

ORIGINAL ARTICLE

Combining Human Epigenetics and Sleep Studies in *Caenorhabditis elegans*: A Cross-Species Approach for Finding Conserved Genes Regulating Sleep

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Study Objectives: We aimed to test a combined approach to identify conserved genes regulating sleep and to explore the association between DNA methylation and sleep length.

Methods: We identified candidate genes associated with shorter versus longer sleep duration in college students based on DNA methylation using Illumina Infinium HumanMethylation450 BeadChip arrays. Orthologous genes in *Caenorhabditis elegans* were identified, and we examined whether their loss of function affected *C. elegans* sleep. For genes whose perturbation affected *C. elegans* sleep, we subsequently undertook a small pilot study to re-examine DNA methylation in an independent set of human participants with shorter versus longer sleep durations.

Results: Eighty-seven out of 485,577 CpG sites had significant differential methylation in young adults with shorter versus longer sleep duration, corresponding to 52 candidate genes. We identified 34 *C. elegans* orthologs, including *NPY/flp-18* and *flp-21*, which are known to affect sleep. Loss of five additional genes alters developmentally timed *C. elegans* sleep (*B4GALT6/bre-4*, *DOCK180/ced-5*, *GNB2L1/rack-1*, *PTPRN2/ida-1*, *ZFYVE28/lst-2*). For one of these genes, *ZFYVE28* (also known as *hLst2*), the pilot replication study again found decreased DNA methylation associated with shorter sleep duration at the same two CpG sites in the first intron of *ZFYVE28*.

Conclusions: Using an approach that combines human epigenetics and *C. elegans* sleep studies, we identified five genes that play previously unidentified roles in *C. elegans* sleep. We suggest sleep duration in humans may be associated with differential DNA methylation at specific sites and that the conserved genes identified here likely play roles in *C. elegans* sleep and in other species.

Keywords: DNA methylation, sleep, epigenetics, genetics, *C. elegans* sleep, *ZFYVE28*, *lst-2*.

Statement of Significance

This study used an experimental approach that combined human sleep studies and epigenome-wide methylation analysis, with assessment of gene function in a model organism, *Caenorhabditis elegans*. We identified five conserved genes with previously unsuspected roles in sleep in *C. elegans*. An ortholog of one of these genes showed consistent differential DNA methylation in human samples. This work suggests a possible association between sleep duration in humans and DNA methylation at specific CpG sites in conserved genes. Due to the exploratory nature of the human study design, additional research with more participants is required to confirm associations of these genes with sleep length in humans. Nevertheless, validation of this approach and our identification of conserved genes regulating sleep in *C. elegans* is a critical step toward understanding the basic mechanisms underlying sleep.

INTRODUCTION

Sleep is universal and conserved. Many genes have been identified that are critical for sleep, sleep duration, and sleep timing across species.^{1–3} Sleep in humans is also affected by societal and environmental factors, such as occupation, socioeconomic status, and lifestyle choices. Additionally, changes in light, temperature, pathological conditions, sleep/wake history, or stress affect sleep. Stress can lead to epigenetic changes with long-lasting and profound impacts on behavior.^{4–6} Few studies, however, have examined association between sleep patterns and epigenetic modifications.

Epigenetic changes are modifications of DNA or protein with impact on gene expression that do not involve alterations in DNA sequence. Epigenetic modifications can suppress or activate the transcription of individual genes or induce alternative splicing through mechanisms including DNA methylation, histone modifications, and/or nuclear small RNA-associated silencing. It has been suggested that epigenetic modifications, especially DNA methylation, can persist through cell division and may have prolonged effects.^{7,8} Epigenetic mechanisms

impact disease etiology and phenotypic variation,^{9,10} including cancer¹¹ and depression.^{12,13} However, only a few studies have examined epigenetic changes related to sleep outside of clinical syndromes.

Epigenome-wide DNA methylation changes were found in shift workers; methylation at more than 5000 CpG sites differed in whole blood samples of long-term night shift workers compared to day workers.¹⁴ Manipulations in mice revealed that short-term, acute sleep deprivation altered the epigenetic landscape in the cerebral cortex.¹⁵ Combined, these studies suggest that epigenetic modifications may arise after sleep pattern changes or sleep restriction. One recent study showed that monozygotic twins with differential diurnal preferences have significantly different methylation patterns in DNA extracted from their buccal cells; these could result from differential circadian rhythms or contribute to sleep–wake cycle regulation.¹⁶ However, identification of specific genes whose methylation directly impacts sleep has been virtually impossible due to the large number of methylation changes observed and the difficulty of large-scale sleep studies in vertebrate models. One

exception is opposing “imprinted” DNA methylation status at chromosome 15q11–13, which correlates with the contrasting sleep defects seen in Angelman and Prader-Willi Syndrome patients.¹⁷ It has been suggested that these sleep changes are driven by altered methylation that consequently decreased or increased expression of *UBE3A*, *SNORD116A*, and other genes in the imprinted region.^{18,19} To circumvent challenges in a context not related to disease, we adopted a new experimental strategy that combined DNA methylation analysis in college students with analysis of gene function in an invertebrate animal model.

Caenorhabditis elegans is a free living nematode with well-characterized behaviors, a compact nervous system, conserved neurotransmitter systems, and powerful genetic tools. Sleep has been studied in *C. elegans* for almost a decade.^{20,21} *C. elegans* sleep shares most of the behavioral characteristics of sleep in other species, that is, cessation of movement and feeding, heightened arousal threshold, rapid reversibility, homeostatic regulation, and regulated timing.^{2,22} Deeply conserved genes and pathways regulate *C. elegans* sleep. Virtually every *Drosophila* gene previously implicated in sleep also regulates *C. elegans* sleep.²³ Genes affecting mammalian sleep, including epidermal growth factor (EGF) and *NPY*, also regulate *C. elegans* sleep.^{2,20,24,25} Although developmentally timed sleep is regulated by a conserved *PERIOD* ortholog in *C. elegans*, the timing is not set by circadian rhythm but rather the pace of development.²⁶

We hypothesized that differential methylation patterns in sleep-related human genes might be associated with sleep length differences in young adults, either contributing to duration of sleep or responding to altered sleep quantity. We identified differentially methylated genes in humans associated with shorter or longer sleep duration during the first semester of college (Discovery Study). Then, we tested the functional role of orthologous genes in *C. elegans*, examining the impact of gene loss of function on developmentally timed sleep to identify genes required for normal sleep (Animal Study). Finally, in a small pilot study with an independent set of human participants, we determined which of these conserved genes were consistently hypermethylated or hypomethylated in shorter sleepers (Confirmation Study) (Figure 1A).

METHODS

Participants

First-year undergraduate students from Brown University were enrolled in the study for the first 9 weeks of their first semester. Participants were asked to complete a daily diary online, including bedtime and rising time, as well as sleep onset latency and nighttime arousals. A new cohort of participants was added every year for 3 years. All participants were 18 or 19 years old at the time of the study. Mood symptoms were assessed with the Center for Epidemiologic Studies Depression Scale (CES-D) questionnaire²⁷ at the start of the study and during the ninth week. Participants were considered for inclusion in this sleep/methylation study if they provided blood samples and mood assessments at both start and end of the longitudinal study and they also completed more than 50% of daily diaries. In the first year, 320 initially enrolled, 192 gave both blood samples and completed more than 50% of daily diaries, and of that

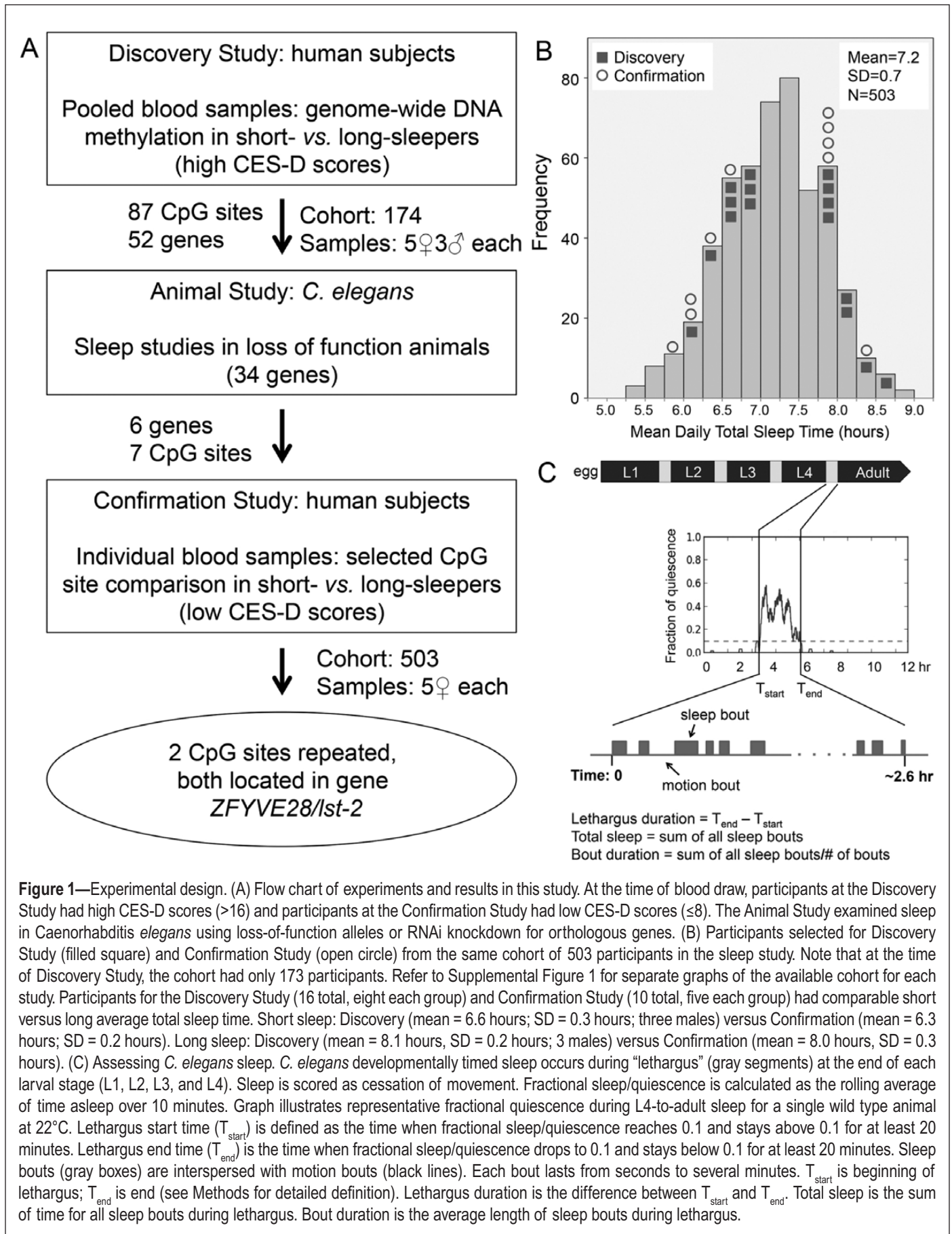
number, 173 provided final assessments of behavior and mood. Combined over 3 years of data collection, 796 individuals initially enrolled, of whom 503 provided two blood samples, more than 50% of daily diaries, and the final assessments. Previous comparisons of daily diary and actigraphy sleep reports suggest that average diary-estimated total sleep estimates are larger than actigraphy-estimated total sleep by roughly 1 hour, although results using the two methods are well correlated, except in insomnia patient populations.^{28,29}

Samples in the Discovery Study were drawn from the initial cohort of 173 participants, enrolled in year 1 (2012) and were selected to maximize differences in daily total sleep time. As mood has a strong association with DNA methylation and sleep,^{12,30} for inclusion in the Discovery Study, we required a low depressed mood score on the CES-D questionnaire²⁷ at the beginning of the study and a moderate to high (>16) depressed mood score on the CES-D questionnaire.²⁷ DNA samples were pooled for participants with short or long sleep. Eight participants with longer (>7.8 hours; mean = 8.1 hours, standard deviation (SD) = 0.2 hours; three males) and eight with shorter (<6.8 hours; mean = 6.6 hours; SD = 0.3 hours; three males) mean daily total sleep time across the semester were included in this study for DNA methylation analysis (Figure 1B).

Participants for the Confirmation Study were selected to match the difference in group mean daily total sleep times in the Discovery Study. These participants were drawn from those enrolled from year 1 to year 3 (2012–2014). Cognizant of the impact of mood on sleep,^{12,30} a nonoverlapping set of participants was selected with low (≤ 8) initial and final depressed mood scores on the CES-D questionnaire. Five female participants reporting longer (>7.7 hours; mean = 8.0 hours, SD = 0.3 hours) and five with shorter (<6.6 hours; mean = 6.3 hours; SD = 0.2 hours) total sleep time across the semester (Figure 1B) were selected for individual DNA methylation analysis below. Other variables, including body mass index, smoking, or reported alcohol use, were not controlled. Note that Figure 1B combines participants from all years and illustrates the full cohort of 503 participants.

Pooled and Individual Epigenome-Wide Methylation Analysis

Genomic DNA was isolated (DNEasy Blood and Tissue Kit, Qiagen) from whole blood collected from participants at the end of the study period. For the Discovery Study, epigenome-wide methylation was determined from pooled samples of eight shorter and eight longer sleepers (three males in each group). DNA concentration for each participant was determined using NanoDrop and an equal quantity of DNA from individuals in the same group was pooled before bisulfite DNA conversion (EZ DNA methylation kit, Zymo Research). DNA methylation level was determined for each group using the Infinium HumanMethylation450 platform (Illumina, San Diego). A methylation index was calculated for each CpG site, which is a continuous variable between 0 and 1, representing the ratio of the intensity of the methylated signal to the total intensity. Zero corresponds to a completely unmethylated site, whereas one corresponds to complete methylation. Illumina's GenomeStudio software was used to calculate the degree of differential methylation, by group, for each CpG site on the array. Specifically,



differential methylation analyses were conducted with an Illumina Custom Methylation Model, which generates p -values for differential methylation of each CpG site based on mean β values and variance across replicate probes. Individual CpG sites were considered differentially methylated between the different sleep groups at false discovery rate (FDR)-adjusted $p < .05$.

For the Confirmation Study, individual samples were assayed for epigenome-wide DNA methylation using the same Infinium HumanMethylation450 platform (Illumina). Methylation betas were preprocessed and normalized using the R package minfi³¹ and the function preprocessFunnorm to perform functional normalization. We removed loci with detection p -value $> .05$, sex chromosomes, loci with single nucleotide polymorphism (SNP) in probe, and cross-reactive and polymorphic probes.³²

Ortholog Identification

C. elegans orthologs of human genes were identified using BLASTP (<http://blast.ncbi.nlm.nih.gov>). The best reciprocal hits between the two species were retained as orthologous gene pairs. In the case of *YES1*, *src-2* is the best *C. elegans* ortholog. We have previously tested *src-2* mutant animals, and they had normal sleep (Supplemental Table 2). Therefore, we instead tested *src-1*, which is the other ortholog of *YES1*.

Strain Information

Caenorhabditis elegans strains were cultured on standard nematode growth media (NGM) seeded with OP50 bacteria and grown at 25°C. However, strains VC3013 and VC226 were grown at 15°C, as they become sterile at 25°C. The standard *C. elegans* wild-type strain, N2, was used as a control for mutant animals. RNA interference (RNAi) feeding was performed using the neuronal RNAi sensitive strain HA2581 *uls72* [pCFJ90(*myo-2p::m-Cherry*) + *unc-119p::sid-1* + *mec-18p::mec-18::GFP*], which was backcrossed to N2 from TU3595 *sid-1(pk3321)* *him-5(e1490)* *V*; *lin-15B(n744)* *X*; *uls72*.^{23,33} All other strains are listed in Supplemental Table 2. All strains were confirmed by genotyping (Supplemental Table 4) except for HY485,³⁴ for which the molecular lesion is not curated.

Selected loss-of-function mutations were either large deletions removing exonic regions or nucleotide substitutions disrupting splicing or creating early stop codons. If appropriate loss-of-function alleles were not available or homozygous lethal, we used long double-stranded RNAi in a neuronal RNAi-sensitive background³³ to knockdown mRNA transcripts for each gene in *C. elegans* neurons. RNAi in *C. elegans* is a well-established and widely used method to knockdown gene expression³⁵ and using long double-stranded RNA increases effective targeting of the mRNA transcripts.

Before RNAi studies, TU3595 was backcrossed into the N2 wild-type strain five times, which eliminated the *sid-1(pk3321)* loss-of-function allele present in the original strain and resulted in strain HA2581. We find that animals with the *sid-1(pk3321)* have abnormal behavior in some assays. After backcross, we confirmed eviction of *sid-1(pk3321)* based on loss of the corresponding *Apo I* restriction fragment length polymorphism.

Caenorhabditis elegans Sleep Analysis

The *C. elegans* sleep assay was adapted from previous studies and relies on cessation of locomotion to detect sleep.³⁶ Briefly,

overnight OP50 *E. coli* culture, treated with kanamycin, was resuspended in liquid NGM and applied to a 10-chamber microfluidic artificial dirt chip. Mid-L4 stage animals were loaded into each chamber and covered with a coverslip. To avoid evaporation of liquid, molten 2% agar was used to seal the coverslip with the chip. Images were recorded every 10 seconds for 12 hours. Sleep data were extracted with a MatLab image subtraction script, and a Python script was used to calculate lethargus duration, total sleep, and average bout duration (deposited to GitHub, <https://github.com/Huiyan-Huang>). The onset of lethargus (T_{start}) was defined as the point at which the fraction of sleep/quiescence (over 10 minutes) stayed above 0.1 for at least 20 minutes. The end of lethargus (T_{end}) was defined as the point at which the fraction of sleep/quiescence stayed below 0.1 for at least 20 minutes. Lethargus duration is the time between the onset and end of lethargus. Total sleep is the sum of all sleep bouts during lethargus. Average bout duration is the average bout length during lethargus (Figure 1C). At least two trials were incorporated into each determination; at least 10 animals were assayed for each strain along with at least 10 wild-type control animals run simultaneously.

RNAi Constructs and Culturing Conditions

RNAi clones of *ced-5*, *Y54E5A.6*, *sel-8*, *ocr1-1*, *oxa-1*, *C18B12.4*, *acl-3*, *maco-1*, and *cct-6* were from the Ahringer library.³⁷ The RNAi clones of *gly-5* and *R11A8.2* were from the Vidal library (GE Dharmacon).³⁸ RNAi clones for *src-1*, *bre-4* and nonoverlapping *cct-6* (N-terminal) were made by PCR, amplifying 493 bp (with primers CGCGCCGCGGCCGCGCA GAGAAGCCCAAATAATGAAGC and CGCGCCGCTAGCA ACTC ACCCGGATACGGAAC), 1178 bp (with primers CGC GCCGCGGCCGCGG ATCTTCGGCGGAATCAGTG and CG CGCCGCTAGCAAAC AAATGACAACCCGGCAAG), and 749 bp (with primers CGCGCCGCGGCCGCCC AGTGCCTG AACCCGAAAG and CGCGCCGCTAGCTCT GGTTCTCT CCATCACGAC) sequences from each gene, followed by insertion between NotI and NheI sites of pL4440 (gift from Andrew Fire [Addgene plasmid # 1654]). The Ahringer *cct-6* RNAi construct and the *cct-6* (N-terminal) RNAi construct had no overlapping sequences. All RNAi bacterial cultures were grown overnight and concentrated 10-fold before seeded onto plates (NGM agar with 1 mM IPTG and 100 μ g/ml ampicillin). All RNAi studies use animals overexpressing the SID-1 double-stranded RNA channel in neurons to increase sensitivity to RNAi in neurons. RNAi effects are predominantly in neurons but may impact other tissues as well. Animals were raised on RNAi plates for at least two generations before sleep assessment. Since RNAi in *C. elegans* is achieved with long double-stranded RNA, which corresponds to at least several hundred base pairs of coding sequence, experimental confirmation of knockdown is rarely performed.

Caenorhabditis elegans Transheterozygotes Sleep Assays

Transheterozygous animals were generated by crossing males to functional females carrying another mutant allele for the same gene. Functional females were generated by raising hermaphrodites on 10 \times concentrated overnight cultures of bacteria expressing *fem-3* dsRNA (from Vidal library), which prevents

sperm development. Males were generated by incubating L4 animals in 10% ethanol for 30 minutes and maintained by crossing to siblings. *fem-3* dsRNA-treated adult females for crosses were selected based on presence of stacked oocytes and an empty uterus. Before setting up crosses, females were left on the plate for at least 4 hours to confirm no embryos were laid. First generation transheterozygous animals were tested for sleep as described above. Animals from at least two independent crosses were tested.

Statistical Analysis

For the Animal Study, animals carrying *C. elegans* alleles or RNAi-treated animals were tested in at least four different microfluidic chips. Each chip was loaded with animals from at least two different strains, one being the appropriate control. Control animals for each strain were tested on the same day; these are not confined to control animals on the same chips, as there was no significant difference in control animals from different chips on the same day. At least two independent trials were examined for each allele/RNAi treatment. Welch's *t*-test was used to calculate the *p* values, which were corrected using FDR to obtain *q* values. Significance was set at $p < .05$ and $q < 0.10$. For the transheterozygous animal analysis, at least two independent trials were examined. Welch's *t*-test was used to calculate the *p* value. Significance was set at $p < .05$.

For the Discovery Study, Illumina's GenomeStudio software was used to calculate the degree of differential methylation, by group, for each CpG site on the array. FDR-adjusted *p*-values (*q*-values) were calculated in order to adjust for multiple comparisons. Significance was set at $q < 0.05$.

For each gene confirmed in *C. elegans* Animal Study, corresponding CpG sites from the Discovery study were re-examined in the pilot Confirmation Study. Hypermethylation or hypomethylation was compared between short and long sleepers using one-tailed Student *t*-test for the Confirmation Study.

Data Availability

Genome-wide methylation data for the pooled human study are available in the NCBI Gene Expression Omnibus (GEO), under accession number GSE80559 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80559>).

RESULTS

Differentially Methylated CpG Sites in Short Sleepers Versus Long Sleepers: Discovery Study

To look for associations between sleep length and differential methylation in specific genes, we undertook a naturalistic study in college students. DNA samples were pooled for participants with short or long sleep, considering previous successes with pooling in estimating the sample mean.^{39,40} Eight participants with longer (>7.8 hours; mean = 8.1 hours, SD = 0.2 hours; three males) and eight with shorter (<6.8 hours; mean = 6.6 hours; SD = 0.3 hours; three males) mean daily total sleep time across the semester were included in this study (Figure 1B and Supplemental Figure 1). Equal quantities of DNA from individuals were pooled into each group, and DNA methylation was assessed using Illumina Infinium HumanMethylation450 BeadChip arrays.

Methylation status was examined at 485 577 CpG sites. Each site was assigned a beta value (β), ranging from 0 to 1, which corresponds to fractional methylation. Comparing shorter sleepers with longer sleepers, we identified 87 sites with statistically significant differential methylation (Table 1 and Supplemental Table 1). In the shorter sleep pool, 59 sites were hypermethylated (increased methylation level) and 28 hypomethylated (reduced methylation level) relative to the longer sleep pool. Differentially methylated CpG sites corresponded to 52 unique candidate genes (Table 1). These include several human leukocyte antigen (HLA)-related genes and *NPY*, a neuropeptide with diverse impacts on behavior across species, including sleep.^{24,25,41,42} Increased methylation at CpG sites may lead to mRNA transcription repression, activation, or alternative splicing. Therefore, we do not draw conclusions here regarding the impact of methylation status on transcription or gene function but suggest that methylation in these genes may be a response to sleep length or contribute to sleep differences in these young adults. Consequently, we expect that a subset of these 52 candidate genes encode proteins whose function impacts sleep.

A Subset of Differentially Methylated Genes May Play Conserved Roles in Sleep: Animal Study

Assessing functional roles in sleep for these 52 candidate genes would be challenging in mammals. Therefore, we took advantage of reagents and tools available in the nematode *C. elegans* and the clear conservation of mechanisms underlying sleep. For each human gene, we identified the closest *C. elegans* ortholog based on protein similarity in reciprocal BLAST similarity searches. Thirty-four out of the 52 candidate genes identified in the human study had clear *C. elegans* orthologs (Supplemental Table 2). *NPY* has already been extensively studied and is involved in sleep regulation in multiple species, including *C. elegans*.^{24,25,42}

C. elegans and vertebrates share conserved transcriptional regulatory pathways, intron/exon splicing mechanisms, and translational control pathways. For example, regulation of transcription by histone methylation and acetylation also occurs in *C. elegans* via conserved pathways. But *C. elegans* and mammalian DNA methylation are not exactly the same. *C. elegans* DNA is methylated only at adenines,⁴³ whereas mammalian DNA is predominantly methylated at cytosines.^{44,45} Therefore, we do not assume that orthologous *C. elegans* genes are differentially methylated when sleep length changes. Instead, we use *C. elegans* only to probe the roles of these conserved genes in sleep and do not intend to draw any functional consequences of differential methylation in human or response to sleep restriction, which are complex processes that require future investigation. We tested 33 genes by measuring sleep in nematodes carrying loss-of-function mutations in the corresponding *C. elegans* genes or RNAi by feeding in a neuronal RNAi-sensitized strain. Twenty-seven genes were tested with loss-of-function mutants, and six genes were tested using RNAi knockdown (Supplemental Table 2).

C. elegans developmentally timed sleep was examined during the transition to adulthood. This developmental period at the end of the last larval stage is called L4/A (L4 larval stage to adult) lethargus. During lethargus, animals stop feeding and sleep in short bouts characterized by cessation of locomotion

Table 1—Genes With Differential Methylation at CpG Sites in Low Sleep Participants.

Gene name	Hypermethylated	Hypomethylated	Gene name	Hypermethylated	Hypomethylated
AGPAT4	2	—	<i>HLA-DRB1</i>	4	5
ANO10	1	—	<i>HLS-DRB5</i>	5	—
ARHGAP22	1	—	<i>HLA-DRB6</i>	3	—
ATP10B	—	1	<i>LOC285830</i>	—	1
B4GALT6	1	—	<i>LRWD1</i>	1	—
<i>C17orf97</i>	1	—	MAGI2	—	1
<i>C21orf56</i>	1	—	MAML2	1	—
<i>CASS4</i>	1	—	NOS1AP	1	—
CBX4	1	—	NPY	—	1
DIP2C	1	—	NR2C2	1	—
DOCK1	1	—	OCRL	1	—
<i>DUS2L</i> ;DDX28	1	—	OXA1L	—	1
ETV3	1	—	PTPRN2	—	1
<i>FAIM3</i>	—	1	RNF103	1	—
FAM102A	—	1	<i>SDHA</i> ;CCDC127	—	1
<i>FAM59B</i>	1	—	SDHAP3	—	3
GALNT9	1	—	<i>SNORA15</i> ;CCT6A	1	—
GGA1	1	—	TAZ	1	—
<i>GNB2L1</i> ;SNORD95	1	—	TMEM57	—	1
GPKOW	1	—	XRCC2	1	—
GPR83	—	1	YES1	—	1
<i>GYPC</i>	1	—	YPEL2	1	—
<i>HLA-C</i>	—	1	ZDHHC14	1	—
<i>HLA-DQA1</i>	3	—	ZFYVE28	—	2

Pooled DNA samples from eight short-sleep participants and eight long-sleep participants were assayed for genome-wide DNA methylation. Differentially methylated CpG sites with q (FDR-adjusted p) < .05 are considered significant. The total number of CpG loci within each human gene that are significantly hypermethylated or hypomethylated in short sleepers is listed. Genes with *Caenorhabditis elegans* orthologs are listed in bold font.

and altered posture.^{21,46} These sleep bouts are interspersed with motion bouts.⁴⁷ We measured lethargus duration (normally lasting ~2.6 hours), total sleep (sum of all sleep bouts, usually 50–60 minutes), and average sleep bout duration (usually 20–30 seconds) (Figure 1C). *C. elegans* sleep was considered defective when at least two of the three metrics were significantly different from control animals examined simultaneously. Twelve mutant strains corresponding to 12 *C. elegans* genes had significant sleep defects (Supplemental Table 2), based on testing one loss-of-function allele or the impact of an RNAi knockdown.

Realizing that mutant *C. elegans* strains might carry unidentified background mutations in other genes, we obtained additional alleles or used RNAi knockdown for these 12 genes to confirm that gene disruption altered sleep. Five genes failed to alter sleep in this rescreening: *gly-5*/*GALNT9*, *ocrl-1*/*OCRL*, *cct-6*/*CCT6A*, *maco-1*/*TMEM57*, and *src-1*/*YES1*, presumably due to differences in background mutations or insufficient

knockdown by an independent RNAi. We do not rule out a role for these genes in sleep, but further analysis would be required to assess their roles.

Genes whose loss of function consistently perturbed sleep were *bre-4*, *ced-5*, *sym-3*, *rack-1*, *gkow-1*, *ida-1*, and *lst-2* (Table 2A). We did not observe any locomotion defects or overt vulval development defects in mutant animals; sleep bouts outside of lethargus were also not observed. We found that the impact of decreased *C. elegans* gene function on sleep was consistent for alleles of *sym-3*, *rack-1*, *ida-1*, and *lst-2*. For the other genes impacting sleep, that is, *bre-4*, *ced-5*, and *gkow-1*, different alleles or RNAi caused reduced or increased sleep, which initially seemed discordant. However, loss-of-function alleles are not equal in their impact on gene function. Different alleles of the same gene can cause complete loss of protein function, loss of specific transcripts, or partial loss of function; these can result in very different behavioral outcomes. Also, background mutations in other genes can affect sleep. Additionally, for

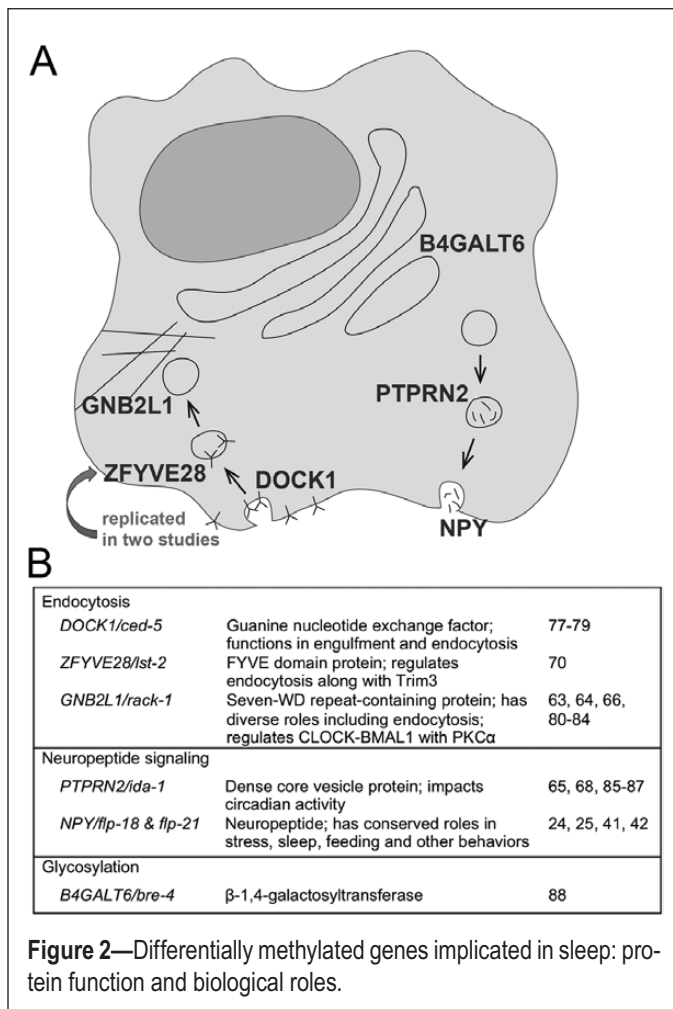
Table 2—*Caenorhabditis elegans* Genes Whose Perturbation Resulted in Sleep Defects.

A					
<i>H. sapiens</i> gene	<i>C. elegans</i> orthologs	Alleles or RNAi	Lethargus duration	Total sleep	Bout duration
B4GALT6	<i>bre-4</i>	<i>ok3167</i>	-88%	-96%	-53%
		<i>ye13</i>	7%	14%	4%
		<i>ye27</i>	23%	34%	6%
		RNAi	-2%	10%	5%
DOCK1	<i>ced-5</i>	<i>n1812</i>	-10%	-46%	-28%
		<i>n2002</i>	73%	26%	-26%
		<i>tm1949</i>	14%	48%	34%
		RNAi	11%	1%	3%
FAM102A	<i>sym-3</i>	<i>mn618</i>	26%	8%	-15%
		<i>tm6929</i>	14%	33%	14%
GNB2L1	<i>rack-1</i>	<i>ok3676</i>	43%	21%	-29%
		<i>tm2262</i>	72%	133%	59%
GPKOW	<i>gkow-1</i>	<i>gk451237</i>	-20%	-56%	-35%
		RNAi	99%	53%	-18%
NPY	<i>flp-18, flp-21</i>		Choi et al. ²⁴ ; Nagy et al. ²⁵		
PTPRN2	<i>ida-1</i>	<i>ok409</i>	56%	54%	-11%
		<i>tm334</i>	2%	29%	33%
ZFYVE28	<i>lst-2</i>	<i>gk721802</i>	50%	138%	79%
		<i>gk839738</i>	59%	120%	51%
B					
Transheterozygotes genotype	Lethargus duration	Total sleep	Bout duration		
<i>bre-4(ok3167)/bre-4(ye13)</i>	2%	-31%	-27%		
<i>ced-5(n1812)/ced-5(tm1949)</i>	-19%	-38%	-26%		
<i>sym-3(tm6929)/sym-3(mn618)</i>	40%	18%	-3%		
<i>rack-1(ok3676)/rack-1(tm2262)</i>	71%	44%	0%		
<i>ida-1(ok409)/ida-1(tm334)</i>	32%	-8%	-25%		
<i>lst-2(gk721802)/lst-2(gk839738)</i>	40%	30%	-5%		

C. elegans orthologs were identified for human genes from the discovery study (see Supplemental Table 2 and Supplemental Table 3 for the full analysis). Percent change for lethargus duration, total sleep, and sleep bout duration was calculated for each strain comparing to corresponding control animals assayed on same days. Dark gray highlight: significantly increased. Light gray highlight: significantly decreased. (A) Genes with majority of alleles and RNAi affecting sleep are shown here. Welch's *t*-test was used to calculate the *p* values, which were FDR corrected to obtain the *q* values (see full analysis in Supplemental Table 2). Significance: *p* < .05 and *q* < 0.10. *n* ≥ 10 animals for each strain. (B) Transheterozygous animals were examined for genes with at least two independent viable alleles. To generate transheterozygous animals, females homozygous for indicated allele listed on the left were crossed to males homozygous for allele listed on the right. However, *rack-1(tm2262)* males were balanced using *hT2* for this cross. Welch's *t*-test was used to calculate the *p* values. (Full Analysis in Supplemental Table 3.) Note that the single heterozygote phenotypes were not reported here. Since dominance is extremely rare, our analysis rests on the assumption that the mutants were not dominant. Significance: *p* < .05. *n* ≥ 10 animals for each strain. For some genes in Panel A, sleep changes were discordant for different mutant alleles or RNAi knockdown. See text for discussion. Note that *ced-5(n1812)* is an early nonsense allele creating a stop codon at E28, whereas the *ced-5(tm1949)* deletion does not interrupt the protein until after G151. And, the nonsense mutation in *ced-5(n2002)* is farther downstream, at R962. For *gkow-1*, the allele *gk451237* disrupts a splice acceptor site for the last exon and presumably has a more drastic impact on GKOW-1 protein function than RNAi knockdown. For *bre-4*, the molecular lesion for *bre-4(ye27)* is unknown. However, *bre-4(ye13)* should yield a truncated protein that is at least 30 amino acids shorter than *bre-4(ok3167)*, which does not correlate with respective sleep changes. We speculate that allele-specific protein interactions or strain-specific background mutations may contribute to the dramatically decreased sleep seen only in *bre-4(ok3167)*.

some genes or alleles, sleep defects may induce homeostatic responses, which can complicate analysis. For example, different levels of *C. elegans* Notch activity lead to very different sleep defects. When both OSM-7 and OSM-11 Notch co-ligands are lost, for example, *C. elegans* exhibit reduced total sleep with decreased arousal thresholds; however, losing only one of these ligands causes increased sleep due to compensatory homeostatic sleep mechanisms.³⁶ Therefore, a detailed analysis for each gene will be required for definitive description of their role in sleep.

To rule out the possible contributions of background mutations to *C. elegans* sleep changes in animals carrying these alleles, we generated transheterozygous animals for genes if two viable alleles were available. We tested transheterozygous animals for *bre-4*, *ced-5*, *sym-3*, *rack-1*, *ida-1*, and *lst-2*. Only *sym-3* heterozygous animals failed to recapitulate sleep defects seen in homozygous mutant animals (Table 2B and Supplemental Table 3), transheterozygous animals showed sleep defects for the five other genes tested. Using transheterozygous animals was not possible for *gkpw-1* as only one viable mutant allele is available; we do not rule out a possible role in sleep for this gene. We note that the heterozygote animals were not examined here. Our analysis rests on the knowledge that dominance is extremely rare, and the correlative assumption that sleep defects reported here were likely not caused by dominant mutations.



Based on previously published work with *C. elegans*, *NPY* orthologs *flp-18* and *flp-21*^{24,25,42} are required for normal L4/A lethargus sleep. Here, we identify roles in sleep for five additional genes, which had not been characterized previously in *C. elegans* sleep: *bre-4*, *ced-5*, *rack-1*, *ida-1*, and *lst-2* (See Figure 2 for protein functions). Since many conserved genes regulate sleep across species, we suggest that these newly identified genes may affect sleep in other species, including humans.

As human orthologs of these genes were differentially methylated in young adults with short versus long sleep duration in the Discovery Study (Table 1 and Supplemental Table 1), it is possible that epigenetic modification of CpG sites in these human genes might contribute to sleep duration differences or that these genes are involved in homeostatic response to insufficient sleep.

Independent Verification of Differential Methylation in a Different Set of Participants: Confirmation Study

We looked for independent verification of DNA methylation changes in human participants for the five genes with roles in *C. elegans* sleep described above, as well as for *NPY*. These genes correspond to seven differentially methylated CpG sites. The Discovery Study examined methylation and sleep differences in young adults with moderate to high depressed CES-D scores. For replication of hypermethylation or hypomethylation changes at these CpG sites, we undertook a pilot confirmation study and selected an independent set of short and long sleepers (independent individuals from a larger cohort that included the initial study) with similar sleep differences and initial mood scores. Five female participants reporting longer (>7.7 hours; mean = 8.0 hours, SD = 0.3 hours) and five females with shorter (<6.6 hours; mean = 6.3 hours; SD = 0.2 hours) total sleep time across the semester (Figure 1B) were examined for individual DNA methylation using the Illumina Infinium HumanMethylation450 BeadChip arrays. The six genes impacting *C. elegans* sleep corresponded to seven CpG sites identified in the pooled analysis in the Discovery Study; we determined if they were similarly hypermethylated or hypomethylated in the Confirmation Study. Two CpG sites replicated ($p < .05$, one-tailed Student *t*-test), despite the difference in CES-D mood scores between the two studies (Table 3). Moreover, the two replicating CpG sites reside in the same gene: *ZFYVE28*, also known as *hLst2* (see Supplemental Figure 1 for amino acid sequence alignment between human and *C. elegans* proteins). We note that if significance was examined using a two-tailed test, assuming no previous knowledge from the Discovery Study, then only one CpG site in *ZFYVE28* reached significance ($p = .05$). None of the differences in methylation were statistically significant after FDR correction, when all 87 CpG sites were examined, likely due to the small sample size in this pilot study.

Both methylation sites for *ZFYVE28* are located in the first intron; they lie within 0.5 kb of each other (Supplemental Figure 2). In the simplest scenario, decreased methylation increases mRNA transcription and gene function. However, we note that different methylation can affect splicing or impact selection of N-terminal exons. *ZFYVE28* CpG sites identified here might influence selection of *ZFYVE28* exon 1a versus 1b,

Table 3—Independent Replication of Hyper- or Hypo-Methylation at Selected CpG Sites.

Gene	CpG ID	Confirmation Study				Discovery Study
		Short sleep mean β	Long sleep mean β	$\Delta\beta$	p	$\Delta\beta$
ZFYVE28	cg11834635	0.21	0.60	-0.39	.025*	-0.54
ZFYVE28	cg20213329	0.18	0.51	-0.33	.035*	-0.43
NPY	cg21097881	0.05	0.03	0.02	.205	-0.30
GNB2L1	cg22306009	0.06	0.06	0.01	.216	0.29
PTPRN2	cg13523718	0.06	0.05	0.01	.332	-0.24
DOCK1	cg06406458	0.83	0.89	-0.05	.360	0.23
B4GALT6	cg11986743	0.64	0.62	0.03	.440	0.30

In the Confirmation Study, CpG sites corresponding to genes with confirmed sleep defects in *Caenorhabditis elegans* were re-examined for replication of predicted hypermethylation or hypomethylation in an independent group of participants with low CES-D scores (five female short-sleep participants and five female long-sleep participants). β Values for individual participants were determined using genome-wide DNA methylation analysis and compared between groups only at selected loci using one-tailed Student *t*-test. * $p < .05$. None of the differences in methylation were statistically significant after FDR correction when all 87 CpG sites were examined, presumably due to the small sample size in this small pilot study.

which encode proteins with different N-terminal amino acid sequences. Nonetheless, these ZFYVE28 CpG sites were relatively hypomethylated in short-sleep participants, which might drive increased mRNA expression. The prediction from this simple model would be that *C. elegans lst-2* loss-of-function alleles would decrease gene function and would increase sleep. Indeed, loss of *lst-2* consistently led to increased *C. elegans* sleep for both alleles, which agrees with this simple model (Figure 3).

DISCUSSION

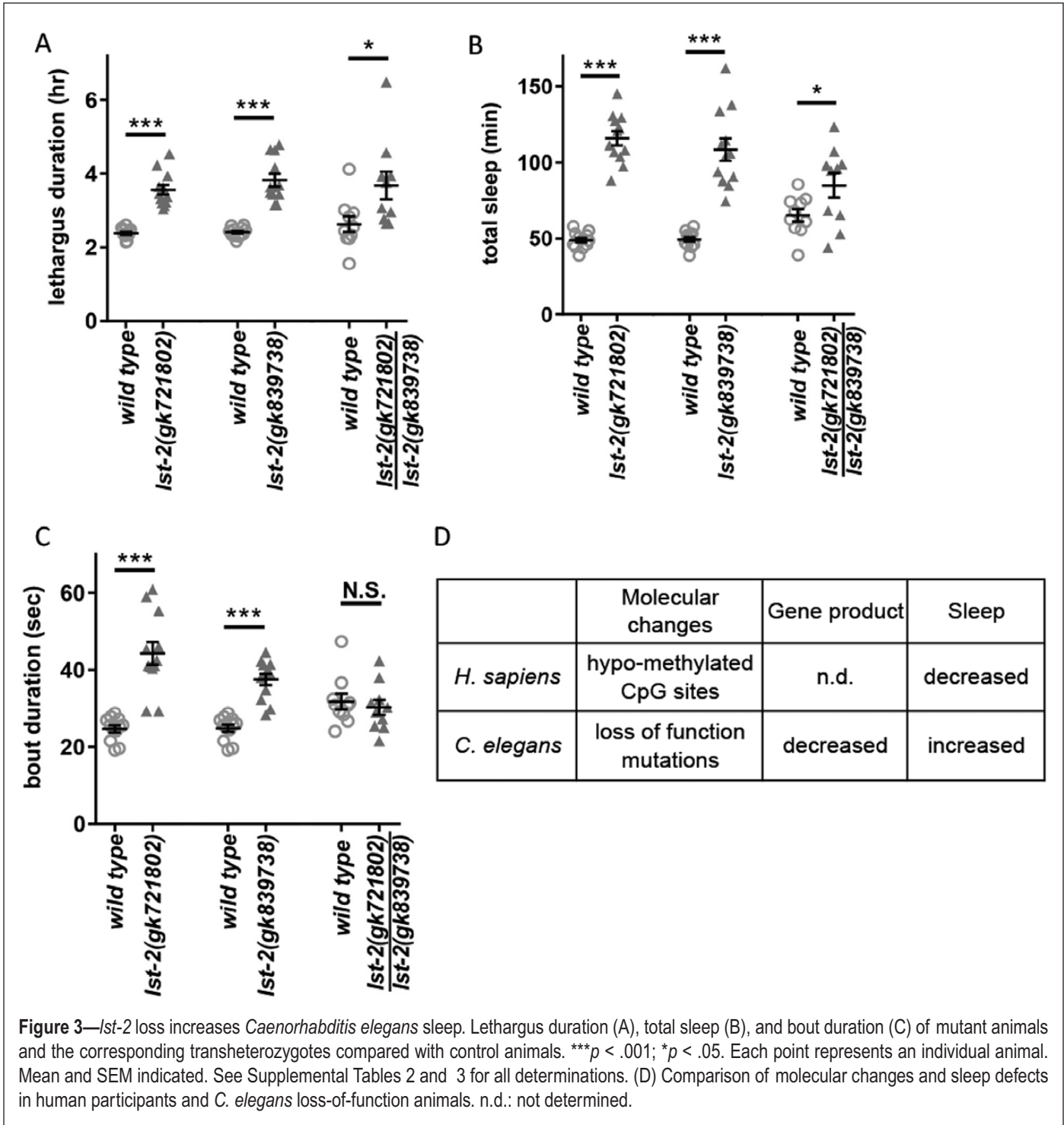
Here, by combining human epigenetic and *C. elegans* sleep studies, we identified six genes that likely play conserved roles in sleep regulation: B4GALT6/*bre-4*, DOCK180/*ced-5*, GNB2L1/*rack-1*, PTPRN2/*ida-1*, ZFYVE28/*lst-2*, as well as NPY/*flp-18* and *flp-21*. Also, despite our small sample size, we detected differential DNA methylation at specific genes that may be associated with specific sleep differences in nonclinical human populations. Eighty-seven loci corresponding to 52 unique candidate genes were differentially methylated in short-versus long-sleeping young adults. Moreover, hypomethylation in short sleepers at two CpG sites was confirmed within ZFYVE28 in an independent set of human participants, albeit with a small sample size. It is premature to predict the clinical utility of this work, because identification of these CpG sites could lead to development of biomarkers for sleep disorders or sleep disruption, or could eventually lead to the development of therapeutic strategies.

In the Discovery Study, we pooled DNA samples for analysis from carefully phenotyped mixed gender participants with large differences in average sleep length and low initial CES-D scores indicating nondepressed mood. Final CES-D scores were high in these participants. To re-examine CpG site methylation changes for genes that play roles in *C. elegans* sleep, we undertook a Confirmation Study. A different set of participants were selected with large differences in average total daily sleep length and low initial CES-D scores. We also required female

gender and low final CES-D scores, which may provide a more stringent test of the association between methylation and sleep in this group. Both human studies found changes in ZFYVE28 CpG site methylation. We attribute the successful identification of CpG sites/genes reported here to our approach of examining DNA methylation.

Power/sample size is a critical parameter for genetic linkage studies. Genome-wide association study and/or SNP analyses usually require large numbers of participants to reach significance at a given genetic locus, especially in behavioral studies. Here, we took a different approach. Genome-wide DNA methylation was measured at CpG sites in participants with large differences in sleep quantity, who were selected from a much larger total participant pool. We note that (1) we do not rely on linkage to specific loci or association with rare SNPs, which would require very large participant pools; (2) DNA methylation at specific CpG sites may be a reaction to inadequate sleep; therefore; we hypothesize that, for some CpG sites, DNA methylation occurs in most participants—in proportion to the amount of sleep lost; (3) here we selected for participants who had large differences in sleep quantity (~1.5 hours), which maximizes methylation changes. With the current sample size, however, we expect to find only specific CpG sites with large differences in methylation, correlated with large differences in average sleep quantity. CpG sites with small differences in methylation will require larger studies. Also, since we only examined freshman year undergraduate students and did not follow participants after the study ended, we cannot provide any assessment of phenotype stability for either their sleep length or DNA methylation.

Mood and sleep may interact at multiple levels. Here, differentially methylated genes were first identified in participants with high final depressed mood scores. Then, nondepressed participants with low final mood scores were used for independent confirmation of methylation status associations with differences in mean daily total sleep time. If methylation at specific CpG sites was specifically associated with short sleep only in depressed participants, methylation at these sites might not



be expected to replicate in the second study. Conversely, CpG sites with differential methylation in both depressed and non-depressed participants are likely associated with sleep quantity, irrespective of mood.

Whole blood was used here for methylation studies due to ease of collection. We remain agnostic as to the site of gene activity; the tissues where methylation changes might impact sleep are unknown. A subset of genes involved in sleep or response to sleep length will likely act in peripheral tissues.^{48,49} We can directly identify DNA methylation within cells found in blood. But, other genes may be required in neurons and DNA

methylation changes elsewhere may be pertinent. Without postmortem samples, we cannot examine DNA methylation in human cortical tissues. For a significant fraction of genes, however, similar DNA methylation/epigenetic changes were found in both peripheral and neuronal tissues associated with Parkinson's disease.⁵⁰ Examples also include the neuropeptide brain-derived neurotrophic factor, which has been implicated in schizophrenia.^{51–55} These systemic DNA methylation changes might be driven by stress response, coordinated by the hypothalamic-pituitary-adrenal (HPA) axis. There has been speculation that in utero or early-life stress might lead to adult insomnia,

via modulation of HPA-axis activity and consequent DNA methylation.^{56,57} Finally, we do not exclude the possibility that differential DNA methylation changes observed were inherited from previous generation(s). The results presented here warrant future studies examining the DNA methylation landscapes of individual participants with a larger cohort and additional studies of epigenetic changes associated with sleep. This should accelerate identification of genes involved in sleep, reveal biomarkers for sleep disturbance, and yield insight into pathways critical for sleep regulation.

In this study, we started with association between differentially methylated genes and sleep length in an exploratory study of human participants and identified conserved genes that regulate sleep in *C. elegans*. If these corresponding human genes are involved in sleep, then epigenetic modifications and presumed expression changes in these genes might (1) drive different sleep length or (2) respond to sleep differences. Explicitly, in the first case, differential methylation in sleep-related genes between the two study groups may drive different sleep length. In the second case, sleep length may drive methylation changes in genes that respond to the differences in sleep. For example, sleep might drive differential methylation in genes that play roles in immune response. This is difficult to test in *C. elegans*, as they lack an active immune system and rely on innate immunity. We detected methylation differences at human CpG sites in many *HLA* genes. At least one of these genes has concordant methylation between blood and brain (ie, *HLA-DQA1*).⁵⁸ SNPs in the *HLA-DR* and *HLA-DQ* region have been associated with differences in narcolepsy and/or human longevity, presumably by regulating immune response.^{59,60} Our results are consistent with previous work delineating the intricate relationship between sleep length, immune function, and longevity.⁶¹ However, from our cross-sectional study design and the small number of participants sampled, it is difficult to determine causal relationship between observed methylation changes and sleep patterns.

In this study, we identified 52 differentially methylated human genes, examined 33 orthologous genes for roles in *C. elegans* sleep, and confirmed five genes. This corresponds to a 15% success rate, which is not unprecedented. A previous *Drosophila* study examined 136 P-element insertion lines, presumably affecting 136 genes, and found 21 insertions affect sleep time,⁶² yielding the same 15% success rate. Therefore, we do not suggest that the cross-species approach presented here enriches for genes involved in sleep. We do suggest that human genes identified here be considered as candidate genes in future DNA methylation studies and that higher priority be assigned to genes whose invertebrate orthologs play important roles in sleep.

Caenorhabditis elegans functional studies presented here identified previously unknown roles for five genes that impact sleep in this model organism. These likely play conserved roles in sleep across the animal kingdom. A surprising convergence arose in the pathways associated with these five genes, together with *NPY* (Figure 2). Two of these genes play conserved roles in neuropeptide signaling (*NPY*, *PTPRN2*). Three are involved in endocytic pathways (*DOCK1*, *GNB2L1*, *ZFYVE28*). Studies in mammals suggest that perturbation of *GNB2L1* and *PTPRN2* alters circadian rhythm.^{63–65} In *Drosophila*, the *GNB2L1* ortholog has also been shown to affect circadian behavior,⁶⁶ and

the expression level of the *DOCK1* is influenced by the circadian clock.⁶⁷ As for sleep, *NPY* neuropeptides affect sleep latency, arousal, and homeostatic response to mild sleep perturbations.^{24,25,42} The release of neuropeptides is heavily regulated and requires *PTPRN2/IDA-1*, which may have a role in EGF-induced sleep in *C. elegans*.^{68,69} A P-element transposon perturbing the *Drosophila* ortholog of *B4GALT6* may alter sleep, although the impact of gene perturbation was not confirmed.⁶² To our knowledge, the other three genes identified here have not been previously implicated in sleep.

ZFYVE28 is consistently hypomethylated in short sleepers in two independent studies reported here. *ZFYVE28/hLst2* has been shown to regulate endocytosis. In HeLa cells, de-ubiquitinated *hLst2* helps internalize activated EGF receptors, diverting them for degradation.⁷⁰ The *C. elegans* ortholog, *lst-2*, is a direct transcriptional target of Notch signaling and acts as a negative regulator of the epidermal growth factor receptor-mitogen-activated protein kinase pathway.⁷¹ Both Notch^{36,72} and EGF^{20,73,74} signaling play important roles regulating sleep in multiple species and *lst-2/hLst2* may be a pertinent target. Future studies will be required to determine if *lst-2/hLst2* loss affects sleep by regulating EGF receptor levels. It is unknown whether decreased sleep alters *ZFYVE28* expression, but our examination of GEO expression databases suggests that mouse *ZFYVE28* transcript levels may be sensitive to sleep quantity. *ZFYVE28* transcripts appear to rise during sleep, but forced sleep deprivation may eliminate this increase⁷⁵ (Supplemental Figure 3). None of these large-scale transcriptomic results were verified here, but they would be consistent with a role for *ZFYVE28/lst-2* in sleep or response to less sleep. Intriguingly, a recent epigenomics study in human reveals the enrichment of genes in the Notch signaling pathway to be differentially methylated after total acute sleep deprivation.⁷⁶

In conclusion, using a cross-species approach, we identified five *C. elegans* genes, which had not been previously implicated in sleep. These genes likely affect sleep in other species as well. It is possible that epigenetic modifications in orthologous human genes may be associated with sleep length differences in humans; additional studies will be required to test this. With the results presented here, we suggest that epigenetic regulation of specific genes may contribute to differences in sleep.

REFERENCES

1. Cirelli C. The genetic and molecular regulation of sleep: from fruit flies to humans. *Nat Rev Neurosci*. 2009; 10(8): 549–560.
2. Trojanowski NF, Raizen DM. Call it worm sleep. *Trends Neurosci*. 2016; 39(2): 54–62.
3. Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet*. 2008; 9(10): 764–775.
4. McEwen BS, Bowles NP, Gray JD, et al. Mechanisms of stress in the brain. *Nat Neurosci*. 2015; 18(10): 1353–1363.
5. Hunter RG. Epigenetic effects of stress and corticosteroids in the brain. *Front Cell Neurosci*. 2012; 6: 18.
6. Stankiewicz AM, Swiergiel AH, Lisowski P. Epigenetics of stress adaptations in the brain. *Brain Res Bull*. 2013; 98: 76–92.
7. Bowers ME, Yehuda R. Intergenerational transmission of stress in humans. *Neuropsychopharmacology*. 2016; 41(1): 232–244.
8. Champagne FA, Curley JP. Epigenetic mechanisms mediating the long-term effects of maternal care on development. *Neurosci Biobehav Rev*. 2009; 33(4): 593–600.

9. Portela A, Esteller M. Epigenetic modifications and human disease. *Nat Biotechnol.* 2010; 28(10): 1057–1068.
10. Abel T, Poplawski S. Epigenetic advances in clinical neuroscience. *Dialogues Clin Neurosci.* 2014; 16(3): 273–275.
11. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis.* 2010; 31(1): 27–36.
12. Lolak S, Suwannarat P, Lipsky RH. Epigenetics of depression. *Prog Mol Biol Transl Sci.* 2014; 128: 103–137.
13. Lockwood LE, Su S, Youssef NA. The role of epigenetics in depression and suicide: a platform for gene-environment interactions. *Psychiatry Res.* 2015; 228(3): 235–242.
14. Zhu Y, Stevens RG, Hoffman AE, et al. Epigenetic impact of long-term shiftwork: pilot evidence from circadian genes and whole-genome methylation analysis. *Chronobiol Int.* 2011; 28(10): 852–861.
15. Massart R, Freyburger M, Suderman M, et al. The genome-wide landscape of DNA methylation and hydroxymethylation in response to sleep deprivation impacts on synaptic plasticity genes. *Transl Psychiatry.* 2014; 4: e347.
16. Wong CC, Parsons MJ, Lester KJ, et al. Epigenome-wide DNA methylation analysis of monozygotic twins discordant for diurnal preference. *Twin Res Hum Genet.* 2015; 18(6): 662–669.
17. Cassidy SB, Dykens E, Williams CA. Prader-Willi and Angelman syndromes: sister imprinted disorders. *Am J Med Genet.* 2000; 97(2): 136–146.
18. Tucci V. Genomic imprinting: a new epigenetic perspective of sleep regulation. *PLoS Genet.* 2016; 12(5): e1006004.
19. Ehlen JC, Jones KA, Pinckney L, et al. Maternal Ube3a loss disrupts sleep homeostasis but leaves circadian rhythmicity largely intact. *J Neurosci.* 2015; 35(40): 13587–13598.
20. Van Buskirk C, Sternberg PW. Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. *Nat Neurosci.* 2007; 10(10): 1300–1307.
21. Raizen DM, Zimmerman JE, Maycock MH, et al. Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature.* 2008; 451(7178): 569–572.
22. Campbell SS, Tobler I. Animal sleep: a review of sleep duration across phylogeny. *Neurosci Biobehav Rev.* 1984; 8(3): 269–300.
23. Singh K, Ju JY, Walsh MB, DiIorio MA, Hart AC. Deep conservation of genes required for both *Drosophila melanogaster* and *Caenorhabditis elegans* sleep includes a role for dopaminergic signaling. *Sleep.* 2014; 37(9): 1439–1451.
24. Choi S, Chatzigeorgiou M, Taylor KP, Schafer WR, Kaplan JM. Analysis of NPR-1 reveals a circuit mechanism for behavioral quiescence in *C. elegans*. *Neuron.* 2013; 78(5): 869–880.
25. Nagy S, Tramm N, Sanders J, et al. Homeostasis in *C. elegans* sleep is characterized by two behaviorally and genetically distinct mechanisms. *Elife.* 2014; 3: e04380.
26. Monsalve GC, Van Buskirk C, Frand AR. LIN-42/PERIOD controls cyclical and developmental progression of *C. elegans* molts. *Curr Biol.* 2011; 21(24): 2033–2045.
27. Radloff LS. The CES-D scale: A self-report depression scale for research in the general population. *Applied Psychological Measurement* 1977; 1: 385–401.
28. Acebo C, Sadeh A, Seifer R, Tzischinsky O, Hafer A, Carskadon MA. Sleep/wake patterns derived from activity monitoring and maternal report for healthy 1- to 5-year-old children. *Sleep.* 2005; 28(12): 1568–1577.
29. Ancoli-Israel S, Martin JL, Blackwell T, et al. The SBSM guide to actigraphy monitoring: clinical and research applications. *Behav Sleep Med.* 2015; 13 Suppl 1: S4–S38.
30. Rumble ME, White KH, Benca RM. Sleep disturbances in mood disorders. *Psychiatr Clin North Am.* 2015; 38(4): 743–759.
31. Aryee MJ, Jaffe AE, Corrada-Bravo H, et al. Minfi: a flexible and comprehensive bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics.* 2014; 30(10): 1363–1369.
32. Chen YA, Lemire M, Choufani S, et al. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. *Epigenetics.* 2013; 8(2): 203–209.
33. Calixto A, Chelur D, Topalidou I, Chen X, Chalfie M. Enhanced neuronal RNAi in *C. elegans* using SID-1. *Nat Methods.* 2010; 7(7): 554–559.
34. Marroquin LD, Elyassnia D, Griffiths JS, Feitelson JS, Aroian RV. Bacillus thuringiensis (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. *Genetics.* 2000; 155(4): 1693–1699.
35. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature.* 1998; 391(6669): 806–811.
36. Singh K, Chao MY, Somers GA, et al. *C. elegans* Notch signaling regulates adult chemosensory response and larval molting quiescence. *Curr Biol.* 2011; 21(10): 825–834.
37. Kamath RS, Fraser AG, Dong Y, et al. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature.* 2003; 421(6920): 231–237.
38. Rual JF, Ceron J, Koreth J, et al. Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Res.* 2004; 14(10B): 2162–2168.
39. Docherty SJ, Davis OS, Haworth CM, Plomin R, Mill J. Bisulfite-based epityping on pooled genomic DNA provides an accurate estimate of average group DNA methylation. *Epigenetics Chromatin.* 2009; 2(1): 3.
40. Gallego-Fabrega C, Carrera C, Muiño E, Montaner J, Krupinski J, Fernandez-Cadenas I; Spanish Stroke Genetics Consortium. DNA methylation levels are highly correlated between pooled samples and averaged values when analysed using the Infinium HumanMethylation450 BeadChip array. *Clin Epigenetics.* 2015; 7: 78.
41. Wu G, Feder A, Wegener G, et al. Central functions of neuropeptide Y in mood and anxiety disorders. *Expert Opin Ther Targets.* 2011; 15(11): 1317–1331.
42. Dyzma M, Boudjeltia KZ, Faraut B, Kerkhofs M. Neuropeptide Y and sleep. *Sleep Med Rev.* 2010; 14(3): 161–165.
43. Greer EL, Blanco MA, Gu L, et al. DNA Methylation on N6-Adenine in *C. elegans*. *Cell.* 2015; 161(4): 868–878.
44. Wu TP, Wang T, Seetin MG, et al. DNA methylation on N(6)-adenine in mammalian embryonic stem cells. *Nature.* 2016; 532(7599): 329–333.
45. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci.* 2006; 31(2): 89–97.
46. Nelson MD, Raizen DM. A sleep state during *C. elegans* development. *Curr Opin Neurobiol.* 2013; 23(5): 824–830.
47. Iwanir S, Tramm N, Nagy S, Wright C, Ish D, Biron D. The microarchitecture of *C. elegans* behavior during lethargus: homeostatic bout dynamics, a typical body posture, and regulation by a central neuron. *Sleep.* 2013; 36(3): 385–395.
48. Maret S, Dorsaz S, Gurcel L, et al. Homer1a is a core brain molecular correlate of sleep loss. *Proc Natl Acad Sci USA.* 2007; 104(50): 20090–20095.
49. Anafi RC, Pellegrino R, Shockley KR, Romer M, Tufik S, Pack AI. Sleep is not just for the brain: transcriptional responses to sleep in peripheral tissues. *BMC Genomics.* 2013; 14: 362.
50. Masliah E, Dumaop W, Galasko D, Desplats P. Distinctive patterns of DNA methylation associated with Parkinson disease: identification of concordant epigenetic changes in brain and peripheral blood leukocytes. *Epigenetics.* 2013; 8(10): 1030–1038.
51. Mitchelmore C, Gede L. Brain derived neurotrophic factor: epigenetic regulation in psychiatric disorders. *Brain Res.* 2014; 1586: 162–172.
52. Ewald ER, Wand GS, Seifuddin F, et al. Alterations in DNA methylation of Fkbp5 as a determinant of blood-brain correlation of glucocorticoid exposure. *Psychoneuroendocrinology.* 2014; 44: 112–122.
53. Kaminsky Z, Tochigi M, Jia P, et al. A multi-tissue analysis identifies HLA complex group 9 gene methylation differences in bipolar disorder. *Mol Psychiatry.* 2012; 17(7): 728–740.
54. Ikegame T, Bundo M, Murata Y, Kasai K, Kato T, Iwamoto K. DNA methylation of the BDNF gene and its relevance to psychiatric disorders. *J Hum Genet.* 2013; 58(7): 434–438.
55. Smith AK, Kilaru V, Klengel T, et al. DNA extracted from saliva for methylation studies of psychiatric traits: evidence tissue specificity and relatedness to brain. *Am J Med Genet B Neuropsychiatr Genet.* 2015; 168B(1): 36–44.
56. Palagini L, Biber K, Riemann D. The genetics of insomnia—evidence for epigenetic mechanisms? *Sleep Med Rev.* 2014; 18(3): 225–235.
57. Palagini L, Drake CL, Gehrman P, Meerlo P, Riemann D. Early-life origin of adult insomnia: does prenatal-early-life stress play a role? *Sleep Med.* 2015; 16(4): 446–456.

58. Walton E, Hass J, Liu J, et al. Correspondence of DNA methylation between blood and brain tissue and its application to schizophrenia research. *Schizophr Bull.* 2016; 42(2): 406–414.
59. Fortney K, Dobriban E, Garagnani P, et al. Genome-wide scan informed by age-related disease identifies loci for exceptional human longevity. *PLoS Genet.* 2015; 11(12): e1005728.
60. Mignot E, Lin L, Rogers W, et al. Complex HLA-DR and -DQ interactions confer risk of narcolepsy-cataplexy in three ethnic groups. *Am J Hum Genet.* 2001; 68(3): 686–699.
61. Luyster FS, Strollo PJ Jr, Zee PC, Walsh JK; Boards of Directors of the American Academy of Sleep Medicine and the Sleep Research Society. Sleep: a health imperative. *Sleep.* 2012; 35(6): 727–734.
62. Harbison ST, Sehgal A. Quantitative genetic analysis of sleep in *Drosophila melanogaster*. *Genetics.* 2008; 178(4): 2341–2360.
63. Zeidner LC, Buescher JL, Phiel CJ. A novel interaction between glycogen synthase kinase-3 α (GSK-3 α) and the scaffold protein receptor for activated c-kinase 1 (RACK1) regulates the circadian clock. *Int J Biochem Mol Biol.* 2011; 2(4): 318–327.
64. Robles MS, Boyault C, Knutti D, Padmanabhan K, Weitz CJ. Identification of RACK1 and protein kinase Calpha as integral components of the mammalian circadian clock. *Science.* 2010; 327(5964): 463–466.
65. Kim SM, Power A, Brown TM, et al. Deletion of the secretory vesicle proteins IA-2 and IA-2beta disrupts circadian rhythms of cardiovascular and physical activity. *FASEB J.* 2009; 23(9): 3226–3232.
66. Price JL, Fan JY, Keightley A, Means JC. The role of casein kinase I in the *Drosophila* circadian clock. *Methods Enzymol.* 2015; 551: 175–195.
67. Ceriani MF, Hogenesch JB, Yanovsky M, Panda S, Straume M, Kay SA. Genome-wide expression analysis in *Drosophila* reveals genes controlling circadian behavior. *J Neurosci.* 2002; 22(21): 9305–9319.
68. Van Buskirk C, Sternberg PW. Paired and LIM class homeodomain proteins coordinate differentiation of the *C. elegans* ALA neuron. *Development.* 2010; 137(12): 2065–2074.
69. Fry AL, Laboy JT, Huang H, Hart AC, Norman KR. A conserved GEF for Rho-Family GTPases acts in an EGF signaling pathway to promote sleep-like quiescence in *Caenorhabditis elegans*. *Genetics.* 2016; 202(3): 1153–1166.
70. Mosesson Y, Chetrit D, Schley L, et al. Monoubiquitinylation regulates endosomal localization of Lst2, a negative regulator of EGF receptor signaling. *Dev Cell.* 2009; 16(5): 687–698.
71. Yoo AS, Bais C, Greenwald I. Crosstalk between the EGFR and LIN-12/Notch pathways in *C. elegans* vulval development. *Science.* 2004; 303(5658): 663–666.
72. Seugnet L, Suzuki Y, Merlin G, Gottschalk L, Duntley SP, Shaw PJ. Notch signaling modulates sleep homeostasis and learning after sleep deprivation in *Drosophila*. *Curr Biol.* 2011; 21(10): 835–840.
73. Kushikata T, Fang J, Chen Z, Wang Y, Krueger JM. Epidermal growth factor enhances spontaneous sleep in rabbits. *Am J Physiol.* 1998; 275(2 Pt 2): R509–R514.
74. Kramer A, Yang FC, Snodgrass P, et al. Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. *Science.* 2001; 294(5551): 2511–2515.
75. Mackiewicz M, Shockley KR, Romer MA, et al. Macromolecule biosynthesis: a key function of sleep. *Physiol Genomics.* 2007; 31(3): 441–457.
76. Nilsson EK, Boström AE, Mwinyi J, Schiöth HB. Epigenomics of total acute sleep deprivation in relation to genome-wide DNA methylation profiles and RNA expression. *OMICS.* 2016; 20(6): 334–342.
77. Wu YC, Horvitz HR. *C. elegans* phagocytosis and cell-migration protein CED-5 is similar to human DOCK180. *Nature.* 1998; 392(6675): 501–504.
78. Reddien PW, Horvitz HR. The engulfment process of programmed cell death in *Caenorhabditis elegans*. *Annu Rev Cell Dev Biol.* 2004; 20: 193–221.
79. Stavoe AK, Colón-Ramos DA. Netrin instructs synaptic vesicle clustering through Rac GTPase, MIG-10, and the actin cytoskeleton. *J Cell Biol.* 2012; 197(1): 75–88.
80. Gallo S, Manfrini N. Working hard at the nexus between cell signaling and the ribosomal machinery: An insight into the roles of RACK1 in translational regulation. *Translation (Austin).* 2015; 3(2): e1120382.
81. Swaminathan G, Cartwright CA. Rack1 promotes epithelial cell-cell adhesion by regulating E-cadherin endocytosis. *Oncogene.* 2012; 31(3): 376–389.
82. Jannot G, Bajan S, Giguère NJ, et al. The ribosomal protein RACK1 is required for microRNA function in both *C. elegans* and humans. *EMBO Rep.* 2011; 12(6): 581–586.
83. Patterson RL, van Rossum DB, Barrow RK, Snyder SH. RACK1 binds to inositol 1,4,5-trisphosphate receptors and mediates Ca²⁺ release. *Proc Natl Acad Sci U S A* 2004; 101: 2328–2332.
84. Sklan EH, Podoly E, Soreq H. RACK1 has the nerve to act: structure meets function in the nervous system. *Prog Neurobiol.* 2006; 78(2): 117–134.
85. Zahn TR, Macmorris MA, Dong W, Day R, Hutton JC. IDA-1, a *Caenorhabditis elegans* homolog of the diabetic autoantigens IA-2 and phogrin, is expressed in peptidergic neurons in the worm. *J Comp Neurol.* 2001; 429(1): 127–143.
86. Zahn TR, Angleson JK, MacMorris MA, et al. Dense core vesicle dynamics in *Caenorhabditis elegans* neurons and the role of kinesin UNC-104. *Traffic.* 2004; 5(7): 544–559.
87. Harashima S, Clarke A, Christie MR, Notkins AL. The dense core transmembrane vesicle protein IA-2 is a regulator of vesicle number and insulin secretion. *Proc Natl Acad Sci U S A* 2005; 102: 8704–8709.
88. Griffiths JS, Huffman DL, Whitacre JL, et al. Resistance to a bacterial toxin is mediated by removal of a conserved glycosylation pathway required for toxin-host interactions. *J Biol Chem.* 2003; 278(46): 45594–45602.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *SLEEP* online.

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THE INSTITUTION AT WHICH THE WORK WAS PERFORMED

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DISCLOSURE STATEMENT

None declared.