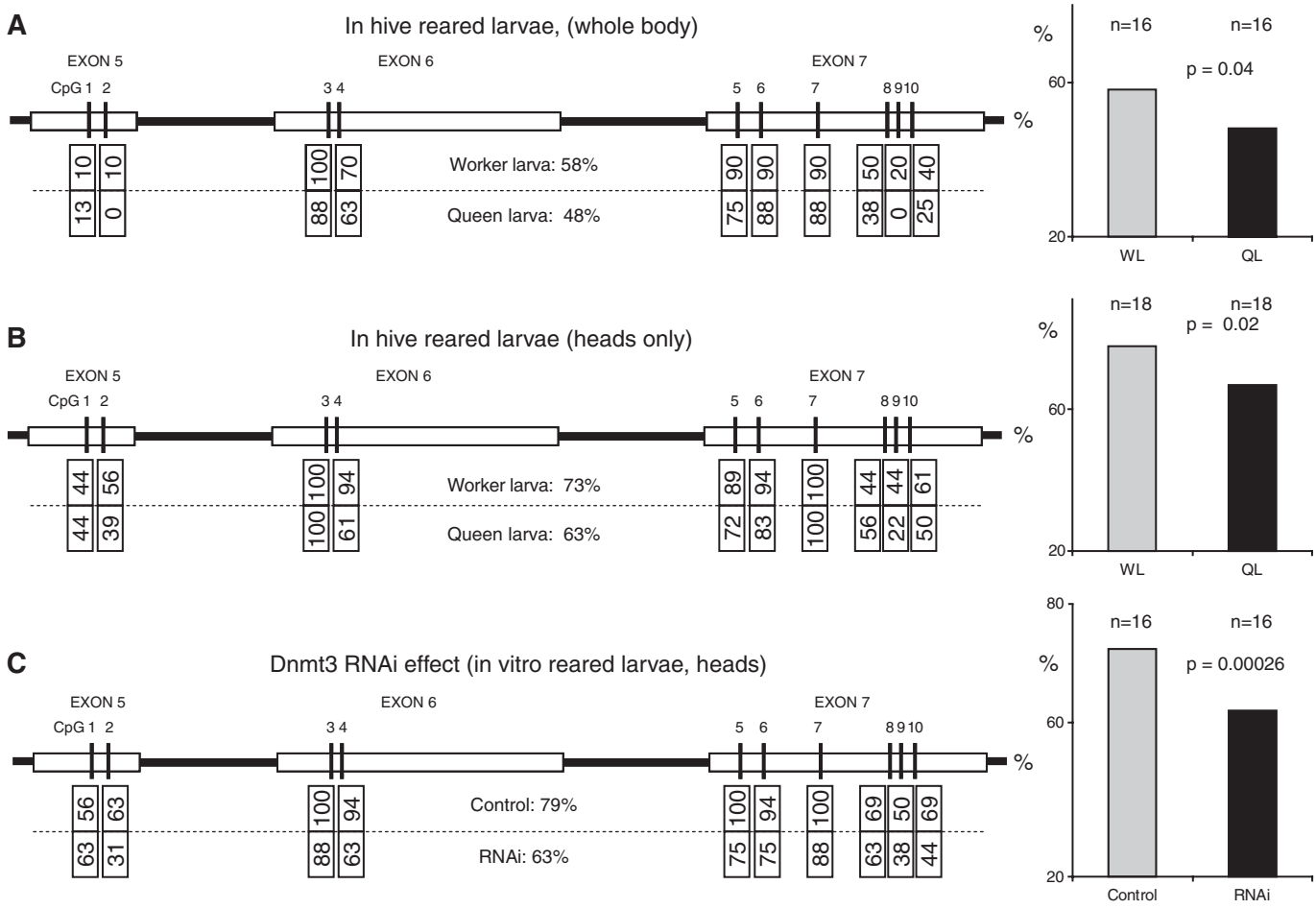

Fig. 3. Methylation status of cytosines in CpG dinucleotides of *dynactin p62*. The percentage of methylation for individual CpGs is shown in boxes, and the overall methylation in the right-hand graphs. DNA was isolated by using larvae collected (**A** and **B**) from the hive [for (A), pooled whole late-L3 larvae, $n = 7$; for (B), heads only, $n = 20$ for workers and $n = 14$ for queen larvae] and (**C**) from pooled heads of late-L3 in vitro reared larvae ($n = 7$). The number of clones sequenced for each category is shown above the bars in the right-hand graphs. Methylation quantities along this gene were analyzed with a general linear model of the binomial family (*31*) by using treatment (diet or RNAi) and position as factors to model the state of each CpG. The differences between queen larvae (QL) and worker larvae (WL) as well as the effect of RNAi are statistically significant.

vae (table S1, ArrayExpress accession number E-MEXP-1394). This revealed a battery of differentially expressed genes associated with lipid transport, hormonal regulation, posttranslational modification, protein turnover, ribosomal biogenesis, energy transfer, and other physiometabolic processes, as well as a number of novel, possibly *Apis*-specific genes with unknown functions (table S1). One differentially expressed gene predicted to encode an ortholog of the adenosine triphosphatase (ATPase) Belphegor has been shown in *Drosophila* to be regulated by TOR, a serine/threonine kinase that is central to a signaling cascade controlling growth (*27*). TOR is believed to be at the core of an ancient gene network that senses nutrient levels, and its involvement in honeybee queen development has already been considered (*7*, *28*). We also expected that the set of genes responsive to Dnmt3 silencing might contain genes implicated in chromatin remodeling and integrity. It is known that regulation of eukaryotic gene expression requires two classes of chromatin-remodeling enzymes: those that modify histones through acetylation, phosphorylation, or methylation and those that alter chromatin structure through hydrolysis of adenosine triphosphate (ATP) (*29*). From the list shown in table S1, we identified at least two genes belonging to this category, a subunit of the INO80 nucleosome remodeling complex and an ATPase of the type involved in structural maintenance of chromosomes. The transcriptional responses to Dnmt3 silencing are in good agreement with our previous study in which we used a smaller, expressed sequence tag (EST)–based array to compare gene expression between queen and worker larvae extracted from the colony environment (*7*). Thus, both Dnmt3 silencing and feeding with royal jelly induce reprogramming of the larval transcriptome that is characterized by transcriptional shift toward higher expression of physiometabolic genes, including genes coding for metabolic enzymes and the general growth of the organism.

This study shows that in *A. mellifera* DNA methylation is a key component of an epigenetic network controlling a most important aspect of eusociality, the reproductive division of labor (*30*). Further work is required to unravel the causal relation between diet-induced methylation changes and altered gene expression, but our data hold substantial promise for functional methylome analyses in Metazoa using an easily manipulatable insect system. Where appropriate, the relevance of the honeybee findings to mammals can be evaluated to determine the level of data transferability, especially in the context of nutritional processes, longevity, and even drug treatment. Methylation data on a genomic scale combined with genomewide expressional profiling in both social and solitary insects possessing functional CpG methylation systems will be needed to dissect the intricacies of this elaborate regulatory system.

References and Notes

1. M. West-Eberhard, *Annu. Rev. Ecol. Syst.* **20**, 249 (1989).
2. M. J. West-Eberhard, *Developmental Plasticity and Evolution* (Oxford Univ. Press, New York, 2003).
3. E. O. Wilson, *Proc. Am. Philos. Soc.* **123**, 204 (1979).
4. K. Hartfelder, W. Engels, *Curr. Top. Dev. Biol.* **40**, 45 (1998).
5. J. D. Evans, D. E. Wheeler, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 5575 (1999).
6. J. D. Evans, D. E. Wheeler, *Genome Biol.* **2**, 1 (2000).
7. A. R. Barchuk *et al.*, *BMC Dev. Biol.* **7**, 70 (2007).
8. E. H. Colhoun, M. V. Smith, *Nature* **188**, 854 (1960).
9. R. W. Shuel, S. E. Dixon, *Insectes Soc.* **7**, 265 (1960).
10. N. Weaver, *Ann. Entomol. Soc. Am.* **50**, 283 (1957).
11. N. Weaver, *J. Econ. Entomol.* **50**, 759 (1957).
12. R. Jaenisch, A. Bird, *Nat. Genet.* **33** (suppl.), 245 (2003).
13. G. C. Burdge, M. A. Hanson, J. L. Slater-Jefferies, K. A. Lillycrop, *Br. J. Nutr.* **97**, 1036 (2007).
14. G. Kaati, L. O. Bygren, S. Edvinsson, *Eur. J. Hum. Genet.* **10**, 682 (2002).
15. R. A. Waterland, R. L. Jirtle, *Mol. Cell. Biol.* **23**, 5293 (2003).
16. V. K. Rakyan, M. E. Blewitt, R. Druker, J. I. Preis, E. Whitelaw, *Trends Genet.* **18**, 348 (2002).
17. I. C. Weaver *et al.*, *Nat. Neurosci.* **7**, 847 (2004).
18. M. G. Goll, T. H. Bestor, *Annu. Rev. Biochem.* **74**, 481 (2005).
19. Y. Wang *et al.*, *Science* **314**, 645 (2006).
20. M. Schaefer, F. Lyko, *Bioessays* **29**, 208 (2007).
21. Materials and methods are available on *Science* Online.
22. J. Maleszka, S. Forêt, R. Saint, R. Maleszka, *Dev. Genes Evol.* **217**, 189 (2007).
23. FlyAtlas, http://flyatlas.org/atlas.cgi?name=FBgn0033206.
24. GEO (Gene Expression Omnibus), www.ncbi.nlm.nih.gov/sites/entrez?db=geo&cmd=search&term=FBgn0033206
25. J. A. Nelson, *The Embryology of the Honeybee* (Princeton Univ. Press, Princeton, NJ, 1915).
26. G. M. Ulrich, H. Rembold, *Cell Tissue Res.* **230**, 49 (1983).
27. D. A. Guertin, K. V. Guntur, G. W. Bell, C. C. Thoreen, D. M. Sabatini, *Curr. Biol.* **16**, 958 (2006).
28. A. Patel *et al.*, *PLoS ONE* **2**, e509 (2007).
29. C. J. Fry, C. L. Peterson, *Science* **295**, 1847 (2002).
30. M. D. Drapeau, S. Albert, R. Kucharski, C. Prusko, R. Maleszka, *Genome Res.* **16**, 1385 (2006).
31. J. Maindonald, J. Braun, *Data Analysis and Graphics Using R* (Cambridge Univ. Press, Cambridge, 2007).

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Supporting Online Material

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Materials and Methods
Figs. S1 to S4
Tables S1 and S2
References

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The Flavivirus Precursor Membrane-Envelope Protein Complex: Structure and Maturation

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Many viruses go through a maturation step in the final stages of assembly before being transmitted to another host. The maturation process of flaviviruses is directed by the proteolytic cleavage of the precursor membrane protein (prM), turning inert virus into infectious particles. We have determined the 2.2 angstrom resolution crystal structure of a recombinant protein in which the dengue virus prM is linked to the envelope glycoprotein E. The structure represents the prM-E heterodimer and fits well into the cryo–electron microscopy density of immature virus at neutral pH. The pr peptide β-barrel structure covers the fusion loop in E, preventing fusion with host cell membranes. The structure provides a basis for identifying the stages of its pH-directed conformational metamorphosis during maturation, ending with release of pr when budding from the host.

Many viruses, including flaviviruses (*1*), undergo a maturation step immediately before their release from the host; the evident purpose of this step is to maintain stability for the hazardous transfer to a new host while preparing virions for rapid fusion with, and entry into, a cell. Flaviviruses within the *Flaviviridae* family are major human pathogens that include dengue virus, West Nile virus, yellow fever virus, and Japanese encephalitis virus. They have a positive-sense, 11-kb RNA genome that is packaged together with multiple copies of the capsid protein within a lipid envelope (*2*). The genome is translated as a polyprotein that has the capsid protein, the precursor membrane glycoprotein (prM), and the envelope glycoprotein (E) in its N-terminal region (Fig. 1A). The polyprotein is cleaved into component proteins by viral and cellular proteases (*2*). Partially assembled flavivirus nucleocapsids bud from the endoplasmic reticulum, thereby becoming enveloped with a lipid membrane that carries with it the E and prM glycoproteins (*2*). These immature particles are transported through the cellular secretory pathway, where the cellular furin protease cleaves prM, eventually resulting in the release of the pr peptide and formation of mature virions (*3*, *4*).

The dengue virus prM glycoprotein consists of 166 amino acids. Cleavage by furin releases

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