Epigenetic mechanisms modulate differences in *Drosophila* foraging behavior

Ina Anreiter[a,b], Jamie M. Kramer[c,d], and Marla B. Sokolowski[a,b,1]

[a]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON, Canada M5S 3B2; [b]Child and Brain Development Program, Canadian Institute for Advanced Research, Toronto, ON, Canada M5G 1M1; [c]Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Western University, London, ON, Canada N6A 5C1; and [d]Department of Biology, Faculty of Science, Western University, London, ON, Canada N6G 2M1

Edited by Gene E. Robinson, University of Illinois at Urbana–Champaign, Urbana, IL, and approved September 26, 2017 (received for review June 14, 2017)

Little is known about how genetic variation and epigenetic marks interact to shape differences in behavior. The *foraging* (*for*) gene regulates behavioral differences between the rover and sitter *Drosophila melanogaster* strains, but the molecular mechanisms through which it does so have remained elusive. We show that the epigenetic regulator *G9a* interacts with *for* to regulate strain-specific adult foraging behavior through allele-specific histone methylation of a *for* promoter (pr4). Rovers have higher pr4 H3K9me dimethylation, lower pr4 RNA expression, and higher foraging scores than sitters. The rover–sitter differences disappear in the presence of *G9a* null mutant alleles, showing that *G9a* is necessary for these differences. Furthermore, rover foraging scores can be phenocopied by transgenically reducing pr4 expression in sitters. This compelling evidence shows that genetic variation can interact with an epigenetic modifier to produce differences in gene expression, establishing a behavioral polymorphism in *Drosophila*.

histone methylation | epigenetics | behavior | foraging | Drosophila melanogaster

Although it has been shown that variation in human and animal behavior correlates with genetic polymorphisms and epigenetic regulation (1, 2), causal links among genetic variation, epigenetic regulation, and behavior have not been established.

The *foraging* (*for*) gene in *Drosophila melanogaster* is a complex gene that encodes several different isoforms of a cGMP-dependent protein kinase (3). *for* regulates various behavioral and physiological phenotypes in the fly and other organisms, including humans (4–9). Importantly, this gene, with its rover and sitter allelic variants, is known to give rise to naturally occurring behavioral variations in *D. melanogaster*. Larvae with the rover allele move longer distances while foraging than those with sitter alleles, and the rover allele exhibits genetic dominance over the sitter in this trait (3). *for* also affects rover–sitter differences in foraging behavior in adult flies (10, 11). These rover–sitter behavioral differences have been shown to arise from genetic variation in the *for* gene, but until now the molecular mechanisms underlying rover–sitter differences have remained elusive.

for has four separate transcription start sites and one transcription termination site encoding at least 21 different transcripts, which cluster into four transcript classes of similar coding sequences according to promoter: pr1, pr2, pr3, and pr4 (4). This complexity could contain the key to understanding the regulation of rover–sitter behavioral differences as well as *for*'s pleiotropic functions. The function of each of *for*'s transcripts and how they are regulated are currently unknown.

Epigenetic modifiers play an important role in depositing marks that recruit transcription factors and regulate expression. *Drosophila G9a* (*dG9a*, *EHMT*) is an epigenetic modifier known to methylate the regions of the *for* promoters (12). *G9a* is one of three histone methyltransferases that catalyze H3K9me2 in flies. While the other two H3K9me2 methyltransferases, *egg* and *Su(var)3–9*, are involved mainly in the formation of heterochromatin, *G9a* acts predominantly on the 5′ of transcription start sites in euchromatic DNA. This pattern of methylation is usually repressive and associated with transcriptional plasticity of actively transcribed genes (12).

Here we show that *G9a* regulation of *for* is responsible for rover–sitter differences in adult foraging behavior. Our results demonstrate that allele-specific histone methylation drives differences in behavior, a mechanism that has not been addressed experimentally.

Results

***for* Interacts with *G9a* to Mediate Rover–Sitter Differences in Adult Foraging Behavior.** A schematic representation of the *for* gene, including transcription start sites and promoter areas methylated by *G9a*, is shown in Fig. 1*A*. We used rover and sitter flies with [*G9a* wild type (WT)] and without (*G9a* null) functional *G9a* alleles to test for a possible interaction of *G9a* and *for* on the rover–sitter difference in adult foraging behavior. Flies were tested in a foraging arena in which individuals are given the opportunity to search for and ingest sucrose drops (Fig. 1*B*). Rovers bearing *G9a* WT alleles, have significantly greater foraging success (i.e., number of food drops found and ingested) compared with sitters ($P < 0.001$; Fig. 1*C*), but this rover–sitter difference disappears when rovers and sitters carry *G9a* null alleles (Fig. 1 *D* and *E*; $P = 0.751$). Thus, *G9a* is required for this rover–sitter behavioral difference. Notably, the *G9a* null mutation significantly increases foraging behavior in sitters ($P < 0.001$), but not in rovers ($P = 0.285$) (Fig. 1 *D* and *E*), indicating a selective interaction of the sitter *for* alleles with the *G9a* null alleles. The higher sensitivity of sitters to the loss of *G9a* is also seen in starvation resistance, a trait correlated with foraging behavior (Fig. S1). Both rovers and sitters with the *G9a* mutation survive longer under starvation conditions than rovers and sitters with WT *G9a*;

Significance

Individuals of the same species display remarkable variation in behavior even in identical contexts, but the molecular mechanisms that underlie this variation are still poorly understood. Here we present important findings on the regulation of behavioral variation. We show that epigenetic regulation interacts with genetic variation, and provide causal evidence that this mechanism underlies distinct foraging behavioral strategies. More globally, our findings show that individuals within a species may behave differently due to the epigenetic control of gene expression.

Author contributions: I.A., J.M.K., and M.B.S. designed research; I.A. performed research; J.M.K. and M.B.S. contributed new reagents/analytic tools; I.A. analyzed data; and I.A. and M.B.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. CP023329–CP023334 (sitter) and CP023335–CP023340 (rover)].

See Commentary on page 12365.

[1]To whom correspondence should be addressed. Email: marla.sokolowski@utoronto.ca.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1710770114/-/DCSupplemental.

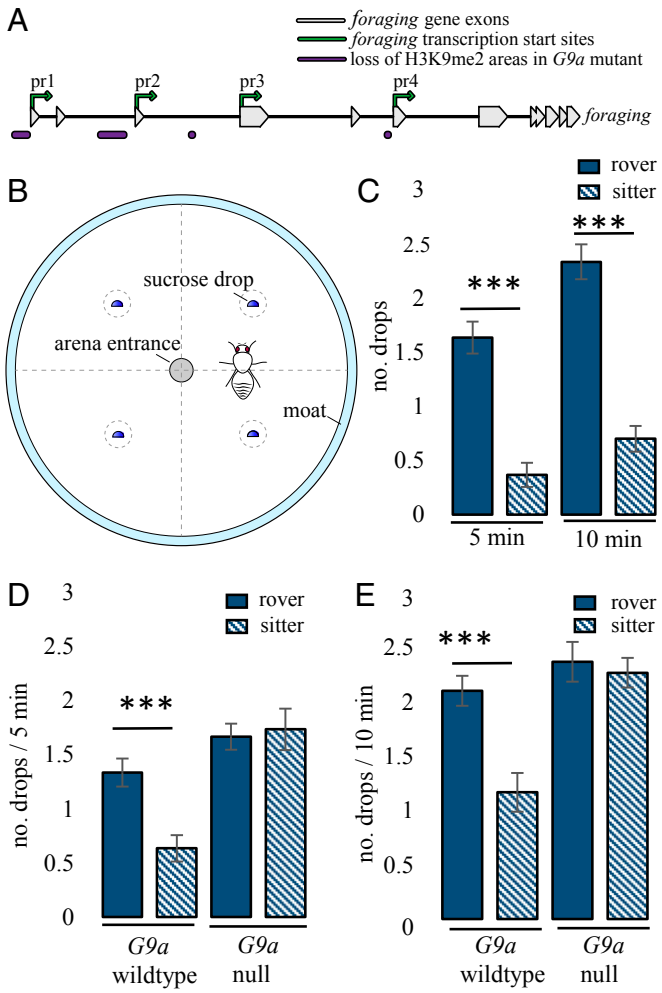

Fig. 1. *foraging* (*for*) interacts with *G9a* to mediate differences in adult feeding behavior. (*A*) The *for* gene model. The four transcription start sites (pr1–4) are marked with green arrows, exons are in gray boxes, and introns are shown as black lines. *G9a*-associated methylation sites are shown in purple. (*B*) Foraging arena schematic. (*C*) Rovers find significantly more sucrose drops than sitters in 5 min ($t_{58} = -6.829$; $P < 0.001$) and 10 min ($t_{58} = -8.523$; $P < 0.001$). (*D*) Rovers with *G9a* WT find significantly more sucrose drops than sitters with *G9a* WT during a 5-min test [$F_{(3,116)} = 10.58$; $P < 0.001$], but this difference disappears in *G9a* null flies ($P = 0.751$). (*E*) Rovers with *G9a* WT find significantly more sucrose drops than sitters with *G9a* WT during a 10-min test [$F_{(3,116)} = 10.88$; $P = 0.004$], but this difference disappears in *G9a* null flies ($P = 0.678$). $n = 20$/strain.

however, the increase in starvation resistance is much greater in sitters.

Differential Expression and Methylation of pr4 Correlates with Differences in Adult Foraging Behavior. We next used qRT-PCR to quantify expression levels from each of the *for* promoter-specific transcript groups (Fig. 2 *A–D*). Rovers bearing the *G9a* WT alleles have significantly lower expression than sitters for pr2 (Fig. 2*B*) and pr4 (Fig. 2*D*); however, the *G9a* null mutation eliminated the rover–sitter expression difference at pr4 (Fig. 2*D*). Thus, *G9a* is required for the rover–sitter expression difference of pr4.

To further explore the interaction between *G9a* and allele-specific *for* expression, we performed chromatin immunoprecipitation-qPCR to assess H3K9me2 levels at the *for* promoters (Fig. 2 *E–H*). pr4 shows a *G9a*-mediated rover–sitter methylation difference (Fig. 2*H*) that agrees with the lower expression of pr4 in *G9a* WT rovers. Rovers have significantly higher pr4 H3K9me2 levels than sitters ($P = 0.002$), but, importantly, this difference disappears in the presence of the *G9a* null allele ($P = 0.476$). This provides further evidence that the rover–sitter difference depends on *G9a*. The fact that some H3K9me2 methylation remains in the *G9a* mutant demonstrates compensation by another H3K9me2 methyltransferase when *G9a* is lost. However, this other H3K9me2 methyltransferase does not discriminate between the rover and sitter alleles, resulting in no difference in H3K9me2 methylation of pr4 in the *G9a* null mutants.

Rovers and sitters also have differing H3K9me2 levels at pr1 (Fig. 2*E*); however, this does not alter the expression of pr1 (Fig. 2*A*). Although histone methylation marks are generally associated with either repression or activation of nearby genes, the relationship between these marks and expression is not necessarily causal or linear. Specifically, H3K9me2 is associated with a wide range of gene repression patterns, and can be found at both active and repressed genes (13). Considering a chain of effect in which methylation regulates expression and altered gene expression regulates behavior, the H3K9me2 methylation difference at pr1 cannot be responsible for the rover–sitter behavioral differences described here, because it does not alter pr1 gene expression.

Differences in H3K9me2 methylation cannot be explained by genetic variation within *G9a*, because our rover and sitter strains were constructed to share identical *G9a* WT alleles (*Materials and Methods*). Furthermore, *G9a* expression levels do not differ in rovers and sitters (Fig. S2*A*). Consequently, the rover–sitter difference in pr4 methylation does not arise from *G9a* expression differences in these strains. To rule out involvement of *egg* and *SU(VAR)3–9*, the only other H3K9me methylases in *Drosophila*, we assessed the expression of *egg* and *SU(VAR)3–9* in rovers and sitters. Like *G9a*, *egg* expression does not differ between rovers and sitters (Fig. S2*B*). On the other hand, *SU(VAR)3–9* is more highly expressed in sitters (Fig. S2*C*). However, since sitters have lower H3K9me2 levels, *SU(VAR)3–9* expression it is not responsible for the *for* pr4 methylation pattern. This further supports the hypothesis that *G9a* mediates the rover–sitter difference in pr4 expression; *G9a* targets pr4 differently in rovers compared with sitters.

Rover Foraging Behavior Can Be Phenocopied in Sitters by Transgenically Reducing pr4 Expression. To establish a causal relationship between *for* pr4 expression and foraging behavior, we designed an RNAi construct that specifically targets pr4 transcripts (Fig. S3 *B–F*). Before knocking down *for*, we show that foraging success is significantly greater in rover–sitter heterozygotes than in sitters ($P = 0.017$) and does not differ significantly from that in rovers ($P = 0.138$) (Fig. 3*B*). Correspondingly, rovers, sitters, and rover–sitter heterozygotes with *G9a* null alleles do not differ in their foraging success (Fig. 3*B*). The fact that the foraging behavior of rover heterozygotes is comparable to that of the rover homozygotes allowed us to perform RNAi experiments in heterozygotes. Since sitters have higher pr4 expression and lower foraging scores than rovers, we predicted that knockdown of pr4 in sitters would result in an increase in foraging scores. As predicted, pr4 knockdown in sitters increases foraging relative to the transgenic controls [Fig. 3*C*; $F_{(4,94)} = 13.487$; Gal4 control, $P = 0.002$; UAS control, $P = 0.014$], while further knockdown in rovers has no effect on foraging. It is possible that foraging behavior might not have increased in rovers because of a ceiling effect. This could be because rovers naturally forage at a physiological maximum, or because there is a limiting step in the activation or repression of a downstream target of *for*. Nevertheless, our finding that a knockdown of pr4 transcripts in sitters results in an increase in foraging success conclusively demonstrates a causal relationship between pr4 expression levels and differences in rover and sitter adult foraging behavior. The differences in adult foraging behavior are also reflected in the proportion

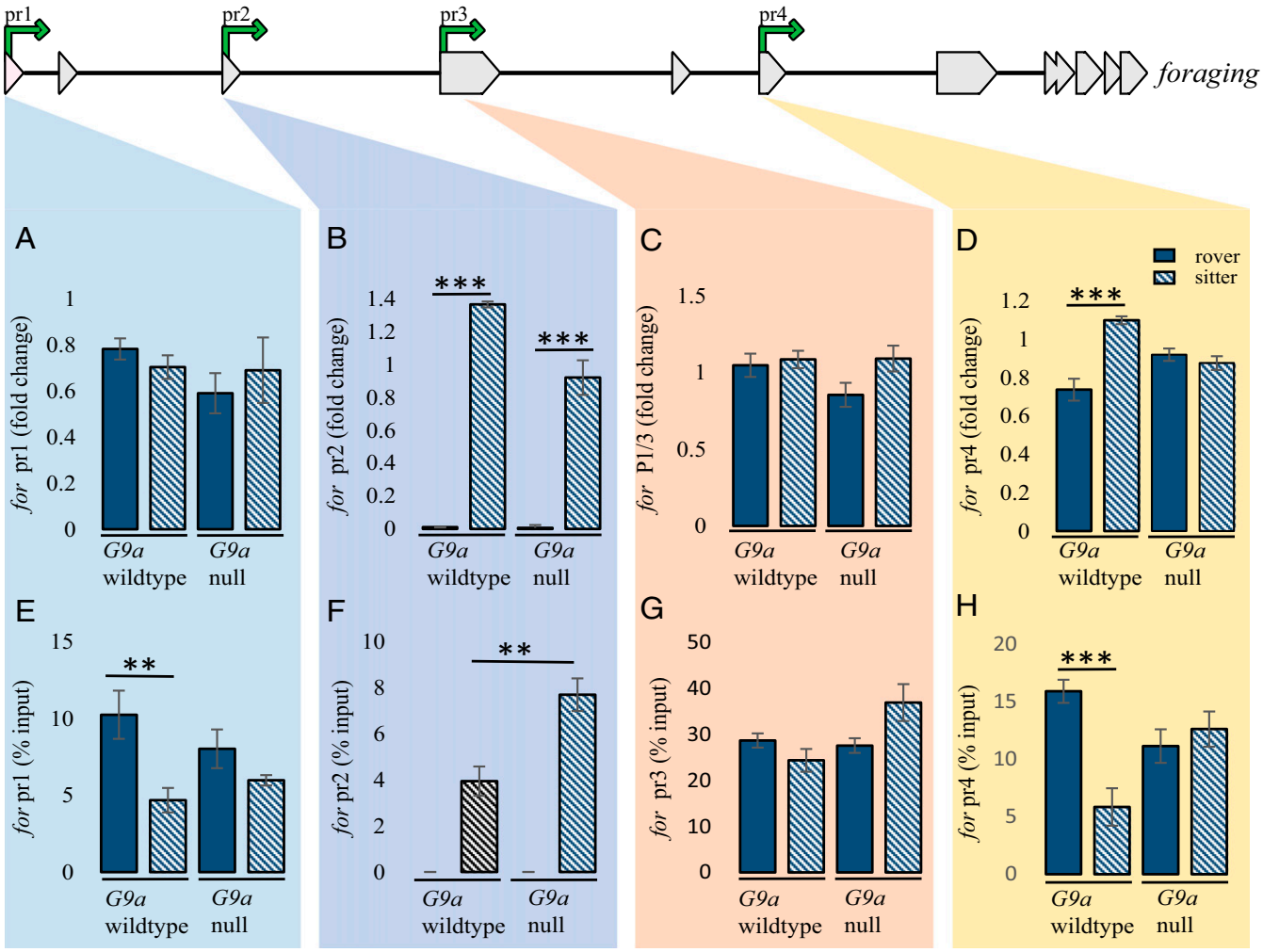

Fig. 2. The *for* promoters are differentially expressed and show *G9a*-dependent methylation differences. (*A*) Rovers and sitters do not differ in *for* pr1 expression [$F_{(3,11)} = 3.96$; $P = 0.053$]. (*B*) Rovers with *G9a* WT have significantly less pr2 expression than sitters with *G9a* WT [$F_{(3,11)} = 42.39$; $P < 0.001$], and this difference is not *G9a*-dependent as it is maintained in *G9a* nulls ($P < 0.001$). (*C*) Rovers and sitters do not differ in *for* P1/3 expression [$F_{(3,11)} = 2.24$; $P = 0.161$]. (*D*) Rovers with *G9a* WT have significantly less pr4 expression than sitters with *G9a* WT [$F_{(3,11)} = 10.62$; $P < 0.003$], and this difference is *G9a*-dependent, as it disappears in *G9a* nulls ($P = 0.2$). (*E*) Rovers with *G9a* WT have significantly more *for* pr1 H3K9me2 than sitters with *G9a* WT [$F_{(3,15)} = 6.59$; $P = 0.007$]. (*F*) Rovers with *G9a* WT have significantly less pr2 H3K9me2 than sitters with *G9a* WT [$F_{(3,15)} = 68.86$; $P < 0.001$], and this difference is not *G9a*-dependent, as it is maintained in *G9a* nulls ($P < 0.001$). (*G*) Rovers and sitters do not differ in *for* pr3 H3K9me2. (*H*) Rovers with *G9a* WT have significantly more pr4 H3K9me2 than sitters with *G9a* WT [$F_{(3,15)} = 8.62$; $P = 0.002$], and this difference is *G9a*-dependent, as it disappears in *G9a* nulls ($P = 0.476$). $n = 3$ for qRT-PCR and $n = 4$ for ChIP-qPCR with 20 adult mated females/biological replicate. *$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P > 0.001$.

of time spent in areas containing food (Fig. 3 *A* and *D*), but not in overall distance traveled during the test (Fig. 3*E*).

The Rover–Sitter *for* pr4 Difference Is Tissue-Specific. Because all of the *for* promoters showed expression in whole adult flies (Fig. 2), we dissected candidate tissues and assessed pr4 expression in rover and sitter flies with WT or null *G9a* alleles (Fig. 4). Notably, highly significant differences between rover and sitter pr4 expression in the brain ($P < 0.001$) disappear in the *G9a* null background ($P = 0.190$) (Fig. 4). Ovaries have a smaller but also significant rover–sitter difference in pr4 expression that also disappears in the *G9a* null background. These findings suggest that rover and sitter adult foraging behavior is likely driven by pr4 expression differences in the brain and the ovaries.

Rover and Sitter *for* Promoter DNA Sequences Are Polymorphic. Since the difference in methylation at pr4 does not originate with *G9a* itself, the most likely explanation for the differences in expression and methylation of pr4 in rovers and sitters are DNA single nucleotide polymorphisms (SNPs) that can affect the recruitment of *G9a* to the promoter region. To address this, we sequenced the rover and sitter *for* alleles and found several SNPs, mostly in the noncoding region; one of these SNPs was in pr4 (Fig. 4*E* and Table S1). We then searched the sequence of pr4 for predicted transcription factor binding sites that coincide with the single SNP found in this region. The pr4 region had predicted transcription factor binding sites for six different factors/classes (*Mad*, *GAGA factor*, *T11*, *Prd*, *Dfd*, and *FTZ*), with the highest confidence for three predicted *mad* sites, one of which falls on the single SNP found in pr4 (Fig. 4*B*). This SNP coincides with a site within the *mad* binding sequence that does not allow substitutions, most likely resulting in no binding of this factor at this site in the rover strain, but not in the sitter strain.

Discussion

We show that rovers and sitters have a natural difference in adult foraging behavior that is caused by differences in *G9a*-dependent

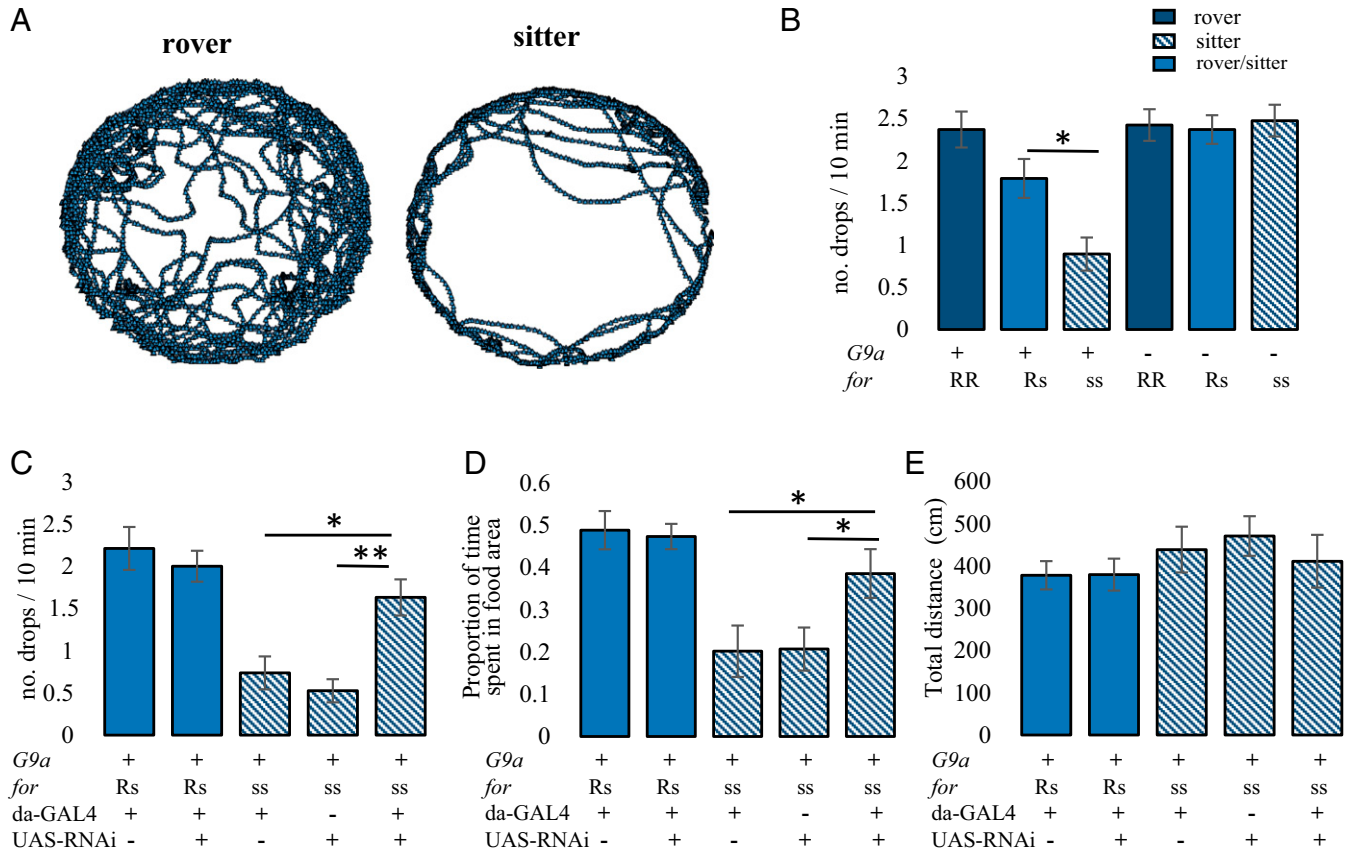

Fig. 3. Rover–sitter foraging behavior is directly regulated by pr4 expression levels. (*A*) Representative images of rover and sitter foraging paths with position coordinates over 10 min plotted as a scatterplot. (*B*) There are significant differences in foraging behavior among rovers, sitters, and rover–sitter heterozygotes [$F_{(4,119)} = 11.09$; $P < 0.001$], driven by *G9a*. Rovers with WT *G9a* forage significantly more in 10 min than sitters with WT *G9a* ($P < 0.001$). rover–sitter heterozygotes with WT *G9a* forage significantly more than sitters with WT *G9a* ($P = 0.017$), and are not significantly different from rovers with WT *G9a* ($P = 0.138$). Rovers, sitters, and rover–sitter heterozygotes with *G9a* null show no differences in foraging behavior. (*C*) Reducing pr4 expression by driving pr4-RNAi with the *da*-GAL4 driver significantly affects foraging behavior [$F_{(4,94)} = 13.49$; $P < 0.001$]. pr4 RNAi expression significantly increases sitter foraging behavior compared with controls ($P = 0.002$ compared with UAS control and $P = 0.014$ compared with GAL4 control), and does not significantly alter rover behavior ($P = 0.719$). (*D*) Reducing pr4 expression by driving pr4-RNAi with the *da*-GAL4 driver significantly affects the proportion of time spent in the interior food-containing area of the arena [$F_{(4,70)} = 6.88$; $P < 0.001$]. (*E*) Reducing pr4 expression by driving pr4-RNAi with the *da*-GAL4 driver does not affect the total distance traveled during foraging [$F_{(4,68)} = 4.16$; $P = 0.005$]. $n = 20$ for all tests. *$0.05 > P > 0.01$; **$0.01 > P > 0.001$.

expression of the *for* pr4 transcripts. pr4 is differentially methylated by *G9a* in rovers and sitters, and we demonstrate that pr4 is solely responsible for the rover–sitter behavioral polymorphism in adult foraging behavior. Nevertheless, *G9a* is not the sole transcriptional regulator, or the sole H3K9 methyltransferase, regulating pr4 expression. Our results show that the loss of *G9a* can result in more or less H3K9me2 at pr4, depending on the *for* allele present. This dual function of *G9a* has been previously shown in mice, where *G9a* is able to both repress and activate gene expression through interactions with other proteins in its regulatory complex (14). While pr4 is responsible for regulating the rover–sitter difference in adult foraging behavior, other *for* promoters likely regulate other *for*-related phenotypes. In fact, our expression data show that other *for* promoters are differentially expressed in rovers and sitters. For example, pr2 is highly expressed in sitters and not expressed at all in rovers (Fig. 2*B*). The pr2 expression difference also correlates with H3K9me2, but cannot be explained solely by *G9a*. pr2 and pr4 transcribe different isoforms of *for* (P1 and P4, respectively) that might differ in function. Our results suggest that the expression of *for*'s four promoters might be regulated by distinct regulatory complexes, and that each promoter might influence distinct behavioral phenotypes.

We also found that the difference in pr4 expression is tissue-specific, being driven by the brain and ovaries. The central nervous system and ovaries might be linked in regulating feeding behavior, since reproduction constitutes the major energy expenditure of female flies, and sex peptide signaling in the reproductive organs affects the feeding behavior of female flies (15). Our work highlights the complex epigenetic architecture that underlies behavioral regulation.

The lack of a DNA-binding domain suggests that *G9a* is targeted to specific DNA regions through interactions with DNA-binding proteins, such as transcription factors. SNPs in the promoter region could lead to differential binding of DNA-binding proteins that recruit *G9a*. For instance, the SNP in pr4 lies within a conserved site of a putative *mad* binding motif, and potentially could affect *mad* binding. If *mad* is one of the elements in the *G9a* complex, then less binding of *mad* in the rover strain (which would be predicted from the SNP) potentially could explain the lower pr4 H3K9me2 levels in rovers. Like *G9a*, *mad* has been shown to act as both a repressor and an activator of gene transcription, depending on context (16). Although *mad* is best known for its role in development (17), some studies suggest that it might have regulatory functions in the mature nervous system (18).

In conclusion, the mechanisms by which epigenetic regulation influences behavioral differences are poorly understood. Epigenetic regulation has been shown to be a mechanism through which animals adjust their behavior and physiology to the environment in

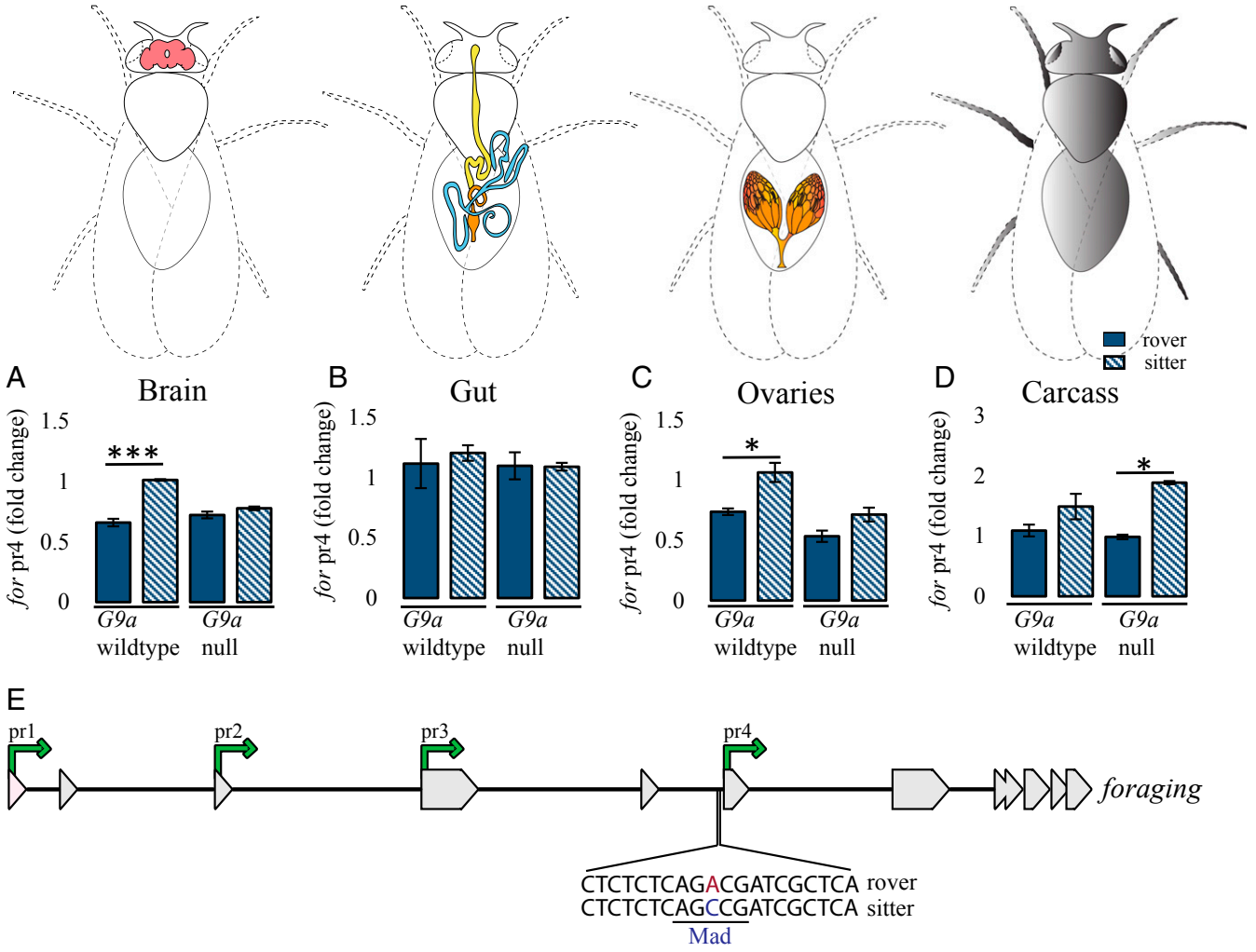

Fig. 4. The rover–sitter difference in *for* pr4 expression arises from the brain and ovaries. (*A*) Rovers with *G9a* WT have significantly less pr4 expression in the brain than sitters with *G9a* WT [$F_{(3,11)} = 32$; $P < 0.001$], and this difference is *G9a*-dependent, as it disappears in *G9a* nulls ($P = 0.19$). (*B*) Rovers and sitters do not differ in *for* pr4 expression in the gut [$F_{(3,11)} = 0.13$; $P = 0.937$]. (*C*) Rovers with *G9a* WT have significantly less pr4 expression in the ovaries than sitters with *G9a* WT [$F_{(3,11)} = 10.34$; $P = 0.004$], and this difference is *G9a*-dependent, as it disappears in *G9a* nulls ($P = 0.19$). (*D*) Rovers and sitters with *G9a* WT do not differ in *for* pr4 expression in the carcass [$F_{(3,11)} = 8.03$; $P = 0.242$], but rovers with *G9a* null have significantly less pr4 expression in the carcass than sitters with *G9a* WT ($P = 0.014$). (*E*) Rovers and sitters differ in one SNP in a 0.1-kb region upstream of the pr4 transcription start site. For qRT-PCR, $n = 3$, with 20 adult mated female tissues/biological replicate for all tissues. *0.05 > P > 0.01; **0.01 > P > 0.001; ***P > 0.001.

which they live. Not all individuals respond similarly to the same environmental cue, however. In this case, epigenetic-by-genetic interaction would be an important but neglected component of gene-by-environment interactions. The deposition of epigenetic marks can depend on underlying genetic differences (19), and genetic variation likely plays an important role in moderating epigenetic differences between individuals. Importantly, epigenetic-by-genetic interactions present an avenue through which genetic variation outside of gene coding regions can modulate phenotypic variability. Two other noteworthy studies in humans and prairie voles have reported associations among genetic variation, DNA methylation, and behavior (1, 2). Here we used the fruit fly to establish molecular causality, and provide definitive evidence for how the complex interactions among genetics, epigenetics, and isoform-specific gene regulation causes variation in naturally occurring behavioral polymorphisms.

Materials and Methods

Fly Strains and Rearing. All flies were reared on a standard cornmeal-molasses medium at 25 °C on a 12-h light/dark cycle with lights on at 0800 h. The rover (*for*) and sitter (*for*^{s}) strains (10) have reisogenized *for*^{R} or *for*^{s} second chromosomes and share reisogenized X and third chromosomes. The *G9a* null and its corresponding *G9a* WT allele were originally designated as *EHMT*^{DD1} and *EHMT*$^+$ (12). The *daughterless*-GAL4 (*da*-GAL4) driver was a gift from Tony Harris, Department of Cell & Systems Biology, University of Toronto, Toronto. The *foraging* pr4 RNAi line was generated in the M.B.S. laboratory, and the UAS-*Dcr* line was acquired from the Bloomington Drosophila Stock Center (24651). A more detailed description of the strains is provided in the *SI Materials and Methods*.

Genomic Sequencing of the *for*^{R} and *for*^{s} Lines. Full genomic sequencing was done on the *for*^{R} and *for*^{s} lines. DNA was extracted from 50 males and 50 females of each strain using a Qiagen DNeasy Blood and Tissue Kit (catalog no. 69504), following the manufacturer's instructions. TruSeq gDNA library preparation and paired-end 100-bp sequencing on the Illumina HiSeq platform was done at the McGill University and Génome Québec Innovation Centre. For reference-guided assembly, the reads were mapped to the *D. melanogaster* reference genome (release 5.57) using the default parameters in bwa v. 0.6.0-r85 (20), and consensus sequences for each line were generated with Samtools v. 0.1.18 (21). Consensus sequences for each chromosome were deposited in GenBank (sitter accession nos. CP023329–CP023334; rover accession nos. CP023335–CP023340). Consensus sequences were aligned and annotated in Geneious v. R10.0.5 (22). Putative transcription factor-binding sites were assessed by submitting 100 bp of the DNA sequence immediately

upstream of the pr4 transcription start site to PROMO v. 3.0.2 (23), specifying both species and factor as *D. melanogaster*.

Generation of the pr4 RNAi Line. For the transgenic knockdown of *foraging* pr4 the *foraging* pr4 RNAi line was generated using the pWIZ RNAi cloning vector (24). A region complementary to the 3′ end of exon 7 was used to amplify a pr4 isoform-specific region of 723 bp. The primers (Table S2) included an NheI restriction site (underscored in the table), which was used to clone the 5′-3′ fragment into the NheI site of pWIZ, and the 3′-5′ fragment into the AvrII (which has complementary sticky ends with NheI) site of pWIZ. P-element injections into *w1118*, performed by BestGene, resulted in insertion of the transgene on the second chromosome.

Adult Foraging Assay. The adult foraging assay (AFA) has been described in detail by Hughson et al. (11). In brief, females were collected at eclosion and housed in groups of 20 females and 10 males. Mated 5- to 6-d-old females were food deprived with a water source for 24 ± 0.5 h before being tested in the AFA. Foraging tests were performed in the afternoon to avoid circadian effects on feeding. A more detailed description of the AFA setup is provided in *SI Materials and Methods*.

qRT-PCR. RNA of whole flies or tissues was extracted using the RNeasy Mini Kit (catalog no. 74104; Qiagen) with RNase-Free DNase (catalog no. 79254; Qiagen). RNA integrity was assessed, and cDNA was synthesized from 1 μg of tRNA with the iScript Advanced cDNA Synthesis Kit for qRT-PCR (catalog no. 1725037; Bio-Rad). qRT-PCR was performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad), using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and gene-specific primers (Table S2). Target gene expression was standardized to three reference genes (*α-tub*, *act5c*, and *1433ε*), and fold change values ($2^{-\Delta\Delta Ct}$) were determined to quantify relative expression differences between genotypes. More details are provided in *SI Materials and Methods*.

Chromatin Immunoprecipitation-qPCR. Crude fly extract was obtained by homogenizing 20 flies in PBS, followed by cross-linking with 1% formaldehyde for 30 min. Nuclei were isolated, and cross-linked chromatin was fragmented by sonicating on ice for 60 cycles (high power, 30 s on/off). Chromatin immunoprecipitation was performed with anti-H3K9me2 antibodies (07-441; Upstate Biotechnology), and Protein A/G beads (Santa Cruz Biotechnology) were used to capture antibody-bound chromatin. Chromatin immunoprecipitated DNA was isolated by phenol/chloroform extraction and ethanol precipitation. qPCR on chipped and input (not chipped chromatin) was performed with primers targeting *foraging* promoter areas (Table S2), and methylation levels were accessed as %input. *moca-cyp*, used as a negative methylation control, showed low methylation (<3%) in all strains (Fig. S4), and *2cta* and *2chi*, used as positive methylation controls, showed high methylation (40–50%) in all strains, with no significant differences among strains (Fig. S4).

Statistical Analysis. All statistical analyses were performed in SigmaPlot 11.0. Data were tested for normality and equal variance, and one- or two-way ANOVA was performed to test for the effects of strain and treatment and their interactions. Post hoc pairwise multiple comparison procedures were done using the Holm–Sidak method. Kaplan–Meier survival analysis (log-rank) with post hoc multiple comparisons by the Holm–Sidak method were performed on the starvation resistance data.

ACKNOWLEDGMENTS. We thank Tony Harris (University of Toronto) for the *da*-GAL4 driver; Ian Dworkin and Thomas Braukmann for help with the genome assemblies; the PNAS editor and reviewers for comments that greatly improved the manuscript; and Joel Levine, Jeff Dason, and Maria Aristizabal for comments on an early draft. This research was supported by a Natural Sciences and Engineering Council of Canada and Canadian Institute for Advanced Research grant (to M.B.S.), and an Ontario Graduate Scholarship (to I.A.). The stocks used in this study were obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537).

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