GENES ASSOCIATED WITH RESILIENCE/VULNERABILITY TO SLEEP DEPRIVATION

Identification of Genes Associated with Resilience/Vulnerability to Sleep Deprivation and Starvation in *Drosophila*

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Background and Study Objectives: Flies mutant for the canonical clock protein cycle (*cyc*^{01}) exhibit a sleep rebound that is ~10 times larger than wild-type flies and die after only 10 h of sleep deprivation. Surprisingly, when starved, *cyc*^{01} mutants can remain awake for 28 h without demonstrating negative outcomes. Thus, we hypothesized that identifying transcripts that are differentially regulated between waking induced by sleep deprivation and waking induced by starvation would identify genes that underlie the deleterious effects of sleep deprivation and/or protect flies from the negative consequences of waking.

Design: We used partial complementary DNA microarrays to identify transcripts that are differentially expressed between *cyc*^{01} mutants that had been sleep deprived or starved for 7 h. We then used genetics to determine whether disrupting genes involved in lipid metabolism would exhibit alterations in their response to sleep deprivation.

Setting: Laboratory.

Patients or Participants: *Drosophila melanogaster*.

Interventions: Sleep deprivation and starvation.

Measurements and Results: We identified 84 genes with transcript levels that were differentially modulated by 7 h of sleep deprivation and starvation in *cyc*^{01} mutants and were confirmed in independent samples using quantitative polymerase chain reaction. Several of these genes were predicted to be lipid metabolism genes, including *bubblegum*, *cueball*, and *CG4500*, which based on our data we have renamed *heimdall* (*hll*). Using lipidomics we confirmed that knockdown of *hll* using RNA interference significantly decreased lipid stores. Importantly, genetically modifying *bubblegum*, *cueball*, or *hll* resulted in sleep rebound alterations following sleep deprivation compared to genetic background controls.

Conclusions: We have identified a set of genes that may confer resilience/vulnerability to sleep deprivation and demonstrate that genes involved in lipid metabolism modulate sleep homeostasis.

Keywords: *Drosophila melanogaster*, lipid metabolism, lipid storage, microarray, sleep homeostasis, transcriptional changes

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INTRODUCTION

Sleep is believed to support a restorative function, whereas extended sleep deprivation can lead to death.[1–4] Between these two extremes, sleep deprivation results in graded cognitive and physiological decrements, suggesting that a lack of sleep exacts a graded toll as sleep debt accumulates.[5–7] Symptoms that may hint at the performance deficits and cause of death have been noted,[3,8–10] but the underlying causes have not yet been determined.

To identify genes that may be responsible for the accumulating deficits during sleep deprivation or that may protect an organism from the deleterious effects of extended waking, we evaluated flies with a mutation in the canonical clock protein *cycle* (*cyc*^{01}). The *cyc*^{01} mutant is particularly sensitive to sleep deprivation as evidenced by an extremely large sleep rebound, cognitive deficits, and death after only 10 h of sleep deprivation, nearly eight times faster than wild-type flies.[2,11,12] In contrast to sleep deprivation, when *cyc*^{01} mutants are starved they show an immediate and sustained increase in waking that is not compensated by a homeostatic response[11,13] and does not induce cognitive impairment.[11] Importantly, *cyc*^{01} mutants can sustain periods of waking 2.6 times longer when starved than when sleep deprived, suggesting that the graded effect of sleep loss may be attenuated during starvation.[11] Interestingly, removing food also induces waking in humans[14] and rats.[15]

Recent data suggest that lipid metabolism and sleep have a reciprocal interaction. Shorter sleep times are associated with increased weight,[6] possibly due to changes in food preferences[16,17] or in energy processing.[18] Likewise, lipid metabolic enzymes have been associated with changes in theta oscillations during rapid eye movement (REM) sleep,[19] and they play a critical role in the response to sleep deprivation.[11] Indeed, mutations in the *brummer* (*bmm*) gene that reduces lipolysis result in a substantially increased sleep rebound following one night of sleep loss.[11] In contrast, mutants in *Lipid storage droplet-2* (*Lsd2*) that exhibit an increase fatty acid release, do not respond to sleep deprivation with either a sleep rebound or deficits in cognitive performance.[11] Thus, lipid metabolism does not simply respond to sleep loss, but it plays an active role in sleep regulation and how an animal can cope with sleep deprivation. The mechanism for how the control of lipids modulates sleep and wake states remains unclear.

We therefore exploited the phenotypic difference in the *cyc*^{01} mutants' response to sleep deprivation and starvation to

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identify genes associated with performance and physiological decrements (those changed with sleep deprivation) or those that allow flies to remain awake without accruing negative consequences or cognitive impairment (those changed due to starvation-induced waking). Microarray analyses revealed transcriptional changes in multiple gene ontology categories, including channels and lipid metabolism genes. Given the limited exploration of lipid metabolism genes and the role that energy management is thought to play in sleep regulation,[20] we tested the functional relevance of lipid metabolic enzymes in the response to sleep deprivation. We found that several putative lipid metabolism genes are involved in mediating the response to sleep deprivation. Moreover, using lipidomics, we demonstrate that one of the genes with the largest transcriptional changes, *heimdall* (*hll*, formerly *CG4500*) also alters lipid metabolism. Heimdall is the fictional character that for ages guarded the bridge to Asgard without sleep and without the consequences of sleep deprivation, similar characteristics to the *hll* knockdown. Together, these data highlight the role that the storage and catabolism of lipids play in sleep regulation after sleep deprivation. These results also confirm the utility of this array strategy in identifying genes that play a role in sleep homeostasis.

METHODS

Flies and Husbandry

Flies were reared in standard laboratory conditions, 12:12 light:dark (LD) schedule, standard food (yeast, sucrose, corn syrup, molasses, and agar), 25°C and 50% humidity. cyc^{01} and $period^{01}$ (per^{01}) mutant flies were obtained from Dr. Jeff Hall. *UAS-hll RNAi* was obtained from the Vienna *Drosophila* Resource Center.[21] Wild-type *Canton-S* (*CS*), *Actin-GAL4/CyO* (*Act-GAL4*), $bubblegum^{EY01376}$ ($bgm^{EY03176}$), $cueball^{2}$ (cue^{2}), *Df(3L)Ar14-8* and *Df(3L)kto2/TMB,Tb*[1] were obtained from the Bloomington Stock Center (Bloomington, IN, USA). *Tubulin GeneSwitch-Gal4* (*TubGSw*)[22] was obtained from Dr Marc Tatar. Revertants for cue^{2} were generated using standard techniques that introduce the *Δ2-3* version of the transposase to mobilize the P-element. Precise excision was confirmed by sequencing with primers designed using Primer3.[23] The $bubblegum^{1}$ (bgm^{1}) deletion mutant and the precise excision (bgm^{rev}) genetic background control were obtained from Dr Kyung-Tai Min. GeneSwitch GAL4 expression was induced by rearing adult flies immediately upon eclosion on either 100 μL/mL of RU486 (mifepristone, Sigma, Saint Louis, MO) or an equal volume of ethanol. A 50 mg/mL stock was diluted into normal food to a final concentration of 100 μL/mL.

Sleep Recording

Three-day-old flies were placed into 65-mm glass tubes containing standard laboratory food and monitored using the *Drosophila* activity monitoring system (Trikinetics, Waltham, MA, USA) as previously described.[1,2] Briefly, activity was recorded in 1-min bins and episodes of quiescence ≥ 5 min were considered sleep. Total sleep time, sleep architecture, and sleep homeostasis were calculated using an in-house program according to criteria previously established.[1,2,24]

Sleep Deprivation

Flies were sleep deprived using the sleep-nullifying apparatus, which asymmetrically tilted −60° to +60° such that the sleeping flies were displaced during the downward movement six times/min.[1,2] The clock mutants, cyc^{01} and per^{01}, were maintained and sleep deprived under constant darkness, or dark:dark (DD); sleep deprivation occurred for 7 h during the day between CT0 and CT12. For transcriptional analysis, *CS* flies were maintained for 3 days under DD conditions and then deprived of sleep for 12 h during the primary sleep period. The primary sleep period was identified from the previous day's data based upon the average time that the *CS* flies initiated their longest sleep bout. All other flies were maintained on a 12:12 LD schedule and deprived of sleep for 12 h between ZT12 (lights out) to ZT0 (lights on). The standard sleep homeostasis protocol consisted of 2 days of baseline followed by sleep deprivation and then flies were released into recovery where they remained unperturbed for 48 h. Sleep homeostasis was calculated for each individual as a percentage of the minutes of sleep gained above baseline during the 48 h of recovery divided by the total min of sleep lost during 12 h of sleep deprivation (minutes gained/minutes lost).

Starvation

Starvation is defined as the desire for food without access to nutrients.[25] Flies were starved according to the previously published protocol.[11] Briefly, flies were moved from a tube containing standard *Drosophila* food to a tube with a 1% agar solution. For all genotypes, baseline and starvation were carried out in constant darkness for controls and starved conditions equivalent to sleep deprived animals. For flies with a functional clock, 3 days of baseline were taken before waking was induced for 12 h during the primary sleep period, which was determined using the previous day's average sleep time and longest sleep bout. cyc^{01} and per^{01} flies were starved or sleep deprived for 7 h so as not to induce lethality.

Microarray

Three-day-old female cyc^{01} mutants were monitored under baseline conditions for 2 days in DD. On the third day, they were either sleep deprived for 7 h according to standard procedures[2] or starved for 7 h by replacing their food with 1% agar. The flies' behavior was evaluated during the treatment at which time, two thirds of the flies and their untreated controls were frozen so that RNA could be extracted from whole heads. The remaining one third of the flies were placed into tubes containing fresh food and their behavior was monitored for an additional 24 h to assay the size of the homeostatic response. Thus, we have behavioral data from siblings that were treated concurrently with flies that contributed to the microarray. Each replicate was made up of 20 pooled heads and processed for microarray analysis. Eight independent experiments were conducted over 4 mo to ensure that the results would be reproducible. Partial complementary DNA (cDNA) microarrays containing 6,240 elements that included > 4,500 unique cDNA expressed sequence tag (EST) clones representing approximately 30–40% of the total estimated number of genes in the *Drosophila* genome were evaluated.[26,27] cDNA arrays were processed at the University of California, San Diego

Biogem Biomedical Microarray Facility. Data from the 4,659 ESTs present were background subtracted, mean normalized, and subjected to loess transformation using Standardization and Normalization of MicroArray Data (http://pevsnerlab.kennedykrieger.org/snomadinput.html). The two most extreme values were identified from each condition (control, sleep deprived, and starved) and removed. For each gene, we calculated the median value of the eight replicates. Each value was then subtracted from the median to yield a difference score. The absolute values of the eight difference scores were ranked and the two highest values were excluded. Thus, statistical analysis was conducted for six replicates/group for each EST present. We were specifically interested in identifying differences between *cyc*^{01} flies that were kept awake by sleep deprivation versus those that were kept awake by starvation. Statistical differences between sleep deprived and starved flies were identified using the Cyber-T Bayesian statistical framework.[28,29] A Bonferroni correction for multiple comparisons was performed resulting in significance threshold of $P < 1.07 \times 10^{-5}$. Untreated controls are presented to provide information on the direction of the change that occurred in the sleep deprived and starved conditions. An independent replicate of sleep deprived, starved, and untreated control was collected from *cyc*^{01} flies for confirmation using quantitative polymerase chain reaction (qPCR). Genes are annotated using FB2014_04. Microarray results have been deposited in the GEO database under identification GSE18550.

Quantitative Polymerase Chain Reaction

Total RNA was isolated from 20 fly heads with Trizol (Invitrogen, Carlsbad, CA) and DNAse I digested. In the case of whole flies, three to five flies were frozen and homogenized. cDNA synthesis was performed in quadruplicate using Superscript III (Invitrogen), according to manufacturer protocol. In order to evaluate the efficiency of each reverse transcription, equal amounts of cDNA were used as a starting material to amplify *RP49* as previously described.[2] cDNA from comparable reverse transcription reactions were pooled and used as a starting material to run four qPCR replicates. Expression values for *RP49* were used to normalize results between groups. For flies maintained on an LD schedule, both experimental and untreated controls, were collected at the exact same circadian time ZT0-1 or CT0-1 for flies maintained in DD. For clock mutants, the control, sleep deprivation, and starvation experiments were run in parallel and the flies were collected at the same time.

Mass Spectrometric Analyses of Lipids

Samples for lipid spectra consisted of five flies homogenized in 1 mL of 0.63% LiCl. Aliquots (200 μL) of the homogenates were removed for protein measurements. Lipids were extracted from the remainder by the method of Bligh and Dyer. The extract was concentrated to dryness under nitrogen and reconstituted in chloroform/methanol (1:1) to which LiCl was added (final [Li], 2 mM), and lipids in the extract were infused (1 μl/min) with a Harvard syringe pump into the ESI source of a Finnigan (San Jose, CA) TSQ-7000 triple stage quadrupole mass spectrometer controlled by Finnigan ICIS software, as previously described.[30–34] Glycerophosphocholine (GPC) lipids and triacylglycerols (TAG) were analyzed as Li^+ adducts in positive ion mode. Free fatty acids (FFA) and glycerophosphoethanolamine (GPE), -glycerol (GPG), -serine (GPS), and -inositol (GPI) lipids were analyzed as $[M-H]^-$ ions in negative ion mode. For tandem MS, precursor ions selected in the first quadrupole were accelerated into a chamber containing argon to induce collisionally activated dissociation (CAD), and product ions were analyzed in the final quadrupole under described instrumental parameters.[30–34] Identities of lipid species were determined from their tandem spectra, and their quantities were determined relative to an internal standard (e.g., 14:0/14:0-GPC for GPC and TAG species).

TAG Analyses and Identification of Molecular Species

TAG were analyzed as Li^+ adducts by positive ion electrospray ionization mass spectrometry, or ESI/MS^n (n = 1 or 2), as previously described.[32] The identities of the molecular species represented by the parent ions in the full scan tracings (Figure 4) were determined from the MS/MS spectra obtained from ions with the *m/z* value of the peak in question upon CAD. For example, the ion of *m/z* 783 in the TIC tracing corresponds to the Li^+ adduct of TAG species in which the total number of carbon atoms in the fatty acyl chains is 46 and the total number of double bonds is 1 (denoted 46:1-TAG). See the legend of Figure 4 for full explanation and rationale for assigning triglyceride species.

RESULTS

Transcriptional Profiling of Sleep Deprived and Starved Flies

Striking phenotypic differences between flies that are sleep deprived and those that are starved have been previously observed.[11] Thus, we hypothesized that transcripts that are differentially regulated between sleep deprived *cyc*^{01} mutants and their starved siblings should identify genes that confer either vulnerability or resilience to sleep loss, respectively. Sleep was evaluated in 3-day old *cyc*^{01} mutants maintained in constant darkness. Following 2 days of baseline in which baseline sleep was not different between the three groups (Figure S1), siblings were either sleep deprived or starved for 7 h. Six independent experiments were completed for each condition over the course of 3 mo. Two thirds of the flies from an experiment were frozen so that RNA could be extracted from whole heads. The remaining one third of the flies were monitored for an additional 24 h to assess the size of the homeostatic response; these latter behavioral results have been reported previously.[11] Transcription profiling was conducted using partial cDNA microarrays processed at the University of California, San Diego Biogem Biomedical Microarray Facility. Because the cDNA microarray did not represent the entire genome, only 4,659 genes were identified as present in all replicates. Of the 4,659 genes that were present, 84 genes were statistically different between sleep deprived *cyc*^{01} and their starved siblings as assessed using the Cyber-T Bayesian statistical framework [28] and confirmed with qPCR (Table 1). Raw array values are displayed in Table S1 and background subtracted, mean normalized data for all 4,659 present ESTs can be found in Table S2. Identified genes include chaperones, channels, proteolysis, transcription factors, kinases/phosphatases, carbohydrate metabolism, and

Table 1—Gene expression changes in sleep deprived and starved *cyc*^{01} flies identified by microarray analysis.

Fbgn number	Gene name	Control	Sleep deprived	Starved	Assigned molecular gene ontology function
FBgn0028519	hll	0.31 ± 0.03	0.48 ± 0.01	1.71 ± 0.08	long-chain fatty acid-CoA ligase activity
FBgn0034501	CG13868	1.95 ± 0.28	13.80 ± 0.62	4.32 ± 0.35	
FBgn0023129	astray	0.78 ± 0.05	1.62 ± 0.05	0.49 ± 0.01	phosphoserine phosphatase activity
FBgn0037090	Esterase Q	0.43 ± 0.05	0.61 ± 0.02	1.47 ± 0.06	carboxylic ester hydrolase activity
FBgn0038299	Serpin 88Eb	0.41 ± 0.03	1.18 ± 0.04	0.62 ± 0.04	serine-type endopeptidase inhibitor activity
FBgn0005654	latheo	0.39 ± 0.03	1.63 ± 0.09	0.79 ± 0.04	DNA replication initiation; olfactory learning
FBgn0035985	Cuticular protein 67B	0.72 ± 0.02	2.25 ± 0.09	1.17 ± 0.04	structural component of chitin-based cuticle
FBgn0032699	CG10383	0.57 ± 0.03	1.78 ± 0.08	0.95 ± 0.03	GPI anchor metabolic process; intracellular protein transport
FBgn0036449	brummer	0.48 ± 0.04	4.92 ± 0.18	2.46 ± 0.20	triglyceride catabolic process
FBgn0032785	CG10026	0.37 ± 0.02	0.44 ± 0.02	0.77 ± 0.03	retinal binding; vitamin E binding
FBgn0002174	lethal (2) tumorous imaginal discs	0.70 ± 0.06	1.61 ± 0.08	2.80 ± 0.11	heat shock protein binding, unfolded protein binding
FBgn0024289	Sorbitol dehydrogenase 1	0.62 ± 0.03	2.66 ± 0.14	5.01 ± 0.26	oxidation-reduction process;
FBgn0259176	bunched	1.28 ± 0.10	3.28 ± 0.15	1.86 ± 0.09	DNA binding transcription factor activity
FBgn0010786	lethal (3) 02640	0.83 ± 0.05	0.70 ± 0.03	0.42 ± 0.01	hydroxymethylbilane synthase activity
FBgn0031305	Iris	1.26 ± 0.06	1.40 ± 0.07	2.54 ± 0.12	
FBgn0033188	Death resistor Adh domain containing target	1.17 ± 0.12	2.64 ± 0.09	1.41 ± 0.06	zinc ion binding; oxidoreductase activity
FBgn0032169	CG4709	0.68 ± 0.09	0.15 ± 0.01	0.32 ± 0.01	nucleic acid binding; metal ion binding
FBgn0014141	cheerio	1.34 ± 0.07	4.54 ± 0.36	2.40 ± 0.06	actin binding; learning or memory
FBgn0028990	Serpin 27A	0.45 ± 0.03	1.28 ± 0.05	0.81 ± 0.03	serine-type endopeptidase inhibitor; enzyme inhibitor activity
FBgn0038250	CG3505	0.48 ± 0.05	1.79 ± 0.06	1.13 ± 0.07	proteolysis; serine-type endopeptidase
FBgn0039580	Glutamine:fructose-6-phosphate aminotransferase 2	0.32 ± 0.02	1.43 ± 0.02	0.91 ± 0.08	carbohydrate biosynthetic process; carbohydrate binding
FBgn0029969	CG10932	0.47 ± 0.03	0.96 ± 0.05	1.40 ± 0.03	fatty acid biosynthetic process; acetyl CoAC-acetyltransferase activity
FBgn0027657	globin 1	0.66 ± 0.02	1.38 ± 0.02	0.92 ± 0.05	oxygen transport; heme binding
FBgn0031261	nicotinic Acetylcholine Receptor beta3	0.30 ± 0.02	0.64 ± 0.04	0.40 ± 0.00	acetylcholine-activated cation-selective channel activity
FBgn0000477	Deoxyribonuclease II	1.33 ± 0.11	4.27 ± 0.10	2.79 ± 0.07	DNA catabolic process; deoxyribonuclease II activity
FBgn0002719	Malic enzyme	0.79 ± 0.08	2.36 ± 0.08	1.59 ± 0.03	oxidation-reduction process; NAD binding
FBgn0040732	CG16926	0.47 ± 0.02	0.35 ± 0.02	0.19 ± 0.00	
FBgn0003257	rudimentary-like	0.68 ± 0.03	0.51 ± 0.01	0.35 ± 0.02	'de novo' pyrimidine nucleobase biosynthetic process
FBgn0031914	CG5973	1.39 ± 0.07	2.00 ± 0.04	2.52 ± 0.04	retinal binding; transporter activity
FBgn0039209	CG13624	0.63 ± 0.03	0.97 ± 0.03	0.66 ± 0.02	sequence-specific DNA binding transcription factor activity
FBgn0015331	abstrakt	0.34 ± 0.02	0.44 ± 0.01	0.63 ± 0.02	ATP-dependent RNA helicase activity
FBgn0043364	cabut	1.44 ± 0.07	2.94 ± 0.15	1.87 ± 0.06	sequence-specific DNA binding
FBgn0017581	Lk6	1.86 ± 0.24	7.31 ± 0.34	3.98 ± 0.13	protein serine/threonine kinase activity
FBgn0039627	CG11837	0.35 ± 0.02	0.19 ± 0.01	0.32 ± 0.01	rRNA(adenine-N6,N6-)-dimethyltransferase activity
FBgn0031030	Tao	0.59 ± 0.04	1.03 ± 0.02	0.68 ± 0.03	receptor signaling protein serine/threonine phosphorylation
FBgn0027348	bubblegum	1.52 ± 0.10	2.44 ± 0.07	3.42 ± 0.13	long-chain fatty acid metabolic process
FBgn0033395	Cyp4p2	0.97 ± 0.07	1.18 ± 0.03	1.59 ± 0.05	oxidation-reduction process; electron carrier activity
FBgn0052423	alan shepard	1.97 ± 0.10	4.13 ± 0.06	3.00 ± 0.17	mRNA binding
FBgn0033134	Tetraspanin 42El	0.83 ± 0.06	0.96 ± 0.04	0.46 ± 0.02	
FBgn0039135	CG13603	1.13 ± 0.11	1.00 ± 0.05	0.49 ± 0.05	
FBgn0085434	Na channel protein 60E	0.65 ± 0.03	1.17 ± 0.08	0.64 ± 0.02	voltage-gated sodium channel activity
FBgn0012042	Attacin-A	0.56 ± 0.08	0.52 ± 0.04	0.24 ± 0.02	defense response to Gram-negative bacterium
FBgn0039464	CG6330	0.75 ± 0.06	0.95 ± 0.08	0.50 ± 0.01	nucleotide catabolic process;
FBgn0015222	Ferritin 1 heavy chain homologue	1.35 ± 0.10	1.41 ± 0.09	0.79 ± 0.03	ferrous iron binding; cellular iron ion homeostasis
FBgn0038733	CG11407	0.56 ± 0.06	0.38 ± 0.02	0.67 ± 0.04	long-chain fatty acid transporter activity

Ave ± SEM; [a]mean, normalized value.

Table 1 continues on the following page

Table 1 (*continued*)—Gene expression changes in sleep deprived and starved cyc^{01} flies identified by microarray analysis.

Fbgn number	Gene name	Control	Sleep deprived	Starved	Assigned molecular gene ontology function
FBgn0033830	CG10814	0.34 ± 0.02	0.42 ± 0.03	0.21 ± 0.02	oxidation-reduction process; gamma-butyrobetaine dioxygenase activity
FBgn0034084	CG8435	0.44 ± 0.06	0.22 ± 0.01	0.35 ± 0.01	neurogenesis
FBgn0036512	CG16979	0.60 ± 0.04	0.50 ± 0.01	0.67 ± 0.02	thiolester hydrolase activity
FBgn0041203	LIM-kinase1	0.91 ± 0.06	0.85 ± 0.03	0.56 ± 0.03	protein serine/threonine kinase
FBgn0031836	CG11050	1.01 ± 0.09	0.87 ± 0.02	0.60 ± 0.04	phosphoric diester hydrolase activity
FBgn0019643	Dopamine N acetyltransferase	1.14 ± 0.06	0.96 ± 0.01	1.29 ± 0.04	catecholamine metabolic process; N-acetyltransferase activity
FBgn0002778	minidiscs	0.36 ± 0.03	0.32 ± 0.01	0.32 ± 0.01	amino acid transmembrane transporter activity
FBgn0026563	CG1979	0.30 ± 0.02	0.39 ± 0.01	0.28 ± 0.00	
FBgn0260012	pds5	0.42 ± 0.04	0.65 ± 0.01	0.48 ± 0.02	sister chromatid cohesion
FBgn0004055	unzipped	1.23 ± 0,10	0.68 ± 0.07	1.29 ± 0.05	axon guidanc
FBgn0261362	Prophenoloxidase 1	0.54 ± 0.05	0.63 ± 0.07	1.24 ± 0.09	dopamine metabolic process; catechol oxidase activity
FBgn0041188	Ataxin-2	0.68 ± 0.06	1.66 ± 0.16	0.78 ± 0.04	oocyte differentiation; protein binding
FBgn0016122	Angiotensin-converting enzyme-related	1.18 ± 0.16	1.59 ± 0.15	2.92 ± 0.13	peptidyldipeptidase activity
FBgn0000079	Amylase proximal	0.38 ± 0.04	3.48 ± 0.41	1.54 ± 0.05	carbohydrate metabolic process; alpha-amylase activity
FBgn0031220	CG4822	0.72 ± 0.09	0.99 ± 0.06	1.69 ± 0.08	ATPase activity; transport activity
FBgn0025454	Cyp6g1	1.23 ± 0.11	1.18 ± 0.05	2.34 ± 0.27	response to DTT; electron carrier; monooxygenase activity
FBgn0261262	CG42613	1.83 ± 0.18	3.65 ± 0.39	1.67 ± 0.27	
FBgn0262517	l(3)76BDr	0.57 ± 0.05	0.55 ± 0.07	1.01 ± 0.08	zinc ion binding
FBgn0032940	Mlx interactor	1.33 ± 0.06	1.75 ± 0.16	0.94 ± 0.07	sequence-specific DNA binding transcription factor activity
FBgn0266557	kismet	1.47 ± 0.11	0.92 ± 0.07	0.52 ± 0.04	ATP-dependent helicase activity
FBgn0015295	SH2 ankyrin repeat kinase	0.49 ± 0.03	0.29 ± 0.04	0.55 ± 0.05	protein tyrosine kinase activity
FBgn0036680	Cuticular protein 73D	0.32 ± 0.05	0.23 ± 0.02	0.43 ± 0.04	structural component of chitin-based cuticle
FBgn0262518	Rab8	1.16 ± 0.07	1.06 ± 0.07	1.71 ± 0.09	protein transport; GTPase activity
FBgn0034709	Secreted Wg-interacting molecule	1.03 ± 0.09	2.64 ± 0.11	1.52 ± 0.18	cysteine-type endopeptidase activity
FBgn0000463	Delta	0.71 ± 0.07	0.61 ± 0.09	1.08 ± 0.07	Notch binding
FBgn0052281	CG32281	1.31 ± 0.09	0.52 ± 0.04	0.27 ± 0.02	tRNA methyltransferase activity
FBgn0001977	CIAPIN1 ortholog	1.27 ± 0.18	7.46 ± 0.80	3.21 ± 0.45	iron-sulfur cluster binding
FBgn0015245	Heat shock protein 60	0.53 ± 0.05	0.68 ± 0.07	0.35 ± 0.02	response to heat; Unfolded protein binding
FBgn0011204	cueball	1.08 ± 0.07	1.41 ± 0.04	0.85 ± 0.09	low density lipoprotein receptor activity
FBgn0262737	mushroom-body expressed	0.83 ± 0.09	1.52 ± 0.06	0.98 ± 0.02	poly(rC) binding; regulation of alternative mRNA splicing
FBgn0034854	Golgin-245 ortholog (H. sapiens)	0.67 ± 0.05	0.67 ± 0.02	0.83 ± 0.01	Golgi vesicle transport; ADP-ribosylation factor binding
FBgn0039465	Tetraspanin 97E	0.65 ± 0.06	1.04 ± 0.04	0.74 ± 0.02	
FBgn0033949	CG10131	0.90 ± 0.08	1.03 ± 0.03	0.78 ± 0.01	oxidation-reduction process; 3-hydroxyacyl-CoA dehydrogenase activity
FBgn0037760	FBX011 ortholog	0.71 ± 0.08	1.26 ± 0.04	0.82 ± 0.02	regulation of gene silencing by RNA; ubiquitin-protein ligase activity
FBgn0036022	CG8329	1.11 ± 0.14	1.23 ± 0.04	1.53 ± 0.02	proteolysis; serine-type endopeptidase activity
FBgn0025633	CG13366	0.48 ± 0.06	0.43 ± 0.02	0.68 ± 0.02	adult somatic muscle development
FBgn0025678	calcium-binding protein 1	0.68 ± 0.05	1.01 ± 0.05	0.63 ± 0.03	cell redox homeostasis; protein disulfide isomerase activity
FBgn0016977	split ends	1.55 ± 0.07	1.64 ± 0.03	1.25 ± 0.03	nucleic acid binding; axon guidance
FBgn0086694	Bre1	0.77 ± 0.05	0.69 ± 0.04	1.09 ± 0.04	histone modification; zinc ion binding

Ave ± SEM; [a]mean, normalized value.

lipid metabolism, suggesting multiple pathways are altered in the response to sleep deprivation.

To further confirm the microarray results, we used qPCR to evaluate gene expression in an independent replicate consisting of sleep deprived and starved cyc^{01} flies. In addition, we extracted messenger RNA (mRNA) from heads of both per^{01} and *CS* flies under baseline and after either sleep deprivation or starvation. These latter experiments were designed to determine the extent to which the genes identified in the microarray on cyc^{01} mutants would be modified in a different clock mutant that responds differently to sleep loss[2] and to a common background strain with an intact clock. cyc^{01}, per^{01}, and *CS* flies were maintained in DD to avoid differentially activating genes with light exposure. Because cyc^{01} mutants begin to die after

only 10 h of sleep deprivation, both cyc^{01} and per^{01} flies were sleep deprived or starved for 7 h while *CS* flies were deprived of sleep or starved for 12 h during their primary sleep period. The primary sleep period was identified from the previous days' data based on the average time that the *CS* flies initiated their longest sleep bout.[24] Changes in gene expression as assessed by qPCR can be seen in Figure 1A. To simplify the presentation, only those genes that show similar relative patterns in cyc^{01} flies and either *CS* and/or per^{01} flies are graphed in Figure 1A. Note that the cyc^{01}, per^{01}, and *CS* flies are in different genetic backgrounds such that it is not possible to directly assess quantitative changes in gene expression between the genotypes. However, within-genotype comparisons can be made by evaluating the relative direction of changes seen in siblings following sleep loss or starvation. After the relative changes in gene expression have been quantified within a genotype, it is informative to ask whether the relative changes are similar in the other genotypes, per^{01} and *CS* flies. Of the 84 transcripts identified on the microarray, numerous genes maintained the same relative relationship between sleep deprived and starved states seen in cyc^{01} flies in per^{01} or *CS* flies (Figure 1B), suggesting that changes in these transcripts withstand changes in background. Relative changes are highlighted for each genotype in gray. Together these data suggest that the identified genes may play a role in regulating the response to sleep loss.

Many of the genes with the greatest transcriptional differences between the sleep deprived and starved flies were genes involved with lipid metabolism. The gene with the largest change was *hll*, but also near the top of our list was *brummer* (*bmm*). This latter observation is of interest in view of our previous finding that mutants of *bmm* have an increased sleep rebound after sleep deprivation.[11] In addition, other lipid metabolism genes including *CG11407*, *bubblegum* (*bgm*), and *cueball* (*cue*) were present on our list. Thus, there appear to be broad alterations in transcription of lipid metabolic enzymes with sleep deprivation.

Behavioral Validation of Microarray

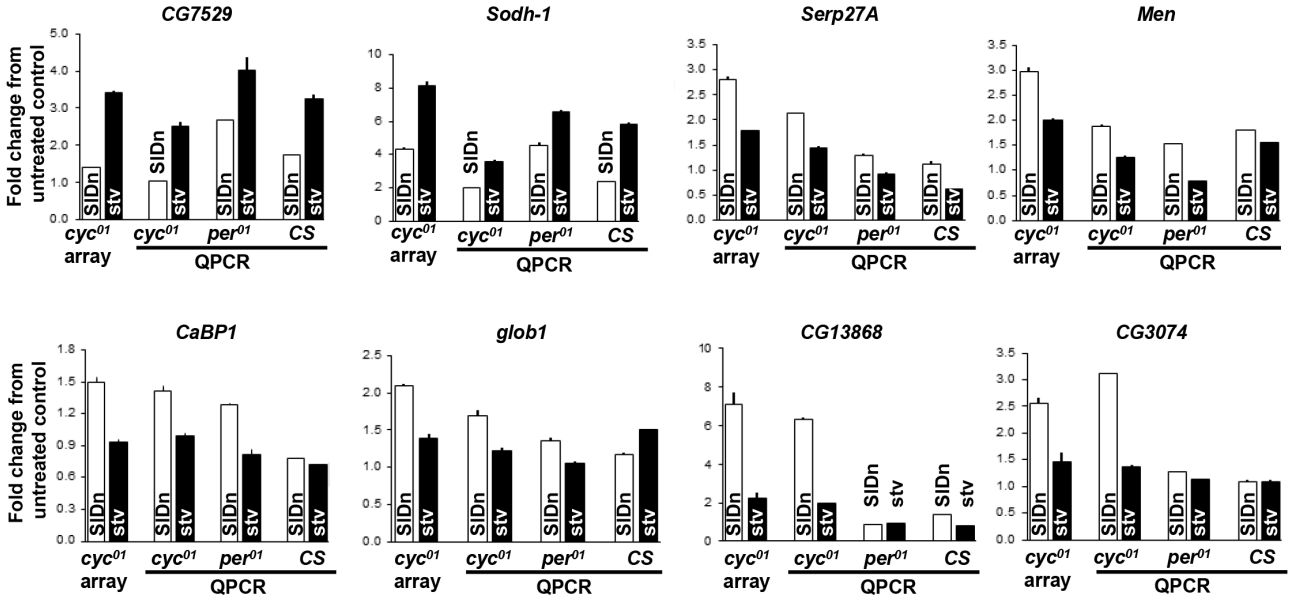
All gene profiling experiments are correlational in nature.[35] Thus, transcriptional changes that result from extended waking induced by either sleep deprivation or starvation may represent changes that (1) regulate the homeostatic response, (2) induce physiologic impairments during sleep deprivation, (3) protect flies from the negative consequences of waking, or (4) have no direct involvement. Ideally, the precise role these genes play should be relevant for sleep regulation in general. That is, our goal is not to investigate the molecular mechanisms underlying *cycle* function, but rather it is to identify genes that influence sleep homeostasis. Given that many of the transcriptional changes we report between sleep deprived and starved flies are small and affect molecular pathways that are modified by post-transcriptional modifications, we have evaluated their role using genetics. If any of the genes we have identified play an active role in sleep homeostasis, their manipulation should alter the response to sleep deprivation. Thus, we used genetics to evaluate three genes, *bubblegum*, *cueball,* and *hll*, given their putative role in the processing of complex lipids.

bubblegum (*bgm*) is an acyl-coenzyme A (CoA) synthetase differentially expressed during sleep deprivation and starvation. Several mutations for *bgm* are available, including bgm^{1} in which a PlacZ element is inserted in the first intron. This insertion results in large reductions in the expression of the *bgm* gene.[36] Although young bgm^{1} flies show normal brain morphology, 15-day-old mutants show signs of degeneration in the optic lobe and retina. Precise excision of the P-element (bgm^{rev}) restored the normal phenotype.[36] The *bgm* mutants have a disruption in the processing of very long chain fatty acids (VLCFA) that results in their accumulation throughout the fly.[36] Sleep parameters for bgm^{1} and their genetic controls bgm^{rev} were consistent with the normal variation seen for wild-type flies and can be found in Table S3. bgm^{1} and bgm^{rev} were sleep deprived for 12 h during the dark period when they were 5 days old so that the recovery period was completed by age 7 days. Sleep homeostasis is calculated for each individual as a ratio of the minutes of sleep gained above baseline during the 48 h of recovery divided by the total min of sleep lost during 12 h of sleep deprivation (min gained/min lost). As seen in Figure 2A, bgm^{1} mutants displayed a significantly larger sleep rebound than their genetic background control, bgm^{rev}.

To confirm that decreased *bgm* resulted in an increased sleep rebound, we employed the *Drosophila* GAL4- Upstream Activating Sequence (UAS) system.[37] In this system, a yeast transcription factor, GAL4, is used to drive tissue-specific expression of a given transcript by binding and transcribing genetic elements downstream of the yeast UAS. Because it has yet to be determined which are the critical tissues for modulating *bgm* expression, we used a driver that would express throughout the fly, *Act-GAL4*.[38] To further evaluate *bgm*, we examined sleep homeostasis in a second allele of *bgm*, $bgm^{EY03176}$. $bgm^{EY03176}$ flies contain a P-element inserted into the 5' region of the *bgm* gene, just upstream of a putative transcript start site for one form of the *bgm* transcript. The P-element contains an integrated UAS element such that GAL4 drivers can be used to exogenously express *bgm*. Using *Act-GAL4*, we increased the expression of *bgm* throughout the fly compared to the background control, $bgm^{EY03176}$/+. qPCR reveals that *bgm* mRNA expression is increased in *Act-GAL4*/+ > $bgm^{EY03176}$/+ flies compared to $bgm^{EY03176}$/+ controls (Figure 2B). Consistent with the findings from the loss-of-function in which bgm^{1} mutants have a large sleep rebound, gain-of-function *Act-GAL4*/+ > $bgm^{EY03176}$/+ flies exhibited a reduced sleep rebound compared to $bgm^{EY03176}$/+ controls (Figure 2C). The increased sleep rebound observed in $bgm^{EY03176}$/+ is likely caused by the reduced levels of *bgm* compared to *CS* flies (Figure S2). Thus, the exogenous expression of *bgm* using the *Act-GAL4* likely rescues a fly hypomorphic for *bgm*. Both the deletion mutant and the GAL4-UAS experiments support the conclusion that reduced levels of *bgm* result in an increased homeostatic response to sleep deprivation.

To further evaluate the possibility that genes associated with low-density lipoprotein receptor activity can influence sleep homeostasis, we identified a gene from our microarray that is predicted to have such activity, *cueball* (*cue*) (http://flybase.org/). The P-element mutant cue^{2} has been reported to be a hypomorphic allele resulting from a P-element insertion.[39] As seen in Figure 2D, we confirmed that cue^{2} had decreased levels of *cue* mRNA compared to the precise excision genetic control (cue^{rev}). Sleep data for cue^{2} and cue^{rev} were within the normal variation

A



B

		cyc 01 microarray		Fold	cyc 01 QPCR		Fold	per 01 QPCR		Fold	Canton-S QPCR		Fold
FBgn number	Gene	SIDn	stv	SIDn/stv	SIDn	stv	SIDn/stv	SIDn	stv	SIDn/stv	SIDn	stv	SIDn/stv
FBgn0028519	hll	153 ± 1	543 ± 7	0.3	545 ± 7	1400 ± 48	0.4	2750 ± 95	8370 ± 381	0.3	1570 ± 73	7880 ± 70	0.2
FBgn0038299	CG6687	288 ± 4	150 ± 3	1.9	518 ± 18	185 ± 3	2.8	225 ± 0.4	186 ± 0.6	1.2	180 ± 1	89 ± 1	2.0
FBgn0037090	CG7529	140 ± 2	340 ± 6	0.4	101 ± 0.8	251 ± 11	0.4	266 ± 1	401 ± 37	0.7	172 ± 1	324 ± 12	0.5
FBgn0025454	Cyp6g1	96 ± 4	190 ± 27	0.5	64 ± 0.7	119 ± 1	0.5	76 ± 0.3	161 ± 0.7	0.5	121 ± 0.4	165 ± 4	0.7
FBgn0024289	Sodh-1	432 ± 13	814 ± 26	0.5	199 ± 0.2	356 ± 7	0.6	452 ± 21	658 ± 8	0.7	233 ± 6	584 ± 12	0.4
FBgn0023129	aay	207 ± 4	62 ± 1	3.3	365 ± 5	128 ± 0.5	2.9	195 ± 2	160 ± 3	1.2	319 ± 12	221 ± 1	1.4
FBgn0000477	DNAseII	321 ± 9	210 ± 7	1.5	184 ± 9	73 ± 0.1	2.5	113 ± 1	83 ± 3	1.4	137 ± 0.5	87 ± 3	1.6
FBgn0043364	cabut	204 ± 15	130 ± 6	1.6	136 ± 5	57 ± 2	2.4	72 ± 0.8	60 ± 0.6	1.2	109 ± 3	73 ± 2	1.5
FBgn0012042	AttA	92 ± 3	42 ± 1	2.2	827 ± 12	77 ± 5	10.7	689 ± 37	65 ± 0.7	10.5	111 ± 0.5	20 ± 0.1	5.5
FBgn0039580	Gfat2	452 ± 2	286 ± 7	1.6	175 ± 0.3	107 ± 2	1.6	121 ± 0.7	98 ± 0.9	1.2	166 ± 0.8	133 ± 13	1.2
FBgn0038733	CG11407	67 ± 1	121 ± 3	0.6	57 ± 1	91 ± 2	0.6	81 ± 1	93 ± 0.2	0.9	66 ± 1	135 ± 2	0.5
FBgn0031220	CG4822	137 ± 6	234 ± 7	0.6	82 ± 3	126 ± 7	0.7	66 ± 0.2	104 ± 0.7	0.6	87 ± 2	120 ± 4	0.7
FBgn0002719	Men	297 ± 7	200 ± 3	1.5	187 ± 4	125 ± 5	1.5	152 ± 3	79 ± 0.5	1.9	180 ± 2	155 ± 0.1	1.2
FBgn0038250	CG3505	374 ± 5	236 ± 6	1.6	226 ± 5	118 ± 6	1.9	151 ± 3	123 ± 2	1.2	118 ± 7	106 ± 0.4	1.1
FBgn0032785	CG10026	119 ± 2	209 ± 3	0.6	117 ± 7	172 ± 10	0.7	164 ± 0.7	193 ± 0.1	0.8	95 ± 2	170 ± 0.7	0.6
FBgn0028990	serpin-27A	281 ± 5	178 ± 2	1.6	212 ± 2	144 ± 4	1.5	128 ± 3	91 ± 5	1.4	112 ± 6	61 ± 2	1.8
FBgn0025678	CaBP1	149 ± 4	93 ± 2	1.6	142 ± 4	99 ± 2	1.4	128 ± 1	81 ± 4	1.6	77 ± 0.8	72 ± 0.5	1.1
FBgn0033134	Tsp42El	116 ± 3	56 ± 1	2.1	138 ± 1	90 ± 0.4	1.5	116 ± 1	92 ± 1	1.3	100 ± 0.1	85 ± 3	1.2
FBgn0015222	Fer1HCH	104 ± 8	58 ± 2	1.8	134 ± 0.9	93 ± 0.7	1.4	120 ± 3	106 ± 2	1.1	96 ± 0.2	82 ± 3	1.2
FBgn0026563	CG1979	127 ± 1	93 ± 0.2	1.4	1120 ± 105	341 ± 20	3.3	910 ± 1	394 ± 19	2.3	929 ± 42	725 ± 36	1.2
FBgn0000165	Bc	116 ± 6	230 ± 9	0.5	48 ± 0.1	110 ± 3	0.4	52 ± 6	57 ± 1	0.9	42 ± 2	123 ± 0.1	0.3
FBgn0033949	CG10131	114 ± 2	86 ± 1	1.3	67 ± 0.1	60 ± 0.3	1.1	98 ± 4	55 ± 2	1.8	64 ± 0.5	34 ± 0.1	1.9
FBgn0005654	lat	421 ± 9	204 ± 4	2.1	94 ± 1	74 ± 2	1.3	78 ± 10	62 ± 2	1.3	109 ± 1	107 ± 8	1.0
FBgn0000463	Dl	85 ± 9	152 ± 7	0.6	50 ± 0.6	61 ± 0.4	0.8	44 ± 2	57 ± 0.5	0.8	106 ± 3	136 ± 0.1	0.8
FBgn0027348	bgm	161 ± 7	225 ± 12	0.7	120 ± 2	123 ± 3	1.0	69 ± 2	81 ± 2	0.8	109 ± 3	143 ± 1	0.8
FBgn0034501	CG13868	707 ± 62	221 ± 34	3.2	633 ± 12	195 ± 8	3.2	90 ± 1	95 ± 0.1	0.9	141 ± 1	82 ± 0.1	1.7
FBgn0040732	CG16926	74 ± 1	40 ± 0.3	1.8	386 ± 5	107 ± 1	3.6	117 ± 3	182 ± 2	0.6	142 ± 8	102 ± 8	1.4
FBgn0039464	CG6330	126 ± 8	66 ± 0.7	1.9	175 ± 7	93 ± 3	1.9	136 ± 3	100 ± 2	1.4	116 ± 2	144 ± 5	0.8
FBgn0027657	glob1	209 ± 2	139 ± 5	1.5	169 ± 6	121 ± 5	1.4	135 ± 4	104 ± 3	1.3	117 ± 2	151 ± 0.3	0.8
FBgn0014141	cher	339 ± 36	179 ± 5	1.9	146 ± 3	61 ± 1	2.4	83 ± 1	101 ± 3	0.8	139 ± 0.2	86 ± 3	1.6
FBgn0033830	CG10814	124 ± 2	63 ± 1	2.0	799 ± 12	205 ± 54	3.8	270 ± 1	146 ± 9	1.8	1490 ± 44	2470 ± 193	0.6
FBgn0031261	nAcRβ-21C	208 ± 4	130 ± 0.4	1.6	137 ± 15	93 ± 7	1.5	81 ± 1	69 ± 0.4	1.2	112 ± 0.9	114 ± 2	1.0
FBgn0011204	cue	131 ± 4	78 ± 8	1.7	108 ± 2	86 ± 0.7	1.3	81 ± 0.1	87 ± 2	0.9	142 ± 0.5	122 ± 0.5	1.2
FBgn0029969	CG10932	205 ± 4	301 ± 2	0.7	75 ± 0.1	98 ± 8	0.8	82 ± 3	107 ± 0.1	0.8	153 ± 2	151 ± 4	1.0
FBgn0015796	Rab8	91 ± 6	147 ± 8	0.6	82 ± 4	98 ± 0.9	0.8	95 ± 0.6	89 ± 3	1.1	111 ± 6	154 ± 7	0.7
FBgn0031305	Iris	110 ± 7	201 ± 11	0.5	72 ± 1	84 ± 3	0.9	114 ± 1	135 ± 0.4	0.8	107 ± 0.1	84 ± 2	1.3
FBgn0031914	CG5973	143 ± 3	180 ± 3	0.8	116 ± 6	133 ± 12	0.9	75 ± 0.4	63 ± 1	1.2	120 ± 3	140 ± 3	0.9
FBgn0034084	CG8435	49 ± 0.9	79 ± 1	0.6	61 ± 3	75 ± 6	0.8	88 ± 2	90 ± 6	1.0	113 ± 0.7	166 ± 14	0.6

Figure 1—Gene expression profiles in sleep deprived and starved *cyc*^{01}, *per*^{0}, and Wild-type *Canton-S* (*CS*) flies. **(A)** Relative fold changes versus untreated genetic controls in representative genes derived from complementary DNA arrays (6 samples/condition) or quantitative polymerase chain reaction (qPCR, 1 sample/group, n = 20 flies). All flies were maintained in dark-dark cycle (DD). *cyc*^{01} and *per*^{01} were sleep deprived (SIDn) or starved (Stv) concurrently for 7 h while *CS* flies were deprived for 12 h during their primary sleep period. **(B)** Percent change versus untreated genetic controls expressed as mean ± standard error of the mean. Fold changes between sleep deprived and starved flies are highlighted in gray.

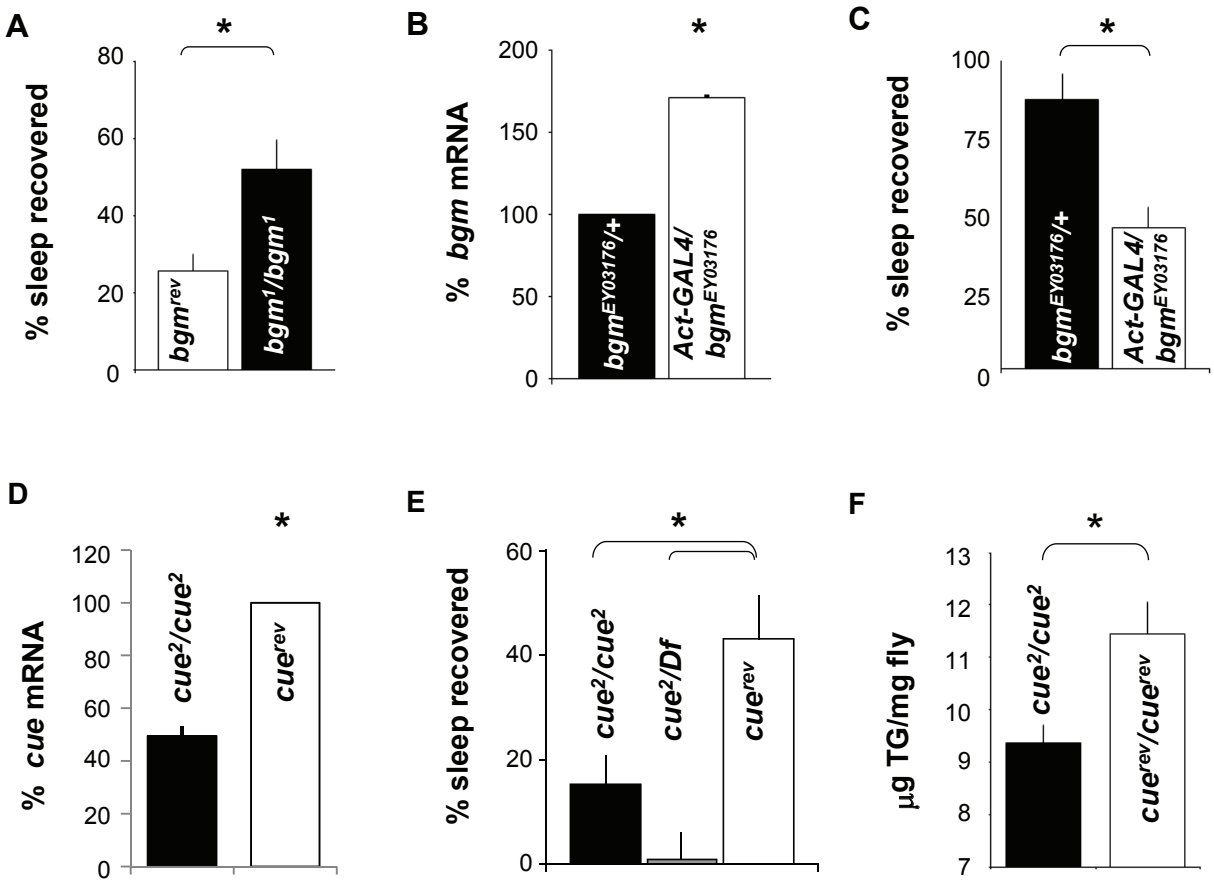


Figure 2—Genetic validation of microarray. **(A)** Sleep homeostasis is increased in *bgm*^{1} mutants (n = 21) compared to its background genetic control, *bgm*^{rev} (n = 24). * P = 0.0045, Student *t*-test. **(B)** *bgm* messenger RNA (mRNA) from flies in *Actin-GAL4/bgm*^{EY03176} compared to *bgm*^{EY01376}/+. mRNA is expressed as a percentage of *bgm*^{EY01376}/+ (n = 5 flies/group). *P < 0.05 by Student *t*-test. **(C)** *bgm*^{EY03176}/+ flies (n = 45) also show an increased homeostatic response in comparison to *Act-GAL4/bgm*^{EY03176} (n = 27). *P = 0.00065 by Student *t*-test. **(D)** *cue* mRNA levels are decreased in *cue*^{2} homozygotes compared with the background control with the P-element precisely excised (*cue*^{rev}). Levels are presented as a percentage of *cue*^{rev}. *P < 0.05 by Student *t*-test. **(E)** Flies homozygous for *cue*^{2} (n = 129), or hemizygous *cue*^{2}*/Df(3L)Ar14-8* (n = 32) have a significantly reduced sleep rebound compared to genetic background controls in which the P-element has been excised, *cue*^{rev} (n = 39). One-way analysis of variance $F_{2,197} = 5.80$; *P < 0.001 modified Bonferroni test. **(F)** Total triglyceride levels were significantly decreased in *cue*^{2}*/cue*^{2} mutants compared to its background control *cue*^{rev}*/cue*^{rev}. *P = 0.017 using Student *t*-test with an n = 5 groups of 10 flies per genotype.

found in wild-type flies and can be found in Table S3. Flies with a homozygous mutation in *cue*^{2} exhibit a low sleep rebound following 12 h of sleep deprivation compared to genetic controls (Figure 2E). To confirm that the low sleep rebound mapped to the *cue* locus, we crossed *cue*^{2} mutants to flies carrying a deficiency (*Df*) covering the *cue* gene. As seen in Figure 2E, *cue*^{2}*/Df* flies also displayed a severely reduced homeostatic response following sleep deprivation. Interestingly, *cue*^{2} mutants show significantly reduced organismal triglyceride stores compared to their genetic background controls, *cue*^{rev} confirming its role in lipid metabolism (Figure 2F).

Finally, we evaluated the role of the gene with the greatest transcriptional change, *hll*, using RNA interference (RNAi). *hll* is a predicted acyl-CoA synthetase. We therefore determined whether knocking down *hll* using RNAi would alter lipid phenotypes. We used the inducible GeneSwitch GAL4 system to knockdown *hll* throughout the fly. In the GeneSwitch system, mifepristone is fed to the flies at the desired time to induce the expression of the RNAi transcript. Thus, adult sibling *tubulin-GeneSwitch* driving *hll* RNAi (*tubGS > hll*^{RNAi}) are given food containing either mifepristone or the vehicle control. Thus, comparisons are made between the induced and uninduced siblings with the same background. Moreover, because induction of transcription occurs at the adult stage, flies avoid any developmental effects of RNA knockdown at earlier stages that may confound results. We analyzed lipid species in extracts from the flies by ESI/MS as previously described.[30–34] Consistent with previous reports that changing acyl-CoA synthase expression results in altered triglyceride stores,[40–42] we report that the global knockdown of *hll* in *tubGS > hll*^{RNAi} flies fed RU486 (RU) results in an overall reduction of triglycerides compared to genetically identical siblings maintained on vehicle (Figures 3A and 3B). It should be noted that we and others have consistently reported that RU does not influence a variety of phenotypes including lifespan, sleep, sleep homeostasis, short-term memory, short-term memory following sleep deprivation, olfactory conditioning, phototaxis, geotaxis, locomotion, and the escape response.[43–46] To determine whether

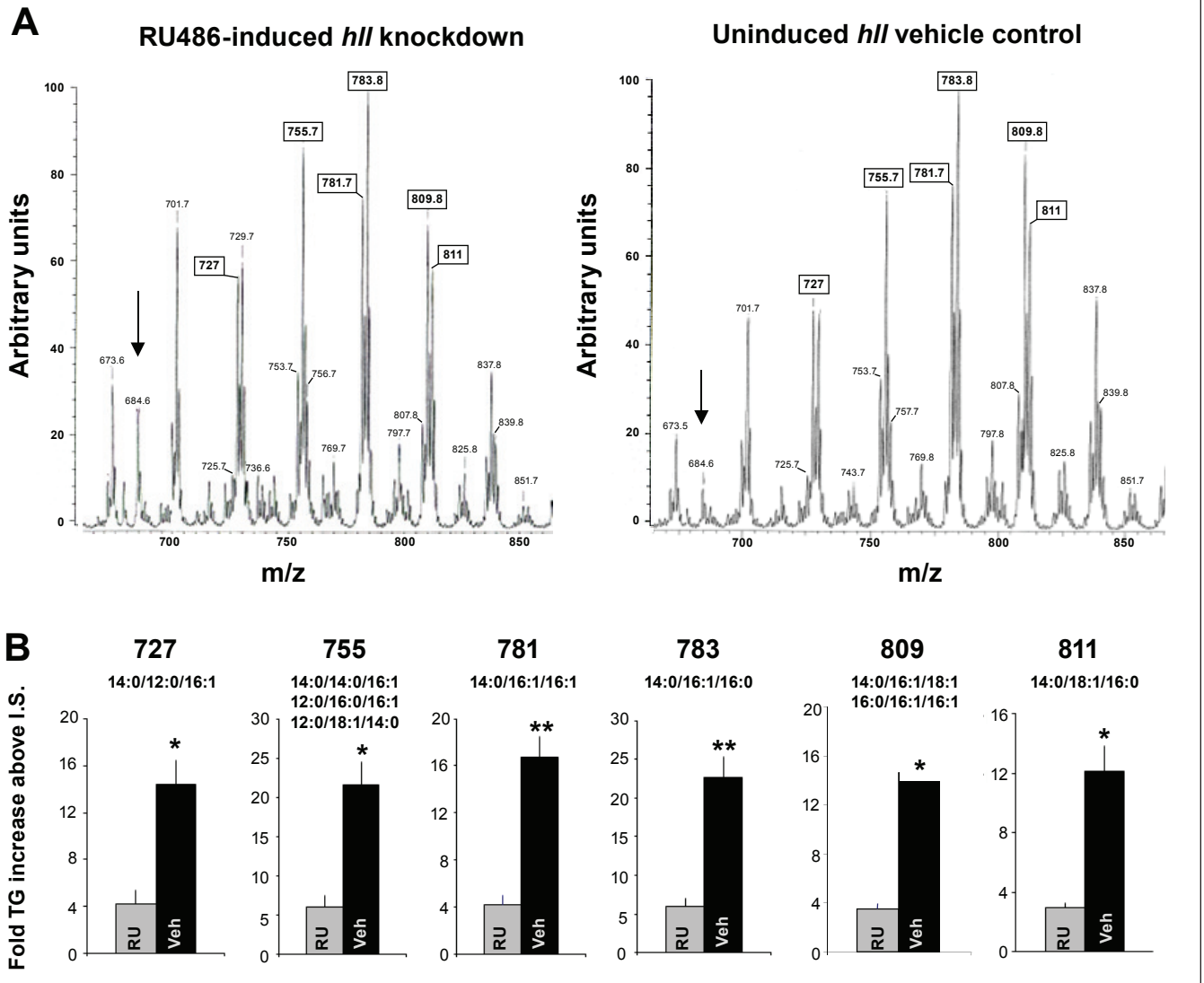


Figure 3—Triglyceride levels are decreased with ubiquitous *hll* knockdown. **(A)** Representative triglyceride profiles from flies with ubiquitous knockdown of *hll* (*tubGS- > UAS-hll*^{RNAi} induced by RU486, left tracing) and the uninduced, genetically identical siblings on the vehicle control (veh, right tracing). We chose to use the GeneSwitch system to minimize the number of groups analyzed and to better control for genetic background. Tracings were generated using positive ion electrospray ionization (ESI) mass spectrometric (MS) analyses of lipid Li^+ adducts from whole flies. Peaks are labeled by their mass to charge (*m/z*) ratios, and the boxed *m/z* values correspond to the peaks quantified in **(B)**. The arrow denotes internal standard (*m/z* 684), and the most intense peak is normalized to 100. Although there is a decrease in overall triglyceride abundance, there is no change in the distribution of triglyceride molecular species. **(B)** Quantification of triglyceride peaks from the mass spectra of *hll* knockdown and the uninduced siblings (four samples per group; n = 5 flies/sample). Numbers above graph represent the *m/z* value from **(A)** and the smaller numbers are the lipid species that could correspond to the given *m/z* value. The intensity of the peak in question was divided by that of the internal standard (I.S.), and the resultant ratio was then normalized to protein content to determine the fold-increase of the triglyceride species represented by the peak. *P < 0.05) or **P < 0.01 as calculated by Student *t*-test with a Bonferroni correction.

the changes in the peaks involved changes in isobaric molecular constituents at a given *m/z* value, we conducted tandem mass spectrometry in which precursor ions selected in the first quadrupole were accelerated into a chamber containing argon to induce CAD and product ions were then analyzed in the final quadrupole. A representative tandem mass is shown in Figure 4. Although there is a decrease in overall triglycerides, we could not detect a shift in the distribution of triglyceride molecular species in *tubGSw > hll* flies compared to vehicle-fed genetically identical siblings. Together these data indicate that *hll* influences lipid metabolism.

We then confirmed that the lipid phenotype was associated with knockdown of *hll* in the RU486 induced flies compared to the vehicle-fed controls. qPCR results demonstrate that *hll* transcript levels are only 14% of the levels observed in controls (Figure 5A). To determine whether the ubiquitous knockdown of *hll* altered sleep homeostasis, we sleep deprived RU-fed *tubGS > hll*^{RNAi} flies and their vehicle-fed siblings. As seen in Figure 5B, sleep rebound was significantly reduced compared to genetic background controls. To further rule out genetic background, we knocked down *hll* using the *Act-GAL4* driver. As seen in Figure 5C, *Act-GAL4/+ > UAS-hll*^{RNAi}*/+*flies showed

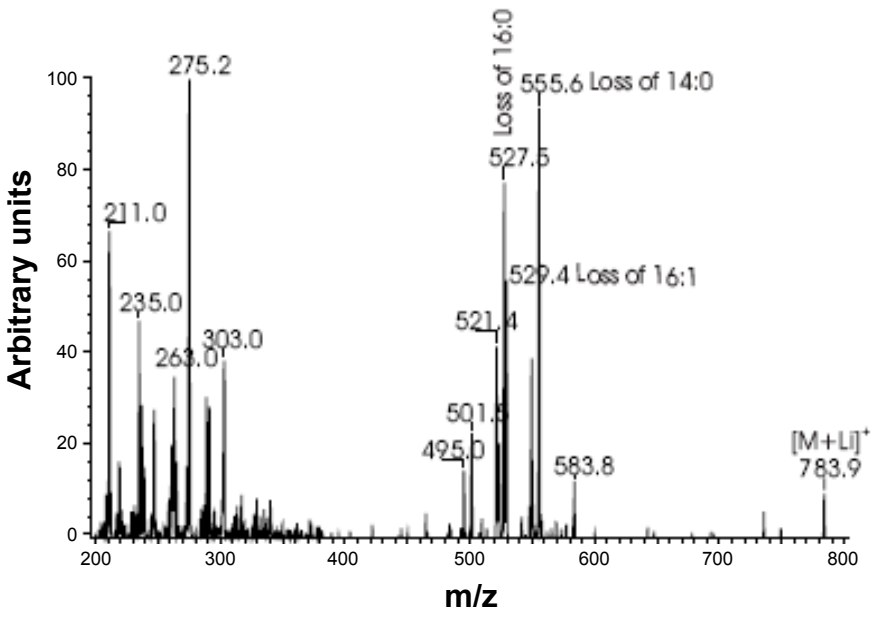


Figure 4—Identification of the constituents that constitute the peak at m/z 783. Mass spectrometric MS/MS spectra obtained from ions with the *m/z* value of 783 in the total ion current tracing (Figure 4) corresponds to the Li^+ adduct of triacylglycerol (TAG) species from the RU-treated flies in which the total number of carbon atoms in the fatty acyl chains is 46 and the total number of double bonds is 1 (denoted 46:1-TAG). The tandem spectrum obtained from collisionally activated dissociation (CAD) of *m/z* 783 is displayed in the figure, and it indicates that the predominant molecular species represented by that peak is 14:0/16:1/16:0-TAG. Features of the spectrum that establish that assignment are the presence of ions that represent neutral losses of each of the substituents as a free fatty acid at *m/z* 527 (loss of 16:0), 529 (loss of 16:1), and 555 (loss of 14:0), respectively. There are also ions reflecting loss of each substituent as a Li^+ salt at *m/z* 521, 523, and 549, respectively. The ions representing loss of the *sn*-2 substituent of TAG-Li^+ species are less abundant than the ions reflecting loss of the *sn*-1 or *sn*-3 substituent,[34] and the *sn*-1 and *sn*-3 positions of TAG molecules are not distinguishable by mass spectrometry. Other ions consistent with this assignment are the acylium ion of 14:0 (*m/z* 211) and ions representing Li^+ adducts of 14:0 (*m/z* 235) and 16:0 (*m/z* 263). There are also ions representing combined losses of 16:1 and 16:0 (*m/z* 275) or 14:0 (*m/z* 303) as an α,β-unsaturated fatty acid. Such combined losses always include the *sn*-2 substituent,[85] indicating that 16:1 is the *sn*-2 substituent in the major TAG isomer contributing ion current to the *m/z* 783 peak. That there are less abundant isomers with the overall composition 46:1-TAG, e.g., 12:0/18:1/16:0-TAG, is reflected by relatively low abundance ions at *m/z* 501 and *m/z* 583 that represent loss of 18:1 and 12:0 as free fatty acids, respectively. MS and MS/MS analyses of lipid extracts of vehicle-treated flies revealed the same major TAG molecules observed for the RU-treated flies.

a substantial reduction in *hll* mRNA compared to *Act-GAL4*/+ and *UAS-hll*^{RNAi}/+ parental controls. Sleep data for parental lines (*Act-GAL4*/+ and *UAS-hll*^{RNAi}/+) and the experimental line (*Act-GAL4*/+ > *UAS-hll*^{RNAi}/+) can be found in Table S3. Sleep homeostasis was evaluated in *Act-GAL4*/+ > *UAS-hll* ^{RNAi}/+ flies and their background controls following 12 h of sleep deprivation. As seen in Figure 5D, flies in which *hll* was knocked down showed a significantly reduced sleep rebound compared to each of the parental lines, *Act-GAL4*/+ and *UAS-hll*^{RNAi}/+. Together with the data presented for *hll*, *bgm*, and *cue*, these data indicate that a subset of the genes identified by transcriptional profiling can indeed influence sleep regulation as measured by sleep homeostasis.

DISCUSSION

We have described a novel strategy to identify genes that are likely to play a role in sleep homeostasis or that protect flies from the negative effects of sleep loss. This strategy takes advantage of the observation that waking induced by starvation leads to different functional outcomes than the same amount of waking induced by sleep deprivation.[11] Our approach also takes advantage of the fact that *cyc*^{01} flies quickly accumulate the negative effects of sleep deprivation.[2] Using this approach we have identified 84 transcripts that are differentially regulated between genetically identical sleep deprived and starved siblings. Importantly, genetic analysis confirms that three of these genes, *bgm*, *cue*, and *hll* modulate sleep homeostasis. Previous genetic studies have confirmed a role for other genes on this list including *brummer*, *Delta*, *bunched,* and positive regulators of *Attacin*.[11,47,48] Interestingly, a tool recently developed to identify orthologs between flies and humans indicates that 68% of the genes identified from this array have human homologs, suggesting that these genes may inform the field about human sleep regulation and adaptation to sleep deprivation.[49] In fact, transcripts for several genes identified on the microarray, including *Amylase*, *malic enzyme*, *cheerio*, and the human homologue of *bunched* (*Tsc22*) are also known to be modulated by sleep deprivation in humans.[44,48,50,51]

Because starvation is not an uncommon occurrence in nature, it is likely that the underlying molecular mechanisms mediating the effects of starvation on the response to sleep loss have been subjected to evolutionary forces. In fact, it has been suggested that animals able to remain alert and vigilant during periods of starvation may have a selective advantage over animals that accrue sleep debt at a normal rate.[52,53] With this in mind, we expect that comparing waking induced by sleep deprivation to waking induced by starvation may expedite the identification of genes whose role in sleep regulation may be evolutionarily conserved and perhaps more related to mechanisms regulating sleep in humans. Our results have identified 84 genes, including channels, immune, signaling, peptide breakdown, and transcription factors, among many other types of functions. Similar types of transcriptional changes have been identified in other microarray studies.[47,54–57] In each instance, many of the specific genes do not overlap but the broader categories are consistent between studies, implicating those pathways in these processes.

We chose to focus on lipid metabolism genes to validate the microarray results because lipids play a number of roles

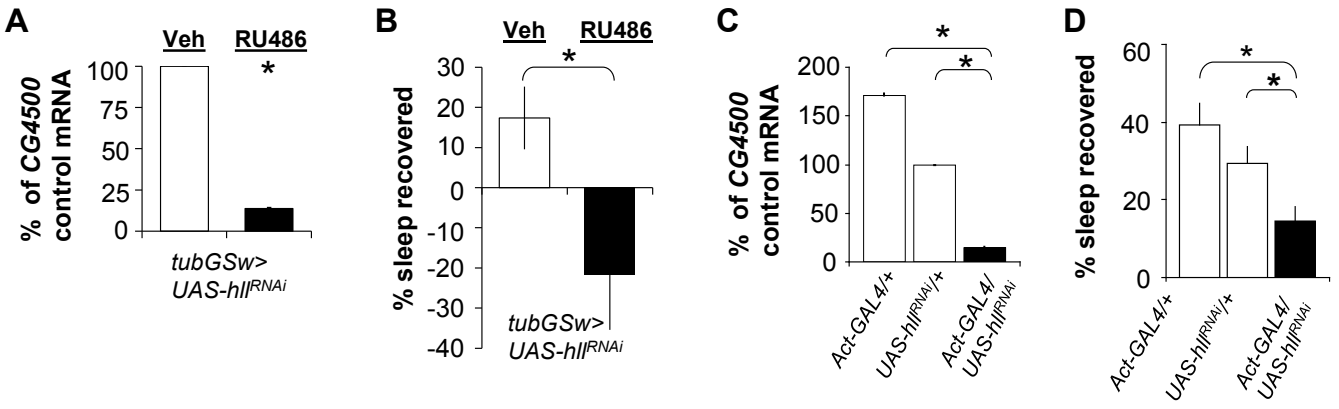


Figure 5—*hll* mutants exhibit reduced sleep homeostasis. **(A)** Levels of *hll* messenger RNA (mRNA) from *tubGS* > *hll*^{RNAi} induced with RU486 are decreased compared to sibling flies uninduced using vehicle control. mRNA is expressed as a percentage of vehicle control (n = 5 flies/group). *P < 0.05 by Student *t*-test. **(B)** Sleep homeostasis is reduced when *tubGS* > *hll*^{RNAi} are induced with the drug mifepristone (RU486, n = 28) compared to when the flies are treated with the vehicle control (veh, n = 28). **(C)** mRNA for *hll* was reduced in whole heads in *Actin-Gal4/UAS-hll*^{RNAi} flies compared to parental lines (*Actin-GAL4/+* and *UAS-hll*^{RNAi}*/+*). mRNA levels were normalized to *UAS-hll*^{RNAi}*/+*; n = 20 heads/group. *P < 0.05 by planned comparison Student *t*-test in indicated comparison. **(D)** Sleep homeostasis is reduced in *Actin-Gal4/UAS-hll*^{RNAi} (n = 66) compared to *Act-GAL4/+* (n = 39) and *UAS-hll*^{RNAi}*/+* (n = 66) parental lines. One-way analysis of variance $F_{2,168} = 7.51$; *P < 0.01 modified Bonferroni Test. *P < 0.01 by Student t-test.

throughout the cell, including energy production, membrane maintenance, and signaling properties.[58] Despite the fact that the first gene to be associated with sleep deprivation in flies was fatty acid synthase (*Fas*),[1] and subsequent microarray studies in flies and mammals have identified lipid metabolism genes as being modulated by behavioral state,[44,55,56] the relationship between sleep loss and lipid processing remains poorly understood. Given the diversity of roles that lipids play in the cell, the precise role of lipid metabolism in sleep regulation is likely to be complex. For example, modest increases in the expression of brain-type fatty acid binding protein (Fabp7) disrupt sleep whereas larger increases in Fabp7 expression result in both an increase in sleep and enhanced memory consolidation.[59] In rodents, a deficiency in short-chain acyl-CoA dehydrogenase causes a slowing in theta frequency during paradoxical sleep without altering other sleep states or modulating the power of other oscillations.[19] Quantitative genetic analysis of sleep in flies suggests a relationship between energy stores and total sleep time.[60] However, as noted by the authors, the relationship between lipid stores and sleep time is likely to be dependent on how these stores are used rather than just their presence.

Interestingly, the lipid metabolism genes we have identified primarily influence sleep homeostasis while having little, if any, effect on baseline sleep time[11] (Table S3). Thus, mutations in the previously characterized *bmm* mutant (which reduce lipolysis and block fatty acid release) result in a substantially increased sleep rebound following a night of sleep loss whereas mutations in *Lsd2* (that exhibit an increase fatty acid release) do not respond to sleep deprivation with either a sleep rebound or deficits in cognitive performance.[11] In this study, *bgm* and *hll* display an opposite response to sleep deprivation, though they both encode for acyl-CoA synthetases. Though male *bgm* mutants exhibit a prominent lipid phenotype, both male and female *bgm* mutants exhibit a neurodegenerative phenotype.[36] These results, in combination with the sequence homology to proteins in humans and mice that show functional long chain fatty acid (LCFA) and VLCFA acyl-CoA synthase activity, indicate that *bgm* mutant animals suffer from a lipid defect.[61–63] *Hll* currently does not have any published information on its specificity. Although the underlying mechanisms are not entirely clear, individual acyl-CoA synthetases are selective for FFA based on carbon chain length and they achieve additional specificity by cellular and subcellular localization.[64,65] In fact, the expression pattern of these two genes is different according to Fly–FISH and BDGP expression pattern projects.[66,67] Moreover, as a VLCFA transporter, *bgm* is likely active in the peroxisome whereas *hll* is likely active at the lipid droplet.[36,65] These localization, developmental, and functional differences may account for the distinct responses to sleep deprivation. Unfortunately, we did not detect reductions in specific lipids upon RNAi knockdown of *hll* using lipidomics. Rather, the knockdown of *hll* resulted in the general reduction of all lipid species assayed. Therefore, future studies will be required to determine if the modulation of specific lipids are responsible for either enhancing or slowing the negative effects of sleep loss.

The interaction between sleep and metabolism has regained attention in recent years.[68–71] For example, both insufficient sleep and sleep fragmentation correlate with increased body mass index (BMI).[6,72] The increased BMI may be driven by a reversal of satiety hormones[73] which could explain the observation that sleep deprived subjects eat more calorically dense snacks[16,17,71,74] or potentially through increases in reactive oxygen species.[75] Moreover, sleep deprivation alters molecular signaling in human adipose tissue, increases plasma nonesterified fatty acids and increases ketone bodies.[76–77] Thus, inadequate sleep appears to alter the way that energy stores are used. Changes in metabolism are also known to alter sleep. That is, starvation induces waking in multiple species.[11,13–15] In addition, mutations in lipid metabolism genes, such as *Lsd2*, have previously been shown to alter the sensitivity to sleep

deprivation.[11] Mutations in *Lsd2* result in an increase in lipolysis which is predicted to increase energy availability.[78,79] Interestingly, the increased presence of adenosine triphosphate and adenosine derived from neuronal activity and energy expenditure has been hypothesized to increase sleep drive.[20,80,81] Thus, although the precise molecular mechanism translating metabolic signals into sleep need are not known, factors resulting from lipid metabolism are well situated to translate metabolic signals to sleep regulatory centers.

It is commonly recognized that sleep deprivation studies are correlational in nature: one applies a stimulus to keep animals awake and then measures a particular outcome.[82,83] The outcome may be either due to the direct effects of sleep loss or the method used to keep the animal awake. Even if a confounding role of the stimulus used to keep the animal awake is excluded, it remains possible that the animal is forced to initiate adaptive mechanisms to defend against the sleep deprivation-induced impairment, and as a consequence, the adaptation rather than the direct effects of sleep loss *per se* may be primarily investigated.[83] With this in mind, an advantage of evaluating the effects of sleep loss in the fly in general, and cyc^{01} mutants in particular, is the rapidity to which sleep deprivation results in negative outcomes in these animals.[2,11,12,45,46,84] Given the speed with which deficits accrue, especially in cyc^{01} mutants, it is unlikely that they are able to mount an adaptive response of sufficient magnitude to mask the underlying pathology. If this proves to be the case, then the genes on our microarray that respond to sleep loss may be particularly interesting for elucidating the mechanisms associated with the negative effects of sleep loss. Similarly, flies also respond very rapidly to the absence of food and can sustain extended periods of waking without accruing adverse outcomes. Thus, the genes that are quickly modulated during waking induced by starvation may be particularly useful for understanding how to offset or slow the harmful effects sleep loss. Nonetheless, there is unlikely to be a magic bullet that can be used to protect animals from the effects of sleep deprivation. Indeed, we have previously shown that polymorphisms in a gene that confers resilience to sleep loss also increase the vulnerability of those animals to starvation.[53] Thus, although these results indicate that lipids can be either positive or negative regulators of sleep homeostasis, it remains unclear how sleep regulatory centers process this information along with circadian and homeostatic signals, to adjust their outputs to match sleep need with environmental demands.

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SUPPLEMENTAL MATERIAL

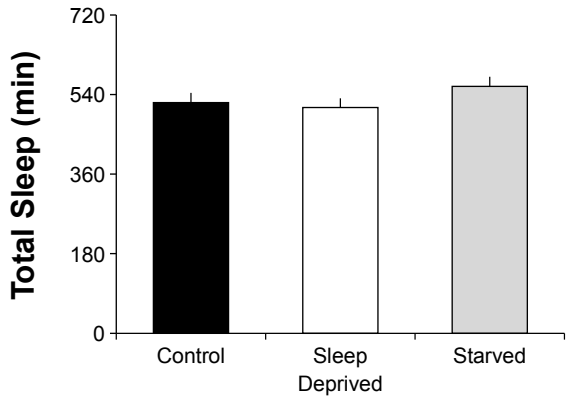

Figure S1—Baseline sleep is similar in *cyc*^{01} flies prior to being sleep deprived, starved or serving as controls. One-way ANOVA for condition $F_{2,766}$ = 1.3; P = 0.26. No significant differences were found between conditions using a modified Bonferroni Test.

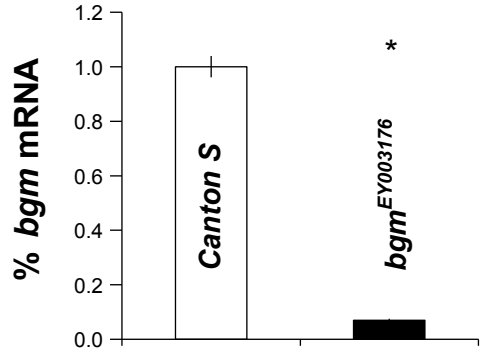

Figure S2—*bgm* RNA levels in wildtype (*Canton S*) and the *bgm* mutant (*bgm*^{EY03176}). *P < 0.05.

Visit www.journalsleep.org to download the supplemental tables Tables S1–S3 (Microsoft Excel format).