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## Identifying sleep regulatory genes using a *Drosophila* model of insomnia

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### Abstract

Although it is widely accepted that sleep must serve an essential biological function, little is known about molecules that underlie sleep regulation. Given that insomnia is a common sleep disorder that disrupts the ability to initiate and maintain restorative sleep, a better understanding of its molecular underpinning may provide crucial insights into sleep regulatory processes. Thus, we created a line of flies using laboratory selection that share traits with human insomnia. After 60 generations *insomnia-like* (*ins-I*) flies sleep 60 min a day, exhibit difficulty initiating sleep, difficulty maintaining sleep, and show evidence of daytime cognitive impairment. *ins-I* flies are also hyperactive and hyper responsive to environmental perturbations. In addition they have difficulty maintaining their balance, have elevated levels of dopamine, are short-lived and show increased levels of triglycerides, cholesterol, and free fatty acids. While their core molecular clock remains intact, *ins-I* flies lose their ability to sleep when placed into constant darkness. Whole genome profiling identified genes that are modified in *ins-I* flies. Among those differentially expressed transcripts genes involved in metabolism, neuronal activity, and sensory perception constituted over-represented categories. We demonstrate that two of these genes are upregulated in human subjects following acute sleep deprivation. Together these data indicate that the *ins-I* flies are a useful tool that can be used to identify molecules important for sleep regulation and may provide insights into both the causes and long-term consequences of insomnia.

### Keywords

*Drosophila*; behavior; sleep; insomnia; learning; genomics

### Introduction

Sleep has been observed in all mammals, birds, reptiles and invertebrates in which it has been thoroughly investigated (P. J. Shaw and P. Franken, 2003; R. Allada and J. M. Siegel,

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#### Author Contributions

LS designed the research, performed research, analyzed data and wrote the paper; YS LG SI SPD all performed research. MT performed research and wrote the paper. PS designed the research, performed research, analyzed data and wrote the paper. All authors commented on the manuscript.

2008). In mammals, sleep is not regulated from a single brain center or by a solitary neurotransmitter (C. B. Saper et al., 2005). Rather sleep is modulated by a variety of neurochemicals operating through multiple neuronal systems distributed throughout the neuraxis (A. A. Borbely and I. Tobler, 1989; F. Obal, Jr. and J. M. Krueger, 2003). Given the complexity of sleep regulation, it is not surprising that sleep disorders are highly prevalent in the general population. Although sleep disorders are believed to have a strong genetic component, it is rare for these disorders to be attributed to single gene defects (M. Tafti et al., 2005). Thus, sleep is a complex behavior that is regulated by many genes and their interactions.

This principle of a distributed sleep regulatory network also appears to be true for the genetic model organism *Drosophila melanogaster* (W. J. Joiner et al., 2006; J. L. Pitman et al., 2006; K. Foltenyi et al., 2007; V. Sheeba et al., 2008). Moreover, sleep in flies is also influenced by a variety of molecular pathways (P. J. Shaw et al., 2002; R. Andretic and P. J. Shaw, 2005; C. Cirelli et al., 2005; K. Kume et al., 2005; K. Foltenyi et al., 2007; K. Koh et al., 2008). Interestingly, a closer inspection of large populations of wild-type flies reveals that many individuals display disrupted sleep (R. Andretic and P. J. Shaw, 2005; L. Seugnet et al., 2008). As with humans, it is likely that the changes in sleep regulation observed in these individuals is due to modifications in many genes which may also increase the vulnerability of these individuals to sleep disruption (e.g. gene  $\times$  environment interactions). Thus, populations of wild-type flies naturally possess sufficient genetic variability to induce complex sleep phenotypes.

Recently, several labs have used laboratory selection as an efficient strategy to identify genes that underlie complex behavior (H. A. Dierick and R. J. Greenspan, 2006; A. C. Edwards et al., 2006; J. G. Sorensen et al., 2007). Given that insomnia is a highly prevalent, naturally occurring condition that disrupts the ability to initiate and maintain restorative sleep, a better understanding of its molecular underpinning may provide crucial insights into sleep regulatory processes. Thus, we have used laboratory selection to create a unique line of flies that share many traits with human insomnia and have identified genes that are both important for sleep regulation and may provide insights into the causes and long-term consequences of insomnia.

## Methods

### Fly stocks, sleep monitoring and sleep deprivation

*Cs* flies were obtained from the Bloomington *Drosophila* stock center. *ins-1* flies used for behavioral and molecular evaluation were obtained from generation 65 to 85 of the selection. Flies were cultured at 25°C, 50 to 60% humidity, in a 12hr:12hr Light:Dark cycle, on a standard food containing yeast, dark corn syrup, molasses, dextrose and agar. Newly eclosed female adult flies were collected from culture vials daily under CO<sub>2</sub> anesthesia. Three day old flies were then individually placed into 65mm glass tubes so that sleep parameters could be continuously evaluated using the Trikinetics activity monitoring system as previously described (P. J. Shaw et al., 2000). Flies were sleep deprived using an automated sleep deprivation apparatus that has been found to produce waking without nonspecifically activating stress responses (P. J. Shaw et al., 2002). The Sleep Nullifying Apparatus (SNAP) tilts asymmetrically from -60° to +60° such that sleeping flies are displaced during the downward movement 6 times per minute. This stimulus is effective presumably because it initiates a geotactic response. The SNAP effectively eliminates all sleep (P. J. Shaw et al., 2000). Thus, 100% of the sleep that would normally occur during stimulation is abolished. Flies were sleep deprived using the SNAP from ZT 12 (beginning of the dark phase) to ZT 0 (beginning of the light phase). Unless otherwise stated, at least 32 flies were analyzed for each experimental condition. Differences in sleep time were assessed

using either a Student's t-test or Analyses of variance (ANOVA) which were followed by planned pair-wise comparisons with a Tukey correction.

### Locomotor rhythms

Flies were individually placed into 5×65mm tubes with regular food and placed into constant conditions for 10 days. Locomotor activity was continuously recorded in 30min bins using the Trikinetics system. Locomotor rhythms were analyzed for 6 or 9 days using MATLAB based computational tools designed by J. Levine (J. D. Levine et al., 2002). Flies that did not show a significant peak on the periodogram were considered arrhythmic. At least 30 flies were analyzed for each genotype.

### Falls

5 flies aged 4–5 days and placed in 5×65mm tubes were simultaneously observed by one experimenter during 30 min. Experimenters were blind to the condition. Data obtained with two different experimenters and two generations of *ins-1* flies was similar and pooled for the analysis.

### Learning

Learning was evaluated using Aversive Phototaxic Suppression (E. Le Bourg and C. Buecher, 2002). Flies are individually tested in a T-maze where they are allowed to choose between a dark and a lighted vial. Adult flies are phototaxic and choose the lighted alley in the absence of reinforcer. During the test a filter paper soaked with a quinine solution is placed in the lighted vial to provide an aversive association. In the course of 16 trials through the maze flies learn to make more frequent choices to the dark vial (photonegative choices). The number of photonegative choices is tabulated during 4 successive blocks of 4 trials and the performance score is the % of photonegative choices made in the last block of 4 trials. At least 8 flies were evaluated for each condition. For each experiment, learning was evaluated by the same experimenter who was blind to genotype and condition. All flies were tested in the morning between ZT0 and ZT4. Flies were sleep deprived using the SNAP from ZT 12 (beginning of the dark period) to ZT 0 (beginning of the light period) and until each flies was tested for learning. Learning scores are normally distributed (L. Seugnet et al., 2008). Differences between scores were assessed using either a Student's t-test or Analyses of variance (ANOVA) which were followed by planned pair-wise comparisons with a Tukey correction. Photosensitivity was evaluated using the T-maze with no filter paper. The average proportion of choices to the lighted vial during 10 trials was calculated for each individual fly. The phototaxis index (PI) is the average of the scores obtained for at least 5 flies ±SEM. Sensitivity to quinine/humidity was evaluated as in (E. Le Bourg and C. Buecher, 2002) with the following modifications: each fly was individually placed in a 14 cm transparent cylindrical tube covered with filter paper, uniformly lighted and maintained horizontal. In one half of the apparatus the filter paper is soaked with quinine solution while the other half is kept dry. The quinine/humidity sensitivity index (referred to as Quinine Sensitivity Index or QSI) was determined by calculating the time in seconds that the fly spent on the dry side of the tube when the other side had been wetted with quinine, during a 5 min period.

### Lifespan

Three day old flies were individually placed into 5×65mm tubes and sleep was monitored until death. Food was replaced every 4 days. 3 independent groups of 30 flies were analyzed for each genotype. Similar results were obtained by aging 3 groups of 10 flies in regular vials and visually monitoring dead flies every day.

### Stress resistance

Three day old flies were placed in 5×65mm tubes for 2 baseline days and then placed in empty tubes (desiccation) or tubes with 1% agar (starvation) at ZT 2 and locomotor activity was monitored until all flies died. Three independent groups of 30 flies were analyzed for each genotype.

### Arousal

To evaluate arousal during the day, *Cs* and *ins-1* flies were transferred in groups of 10 to an empty vial. All flies were awake both before and following the transfer. The vial was then subjected to a rapid shock to induce geotaxis. The percentage of flies present in the top half of a 50ml tube was calculated following 5, 10, 15, 30, 60 and 120-s. To evaluate responsiveness during the flies primary sleep period, we exposed *Cs* and *ins-1* flies to a 1 min pulse of light at CT-15; flies that were awake did not contribute to the score. The level of light was sufficiently strong to awaken all animals. The amount of sleep was then calculated for the following hour and expressed as a percentage of baseline sleep.

### HPLC

For each condition independent sets of 20 fly heads (n=4 replicates) were frozen and collected at age 5–6 days and at ZT0. Samples were sent to Dr. Raymond F Johnson, Neurochemistry Core Lab, Nashville, for HPLC analyses.

### Lipid analysis

10–15 whole flies were collected for each condition (ZT0). Samples were frozen at –80C and homogenized in a 2:1 methanol:chloroform solution for lipid extraction. Lipid content was evaluated using colorimetric assays according to kit manufacturer's instructions. Triglycerides and cholesterol were evaluated with the Infinity kit (Thermo Electron), and non-esterified free fatty acids with the NEFA C kit (Wako). Lipid measurements were normalized to the initial weight of the flies. (n=3 replicates).

### QPCR

Total RNA was isolated from sets of 20 fly heads with Trizol (Invitrogen, Carlsbad, California) and DNase I digested. 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* a gene which expression is not changing upon sleep deprivation (P. J. Shaw et al., 2002; L. Seugnet et al., 2008). 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.

### Microarray Gene Profiling

Three groups of 20 short sleeping *ins-1* flies (criteria: total daily sleep < 60min, daily sleep average: 16±5min) and three groups of *Cs* flies (daily sleep average: 725±5min) were collected at age 5–6 days. Total RNA was extracted from whole heads using the Trizol protocol (Invitrogen, Carlsbad, CA, USA) and processed for cDNA synthesis and cRNA amplification according to the Affymetrix protocol. Over-represented GO categories were identified using the GOToolBox software (D. Martin et al., 2004) with the hypergeometric statistical method. The following criteria were used to select over-represented GO categories shown in Figure 5A: 1) a representation increased at least 2 fold in the experimental set compared to the reference; 2) at least 20 genes present in the given category in the experimental data set; and 3) over-represented with a p<0.01 after false discovery rate correction.

## Human samples

Nine healthy human adult volunteers (seven men and two women) were enrolled in the study after providing their consent. The sleep protocol was carried out at the Sleep Medicine Center, Department of Neurology, Washington University School of Medicine and was approved by the Institutional Review Board at Washington University School of Medicine. The subjects were randomly separated in two groups, which were scheduled to alternate 2 weekends of either normal sleep or 28 h of continuous waking. On the normal-sleep weekend, the volunteers were allowed to fall asleep at 10:00 p.m. Normal sleep architecture was confirmed by standard polysomnography. The SD group remained awake and was allowed free access to water during the night. Saliva was collected from plain (noncitric acid) cotton Salivettes (Sarstedt, Newton, NC), rapidly frozen over dry ice and kept at  $-80^{\circ}\text{C}$  until assayed. RNA was isolated from cell-free supernatant and processed for QPCR as described (L. Seugnet et al., 2006). Control experiments removing either mRNA or reverse transcriptase from the reaction failed to result in amplification (data not shown) indicating that the signal was mRNA and not due to contamination. Results were evaluated using the Wilcoxon signed rank test.

## Results

### Isolation of flies presenting insomnia-like traits using laboratory selection

Evaluation of a normative dataset of wild-type *Canton-S* (*Cs*) flies indicates that they display a sufficient range of sleep times and activity levels to make them suitable for laboratory selection (Figure 1A, black bars). In humans, insomnia starting earlier in life is believed to be under greater genetic influence than insomnia that is associated with aging (N. F. Watson et al., 2006). Thus, sleep was evaluated in three day old male and female *Cs* flies for 4 days according to standard protocols (P. J. Shaw et al., 2000). For each generation, we identified 8–12 individual male and female flies that simultaneously demonstrated reduced sleep time in combination with increased sleep latency, reduced sleep bout duration, and elevated levels of waking activity (*insomniac-like* flies, referred to as *ins-I* flies). These flies were then bred over successive generations. As seen in Figures 1B,C, total sleep time was progressively reduced during selection and stabilized after ~30 generations. Although sleep time was reduced both during the day and night, the increase in total-wake time in selected flies came primarily at the expense of night time sleep (Figure 1C). While total sleep time averaged 100 minutes or less by generation 65 (Figure 1D), ~50% of *ins-I* flies obtained less than 60 minutes of sleep in a day. Moreover, the distribution of sleep times was shifted dramatically to the left (Figure 1A, white bars). Direct observation of 14 h of fly behavior revealed that all the *ins-I* flies were indeed awake and active with very few episodes of quiescence (see also supplemental video1 for a comparison of *ins-I* and *Cs* flies).

In humans, primary insomnia is a chronic condition that may persist for 10 years (C. Janson et al., 2001). Thus we evaluated sleep in *ins-I* flies over their lifespan. Sleep remains disturbed in *ins-I* flies over time (Figure 1E). Total sleep for three representative *ins-I* flies and one *Cs* fly are shown in Figure 1E. These three *ins-I* flies obtained a total of  $358 \pm 128$  minutes of sleep during their first 20 days of life vs.  $17,567 \pm 655$  min over the same time period in *Cs* flies. Importantly, the shortest sleeping *ins-I* flies do not become longer sleepers or vice versa. To assay for dominant effects, we evaluated sleep in heterozygous *ins-I/+* flies and found an intermediate sleep phenotype indicating the effects are semi-dominant (Figure 1F).

In addition to selecting for short-sleeping flies, we also selected for flies with an increased sleep latency, disrupted sleep consolidation, and hyperactivity. As seen in Figure 2A, *ins-I* flies show increased latency from lights off to the first sleep bout of the night, suggesting



that they have difficulty initiating sleep (R. Andretic and P. J. Shaw, 2005). *ins-1* flies also have difficulties maintaining sleep as evidenced by an inability to consolidate sleep into long bouts (average sleep bout duration, Figure 2B)(R. Andretic and P. J. Shaw, 2005). Indeed, the maximum episode of consolidated sleep that can be generated by an *ins-1* fly is only  $36 \pm 9$  min vs.  $257 \pm 22$  min in *Cs* flies. Although *ins-1* flies exhibit fragmented sleep, they do not compensate by initiating more sleep episodes, ( $6.8 \pm 0.8$  bouts vs.  $10.4 \pm 0.2$  bouts for *ins-1* and *Cs* flies respectively). Since *ins-1* flies do not compensate by initiating more sleep episodes, it is possible that they are simply short-sleepers with a reduced need for sleep. Interestingly, *ins-1* flies exhibit a normal homeostatic response after 12 h of sleep deprivation indicating that they are able to compensate for acute sleep loss (Figure 2C). However human insomniacs also respond to acute sleep loss (W. R. Pigeon and M. L. Perlis, 2006) and thus homeostasis cannot distinguish between short-sleep and insomnia. In humans, insomnia is associated with increased daytime sleepiness(J. D. Edinger et al., 2008). To determine whether *ins-1* flies also exhibit daytime sleepiness we evaluated *Amylase* transcript levels using real-time quantitative PCR (QPCR). We have shown that, in flies, *Amylase* levels are only elevated following waking conditions that are associated with increased sleep drive and are not induced by stress(L. Seugnet et al., 2006). RNA was extracted from whole heads of *Cs* and *ins-1* flies that had been spontaneously awake for 3 h between Zeitgeber Time (ZT) 0 and ZT3. As seen in Figure 2D, *ins-1* flies show elevated levels of *Amylase* mRNA relative to *Cs* flies suggesting that they may experience increased sleep drive during their primary wake-period.

As mentioned above, patients with insomnia are believed to be hyper-aroused. Thus we selected for flies with increased locomotor activity during waking. As seen in Figure 2E, our selection procedure significantly increased the intensity of waking locomotor activity. The levels of activity during waking in *ins-1* flies are high during the day and during the night and these levels are not statistically different ( $p=.56$ , paired t-test). Insomniacs also show greater reactivity to stressors (C. L. Drake et al., 2008). To determine whether this would be true for *ins-1* flies as well, we exposed them to environmental perturbation during both the day and the night. As seen in Figure 2F, *ins-1* flies are more responsive than *Cs* flies to mechanical stimulation during the day. *ins-1* flies are also more responsive to perturbations at night. When *Cs* and *ins-1* flies were exposed to a 1 min light pulse during their primary sleep period at ZT-15, *Cs* flies briefly woke up in response to the light pulse but quickly went back to sleep ( $12.2 \pm 9\%$  of sleep lost during the following hour). In contrast, all the *ins-1* flies woke up and remained awake for an entire hour (100% of sleep lost,  $n=16$  flies per group,  $p<0.05$ ). Together these data suggest that *ins-1* flies are in a state of increased arousal.

### Physiological changes associated with the insomnia-like phenotype

To be considered an insomniac an individual must display daytime impairment or distress in combination with sleep disruption. Human insomniacs report that they have impaired concentration and poor memory (H. J. Orff et al., 2007). Thus we evaluated learning in *ins-1* flies using Aversive Phototaxic Suppression (APS)(E. Le Bourg and C. Buecher, 2002). In this task, flies learn to avoid a light that is paired with an aversive stimulus (quinine / humidity). We have recently shown that APS is sensitive to both sleep loss and sleep fragmentation (L. Seugnet et al., 2008). As seen in Figure 3A, learning is significantly impaired in the shortest sleeping *ins-1<sub>short</sub>* flies compared to *Cs* controls. To determine whether our selection protocol generated poor-learning flies as an independent phenotype from the observed sleep deficit, we evaluated learning in long-sleeping *ins-1* flies (*ins-1<sub>long</sub>*; Figure 1A). Importantly, longer sleeping siblings maintain their ability to learn, indicating that our selection procedure did not inadvertently create a poor learning fly. The time to complete the test, photosensitivity and quinine sensitivity (Control Metrics) were similar in

*Cs*, *ins-I<sub>short</sub>* and *ins-I<sub>long</sub>* flies indicating that the learning deficit was not due to differences in sensory thresholds (Figure 3B, supplemental table 1).

In addition to cognitive impairments, human insomniacs exhibit deficits in motor coordination as indicated by difficulty in maintaining their balance (P. J. Hauri, 1997). Thus, we evaluated the number of spontaneous falls in young age-matched *Cs* and *ins-I* flies walking for 30 minutes in an obstacle free environment. *Cs* flies rarely fall under these conditions. In contrast, *ins-I* flies frequently lose their balance (Figure 3C). Given the role of dopamine in learning and motor control, we evaluated transmitter levels in whole heads of *ins-I* flies. As seen in Figure 3D, dopamine levels are significantly elevated in *ins-I* flies (Figure 3D). Dopamine is present in the brain as well as in peripheral tissues such as the epidermis where it affects pigmentation. However, no obvious change in cuticle pigmentation is observed in *ins-I* flies. Thus the large increase in dopamine level seen in *ins-I* flies most likely reflects a significant change of the neurotransmitter in the brain. Interestingly we have found that learning impairments following acute sleep deprivation are also associated with an increase in dopamine levels (L. Seugnet et al., 2008). Levels of 5-HT and GABA, which are also associated with learning and sleep disruption, were not changed (Figure 3E–F). Together these results further implicate dopamine signaling in cognitive impairments following sleep disruption.

Accumulating evidence in humans indicates that sleep disruption may increase the risk of obesity (K. L. Knutson and E. Van Cauter, 2008). Moreover, insomnia is more frequent in obese than non-obese individuals (C. Janson et al., 2001) and common genetic effects have been observed between insomnia and obesity in a recent twin study (N. F. Watson et al., 2006). Thus we evaluated organismal levels of triglycerides, free fatty acids and cholesterol in *ins-I* flies. As with humans, *ins-I* flies exhibit increased adiposity (Figure 3G–I). These data further suggest that *ins-I* flies are getting less sleep than they need and are not simply short sleepers.

Several recent epidemiological studies have found that sleep duration and insomnia are associated with an increased risk of all-cause mortality and reduced lifespan (K. Tamakoshi and Y. Ohno, 2004). Reduced sleep has also been associated with shortened lifespan in *Drosophila* (C. Cirelli et al., 2005; J. L. Pitman et al., 2006; K. Koh et al., 2008). If our selected lines are getting less sleep than they need, one would predict that they would have a shortened lifespan. As seen in Figure 3J this is indeed the case. The reduced lifespan observed in our *ins-I* flies may be due to reduced sleep or decreased fitness resulting from inbreeding. To determine whether our *ins-I* flies exhibited reduced fitness we exposed them to starvation, desiccation, and paraquat (Figure 3K–L, paraquat data not shown). Survival curves clearly indicate that *ins-I* flies do not exhibit reduced fitness when compared to *Cs* controls. In addition, *ins-I* flies have no discernable morphological, developmental, or fertility phenotypes further indicating that they do not exhibit reduced fitness.

### Sleep in the absence of zeitgebers

The misalignment of sleep and circadian cycles can trigger or aggravate insomnia symptoms in humans (e.g. jet lag). To assess the influence of zeitgebers we evaluated sleep in *ins-I* flies placed into constant darkness (DD). When zeitgebers are removed, *ins-I* flies show a further decrease in sleep time (Figure 4A, white circles), a result not observed in *Cs* flies (Figure 4A, black diamonds). To determine whether the increased wakefulness was due to the absence of light, we placed *ins-I* flies in constant darkness but allowed temperature to maintain a circadian oscillation of  $\pm 1.0$  °C. *ins-I* flies were able to entrain to these small circadian fluctuations in temperature and sleep was not altered compared to LD (Figure 4B). To further evaluate the consequences of sleep loss induced by removing zeitgebers, we evaluated the behavior of longer sleeping *ins-I* flies. Longer sleeping *ins-I* flies were

examined to ensure that all flies would have the opportunity to lose sleep when placed into DD. Moreover, since the shortest sleeping *ins-I* flies cannot learn it is not possible to assess whether additional sleep disruption alters cognitive behavior. As seen in Figure 4C, *ins-I* flies not only lose sleep when placed into DD for 24 h, they initiate a homeostatic response when placed back into LD. In comparison *Cs* flies only lose  $7 \pm 1\%$  of their baseline sleep in DD. Interestingly, *Amylase* mRNA levels are lower in *ins-I<sub>long</sub>* flies compared to their short sleeping siblings when maintained in LD suggesting that they experience lower sleep drive (Figure 4D, left). However, when sleep is disrupted by placing *ins-I<sub>long</sub>* flies into DD *Amylase* mRNA levels increase in comparison with control *ins-I<sub>long</sub>* siblings maintained under LD (Figure 4D, right). Given the mild-nature of placing flies into constant darkness, these data are consistent with our previous results demonstrating that, in flies, *Amylase* levels are responsive to conditions of high sleep drive, do not depend upon the method used to keep the animal awake, and are not simply activated by stress. To determine whether waking induced by removing zeitgebers was associated with learning impairments we evaluated *ins-I<sub>long</sub>* flies under LD and after 5 days in DD. As seen in Figure 4E, *ins-I<sub>long</sub>* flies learn normally under LD but are significantly impaired following the additional sleep disruption induced by removing zeitgebers; learning impairments were not associated with changes in control metrics (Supplemental Table1) and 5 days of DD does not alter learning in *Cs* flies (data not shown). Thus, the absence of zeitgebers appears to interfere with the ability to obtain needed sleep in *ins-I* flies.

Is sleep loss in DD associated with a deficit in the circadian pacemaker? 70% of *ins-I* flies appear to lose locomotor rhythms in constant conditions while the remaining cycling flies have a normal 24h period (Figure 4F). The average RI was  $0.10 \pm 0.02$  for *ins-I* flies for 9 days in DD, compared to  $0.43 \pm 0.03$  for *Cs* flies. We found that RI was not correlated with either counts/waking min ( $r = 0.04$ ,  $n=30$ ) or the amount locomotor activity ( $r = -0.2$ ,  $n=30$ ). In *Drosophila*, circadian rhythms in DD are controlled by a subset of clock neurons, the small ventral lateral neurons (sLN<sub>v,s</sub>). In *ins-I* flies, sLN<sub>v,s</sub> neurons still display circadian oscillations of the Period (PER) protein, similar to *Cs* flies (Figure 4G). Normal circadian oscillations of the TIMELESS (TIM) protein were also observed in the sLN<sub>v,s</sub> of *ins-I* flies: TIM was detected in the cytoplasm at CT18, in the nucleus at CT0, and was not detected at CT6 and CT12 (data not shown). These results suggest that the sleep reduction and loss of rhythmicity observed in *ins-I* flies in constant conditions is not merely linked to a deficit of the core molecular clock.

### Gene profiling of *ins-I* flies

By using laboratory selection we generated a line of flies that exhibit a constellation of unique phenotypes that may be useful for both elucidating molecular mechanisms important for aspects of sleep regulation and that may provide insights into both the causes and long-term consequences of insomnia. However, if the behavioral changes seen in *ins-I* flies are due to changes in many genes, as is the case for other complex traits, it may not be possible to easily map them using classic genetic strategies. Moreover, since polymorphisms in regulatory regions alter expression levels without disrupting gene function (T. F. Mackay, 2004), sequencing strategies may also prove ineffective. Thus, we conducted whole-genome transcript profiling using Affymetrix high density oligonucleotide microarrays to identify genes that are modified in *ins-I* flies. To maximize the comparison between *ins-I* and *Cs* flies and to reduce variability, we monitored sleep for 3 days and identified individuals that displayed stable sleep patterns. Only those *ins-I* flies that slept less than 60 min a day for each of the three days were included in the study (average daily sleep:  $16 \pm 5$ min). *Cs* flies display a large range of sleep times (Figure 1A). To avoid combining *Cs* flies with short, fragmented sleep or flies that displayed excessively long sleep and thus might be unhealthy together with normal-sleeping healthy flies, we identified individuals whose total sleep fell



at the center of the distribution of daily sleep (average daily sleep:  $721 \pm 5\text{min}$ ). Sleep was evaluated in three independent groups of *ins-1* and *Cs* flies on separate days. RNA was collected from whole heads of flies that had been spontaneously awake for 3 h at CT3; 3h of spontaneous waking is sufficient to change gene expression in rodents and flies (P. J. Shaw et al., 2000; C. Cirelli et al., 2004). Statistical differences were identified first using the Cyber-T Bayesian statistical framework followed by Bonferroni correction (<http://cybert.microarray.ics.uci.edu/>) (P. Baldi and A. D. Long, 2001). In addition, the data were independently analyzed using Partek Genomics Suite with a False Discovery Rate test correction set to  $p < 0.01$  (Partek, St. Louis, MO). Only those genes that were identified as being significant using both Cyber-T and Partek were considered further. Approximately 1350 genes are differentially expressed in *ins-1* flies, 755 of which were found to have homology with human genes using the Genomic Information for Eukaryotic Organisms Database ([www.eugenics.org](http://www.eugenics.org)). The mean, SEM, fold change, and both the corrected and non-corrected p-values for all 1350 genes can be found in Supplemental Table 2.

To further evaluate the 1350 genes that were differentially expressed between *Cs* and *ins-1* flies, we conducted a Gene Ontology (GO) analysis using GOToolBox software (D. Martin et al., 2004) with the hypergeometric statistical method followed by False Discovery Rate correction. As seen in Figure 5A, over-represented GO categories include Metabolism, Neuronal activity, Behavior and Sensory Perception. Changes in genes associated with lipid metabolism and synapse transmission are consistent with the increased adiposity and learning deficits observed in *ins-1* flies. Importantly, the overrepresentation of genes associated with sensory perception suggest the possibility that hyperarousal in insomniacs may be due to increased activity within sensory systems and that these processes may be under genetic control.

The genes listed in Supplemental Table 2 were identified from three experimental replicates collected on separate days. Given that the expression data was further analyzed using two independent statistical approaches with extremely conservative adjustments for multiple tests, we believe the results to be reliable. Nonetheless, we collected a fourth independent replicate of the shortest sleeping *ins-1* flies and normal sleeping *Cs* flies for confirmation using QPCR. As above, flies were monitored for 3 days to ensure that sleep was stable and the flies were collected at CT3. Genes were rank ordered by p-value and QPCR confirmation was conducted on candidate genes that fell within the top 0–5%, 5–50% and bottom 50% of all genes. As seen in Figure 5B, we successfully confirmed expression changes across all levels of significance providing further validation that the identified genes are modified in *ins-1* flies.

If these genes are associated with the insomnia phenotypes, their expression should persist even when *ins-1* flies are placed into a different genetic background. Although we do not yet have a genetic marker for the *ins-1* flies our results indicate that *ins-1* is dominant (Figure 1F). Thus, *ins-1* flies were out-crossed to normally sleeping *w<sup>1118</sup>* flies. After each cross, white-eyed progeny that displayed short-sleep, short sleep bouts, and hyperactivity were identified and then crossed again to normal-sleeping *w<sup>1118</sup>* flies. This was repeated 5 times creating a new stock, *ins-1<sup>w</sup>*. *ins-1<sup>w</sup>* flies were then divided into two groups and selected again for 20 generations: one group was re-selected to amplify *insomnia-like* phenotypes while the other was back-selected to obtain normal sleeping flies (*ns<sup>w</sup>* flies, Supplemental Figure 1). Sleep time was modestly increased in *ins-1<sup>w</sup>* flies immediately after the outcross but returned to typical *ins-1* levels within three rounds of selection and remained stable thereafter indicating that the critical loci were maintained in this new line (data not shown). Importantly *ins-1<sup>w</sup>* flies exhibited all of the phenotypes found in *ins-1* flies. After the re-selection we performed real-time quantitative PCR (QPCR) on head RNA extracts obtained from *ins-1<sup>w</sup>* and normal sleeping *ns<sup>w</sup>* flies for a subset of the genes shown in Figure 5B. As

above, flies were monitored for 3 days to ensure sleep stability and RNA was collected at CT3. As seen in Supplemental Figure 1B, the expression changes seen in *ins-I<sup>W</sup>* vs. *ns<sup>W</sup>* are similar to that observed in *ins-I* vs. *Cs* flies. Thus, genes associated with insomnia can be identified in at least two different genetic backgrounds.

### Genes differentially regulated *ins-I* flies are modulated by sleep loss in humans

An important issue about *Drosophila* sleep research is whether it has relevance for human sleep research. We have previously shown that *Amylase*, a biomarker of sleepiness in *Drosophila*, is upregulated in human saliva following sleep loss (L. Seugnet et al., 2006). *Amylase* is not responsive to stress in flies and is only transiently elevated by stress in humans (N. Takai et al., 2004; L. Seugnet et al., 2006; D. Schoofs et al., 2008). Importantly, we demonstrated that *Amylase* levels were elevated following 28 h but not 24 h of waking in humans while cortisol levels remained unchanged (L. Seugnet et al., 2006). Thus, it is possible to identify genes in human saliva that are responsive to sleep loss. Saliva contains thousands of mRNAs and recent studies have demonstrated that they can be used as diagnostic markers for human diseases (V. Palanisamy et al., 2008). To determine whether genes identified in *ins-I* flies could be modified by sleep disruption in humans; we designed primers for the human homologues of 6 genes differentially expressed in *ins-I* flies. Of these 6 genes, only 2, *malic enzyme* (homolog of *Drosophila men*) and *Filamin A* (homolog of *Drosophila cheerio*) are reliably detected by QPCR in saliva RNA extracts. *men* and *cheerio* have elevated mRNA levels in *ins-I* flies (Figure 6A). Interestingly, their human homologues are up-regulated in normal subjects after 28h of waking, indicating that both genes are modulated by sleep loss in humans and flies (Figure 6B).

## Discussion

Using artificial selection we have generated a unique line of flies that exhibit a constellation of stable and heritable phenotypes that are directly relevant for addressing fundamental questions about sleep regulation. These flies display large disruptions in sleep, and as a consequence can be useful in identifying basic mechanisms of sleep regulation. Importantly, the sleep deficits observed in *ins-I* flies are not produced by mechanical interventions. Thus these flies are well suited to evaluate the acute and long-term consequences of sleep disruption at all levels of investigation, including genetic, circuit, physiologic, and behavioral analyses. Our exhaustive characterization of *ins-I* flies indicates that they may be particularly useful for providing additional insight into mechanisms linking sleep with important physiological processes underlying metabolism, learning, and aging.

An additional and important feature of the *ins-I* flies is that the changes in behavior are unlikely to be due to changes in a single gene. The fact that 30 generations were necessary to reach a plateau in the selection process suggests that a large number of genes are involved in the *ins-I* phenotype. Interestingly, a similar number of generations (20–30) allowed the progressive selection of other complex behaviors such as learning (F. Mery and T. J. Kawecki, 2002), locomotion (K. W. Jordan et al., 2007) or aggression (H. A. Dierick and R. J. Greenspan, 2006). In addition, we selected for multiple phenotypes, each of which exhibited slow incremental changes over the course of many generations. Thus, it is unlikely that we inadvertently selected for a mutation at a single locus. Rather the *ins-I* phenotypes are undoubtedly a consequence of individuals inheriting many alleles which exert small but cumulative effects on sleep regulatory circuits (T. F. Mackay et al., 2005). This interpretation is consistent with the observation that it is rare for sleep disorders to be attributed to single gene defects (M. Tafti et al., 2005). Thus, *ins-I* flies more closely approximate conditions found in human populations further increasing the utility of these animals for the efficient dissection of sleep regulation.

We chose to create an animal model of insomnia based upon our hypothesis that sleep disorders are highly prevalent in general populations due to the inherent complexity of sleep regulation. Insomnia is among the most common of all sleep disorders. As a complaint, insomnia occurs in 30–50% of the population annually; when accompanied by daytime impairment insomnia afflicts 9–15% of the population; and when diagnosed using DSM-IV criteria the prevalence is 6% (M. M. Ohayon, 2002). Twin studies suggest that genetics may account for approximately one third of the variance in insomnia complaints (A. C. Heath et al., 1990; N. F. Watson et al., 2006). If insomnia results, in part, from predisposing trait characteristics it should be possible to amplify these traits using laboratory selection. Insomnia is defined as difficulty falling asleep, difficulty staying asleep, or poor sleep quality which is accompanied by daytime impairment or distress (J. D. Edinger et al., 2004). Moreover, patients with insomnia are hyper-aroused and show greater reactivity to stressors than good sleepers (M. L. Perlis et al., 2001; C. H. Bastien et al., 2008). With this in mind, we used artificial selection to generate flies with insomnia-like traits. Our selection created flies with a 10-fold reduction in sleep time. In addition these flies exhibited: 1) difficulty in initiating sleep as defined by increased sleep latency; 2) difficulties in maintaining sleep as indicated by an inability to maintain normal length sleep bouts; 3) hyperarousal, as measured by both an increase in locomotor activity during waking and increased responsiveness to environmental perturbations; 4) reduced lifespan; 5) decreased cognitive performance during the day; 6) evidence for increased sleep drive during the animals' primary wake period and 7) difficulty sleeping following abrupt changes to environmental zeitgebers. To our knowledge, this is the first animal model of insomnia to result in such large, stable and heritable disruptions in sleep regulation that are not brought about by experimentally induced stress.

Recently, several labs have used artificial selection coupled with whole genome arrays as an efficient strategy to identify genes that underlie complex behavior (H. A. Dierick and R. J. Greenspan, 2006; A. C. Edwards et al., 2006; K. W. Jordan et al., 2007; J. G. Sorensen et al., 2007; D. Zhou et al., 2007). Follow up studies using single gene mutations were then evaluated to demonstrate that the identified gene modified the behavior of interest in an otherwise wild-type background. One reason that no attempt was made to use genetic tools to rescue the phenotypes observed in the selected lines is that the behavior observed in these selected lines is likely due to small changes in many genes. As a consequence modifying a change in a single gene may not result in a large change in the behavior in the selected lines. Thus, no gene has yet been shown to be causally involved in producing the behavioral phenotype in the selected flies themselves. Of course the goal of the selection strategy is to find genes whose function can be generalized beyond an isolated and rare genetic line of flies. In that regard, artificial selection combined with whole genome gene profiling has successfully identified candidate genes, many of which have been shown to alter behavior using genetic strategies.

Using whole genome arrays we identified 1350 genes that are differentially expressed in *ins-I* flies compared to their genetic controls. The changes in gene expression are robust and were observed even when *ins-I* was placed into a separate genetic background. *ins-I* flies are dominant and thus we were able to follow specific insomnia-like traits over multiple crosses. The new line, *ins-I<sup>w</sup>*, display all of the characteristic traits found in *ins-I* flies including those that were not actively selected during the outcross, such as an inability to sleep following the removal of zeitgebers. The observation that 2 genes, which were identified in *ins-I* flies, are also responsive to acute sleep deprivation in humans increases our confidence that the genes identified in the microarrays are robust and useful for investigating genes involved in sleep regulation.

It is important to acknowledge that all gene profiling experiments are correlation in nature and thus identify genes that are causative for a given behavior and those that are consequence of the behavioral change (G. E. Robinson et al., 2005). Thus, for each gene identified by transcriptional gene profiling, genetic experiments are required to determine whether the gene activation/inactivation reveals a pathway that regulates sleep, that is regulated by sleep or that is part of a compensatory mechanism for sleep loss. This latter distinction is particularly relevant for the *ins-1* flies. That is, while they display substantial reduction in sleep in combination with cognitive impairments, increased adiposity and reduced lifespan, *ins-1* flies also show signs of resistance to the deleterious effects of waking. For example, a substantial number of *ins-1* flies are able to remain continuously awake for several days without dying or showing overt signs of sickness. Interestingly, the longest sleeping *ins-1* flies retain many insomnia-like traits including severely fragmented sleep. Nonetheless they retain their ability to learn in the face of this challenge while *Cs* flies with similarly fragmented sleep are learning impaired (L. Seugnet et al., 2008). Although the changes in learning may appear to be minor, they are well within the range of effect sizes observed following sleep loss in humans and rodents across a number of cognitive domains (L. A. Graves et al., 2003; D. J. Frey et al., 2004; J. Fu et al., 2007; C. Pierard et al., 2007). Together these data suggest that both sleep disruption and resilience to sleep loss are components of the genetic condition and are likely to be reflected in gene expression changes.

Although we have identified a large number of candidate genes, our extensive phenotypic characterization of *ins-1* flies directs us towards specific classes of genes and neuronal circuits. For example, one of the most highly over-represented category of genes that are differentially modulated in *ins-1* flies is those involved in sensory perception. Thus, the increased amount of waking that is observed in *ins-1* flies may be due, in part, to the continued activity of specific sensory networks during sleep. In that regard, it is interesting to note that human insomniac patients have also been hypothesized to display persistent sensory processing during sleep as indicated by increased metabolism in the thalamus (E. A. Nofzinger et al., 2004; M. Desseilles et al., 2008). Given the established role of sleep in synaptic plasticity and the learning deficits observed in *ins-1* flies it is perhaps not surprising to find that another large class of over-represented genes in *ins-1* flies is involved in cell surface signaling (80 genes) or synaptic transmission (30 genes). Interestingly, human insomniacs show deficits in specific brain structures involved in memory and behavioral flexibility (E. A. Nofzinger et al., 2004; D. Riemann et al., 2007). Similarly, specific structures involved in learning and memory, such as dopaminergic neurons or the Mushroom Bodies (MBs) may play a key role in the deficit observed in *ins-1* flies. MBs regulate total sleep time and play an important role in protecting flies from learning deficits following sleep deprivation (W. J. Joiner et al., 2006; J. L. Pitman et al., 2006; L. Seugnet et al., 2008). Thus, the MBs are likely to play an important yet complex role in the circuitry underlying insomnia. Finally, *ins-1* flies lose their ability to sleep following the removal of zeitgebers suggesting that aspects of insomnia might involve specific subsets of clock neurons. Recent studies have found that artificially increasing the electrical excitability of large ventral-lateral neurons (ILNvs), which are responsive to light, increases waking (K. M. Parisky et al., 2008; Y. Shang et al., 2008; V. Sheeba et al., 2008). Although it is not clear whether more physiologic manipulations will produce similar results, genes affecting this circuitry are of particular interest. Our data suggest that a gene can play multiple roles in the brain thereby producing an individual that exhibits evidence of both sleep disruption and resilience to sleep loss. Identifying genes that confer resilience may provide crucial insight into mechanisms of sleep regulation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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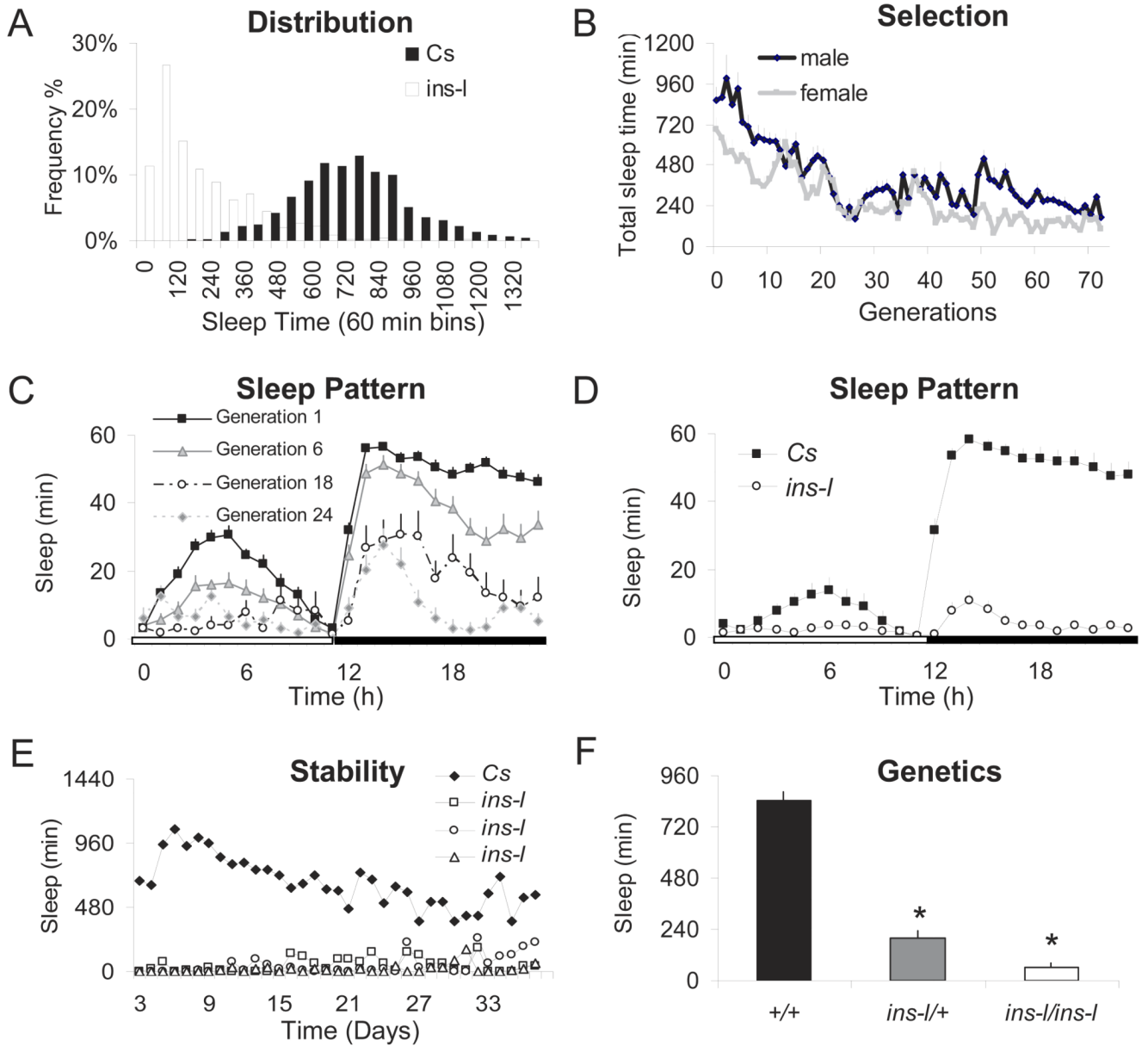
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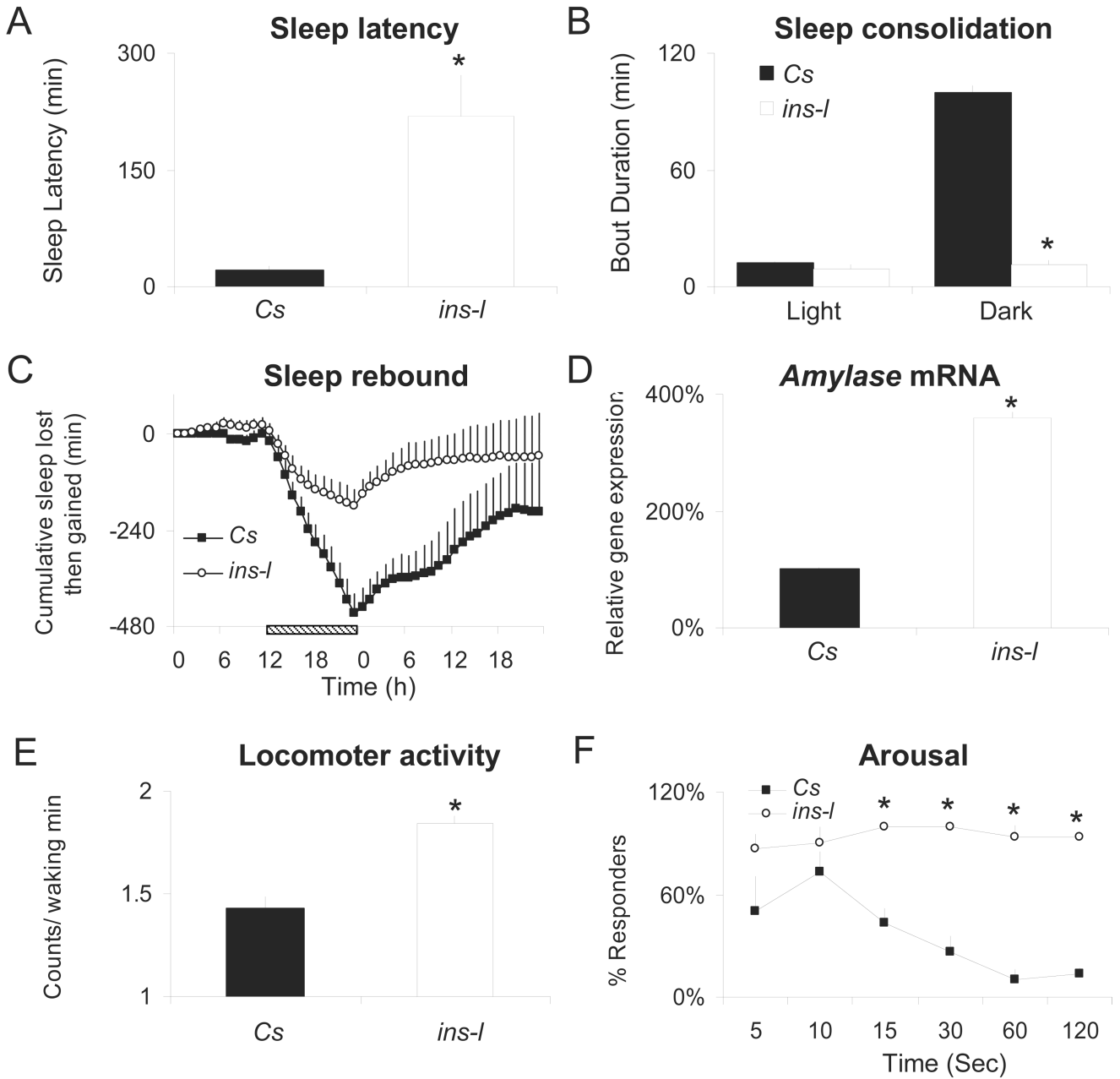
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**Figure 1. Sleep patterns of *ins-I* flies over generations**

(A) Frequency distribution of total sleep time in 60 min bins in wild-type *Cs* flies (n=1000) and in *ins-I* flies at generation 65 (n=364). (B) Total sleep time in males (n=40) and females (n=40) for successive generations of *ins-I* flies. (C) Daily sleep patterns (min/h) for progressive generations of *ins-I* flies (n≈32/generation). (D) Sleep in min/h for 24 h in *Cs* (n=32) and *ins-I* flies (Generation 65; n=32). (E) Daily total sleep time is shown for 37 days in one *Cs* and three *ins-I* flies. (F) Daily total sleep time is decreased in *ins-I/ins-I* as well as in *ins-I/Cs* flies compared to *Cs* (ANOVA  $F_{(1,89)}=5.50E+06$ .  $p<0.0005$ ). Error bars represent s.e.m

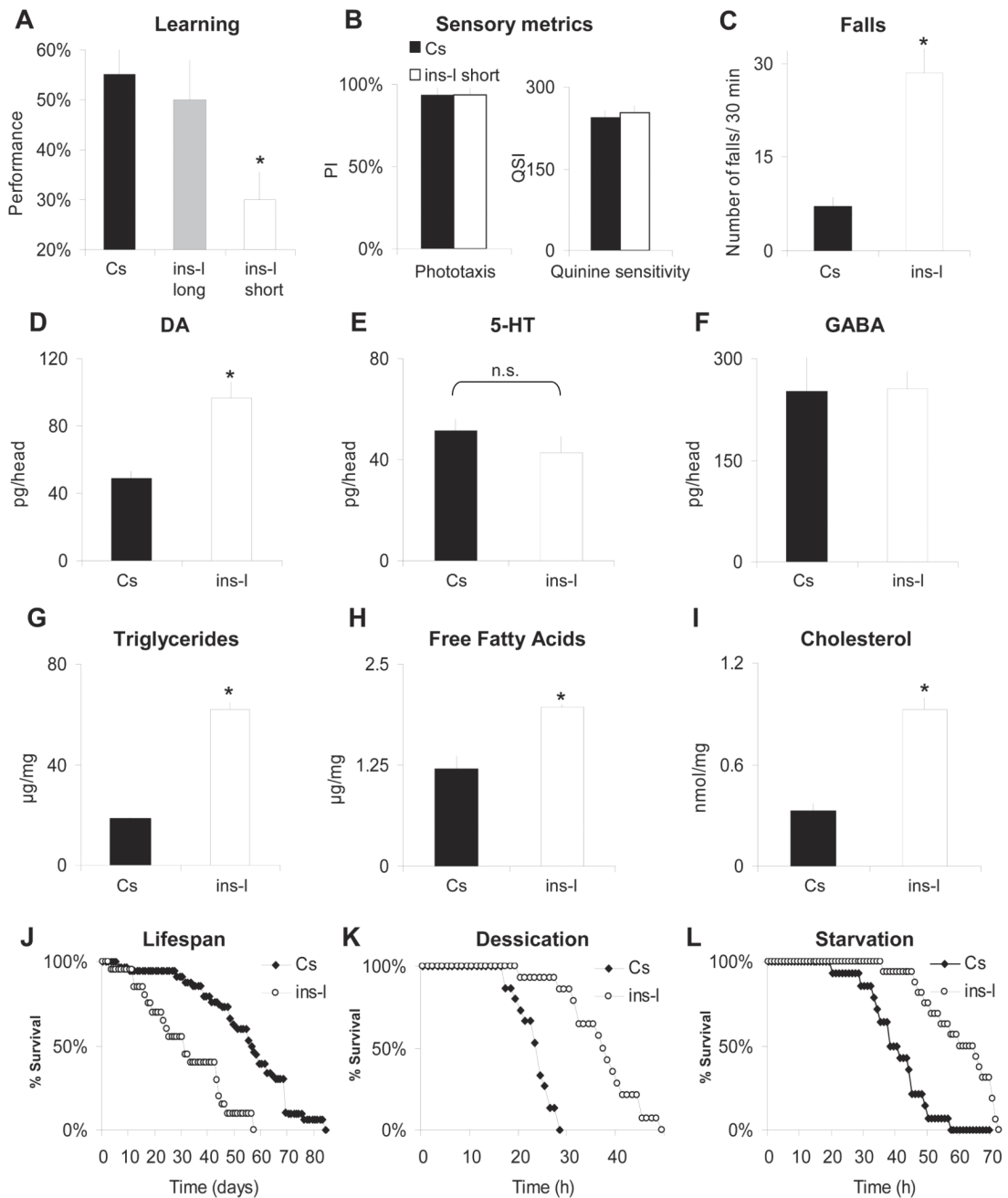


### Figure 2. Characterization of insomnia-like traits

(A) Sleep latency is increased in *ins-1* flies ( $n=28$ ) vs. *Cs* ( $n=33$ ) (\*:  $p=8.78E-08$ , one-tailed ttest). (B) Average sleep bout duration is reduced during the dark period in *ins-1* ( $n=32$ ) vs. *Cs* ( $n=32$ ) flies; ANOVA  $F_{(1,122)}=4.34E+04$ ,  $p<0.0005$ , \* $p<.05$  planned comparison with Tukey correction). (C) Cumulative sleep lost or gained during 12h of sleep deprivation and subsequent recovery in *ins-1* ( $n=32$ ) and *Cs* flies ( $n=32$ ); striped bar indicates sleep deprivation. For each hour, the amount of sleep obtained during baseline is subtracted from the respective amount of sleep time obtained during the corresponding hour of the sleep deprivation and recovery days; the difference scores are then summed across each hour to create the cumulated gained-lost plot. A negative slope indicates sleep lost, a positive slope indicates sleep gained; when the slope is zero recovery is complete. (D); *Amylase* mRNA

levels are elevated in *ins-1* flies (% of Cs expression) at ZT0-1 ( $p=0.0002$ , one-sample ttest). **(E)** Intensity of locomotor activity as measured by the counts/ waking min for 24 h in *Cs* ( $n=57$ ) and *ins-1* flies ( $n=61$ ). (\*:  $p=1.67E-10$ , two-tailed ttest). **(F)** Geotaxic response to a sudden shock is greater in *ins-1* vs. *Cs* flies. Percentage of flies present in the top half of a 50ml tube (responders). 3 groups of 10 flies were tested for each genotype (Genotype  $\times$  Time interaction  $F_{(6,24)}=4.301, p=.0006$ ). Error bars represent s.e.m

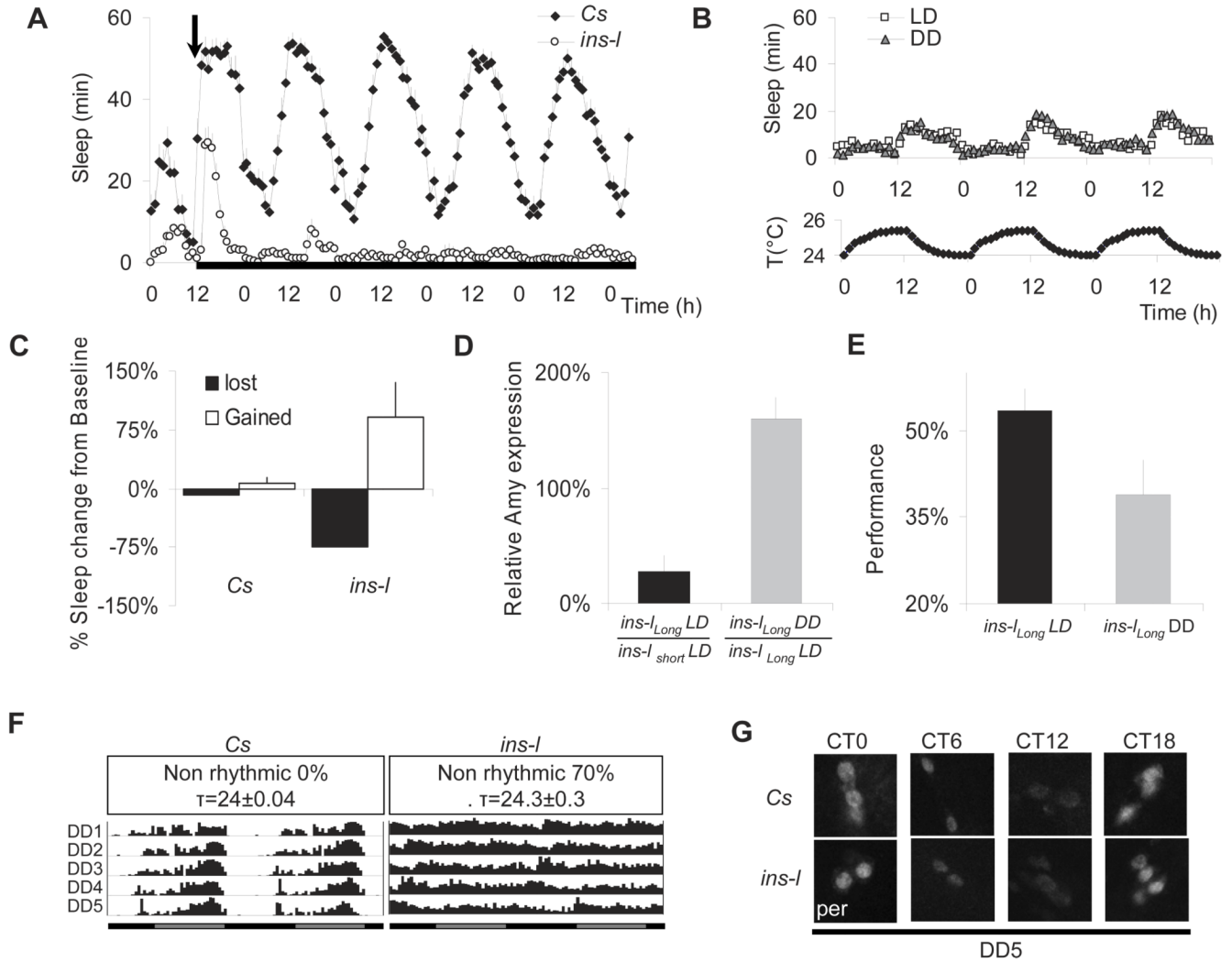




**Figure 3. Physiological changes associated with the insomnia-like phenotype**

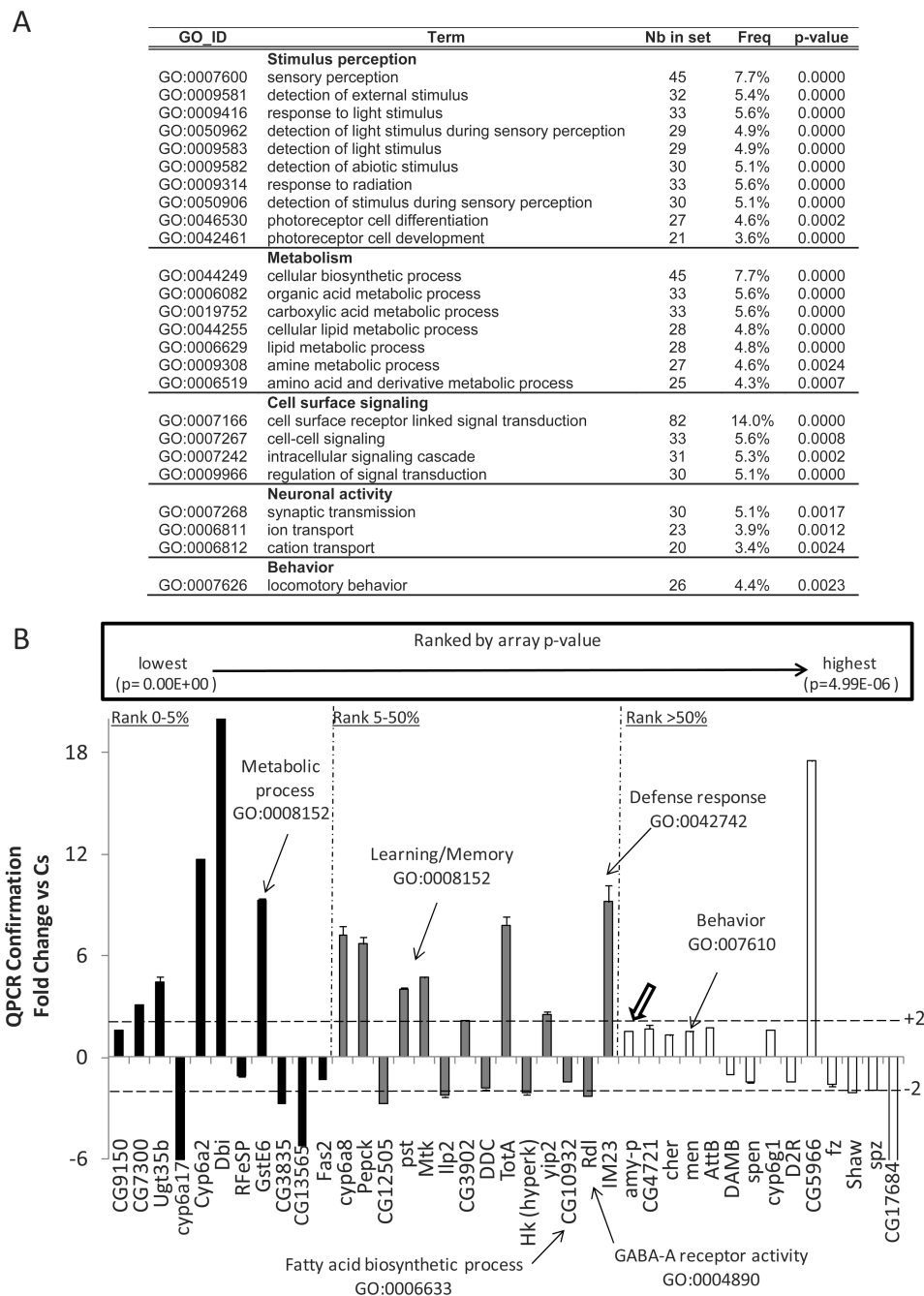
(A) Learning in *Cs* flies, longer sleeping *ins-I* flies (average daily sleep time:  $347 \pm 55$  min), and short sleeping *ins-I* flies (average daily sleep:  $26 \pm 7$  min) ( $n=10$  for each group) (\*ANOVA  $F_{(1,27)} = 10.26$ ,  $p=0.0005$ , \* $p<0.05$ , planned comparison with Tukey correction). (B) Phototaxis Index (PI) and Quinine Sensitivity Index (QSI) in *Cs* ( $n=5$ ) and short sleeping *ins-I* flies ( $n=5$ ). (C) Number of falls during 30 minutes in *Cs* ( $n=20$ ) and *ins-I* flies ( $n=18$ ) (\*:  $p<0.0001$  two-tailed ttest). (D–F) Head neurotransmitter levels in *Cs* and *ins-I* flies, as measured by HPLC ( $n=4$  replicates of 20 heads; \*:  $p=0.02$ , two-tailed ttest). (G–I), Whole body lipid content in *Cs* and *ins-I* flies ( $n=3$  replicates of 10 flies): (G) triglycerides

**(H)**, free fatty acids **(I)** and cholesterol (\*:(G), p=0.0001, (H), p=0.002, (I), p=0.01, two-tailed ttest). **(J)** Representative survival curve of aging *ins-1* compared to *Cs* flies (30 flies/group). **(K–L)**, Representative survival curves of *ins-1* and *Cs* flies after exposure to desiccation **(K)** or starvation **(L)** (30 flies/group). **(D)** Error bars represent s.e.m



**Figure 4. Sleep in the absence of zeitgebers**

(A) Baseline sleep in min/h in *Cs* and *ins-I* flies maintained under LD and following placement into DD (arrow). Black rectangle indicates lights off. (B) Upper graph: sleep in min/h in *ins-I* flies maintained in LD or in DD. Bottom graph: circadian oscillations in temperature used to entrain the flies in DD. (C) Sleep was evaluated in *Cs* and *ins-I* flies placed into DD for 24 h (Black bars) and then into LD for recovery (white bars). Sleep is expressed as a percentage of baseline sleep in LD. (D) *Amylase* levels in *ins-I<sub>Long</sub>* flies under LD (n=20) and after 24 h in DD (n=20); data are presented as % change from age matched *ins-I<sub>short</sub>* and *ins-I<sub>Long</sub>* flies respectively (\*p<.05 two-tailed ttest). (E) Learning in *ins-I<sub>Long</sub>* flies in LD (average daily sleep  $331\pm19$ min) and after 5 days in DD (average daily sleep at DD5:  $72\pm14$ min) (n=10/group; \*p=0.036, one-tailed ttest) (F). Representative actograms for single female flies from DD1 to DD5 are shown. Similar results were obtained with *ins-I* males. (G) Immunolocalization of PER protein in the small ventral lateral neurons (sLN<sub>v,s</sub>) at DD5 (single confocal sections are shown). 10 brains were evaluated for each time point, a representative example is shown. Error bars represent s.e.m

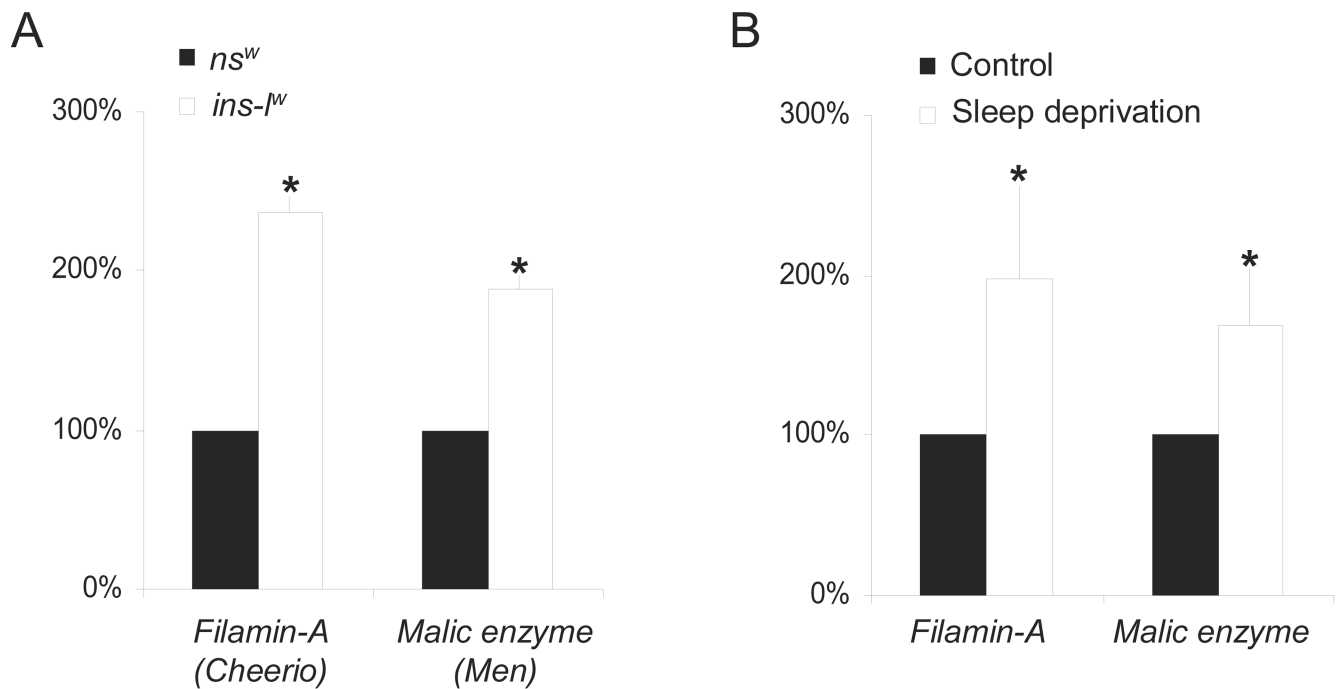


**Figure 5. Gene expression in *ins-I* flies**

(A). GO categories identified using GOToolBox software schematic. Note the overrepresentation of genes involved in sensory perception. Nb in set: number of genes differentially expressed for the corresponding the GO category. Freq: Number of genes with the corresponding GO annotation divided by the total number of genes differentially expressed. (B) QPCR confirmation of gene expression changes in short sleeping *ins-I* flies expressed as fold change from normal sleeping Cs controls. RNA was collected from all flies after 3 h of spontaneous waking at CT3. Genes were rank ordered by p-value and

confirmation was conducted on the top 5% (black), 5–50% (grey) and bottom 50% of all genes. Error bars represent s.e.m





**Figure 6. Gene differentially regulated *ins-1* flies are modulated by sleep loss in humans** (A) *Filamin-A (Cheerio)* and *Malic enzyme (Men)* and are both up-regulated in *ins-1<sup>w</sup>* flies compared to *ns<sup>w</sup>* flies. QPCR data from 20 fly heads collected at ZT0. Levels are expressed as % of *ns<sup>w</sup>* expression. (B) *Filamin-A* and *Malic enzyme* expression are elevated after 28h of waking in humans (n=8; Wilcoxon signed rank test, p=.02 and p=.059, respectively). QPCR data obtained from saliva mRNA extracts. Each saliva sample collected after sleep deprivation was compared to a circadian matched baseline sample from the same subject. Levels are expressed as % of baseline expression. Error bars represent s.e.m.