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Nat Genet. Author manuscript; available in PMC 2009 September 01.

Published in final edited form as:
Nat Genet. 2009 March ; 41(3): 371–375. doi:10.1038/ng.330.

Co-Regulated Transcriptional Networks Contribute to Natural Genetic Contribute Variation in *Drosophila* Sleep

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Abstract

Sleep disorders are common in humans, and sleep loss increases the risk of obesity and diabetes1. Studies in *Drosophila*2, 3 have revealed molecular pathways4–7 and neural tissues8–10 regulating sleep; however, genes that maintain genetic variation for sleep in natural populations are unknown. Here, we characterized sleep in 40 wild-derived *Drosophila* lines and observed abundant genetic variation in sleep architecture. We associated sleep with genome-wide variation in gene expression11 to identify candidate genes. We independently confirmed that molecular polymorphisms in *Catecholamines up* are associated with variation in sleep; and that *P*-element mutations in four candidate genes affect sleep and gene expression. Transcripts associated with sleep grouped into biologically plausible genetically correlated transcriptional modules. We confirmed co-regulated gene expression using *P*-element mutants. Genes associated with sleep duration are evolutionarily conserved. Quantitative genetic analysis of natural phenotypic variation is an efficient method for revealing candidate genes and pathways.

We recorded activity patterns2,3 of 40 inbred *Drosophila* lines11 for seven days, separately for males and females. From these patterns we calculated sleep as any period 5 minutes or longer without activity2,3, a state previously associated with reduced electrical activity in the fly brain12. We quantified the duration of sleep, number of sleep bouts during the day and night, and the number of activity counts per waking minute (waking activity), and observed significant genetic variation for all traits (Fig. 1, Supplementary Table 1). All traits except night sleep bout number were highly sexually dimorphic5, 21 – on average, males slept longer (Fig. 1a–b) and were more active when awake (Fig. 1c) than females. Females

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Author Contributions S.T.H. and T.F.C.M. conceived of the experiment. S.T.H measured sleep phenotypes in all lines and measured expression levels in *P*-element insertions. M.A.C. generated whole-genome expression data and sequenced *Catsup*. S.T.H, J.F.A, and E.A.S. analyzed the data. R.F.L. generated the 40-line reference panel. S.T.H. and T.F.C.M wrote the paper.

Additional data files and information are available at our website: mackaylab.ncsu.edu. Fly stocks are available from the Bloomington *Drosophila* Stock Center.

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tended to have more sleep bouts, and thus more disrupted sleep, than males (Fig. 1d–e). The lines varied by 13 and 10 fold, respectively, for duration of night and day sleep in females; by eight and three fold, respectively, for night and day sleep time in males; and approximately five-fold for night and day bout number in both sexes. Broad-sense heritabilities were high for all traits, ranging from 0.42 – 0.64 in the combined sex analyses. We observed considerable genetic variation in the magnitude and direction of sexual dimorphism for the sleep phenotypes, as reflected in significant line × sex interaction variance components and departure of cross-sex genetic correlations from unity. These extreme differences underscore the importance of genetic variation in sleep homeostasis in natural populations of flies, comparable to inter-individual differences in human sleep[13].

Night and day sleep and bout number were only partially genetically correlated (r_G), indicating that different genes influence the duration and pattern of day and night sleep (Fig. 1f–g). Waking activity was positively correlated with night sleep duration in females, but not in males or in the sexes-pooled analysis. Significant correlations between sleep duration and waking activity have been previously noted in some[14], but not all[15] studies of the effects of new mutations on sleep. Waking activity was negatively correlated with daytime sleep bout number: genotypes with more fragmented daytime sleep are less active.

Other life-history traits assayed in these lines[11] were genetically correlated with sleep. Both night ($r_G = 0.335$, $P = 0.035$) and day ($r_G = 0.326$, $P = 0.040$) sleep were positively correlated with starvation resistance, and night sleep was correlated with lifespan in females ($r_G = 0.338$, $P = 0.033$). Waking activity was correlated with chill coma recovery ($r_G = 0.450$, $P = 0.004$), and night bout number with sensitivity to ethanol exposure ($r_G = 0.323$, $P = 0.042$).

Previously, 10,096 genetically variable transcripts and 3,136 probes containing single feature polymorphisms (SFPs) were identified in these lines[11]. We hypothesized that the genetic variation in transcript abundance would reflect the genetic variation in sleep propensity in these lines. We therefore used association tests for SFP alleles and regressions of sleep phenotypes on transcript abundance to identify candidate genes contributing to genetic variation in sleep phenotypes. We found 153 SFPs in 134 genes ($P < 0.01$, Supplementary Table 2a) and 1,659 unique genes for which quantitative trait transcripts[16] (QTTs, $P < 0.01$, Supplementary Table 2b) are associated with one or more sleep traits; 31 QTTs also contain probes with SFPs. High numbers of transcripts were associated with day and night sleep duration: 1150 and 289, respectively, with false-discovery rates (FDRs) of 0.04 and 0.29. The 151 transcripts associated with waking activity had a much higher FDR of 0.64; however, the the number of transcripts associated with night sleep, day sleep and waking activity exceeded the number expected by chance based on permutation tests at $P < 0.001$. Numbers of transcripts associated with bout number did not exceed chance expectation, yet grouped into biologically meaningful modules as detailed below. Low numbers of overlapping transcripts for sleep duration and waking activity were consistent with the low genetic correlations observed between these traits. Interestingly, transcript levels were negatively correlated with sleep for 87.6% of the QTTs, suggesting that the global regulation of transcription plays a key role in sleep duration.

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Two loci previously implicated to affect sleep[4,7] and 102 genes previously identified in microarray studies[17] were identified as QTTs in this analysis. However, most of the candidate genes associated with sleep phenotypes are computationally predicted or unexpected based on prior functional annotations. We therefore sought to independently validate that the candidate genes affect sleep. First, we noted that an SFP in *Catsup* had a strong association with day sleep (Supplementary Table 2a). *Catsup* encodes a negative regulator of tyrosine hydroxylase[18], the rate limiting step in the production of dopamine. Sleep in flies is altered by changes in dopamine signalling[3,5,17,19], and daytime sleep is modified by the pharmacological inhibition of dopamine synthesis[19] and the ablation of the dopaminergic pathway[20]. We sequenced *Catsup* in all lines and found 33 non-singleton molecular polymorphisms, of which eight were significantly associated with one or more sleep phenotypes (Fig. 2a, Supplementary Table 3). Several of the variants were possibly functional non-synonymous polymorphisms, including a non-synonymous polymorphism in the first of two histidine-rich extracellular loops of the *Catsup* protein, which may bind and transport zinc[21] (Fig. 2b)

Second, we selected four candidate genes affecting sleep duration (*CG17574, bicoid-interacting protein 3 (bin3), Tetraspanin 42Ef (Tsp42Ef)*, and *Akt1*) for which homozygous *P*-element mutations had been generated in an isogenic background[22]. We assessed sleep duration and transcript abundance using quantitative RT-PCR in the mutant lines and their controls. All mutations affected the duration of night and day sleep duration (Fig 3a, Supplementary Table 4a) and had significantly altered gene expression in at least one sex (Fig. 3b). Thus, disruption in the expression of genes associated with variation in sleep patterns in the wild-derived inbred reference panel gives rise to altered sleep phenotypes in a standard laboratory (*Canton-S*) strain.

The *Drosophila* transcriptome is highly genetically correlated, enabling us to group transcripts associated with sleep phenotypes into statistically correlated transcriptional modules[11]. We found 20 modules associated with day sleep, nine for night sleep and night bout number, five for waking activity and three for day bout number (Fig 4a; Supplementary Table 2b). The genomic signatures of day and night sleep were distinct: the day sleep modules were largely positively correlated, but six of the night sleep modules were independent. To independently verify the correlated transcript modules, we used quantitative RT-PCR to assess expression of seven genes with positive intercorrelations in night sleep Module 6 (*CG17574, bin3, Akt1, Aats-asp, CG11306, CG11563*, and *Use1*) in *P*-element insert mutations of *CG17574, bin3, Akt1* and their co-isogenic control. We found high pair-wise correlations between transcript levels in all but four comparisons among these genes (Supplementary Table 4b), consistent with our interpretation that the modules represent co-regulated gene expression networks.

We queried the biological significance of these modules by performing enrichment analyses for gene ontology (GO) categories[23], tissue-specific expression[24], and shared transcription factor motifs. Only night bout number was enriched for genes affecting nervous system development and function. Day and night sleep duration were enriched for genes affecting metabolism, transcription, and protein binding, localization and transport. Waking activity and day bout number were also enriched for genes affecting metabolism (Supplementary

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Table 5). Overall, modules for sleep phenotypes had an over-representation of GO categories describing fundamental cellular processes. Modules of correlated transcripts associated with night sleep duration and bout number were enriched for expression in the midgut and tubule (Supplementary Fig. 1a–b). Notably, all other significant enrichment or depletion of expression involves reproductive tissues (Supplementary Fig. 1a, c–d). Thus, genes impacting sleep phenotypes are widely expressed and may have pleiotropic functions in reproduction. We compared the proportion of genes per module for which a *Drosophila* transcription factor motif was present to the genome-wide proportion and found that several were enriched in sleep modules: *hairy* ($P = 0.000002$) and *Dref* ($P = 0.0004$) in day sleep Modules 13 and 14, respectively; and *pannier* in night sleep Module 7 ($P = 0.0013$). Two transcription factors previously implicated to affect *Drosophila* sleep[6,14] were significantly enriched; *escargot* for day sleep Module 19 ($P = 0.0432$), and *Relish* for both night sleep Module 7 ($P = 0.0427$) and waking activity Module 5 ($P = 0.040$).

Modules of correlated transcripts can be represented as undirected transcriptional networks, where nodes represent transcripts and edges join correlated transcripts. These analyses identify highly correlated 'hub' genes for further functional analyses. One hub gene for both day and night sleep time is *bin3* (Fig. 4b, Supplementary Table 2b), a putative RNA binding protein methyltransferase[25]. Variation in *bin3* transcript abundance is genetically correlated with chill coma recovery and starvation resistance, and mutations in *bin3* affect chill coma recovery[11] and olfactory behaviour[26], suggesting that candidate genes affecting sleep may be highly pleiotropic.

Modules of correlated transcripts have previously been associated with other behavioural and life history traits in the same panel of inbred lines[11], enabling us to quantify substantial pleiotropy of QTTs, SFPs and entire modules. Night sleep duration modules are significantly associated with transcriptional modules affecting lifespan, starvation stress resistance and time to recover from a chill-induced coma, indicating that night sleep is a component of fitness.

Sleep behaviour is widely conserved across taxa[27]; furthermore, 17% of our sleep QTTs have been identified in sleep microarray studies of flies, rats, and mice (Supplementary Table 2b). We therefore assessed the degree to which our candidate genes were evolutionarily conserved. Genes associated with sleep duration, waking activity, and night bout number had more homologues across 12 *Drosophila* genomes[28] than all *D. melanogaster* genes, consistent with evolutionary conservation of sleep genes (Fig. 4c). Candidate genes associated with variation in sleep duration were over-represented for low values of ω[29] (the ratio of non-synonymous to synonymous substitutions) compared to all single-copy orthologous protein-coding genes in the *melanogaster* group, consistent with strong purifying selection (Fig. 4d). In contrast, genes associated with day bout number were under-represented for low values of ω and over-represented for high values of ω (Fig. 4d), indicating they are rapidly evolving. The correlations between ω and H^2 were positive and significant for candidate genes affecting sleep duration and waking activity, as expected if genetic variation within species is correlated with the rate of evolution. The correlations between ω and the degree of connectivity (the average correlation of a transcript with all other genetically variable transcripts) were significant and negative for the duration of sleep

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and waking activity, indicating that 'hub' genes evolve more slowly than less connected genes. In contrast, the correlation between ω and the degree of connectivity was significant and positive for genes associated with day bout number, consistent with the inference that these genes are rapidly evolving.

Integrating natural variation in organismal sleep phenotypes with variation in gene expression identifies segregating allelic variation in novel candidate genes associated with sleep that were not anticipated by screening *de novo* mutations. These candidate genes form highly correlated and evolutionarily conserved transcriptional networks, many of which are pleiotropically associated with other components of fitness.

METHODS SUMMARY

Inbred lines were derived by full sib inbreeding isofemale lines from Raleigh, NC for 20 generations. *P*-element mutations and co-isogenic control lines were obtained from the Berkeley *Drosophila* Gene Disruption Project. Flies were reared on standard medium at 25°C, a 12-hour light/dark cycle, and controlled density. Individual flies were measured for seven days for 16 virgin flies/sex/Raleigh inbred line and 32 flies per sex for *P*-element inserts and controls, using the *Drosophila* Activity Monitoring System (Trikinetics, Waltham, MA). Sleep was defined as five minutes or longer without an activity count2,3. Sleep phenotypes were expressed as a deviation from a contemporaneous w^{1118}; *Canton-S* isogenic control line mean. Whole genome transcript profiles were obtained as described11. ANOVA was used to partition variation of sleep phenotypes between sexes, lines, line-by-sex interaction, and error. Broad sense heritabilities and genetic correlations between sexes and traits were estimated using standard methods. Linear regression was used to identify transcripts associated with sleep. Residuals from the regressions were used to compute genetic correlations among transcripts; modules of correlated transcripts were identified as described11. PCR products including the *Catsup* transcription unit and putative promoter region were sequenced using ABI PRISM Big Dye Terminator chemistry. ANOVA was used to associate sleep with *Catsup* polymorphisms. RT-PCR in an ABI 7000 Thermal Cycler was used to quantify gene expression of *P*-element and controls. Oligonucleotide primers are provided in Supplementary Table 6. ANOVAs accounting for line, sex, and experimental block effects were used to compare transcript levels between genotypes. Fisher's Exact Tests were used to quantify transcripts significantly shared between modules of different phenotypes. Permutation tests determined whether 5' UTR sequences of transcripts in each module were enriched for known transcription factor binding sites.

Raw microarray data are deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress), under accession E-MEXP-1594. *Catsup* sequence GenBank accession numbers are FJ160415-FJ160454.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

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Acknowledgements

This work was supported by National Institutes of Health grants R01 GM 45146, R01 GM 076083 and R01 AA016560 to T.F.C.M., and the National Sleep Foundation Pickwick Fellowship to S.T.H. We thank David Reif for assistance with *Catsup* permutation tests, Katie Jordan for assistance with the genetic correlation data, and Robert Anholt for comments on the manuscript. This is a publication of the W. M. Keck Center for Behavioral Biology.

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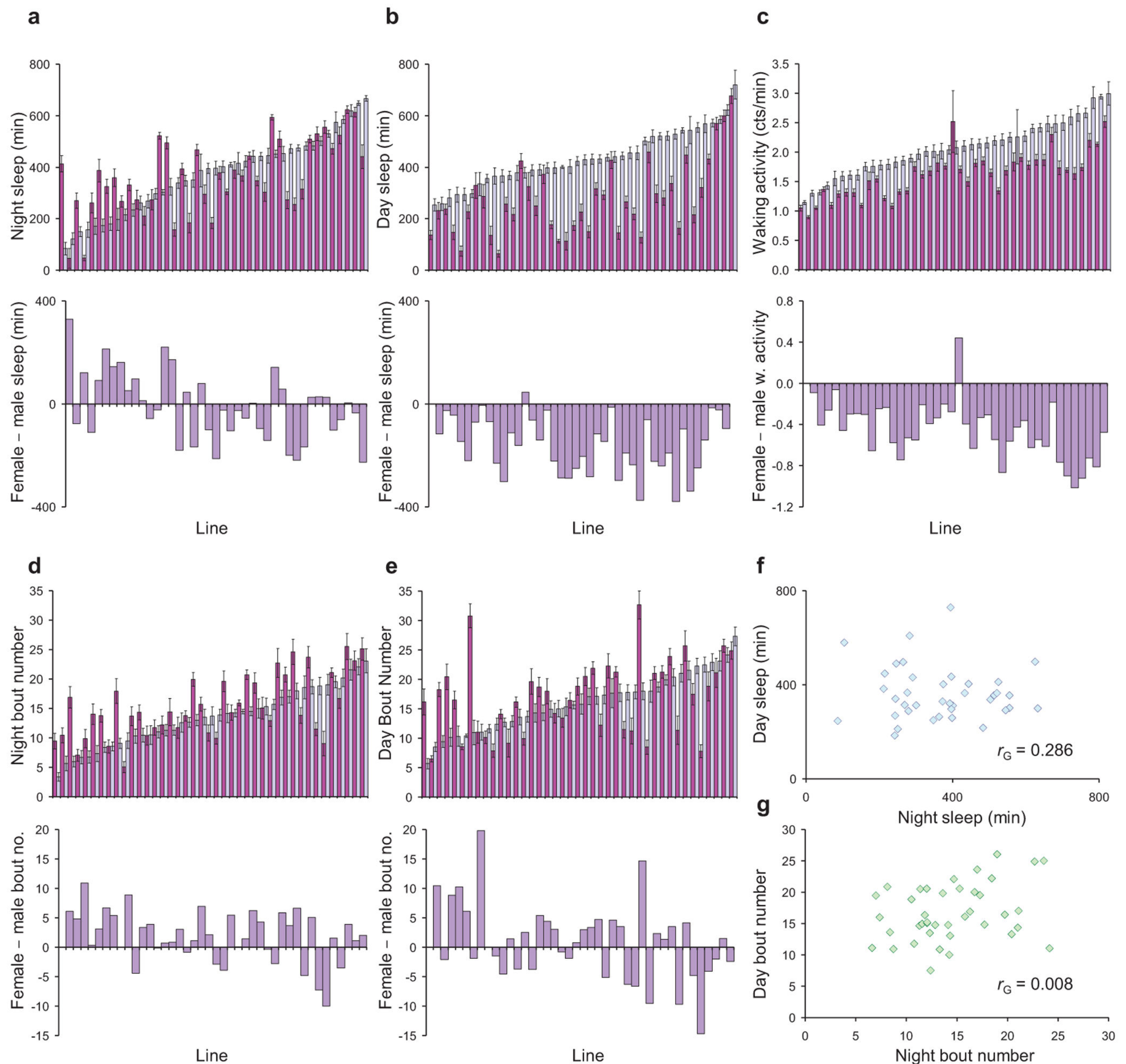

Figure 1. Variation in sleep phenotypes among wild-derived inbred lines

a–e, Line means for sleep phenotypes for males (blue bars) and females (pink bars), and sexual dimorphism (female – male) in sleep phenotypes (purple bars). Error bars are ± standard error of the mean. **a,** Night sleep. **b,** Day sleep. **c**, Waking activity. **d,** Night bout number. **e,** Day bout number. **f,** Genetic correlation (r_G) between night and day sleep time ($P = 0.074$). **g,** Genetic correlation between night bout number and day bout number ($P = 0.962$).

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Figure 2. Association between sleep phenotypes and *Catsup* polymorphisms

a, *P*-values (log(1/*P*), *y*-axis) from association tests of *Catsup* polymorphisms (*x*-axis) and day bout number (teal), day sleep time (light purple), night bout number (blue), night sleep time (dark purple) and waking activity (gold). The black and red dotted lines depict the permutation test-derived *P*-value threshold (0.0412) and the Bonferroni threshold (0.0014), respectively. **b**, Schematic diagram of the *Catsup* protein showing the type and location of significant polymorphisms. Yellow indicates a non-synonymous change, green indicates a deleted region, and pink indicates a synonymous change.

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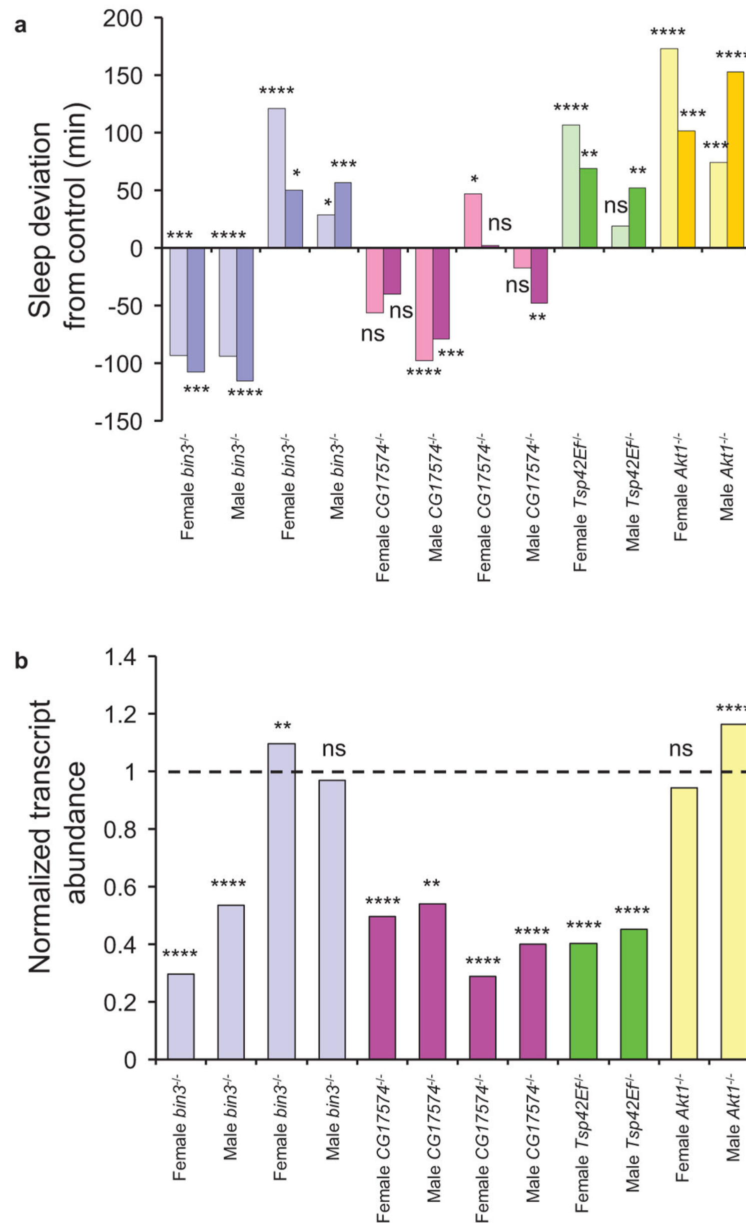

Figure 3. Validation of sleep candidate genes

Mutations in *bin3, CG17574, Tsp42Ef*, and *Akt1* are colour-coded blue, pink, green and yellow, respectively. Specific alleles tested are $bin3^{BG01137}$, $bin3^{BG01416}$, $CG17574^{BG02368}$, $CG17574^{BG00992}$, $Tsp42Ef^{BG00864}$, and $Akt1^{BG00351}$, and are listed in that order on the figure. **a,** Mean day (light shading) and night (dark shading) sleep times for males and females of *P*-element insertion mutations, expressed as deviations from the isogenic control line. **b,** Normalized transcript abundance for each *P*-element insertion line. The dashed line represents the isogenic control; bars indicate the transcript level relative to the isogenic control. *: 0.01 P < 0.05; **: 0.001 P < 0.01; ***: 0.0001 P < 0.001; ****: $P < 0.0001$; ns: $P > 0.05$.

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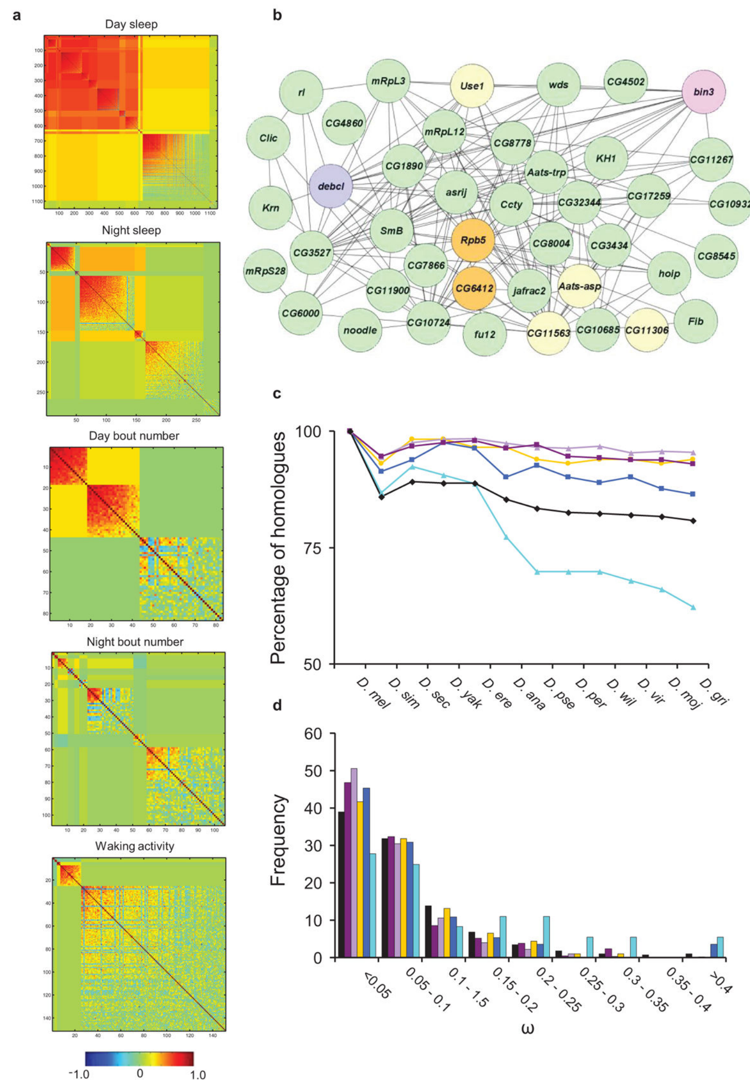

Figure 4. Analyses of candidate genes associated with natural variation in sleep phenotypes

a, Modules of correlated transcripts associated with day sleep (20 modules), night sleep (9 modules), day bout number (3 modules), night bout number (9 modules), and waking activity (5 modules). Each point represents the correlation between two genes. The colour scale bar indicates the value of the correlation. **b,** Network of correlated (|*r*| 0.7) transcripts for night sleep Module 6. The pink circle represents *bin3*, yellow circles represent genes whose connectivity with *bin3* was verified by RT-PCR, and orange circles represent genes for which associations with both transcripts and SFPs are signficant. The blue circle identifies a gene implicated in a previous microarray study of sleeping flies17. **c,** Percentage of homologous genes (including orthologues and paralogues) across the 12 *Drosophila* species for night sleep time (dark purple), day sleep time (light purple), night bout number (blue), day bout number (teal) and waking activity (gold), compared to all *D. melanogaster* genes (black). Species are in order of evolutionary distance from *D. melanogaster*. **d,** Frequency distribution of ω for each sleep phenotype. Colours are the same as in (**d**). Night

sleep ($\chi^2 = 21.9$, $P = 0.005$), day sleep ($\chi^2 = 74.7$, $P < 0.0001$) and day bout number ($\chi^2 = 15.7$, $P = 0.047$) had significantly different distributions from that of all genes.

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