Identifying novel links between cardiovascular disease and insomnia by *Drosophila* modeling of genes from a pleiotropic GWAS locus

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Abstract:

Background: Insomnia symptoms have been associated with CVD, doubling the risk of incident CVD, but specific shared pathways remain poorly understood. Recently, genome-wide association studies (GWAS) identified genetic loci significantly associated with insomnia symptoms, including one locus that was previously associated with CVD in an independent GWAS.

Methods: To evaluate the cell-autonomous role of genes near the CVD- and insomnia-related locus, we used *Drosophila melanogaster* models to perform tissue-specific RNAi knockdown of these genes in the heart and neurons. We also performed suppression of these genes in the heart or neurons, and assessed sleep or cardiac function, respectively, to identify non-cell-autonomous mechanisms.

Results: Our results show that neuronal and cardiac-specific RNAi knockdown of four genes conserved in *Drosophila*, *Lsn, ATPSynC, Bruce*, and *Imp*, contributes to compromised sleep and cardiac performance, respectively. Cardiac-specific knockdown of *Lsn* led to significant cardiac dilation and reduced cardiac performance. Knockdown of *ATPSynC* led to significantly reduced cardiac performance without dilations. Furthermore, *Lsn* and *ATPSynC*-suppressed hearts showed disruption in the actin-containing myofibrillar organization and led to a significantly shortened lifespan. Suppression of *Lsn* increased pericardin deposition, indicative of a fibrotic phenotype. Neuronal-specific knockdown of *ATPSynC, Imp, and Lsn* led to compromised sleep. Moreover, the knockdown of *Imp* in the brain led to a significantly compromised cardiac function characterized by decreased systolic and diastolic intervals and fractional shortening in a non-cell autonomous manner. Furthermore, the knockdown of *Bruce*, *ATPSynC,* and *Lsn* in the heart led to compromised sleep characterized by decreased activity and daytime activity and increased bin number in a non-cell autonomous manner.

Conclusions: Our study provides novel insights into genetic mechanisms linking CVD and insomnia, highlighting the importance of these four conserved genes in the development and association of both diseases.

Key Words: Cardiovascular Disease, Insomnia, Human Genetics, GWAS, Drosophila Model

Nonstandard Abbreviations and Acronyms:

CVD: Cardiovascular disease

GWAS: Genome-Wide Association Study

SNP: Single-Nucleotide Polymorphism

ESCRT: Endosomal Sorting Complexes Required for Transport

HP: Heart Period

AI: Arrhythmia Index

DI: Diastolic Interval

SI: Systolic Interval

DD: Diastolic Diameter

SD: Systolic Diameter

FS: Fractional Shortening

Introduction:

Cardiovascular disease (CVD), one of the leading causes of death worldwide, encompasses several conditions that affect the heart or blood vessels.[1-3] The incidence of CVD continues to rise, with approximately 18.2 million deaths worldwide in 2019,

which contributes to rising healthcare costs and creates a significant socioeconomic burden.[3,4] Many factors that increase the risk of CVD include genetic factors, smoking, and lack of physical activity. One important risk factor for CVD that has recently emerged is sleep dysfunction and insomnia.[4-7] Insomnia is the most common sleep disorder, affecting 10 to 30% of the population, and is defined as persistent difficulty in falling and/or staying asleep or non-restorative sleep resulting in daytime sleepiness, fatigue, or dysfunction.[8-10] Studies suggest that insomnia has a genetic component with heritability estimates ranging between 22 and 25% in adults[11,12], and multiple GWASs have identified genetic loci with links to insomnia.[12-15] Although genetic factors have been identified as contributors to CVD and insomnia, the genetic mechanisms underlying these two diseases remain poorly understood.

Observational studies have demonstrated that insomnia increases the risk of several disorders, especially CVD.[16-19] Moreover, mendelian randomization analyses show that insomnia symptoms double the risk for incident CVD[12,13] Similarly, cardiac dysfunction has been associated with sleep disruptions.[20,21] Furthermore, a recent study found that sleep modifies atherosclerosis through hematopoiesis in mice.[22] Together these findings establish a clear link between cardiovascular traits and sleep. However, the specific underlying causal genetic pathways and mechanisms connecting CVD and insomnia are unknown. Recent genome-wide association studies (GWASs) identified multiple significant loci for self-reported insomnia symptoms in UK Biobank and 23andMe participants.[12,13] From these loci, we identified a single locus, represented by lead SNP rs4643373, that has also been previously associated with coronary artery disease.[23,24] This locus provides a valuable opportunity to identify genes important for CVD and/or insomnia, and dissect potential genetic mechanisms underlying the link between cardiovascular function and sleep. Near this locus, we identified five candidate genes, *ATP5G1*, *UBE2Z*, *SNF8*, *IGF2BP1*, and *GIP*. The known functions of these genes are very diverse, including energy metabolism (ATP5G1), protein ubiquitination (UBE2Z), multivesicular body biogenesis (SNF8), post-transcriptional regulation (IGF2BP1), and lipid metabolism (GIP).[25-29] However, it remains unclear which of these genes, if any, contribute to CVD or insomnia.

To elucidate the impact of these candidate genes on the regulation of cardiac function and sleep, we identified conserved *Drosophila* orthologs for the insomnia and CVD-related candidate genes near rs4643373*. Drosophila* has become a well-established model system for studying both CVD and sleep disturbances.[30-35] The fly heart displays many developmental and functional similarities to the mammalian heart.[30,36] Moreover, many genes causing heart disease in humans are present in *Drosophila* and play similar roles in heart structure and function. Furthermore, manipulation of these genes in Drosophila cause disease phenotypes similar to humans.[30,36,37] Additionally, sleep in flies has been demonstrated to share many characteristics with human sleep, such as consolidation during the night and similar responses to sleep altering drugs .[31,38-40] Therefore, studies investigating the role of human-relevant Drosophila orthologs in the regulation of cardiovascular function and sleep provide an efficient means to identify new causal genes related to CVD and/or insomnia, and understand mechanisms relating both diseases to identify potential future therapeutic targets.

Here characterized the function of the *Drosophila* genes *ATPSynC, Bruce, Lsn and Imp,* orthologs of *ATP5G1*, *UBE2Z*, *SNF8*, and *IGF2BP1,* respectively. To assess the role of these genes in cardiac and sleep physiology, we performed tissue specific knock-down (KD) in the heart and nervous system, respectively. Cardiac- and neuronal-specific KD of these genes led to cardiac and sleep dysfunction, suggesting tissue-specific functions related to each disease. We further characterized the mechanisms through which the two genes with the strongest cardiovascular phenotypes, *ATPSynC* and *Lsn*, lead to CVD. We found that disruption of another component of the ATP Synthase complex and disruption of the ESCRT pathway also lead to cardiac dysfunction, similar to *ATPSynC* and *Lsn*, respectively. After characterizing the role of *ATPSynC*, *Bruce*, *Lsn*, and *Imp* in cardiac function and sleep, we also identified non-cell-autonomous effects of these genes on cardiac and sleep phenotypes, upon KD of these genes neuronally or in cardiac tissue, respectively. In conclusion, were able to uncover novel genetic mechanisms with cell-autonomous effects on the regulation of cardiac function and sleep as well as non-cell autonomous genetic mechanisms linking

cardiac function in the regulation of sleep and sleep on cardiac function. Taken together, our data are among the first functional genetic proofs that link CVD with sleep disorders and provide mechanistic insight into potential therapeutic targets to prevent or attenuate both diseases.

Methods

LocusZoom plots and COJO analysis.

The LocusZoom plots for CVD and insomnia SNP, rs4643373, and secondary CVD SNP, rs46522, were generated using LocusZoom v1.3 (06/20/2014). The COJO analysis was performed using GCTA version 1.94.1, with an LD-reference panel of unrelated EUR samples from the HGDP-1KG reference set.[41,42]

Drosophila Stocks

Drosophila stocks were cultured at 25°C on standard agar media.[43] UAS-RNAi transgenic stocks of CVD- and insomnia-related genes were obtained from Vienna Drosophila Resource Center (VDRC) and Bloomington Drosophila Stock Center (BDSC): *ATPSynC*-RNAi (VDRC: 106834; BDSC: **35464**, 57705), *Lsn*-RNAi (VDRC: 110350, **21658**; BDSC: 38289), *Bruce*-RNAi (VDRC: 107620, 48309; BDSC: **51814**), *Imp*-RNAi (VDRC: 20321, 20322; BDSC: 38219, **55645**, 34977), *Vps25*-RNAi (VDRC: 108105; BDSC: 26286), *Vps36*-RNAi (VDRC: 107417; VDRC: 16846), *shrb*-RNAi (VDRC: 106823; BDSC: 38305), *Vps2*-RNAi (VDRC: 24869; BDSC: 38995), *Vps20*-RNAi (VDRC: 103944; BDSC: 40894), *Vps24*-RNAi (VDRC: 100295; VDRC: 29275), *ATPSynGamma*-RNAi (VDRC: 16538; BDSC: 28723), control lines (w^{1118}: BDSC: *5905*, VDRC: 60100, BDSC: 36303), *Act5C-Gal4* (BDSC: 4414); *24b-Gal4* (BDSC: 1767), *Elav-Gal4* (BDSC: 458). *Hand-Gal4* was obtained from Dr. Olson's lab. Data from RNAi lines were not combined if more than one RNAi line was used. Line 1 is underlined and is the main line used for all experiments. Line 2 is bolded and used only in Fig. S2. *ATPSynC* Line 2 is used for neuronal-specific suppression as Line 1 was lethal.

Ubiquitous- and Tissue-specific knock-down and genetic modulation

The GAL4-UAS system[44] was used to drive the knockdown of CVD- and insomnia-related genes ubiquitously or tissue-specifically. Adult flies possessing UAS RNAi CVD- and insomnia-related genes were crossed to *Hand-Gal4, Elav-Gal4, Act5C-Gal4, Ubi-Gal4,* or *24b-Gal4* flies and incubated at 25°C throughout development. Adult male and female F1 progeny were separated according to sex and allowed to age, with a new food source supplied every three days prior to assays of cardiac function. Age-matched adults from w^{1118} (wild-type), V60100 or BL36303 (VDRC and BDSC RNAi controls) were crossed with each of the *Gal4* drivers as controls. Male and female flies were screened at 1 and 3 weeks of age for cell-autonomous assays and 3 weeks for non-cell-autonomous assays in at least 2 independent experiments. All flies were kept at 25 °C, 50% humidity in a 12 h light/12 h dark (LD) cycle.

Sleep-wake behavioral analysis

Three-to-four-day old male and female progeny of *Elav-Gal4* (cell-autonomous) and 2.5-week-old male progeny of *Hand-Gal4* (non-cell-autonomous) with control and RNAi lines of each of the four genes were collected and individual flies were loaded into glass tubes containing standard fly food (n>16). Sleep-wake behavior was recorded using the Drosophila Activity Monitor (DAM, TriKinetics inc MA, USA) system in a 12L:12D cycle at 25°C. Drosophila activity (or wake) is measured by infra-red beam crosses in the DAM system.[45] Data was analyzed using ClockLab and RStudio. Custom R scripts and methodology used with RStudio can be found on https://github.com/jameswalkerlab/Gill_et.al. One-way ANOVA with Dunnet's multiple comparisons test for DAM system data was performed using GraphPad Prism. Drosophila sleep was defined by a period of at least 5 minutes of inactivity, demonstrated by zero beam breaks recorded.[46] Average sleep per 24 hours (ZT0-ZT24) of each genotype was calculated. Five days were used for analysis of 1-week-old flies, and 3 days were used for 3-week-old fly experiments to overcome decreased viability in older flies. Sleep fragmentation was defined by the number of 1-minute wakes during a 24-hour period. Sleep bouts were quantified by counting the number of periods of sleep as defined above. Sleep bout length was quantified by measuring the length of each

sleep bout. Data for daytime sleep is from ZT0 to ZT12 and nighttime sleep is from ZT12-ZT24.

Cardiac physiological analyses of semi-intact Drosophila hearts

1-week-old male and female progeny of *Hand-Gal4* (cell-autonomous) and 2.5-week-old male progeny of *Elav-Gal4* (non-cell-autonomous) with control and RNAi lines of each of the four genes were collected, and semi-intact hearts were prepared as described (n>30).[47,48] Direct immersion optics was used in conjunction with a digital high-speed camera (at 200 frames/sec, Hamamatsu Flash 4 camera) to record 30 second movies of beating hearts; images were captured using HC Image (Hamamatsu Corp.). Cardiac function was analyzed from the high-speed movies using semi-automatic optical heartbeat analysis (SOHA) software that quantifies heart rate, heart period, diastolic and systolic diameters, diastolic and systolic intervals, cardiac rhythmicity, fractional shortening and produced the Mechanical-mode records [47,48].

Cytological Studies of adult hearts

Dissected hearts from one-week old adults were relaxed by a one-minute treatment with 5 mM EGTA in hemolymph and then fixed with 4% paraformaldehyde in PBS for 30 min as previously described [48]. Fixed hearts were stained with anti-Pericardin antibody overnight (5ug/ml, 1:10; Developmental Biology Hybridoma Bank, University of Iowa) followed by Alexa488-phalloidin for 30 min (1:1000, U0281, Abnova), which stains F-actin containing myofibrils. Samples were then mounted with Diamond Antifade Mountant with DAPI. Confocal images were taken from a Nikon A1R HD microscope (UAB) at 10X for pericardin quantification and 20X for representative images for phalloidin staining. Quantification of pericardin area in the confocal images from three to five independent male hearts per genotype was performed by thresholding images in ImageJ, then percent area was measured.[49]

Viability

Adult flies (n>100, males and females) with suppression of CVD- and insomnia-related genes and controls were collected on the day of eclosion from the pupal case,

designated as day zero. Approximately 30 flies were placed in each vial and transferred to a new vial every three to four days. The numbers of surviving adults were counted twice a week. The numbers of surviving adults were compared to the original number of adults collected on day zero and the percentage for each day was graphed.[50]

Hemocyte Counts

To evaluate inflammation, fly hemolymph was collected from n>100 (per replicate, 3 biological replicates) one-week-old adult male flies with cardiac-specific suppression using *Hand-Gal4* by making an incision in the thorax of flies and centrifuging them.[51] Hemocytes were then counted by staining the hemolymph with 1:1 Trypan blue dilution and using a hemocytometer.[51]

Real-time quantitative PCR

Dissected male hearts (n=10-12 per biological replicate, 3 biological replicates) and heads (n=10, per biological replicate, 3 biological replicates) from 1-week-old flies was placed in the RNA lysis buffer, and flash frozen. RNA from heads was extracted using Zymo Research Quick-RNA Microprep Kit with on column DNase I digestion. RNA from hearts was extracted using the RNeasy kit (QIAGEN). Quantitative PCR was performed using SsoAdvanced Universal SYBR Green supermix (Bio Rad) in a BIO-RAD CFX Opus Real-Time PCR System. Expression was normalized with 60S ribosomal protein (RPL11). Primers for qPCR are listed below: *ATPSynC-F: GCAACAGTCGGTGTCGCT; ATPSynC -R: AGGCGAACAGCAGCAGGAA; Lsn-F: TCACCAAGGAGGACATCCTAATGG; Lsn-R: TCCGGGAATGGACTGAACTATGTA; Bruce-F: AATAGCGCTCCATCTCGACCAT; Bruce-R: ATCGACCATGCACAATGCTGT; Imp-F: AATTCGCCGACCTGGAACTCT; Imp-R: ACTCGACACCGTTCAGACCAA; Upd3-F: AGCCGGAGCGGTAACAAAA; Upd3-R: CGAGTAAGATCAGTGACCAGTTC; Eiger-F: GATGGTCTGGATTCCATTGC; Eiger-R: TAGTCTGCGCCAACATCATC; Vps25-F: TCTCAAATACCTCAGGCACACG; Vps25-R: CACCCAGTCGTACACCATGTT; Vps36-F: CTCACCACACACCGACTGTTT; Vps36-R: GAGGCAGTAGTCTCTTCGCTG; Vps2-F: ATGCTGCGTAAGAATCAGCG; Vps2-R: GGCATCCATTTGACCCTCCT; shrb-F: CGGATGCCCTCAAGAGAGC; shrb-R:*

CGGGTATGCCAATGATTTCCTT; Rpl11-F: CGATCTGGGCATCAAGTACGA; Rpl11-R: TTGCGCTTCCTGTGGTTCAC. Results are presented as $2^{-\Delta\Delta Ct}$ values normalized to the expression of Rpl11 and control samples. All reactions were performed using biological triplicates. The means and standard error of the mean were calculated in GraphPad Prism 9 software.

Statistical Analysis

For all quantitation except transcript levels and lifespan analyses, statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test to determine significance between groups for sleep and cardiac physiological parameters. For expression of transcript levels in heads, statistics were calculated by 1-way ANOVA. For expression of transcript levels in hearts, statistics were calculated by Kruskal-Wallis test and without correcting for multiple comparisons to account for variability. For ESCRT transcript levels, statistical significance was determined using an unpaired t-test. Bar graphs show mean ± SEM. For lifespan studies, data were analyzed using the Kaplan-Meier test followed by multiple comparisons between control and experimental groups. Significance was presented using p-values on figures. All statistical analysis were performed with GraphPad Prism 9.

Results

Shared GWAS locus at 17q21 is associated with coronary artery disease and insomnia.

A GWAS signal at 17q21.32 for coronary artery disease, represented by lead SNP rs4643373 (Fig. 1A, n=60,801 cases and 123,504 controls; OR=1.04 (95% CI=1.028-1.050))[52] was found to co-localize with an association signal for insomnia symptoms (n=593,724 cases and 1,771,286 controls; OR=1.04 (95% CI=1.025-1.046); Fig. 1B; pp = 0.95 that both traits share the same causal SNP)[53]. The genomic region around the co-localized signal encompasses multiple genes including *ATP5MC1, UBE2Z, SNF8, GIP and IGF2BP1*. Notably, a partially independent association signal in the region is observed for coronary artery disease with the lead SNP rs46522 (OR=1.03 (95%

CI=1.02-1.05; r^2=0.184 in 1KG CEU (Fig. 1C), reinforcing the importance of this genomic region in cardiovascular disease. The causal genes and variants at this locus are unknown. Furthermore, it is unclear if the association signals reflect independent contribution of effector genes to sleep and cardiovascular disease, or if effector genes influence sleep through cardiovascular dysfunction or vice versa. Thus, we developed a *Drosophila* screening approach to identify the role of fly orthologs of these genes in sleep and cardiovascular function (Fig. 1D).

Neuronal-specific suppression of CVD- and insomnia- related genes leads to altered sleep phenotypes along with enhanced sleep fragmentation.

We identified fly orthologs of the four of five human genes near rs4643373 with their % similarity shown in Table 1. The fifth gene, GIP, lacks fly ortholog. Firstly, in order to assess whether any of the orthologs of the genes within the CVD- and insomnia-associated locus were essential in *Drosophila*, we performed ubiquitous KD of these genes driven by ubiquitous driver, *Act5C-Gal4.* Ubiquitous KD of *ATPSynC* and *Lsn* led to lethality, while that of *Bruce* and *Imp* did not affect their viability (Table S1). We then performed a pan-muscle *KD* using the *24b-Gal4* driver to determine if the function of these genes was essential in muscle tissue. Like the ubiquitous KD, we found that pan-muscle KD of *ATPSynC* and *Lsn* resulted in lethality (Table S1).

To test the impact of these 4 genes near the CVD- and insomnia- associated locus on sleep, we used the neuronal-specific *Elav-Gal4* driver to KD gene expression. Level of KD of each gene in the head is shown in Fig S1A-D. We quantified total, day and night characterizations, averaged over a 5-day period of: sleep, locomotor activity, sleep fragmentation, sleep bout length and sleep bout number of three-to-four-day-old male flies with pan-neuronal KD of each of the four Drosophila orthologs (progeny of *Elav,* driving each of the four genes).

Compared to control flies, RNAi-mediated inhibition of *ATPSynC* significantly increased overall sleep time which primarily resulted from an increase in nighttime sleep in (Fig. 2A-D). This increased sleep corresponded to a decrease in overall locomotor activity

which decreased during both daytime and nighttime (Fig. 2E-G). KD of *Imp* also showed increased sleep which was primarily due to increased daytime sleep (Fig. 2A-D) with decreased activity (Fi. 2E-G). Moreover, suppression of *Lsn* led to a significant increase in nighttime sleep that was (Fig. 2A-D) and was accompanied by decreased nighttime activity (Fig. 2E-G). However, neuronal KD of *Bruce* decreased daytime sleep but had no effect on activity or fragmentation (Fig. 2A-J). To further characterize these sleep disruptions in each genotype, we assessed the number of one-minute wakes experienced by the flies as a measure of sleep fragmentation. KD of both *ATPSynC* and Imp resulted in an increase total sleep fragmentation. Although only KD of Imp resulted in an increase in daytime sleep fragmentation, KD of *ATPSynC*, Lsn, or Imp resulted in a significant increase in nighttime sleep fragmentation (Fig. 2H-J). Moreover, we observed similar sleep and activity trends in *ATPSynC*, *Lsn* and *Bruce* females, but no change in *Imp* flies compared to controls (Fig. S3A-D). We also observed similar sleep and activity trends in 3-week-old male flies (Fig. S5A-C). Therefore, the neuronal suppression of genes within the CVD- and insomnia-related locus led to a significantly altered sleep phenotype characterized by an increase in fragmented overall and/or nighttime sleep in 3 out of the 4 genes.

Cardiac-specific suppression of CVD- and insomnia-related genes leads to cardiac dysfunction, myofibrillar disorganization, cardiac fibrosis, inflammation, and shortened lifespan.

To determine the effect of suppressing these genes in the heart on cardiac performance, KD of *ATPSynC*, *Bruce*, *Lsn, or Imp,* was carried out using the cardiac-specific *Hand-Gal4* driver. Levels of KD of each gene in the heart is shown in Fig S1E-H. 1-week-old male and female flies were dissected and imaged for assessment of cardiac physiological parameters. Interestingly, suppressing *Lsn* led to a non-beating heart phenotype where only 62.7% of hearts beat at 1 week of age which decreased to 15% by 3 weeks of age in males (Fig. 3A). Upon analyzing beating hearts, cardiac-specific KD of *ATPSynC* showed a significantly increased HP, AI, DI and SD and reduced DD and FS, a measure of cardiac performance, in both male and female flies (Fig. 3B-H, Fig. S3D). Suppression of *Lsn* led to significantly increased DD and SD and

reduced FS without changes in HP, AI and DI in both sexes (Fig. 3B-H, Fig. S3D). Suppression of *Imp* decreased HP and DI without affecting FS in males only (Fig. 3B-H, Fig. S3D). However, suppressing *Bruce* in males and females did not severely affect heart function in one-week-old flies (Fig. Fig. 3B-H, Fig. S3E). To determine if there is an aging-related component to the effects of these genes on the heart, we assessed cardiac function in 3-week-old male flies with cardiac-specific suppression of these genes and observed similar trends to those observed in 1-week-old *ATPSynC* and *Lsn* flies. Interestingly, in 3-week-old flies with KD of *Bruce*, we observed cardiac dysfunction characterized by increased DD and SD and decreased FS (Fig. S4D).

Furthermore, 1-week-old male hearts stained with Phalloidin showed a disruption in actin-containing myofibrillar organization, where KD of *ATPSynC* showed almost complete loss of contractile circumferential muscles (CF) and mostly non-contractile longitudinal muscles (LF) are seen (Fig. 4A, Fig. S5A). KD of *Lsn* showed a dilated heart with more evident LF and CF aggregations along with myofibrillar disarray, while that of Bruce and Imp showed a less severe phenotype (Fig. 4A, Fig. S5A). Moreover, only suppression of *Lsn* led to significantly increased pericardin deposition, which is a collagen-like protein and a component of the extra cellular matrix (ECM) indicative of a fibrotic phenotype (Fig. 4B, C). Interestingly, cardiac suppression of *Lsn* also led to significant increase in Upd3 levels, which is an inflammatory cytokine in flies equivalent to IL-6 (Fig. 4D), while suppression of ATPSynC led to a trend towards increased Upd3 levels ($p=0.0719$). Suppression of both genes individually led to a significant decrease in levels of *Eiger* (Fig. S5B), an ortholog to TNF, which is a trend that has been previously reported.[54] This may suggest that Upd3 is the specific mechanism underlying CVD-related inflammation in these flies. We also performed hemocyte counts as another measure of an inflammatory-like state in flies. Cardiac-specific suppression of Lsn lead to a significantly increased number of hemocytes in the hemolymph (Fig. S5C). Moreover, the cardiac suppression of *ATPSynC, Lsn,* and *Imp* led to a significantly shortened lifespan in both sexes ($p< 0.0001$), while flies with suppressed *Bruce* showed an increased lifespan ($p=0.0057$) (Fig. 4E). Our findings indicate that suppression of CVD- and insomnia-related genes *Lsn* and *ATPSynC* in the heart led to significantly

compromised cardiac function, with myofibril disorganization, increased inflammation and shortened lifespan. Moreover, *Bruce* and *Imp* showed less severe phenotypes with *Bruce* leading to cardiac dysfunction with increased age.

Pathways/complexes involving *ATPSynC* and *Lsn* as potential underlying mechanisms to CVD.

ATP5MC1 (*ATPSynC* in *Drosophila*) is a subunit of mitochondrial ATP synthase, which catalyzes ATP synthesis (Fig. 5A). Suppression of *ATPSynC* in the heart lead to a significantly reduced fractional shortening and increased diastolic interval and arrythmia index (Fig. 3). Therefore, to test if the role of *ATP5MC1* in ATP synthase is the underlying mechanism behind the observed cardiac dysfunction, we suppressed expression of another component of ATP synthase, *ATPSynGamma*, in the heart using the *Hand-Gal4* driver. One-week-old male flies with suppression of *ATPSynGamma* showed an increased AI, SI and DI and SD, and decrease DD and FS (Fig. 5B-H), similar to that observed after *ATPSynC* suppression. Moreover, since ATP Synthase dysfunction and ATP depletion are involved in cell death[55], we measured transcript levels of *Reaper* and *Hid*, *Drosophila* cell death activators.[56] KD *ATPSynGamma* significantly increased the expression of both, Reaper and Hid (Fig. 5I). KD of ATPSynC also increased expression of both genes, but only that of Hid was significant while that of Reaper was not (p=0.1087) (Fig. 5I). Also, *ATPSynGamma* flies had a very short lifespan similar to *ATPSynC* (data not shown). Taken together, these findings suggest that the role of *ATPSynC* in ATP synthase underlies its connection to CVD.

SNF8 (*Lsn* in *Drosophila*) is a component of the endosomal sorting complex required for transport II (ESCRT-II) (Fig. 6A), which regulates the movement of ubiquitinylated transmembrane proteins to the lysosome for degradation. Suppression of *Lsn* in fly hearts leads to a significantly dilated cardiac phenotype and decreased fractional shortening (Fig. 3). We hypothesized that the role of SNF8 in the ESCRT pathway is the reason behind the observed cardiac dysfunction. To test that hypothesis, we suppressed expression of other components of ESCRT-II and III complexes involved in the pathway, using the cardiac-specific driver. *Hand-Gal4* driven one-week-old male flies with suppression of *Vps25* and *Vps36*, components of ESCRT-II complex, led to

significantly decreased HP and DI, increased SI and DI and decreased FS (Fig. 6B-G), similar to the suppression of *Lsn*. Moreover, suppression of *Vps2, Vps20* and *shrb*, components of ESCRT-III, also lead to a similar dilated cardiac phenotype with reduced fractional shortening (Fig. 6H-M). Since *Lsn* is a component of the ESCRT-II complex, we assessed the effects of cardiac-specific *Lsn* KD on transcript levels of ESCRT-II and ESCRT-III components. *Vps2* and *shrb* levels were increased which could serve as a compensation mechanism to the deficiency in Lsn levels, while Vps25 and Vps36 levels were not significantly changed (Fig. S6A, B). These results support the role of *Lsn* in the ESCRT pathway as an underlying mechanism to its connection to CVD.

Non-cell-autonomous mechanisms linking CVD with insomnia.

Mendelian randomization analyses in recent genetic studies confirm a causal role for insomnia on CVD.[12] Moreover, cardiac disfunction has also been associated with sleep disturbances.[20,21,57] Therefore, to assess non-cell-autonomous roles of these genes in influencing cardiac and sleep dysfunction, we suppressed genetic expression in the brain and measured cardiac function, or we suppressed it in the heart and assessed sleep phenotypes (Fig. 7A). Unlike cardiac-specific KD, neuronal suppression of *ATPSynC*, *Lsn* or *Bruce* resulted in no cardiac phenotype, while neuronal suppression of *Imp* in 3-week-old male flies significantly decreased HP, DI and significantly reduced FS (Fig. 7B). This compromised cardiac performance indicates a non-cell-autonomous effect of *Imp* in the causal role of insomnia on cardiac function. (Fig. 7B).

Upon the cardiac-specific KD of *ATPSynC*, 3-week-old male flies showed a significant decrease in nighttime sleep without change in activity (Fig. 7C, D). Similarly, cardiac-specific KD of *Bruce* in 3-week-old flies significantly increased overall and daytime sleep (Fig. 7C). This was also accompanied by a decrease in overall, daytime, and nighttime activity (Fig. 7D). However, KD of *Lsn* did not affect overall sleep, but it significantly decreased overall and daytime activity (Fig.7C, D). To further characterize these sleep disruptions, we assessed the number of sleep bouts as another measure of fragmentation. Only *ATPSynC* and *Lsn* showed a significant increase in overall, daytime and nighttime sleep bout numbers (Fig. 7E). To identify a potential role of inflammation in a connection between CVD and sleep disruption, we measure *Upd3* levels in the

heads of flies of cardiac-specific KD. Remarkably, KD of *Lsn* in the heart led to a significant increase in *Upd3* levels in the head, while that of *ATPSynC* showed an increase that was not significant (Fig. 7F, p=0.33). This indicates an inflammatory state in the brain of these flies, which also had increased Upd3 levels in the heart (Fig. 4D) and were the only 2 genes that led to an increased sleep fragmentation phenotype (Fig. 7E). These novel findings suggest a non-cell-autonomous effect of these genes on CVD and sleep dysfunction through *Upd3*-specific inflammation. In support of our data, cardiac dysfunction has been associated with sleep disruptions in observational studies.[21,57]

Discussion

This study is the first to identify four genes at a single locus that link CVD and insomnia and characterize the two genes with the most severe cardiac phenotype, *ATPSynC* and *Lsn* (ATP5G1 and SNF8 in humans). Genetic screens have been previously applied in different model systems including zebrafish, *Drosophila* and mice to identify genes involved in different CVDs and sleep regulation.[58-64] Despite the numerous advantages of these models for functional and behavioral screening, only few studies have utilized them to test genes identified after human GWASs. A recent study[64] used Drosophila to identify causal variants reported in an insomnia GWAS[13], including our insomnia-related locus, and screen candidate genes to pinpoint those involved in sleep regulation. Another study used fish to screen and identify genes related to CVDs.[65] However, there are no studies to date that identify genes related to both diseases or identify functional genetic mechanisms underlying a connection between CVD and sleep dysfunction.

Here, we used an innovative approach integrating the use of human genetics in conjunction with fly genetics to identify genes related to each disease and advance the understanding of the association between CVD and insomnia. We focused on a genetic locus identified in both CVD and insomnia GWASs[13,15,23] and identified *Drosophila* orthologs of potential nearby causal genes (Fig. 1A, B). The locus we identified presented as a colocalization signal for both diseases. Interestingly, of 554 risk loci for insomnia identified thus far, this locus is among only three loci that colocalize with CAD

(pp >0.90; others include the ApoE region, *and LINGO4/RORC*)[53]. Functional dissection of an independent association signal for CAD in this same genomic region suggests complex contributions of multiple genes at the locus to CAD pathogenesis.[66]

The first objective of our study was to functionally identify a novel role of these genes in CVD and/or insomnia. Thus, we performed tissue-specific, neuronal and cardiac, KD, separately, of each gene. KD of *Imp* (IGF2BP1 in humans) significantly increased sleep which was fragmented (Fig. 2A-D, H-J), thus implicating this gene with sleep regulation. However, KD of *Imp* did not have a severe effect on the heart. Similarly, KD of *Bruce* (UBE2Z in humans) did not affect sleep but started showing a decrease in cardiac function at 3 weeks of age (Fig. S4A).

Neuronal suppression of *ATPSynC* increased overall sleep, however, further characterization of this sleep indicated it was highly fragmented (Fig. 2A-D, H-J). This sleep phenotype is supported by published findings in another study, screening insomnia-related genes identified from GWAS, which demonstrated neuronal-specific KD of *ATPSynC* lead to an increase in total sleep.[64] This increased sleep corresponded with decreased locomotor activity, which has been recently reported in humans and flies with mutations in *ATP5G1*/*ATPsynC*[67]. Cardiac suppression of *ATPSynC* significantly compromised cardiac function characterized by severely increased arrythmia (Fig. 3D) and disrupted structure and fibrosis (Fig.4A-C). It also increased *Upd3*-specific inflammation (Fig. 4D), which is an important indicator of cardiac injury. These findings revealed a novel role of *ATPSynC* in cardiac and sleep regulation in a cell-autonomous manner. Both the brain and heart require large amounts of ATP to perform their functions. In both organs, ATP is essential for electrophysiological activities in resting and active states[68,69], and reduction of ATP levels impairs neural and cardiac functions[68,70,71]. ATP is produced by ATP Synthase, and impairing the function of ATP Synthase is known to be associated with cardiovascular and neurological diseases.[72] *ATPSynC* is a component of ATP Synthase. Therefore, KD of *ATPSynC* potentially disrupts the function of ATP Synthase thus contributing to CVD and sleep disruptions

we observed. In order to identify a potential mechanism underlying the role of *ATPSynC* in cardiac function, we performed a KEGG pathway search and found a potential association of *ATPSynC* in cardiomyocyte death and eventual cardiac dysfunction.[73,74] Since it is well established that mitochondrial function including ATP Synthesis is important for cardiac performance[55,75], and in order to confirm the importance of *ATPSynC* in ATP Synthase, we suppressed *ATPSynGamma*, another component of the enzyme. Remarkably, we observed similar cardiac dysfunction with *ATPSynGamma* KD (Fig. 5C-H) as with *ATPSynC*. Also, KD of *ATPSynC* or *ATPSynGamma* increased cell death markers in the heart (Fig. 5I), supporting our hypothesis that the role of *ATPSynC* in ATP Synthase is important for cardiac function.

The second gene causing severe cardiac phenotypes is *Lsn*. Cardiac suppression of *Lsn* significantly compromised cardiac function characterized by significant dilation (Fig. 3F-H), and evident myofibril disorganization and fibrosis (Fig. 4A-C). It also showed *Upd3*-specific inflammation (Fig. 4D), indicating cardiac injury, along with a unique non-beating heart phenotype that worsened with age (Fig. 3A). These novel findings establish a cell-autonomous role of *Lsn* in cardiac dysfunction. *Lsn* is part of the ESCRT pathway, which is a key mechanism of multivesicular body (MVB) biogenesis.[27,76] MVBs form exosomes which are crucial for intercellular communication and have been implicated in the pathophysiology of CVD and other diseases.[27,76,77] Therefore, in order to study the mechanism underlying the role of *Lsn* in cardiac function, we hypothesized that its involvement in the ESCRT pathway is important for cardiac function. There are four main ESCRT complexes: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. *Lsn* is part of the ESCRT-II complex along with *Vps25* and *Vps36* and this complex signals for ESCRT-III, which is made up of *Vps2*, *shrb*, *Vps20* and *Vps24* (Fig. 6A). Interestingly, the suppression of *Lsn* led to an increase in ESCRT-III components, suggesting a compensatory mechanism to the deficiency in Lsn levels (Fig. S6). Moreover, suppressing both ESCRT-II components and 3 out 4 of the ESCRT-III components in the heart led to a similar cardiac phenotype as that observed upon *Lsn* suppression (Fig. 6B-M); thus, supporting our hypothesis.

Our next objective was to assess associations between CVD and insomnia and assess the effects of one disease on the other through these genes. First, we suppressed these genes neuronally and assessed cardiac function (Fig. 7A). Unlike cardiac KD, neuronal suppression of only *Imp* significantly reduced cardiac function (Fig. 7B) which goes along the strong sleep phenotypes observed upon neuronal *Imp* KD. This novel finding provides a proof of an influence of sleep disfunction on cardiovascular performance, supporting mendelian randomization reports that show an effect of insomnia on CVD.

Although previous observational and genetic studies more commonly report an effect of sleep on CVD, some human studies show an effect of heart failure on sleep interruption.[20] Therefore, we were interested in assessing whether there is an influence in the opposing direction, from heart on the brain. Therefore, we suppressed these genes in the heart and assessed sleep physiology (Fig. 7A). We observed non-cell-autonomous effects on sleep in all genes with cardiac dysfunction after cardiac KD in 3-week-old flies. Although cardiac KD of *Bruce* did not affect heart function in 1-week-old flies, it significantly reduced cardiac function in 3-week-old flies. This is consistent with our findings when assessing non-cell-autonomous effects on sleep, where KD of *Bruce* significantly increased sleep (Fig. 7C-E). This shows an evident effect of cardiac dysfunction on sleep. These finding suggest a non-cell-autonomous influence of *ATPSynC*, *Lsn* and *Bruce* in sleep regulation.

While associations between the heart and nervous system have been reported, underlying mechanisms remain poorly understood.[18,78,79] We hypothesized that inflammation is a mechanism underlying the effects of cardiac KD observed on sleep. Interestingly, KD of *Lsn* and *ATPSynC* increased inflammation in the head after cardiac suppression (Fig. 7F) which supports our hypothesis.

In conclusion, we have identified four novel genes that are associated with CVD and sleep dysfunction cell-autonomously and non-cell-autonomously through elevations in proinflammatory biomarkers. We also demonstrated that two genes, not reported before, *ATPSynC* and *Lsn*, are important for cardiac function through their respective roles in ATP Synthase and the ESCRT pathway. These findings provide basis for future

studies to help develop therapeutic strategies that prevent or attenuate insomnia and coincident CVD.

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Human Symbol	Drosophila Symbol	% Similarity
ATP5G1	ATPSynC	83
UBE2Z	Bruce	54

SNF8	Lsn	71
IGF2BP1	Imp	58
GIP	-	-

Table 1. Human and fly symbols of CVD- and insomnia-related genes with %similarity at a single locus.

Figure 1

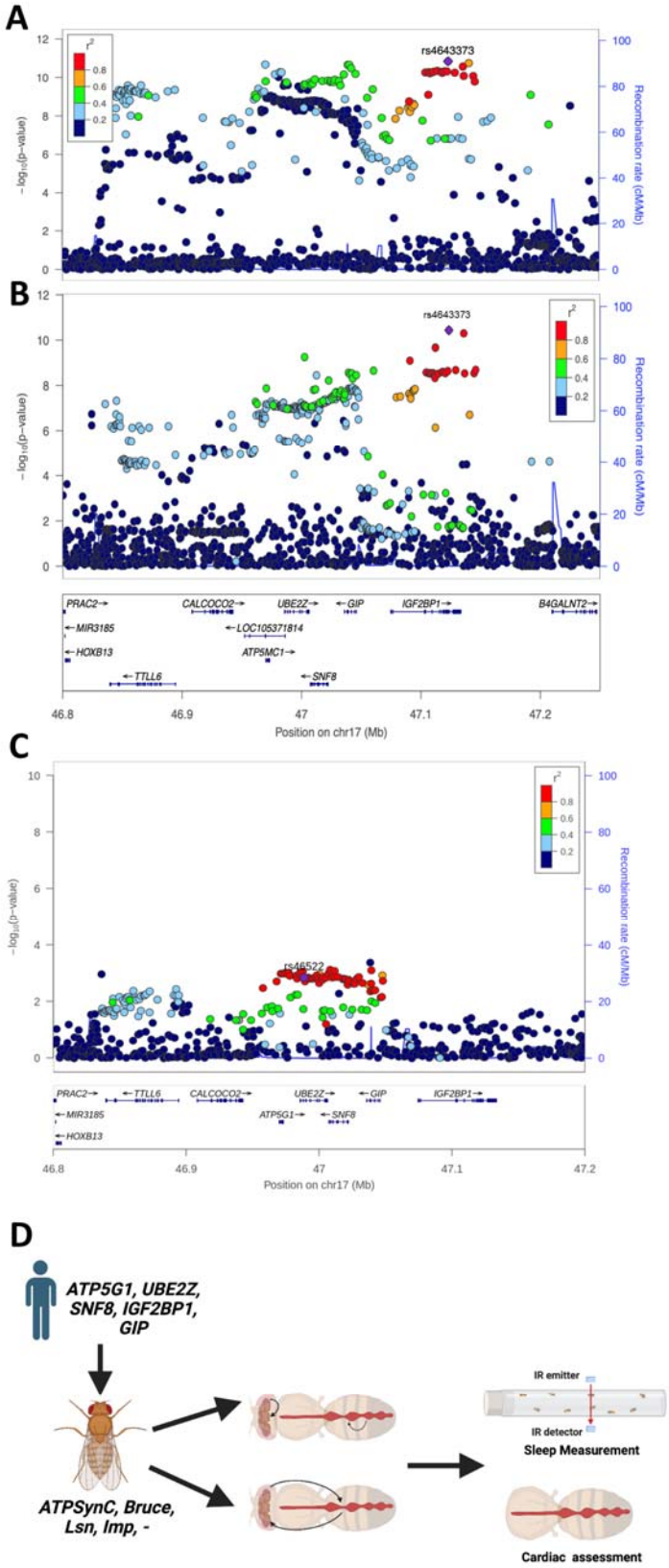
A
rs4643373
r^2
0.8
0.6
0.4
0.2
$-\log_{10}$(p-value)
Recombination rate (cM/Mb)
B
rs4643373
PRAC2
CALCOCO2
UBE2Z
GIP
IGF2BP1
B4GALNT2
MIR3185
LOC105371814
HOXB13
ATP5MC1
TTLL6
SNF8
46.8
46.9
47
47.1
47.2
Position on chr17 (Mb)
C
rs46522
PRAC2
TTLL6
CALCOCO2
UBE2Z
GIP
IGF2BP1
MIR3185
ATP5G1
SNF8
HOXB13
Position on chr17 (Mb)
D
ATP5G1, UBE2Z, SNF8, IGF2BP1, GIP
ATPSynC, Bruce, Lsn, Imp, -
IR emitter
IR detector
Sleep Measurement
Cardiac assessment

Figure1. CVD- and insomnia-related locus and nearby genes eQTL analyses. Manhattan plots (LocusZoom) showing CVD (A) and insomnia (B) SNP association peaks with 5 nearby candidate genes, *ATP5MC1 (ATP5G1), UBE2Z, SNF8, GIP, IGF2BP1*. LocusZoom plot showing lead SNP rs46522 after conditioning out rs4643373 in the CAD GWAS (C). Graphical Scheme showing workflow (D).

Figure 2

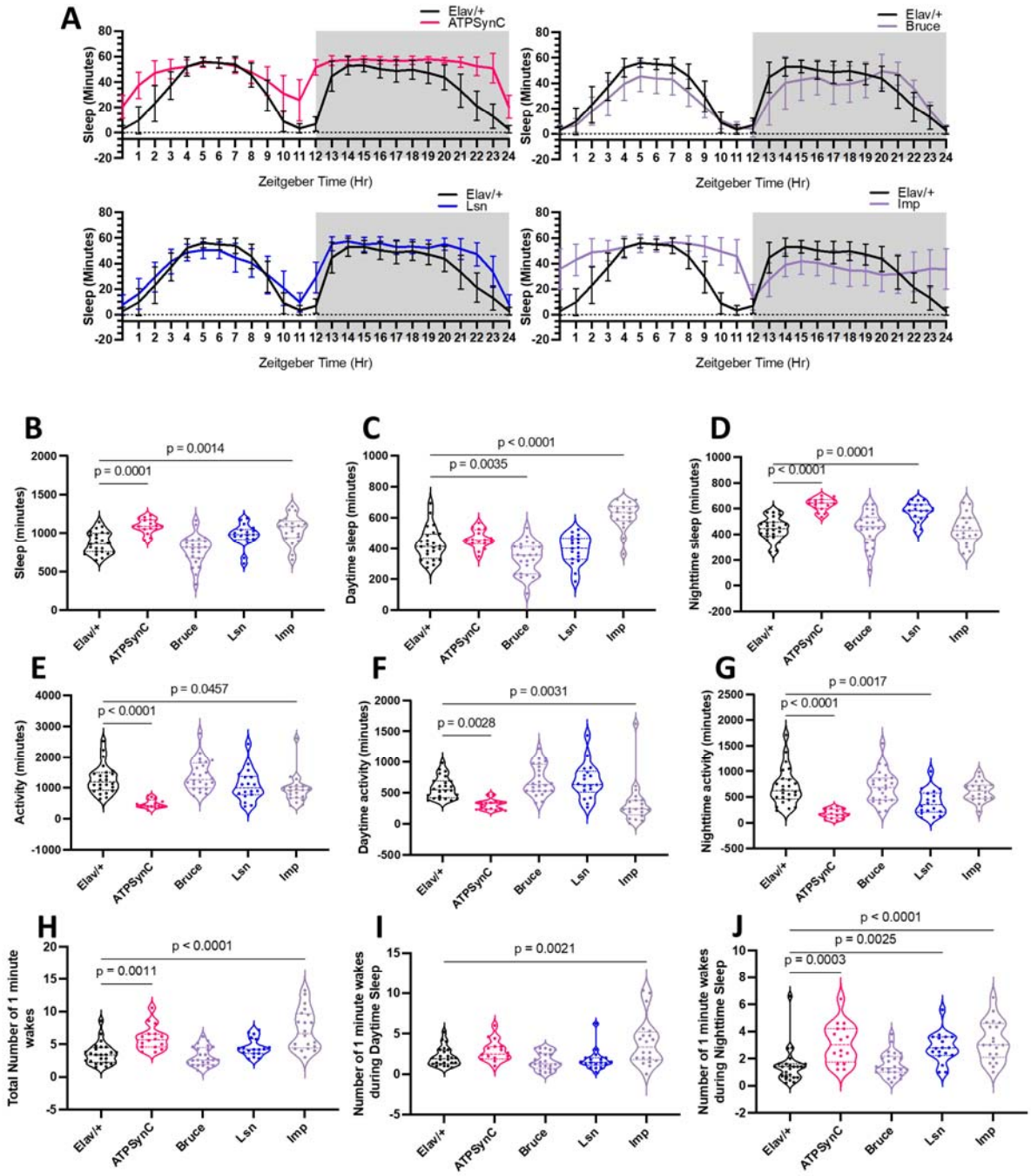
A
Elav/+
ATPSynC
Bruce
Lsn
Imp
Sleep (Minutes)
Zeitgeber Time (Hr)
B
C
D
E
F
G
H
I
J
Sleep (minutes)
Daytime sleep (minutes)
Nighttime sleep (minutes)
Activity (minutes)
Daytime activity (minutes)
Nighttime activity (minutes)
Total Number of 1 minute wakes
Number of 1 minute wakes during Daytime Sleep
Number of 1 minute wakes during Nighttime Sleep
p = 0.0014
p = 0.0001
p < 0.0001
p = 0.0035
p = 0.0001
p < 0.0001
p = 0.0457
p < 0.0001
p = 0.0031
p = 0.0028
p = 0.0017
p < 0.0001
p < 0.0001
p = 0.0011
p = 0.0021
p < 0.0001
p = 0.0025
p = 0.0003

Figure 2. Neuronal-specific suppression of CVD- and insomnia- related genes leads to compromised sleep phenotypes. Sleep profiles showing sleep minutes per hour for 24 hours (A). Violon plots for quantitative sleep parameters, total sleep amount (B), daytime sleep amount (C), nighttime sleep amount (D), total locomotor activity (E), daytime activity (F), nighttime activity (G), total sleep fragmentation (H), daytime sleep fragmentation (I), and nighttime sleep fragmentation (J) from 1-week-old male *Drosophila* with neuronal RNAi knockdown of CVD- and insomnia-related genes with the pan-neuronal *Elav-Gal4* driver. N=16-24 for each group. For ATPSynC, Line 1 was lethal; Line 2 was used (refer to methods section). Data was collected from at least 2 independent experiments from one RNAi line (second line data and statistical analyses shown in Figure S2 and Table S2 respectively). Each data point represents a fly. Statistics were calculated by one-way ANOVA for comparison to controls.

Figure 3

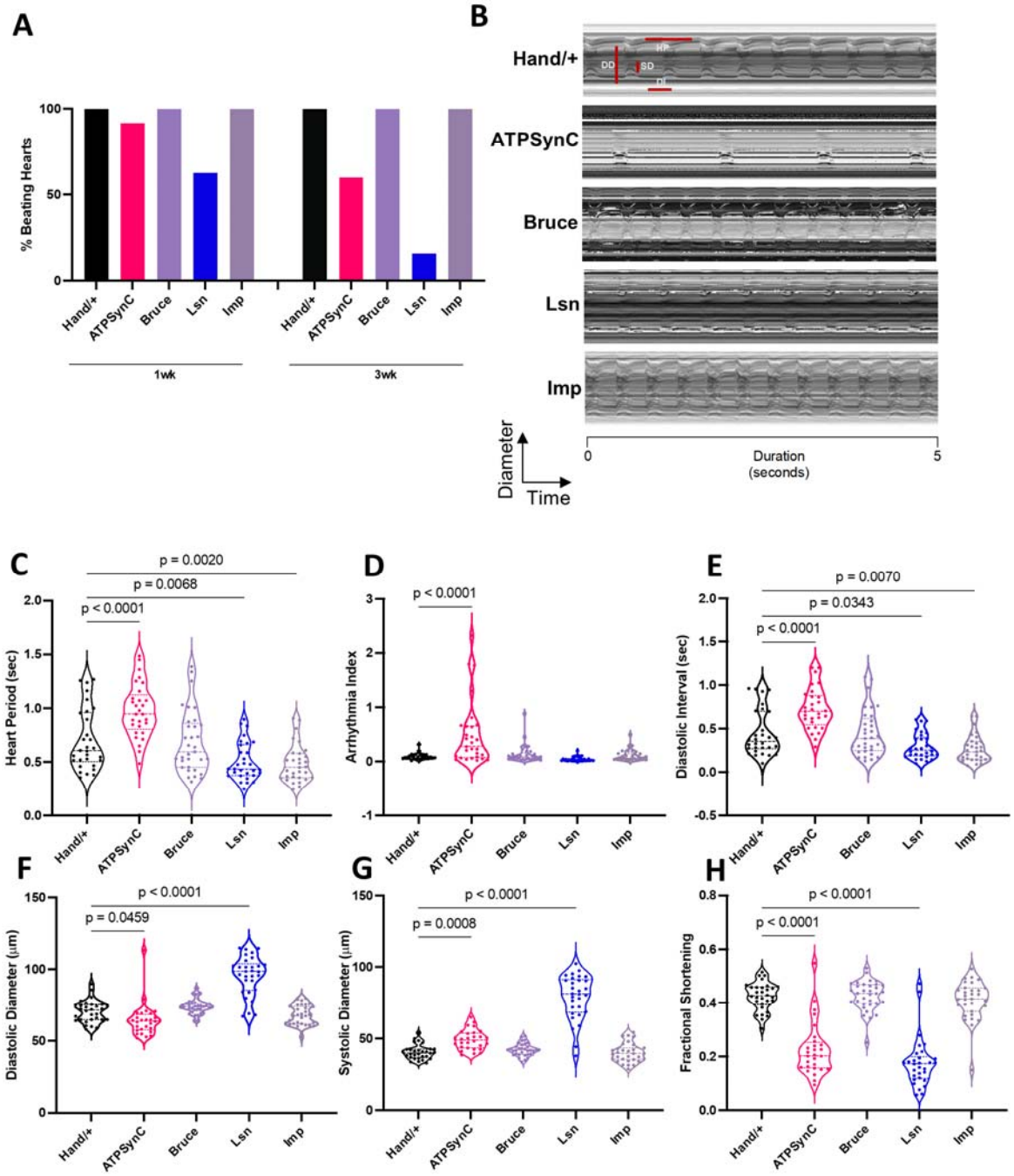
A
% Beating Hearts
100
50
0
Hand/+
ATPSynC
Bruce
Lsn
Imp
1wk
3wk
B
Hand/+
ATPSynC
Bruce
Lsn
Imp
HP
DD
SD
Diameter
Time
0
Duration
(seconds)
5
C
p = 0.0020
p = 0.0068
p < 0.0001
Heart Period (sec)
D
p < 0.0001
Arrhythmia Index
E
p = 0.0070
p = 0.0343
p < 0.0001
Diastolic Interval (sec)
F
p < 0.0001
p = 0.0459
Diastolic Diameter (μm)
G
p < 0.0001
p = 0.0008
Systolic Diameter (μm)
H
p < 0.0001
p < 0.0001
Fractional Shortening

Figure 3. Cardiac-specific suppression of CVD- and insomnia-related genes leads to cardiac dysfunction. Representative 5-second mechanical-modes (A) from 1-week-old male flies with cardiac RNAi knockdown of CVD- and insomnia-related genes with cardiac-specific Hand-Gal4 driver. Percentage of beating hearts at 1 versus 3 weeks of age shows significant effect of Lsn KD with age ($p<0.0001$) (B). Violin plots for cardiac physiological parameters, heart period (C), arrythmia index (D), diastolic interval (E), diastolic diameter (F), systolic diameter (G), fractional shortening (H), and N= 29-33 for each group for C-H, N=32-51 for each group for B. Each data point represents one fly. Data was collected from at least 2 independent experiments from one RNAi line (second line data shown in Fig. S2). Statistics were calculated by 1-way ANOVA for C-H. Fisher's exact test was performed for B.

Figure 4

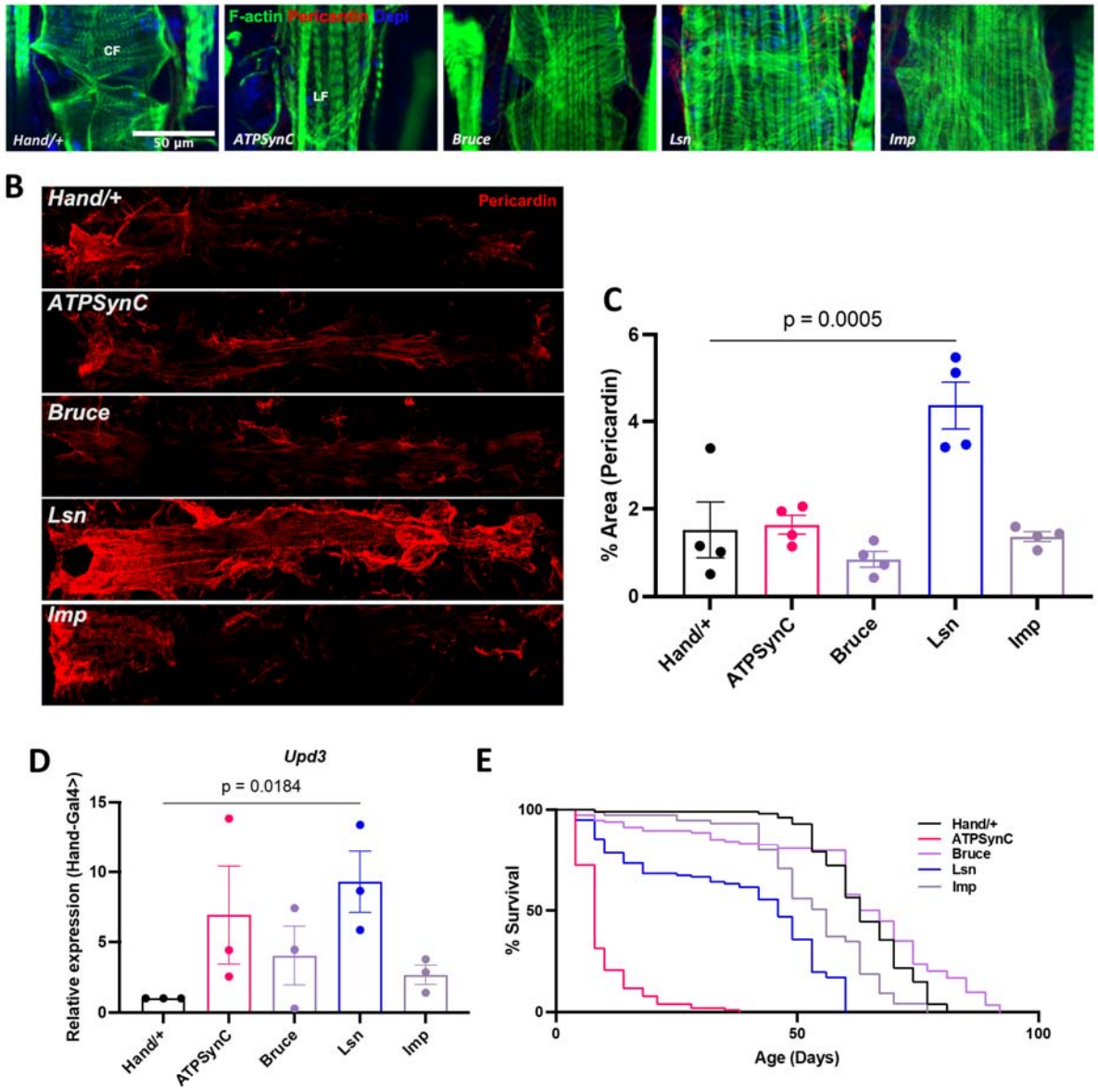
A
F-actin Pericardin Dapi
CF
LF
Hand/+
50 µm
ATPSynC
Bruce
Lsn
Imp
B
Hand/+
Pericardin
ATPSynC
Bruce
Lsn
Imp
C
p = 0.0005
% Area (Pericardin)
Hand/+
ATPSynC
Bruce
Lsn
Imp
D
Upd3
p = 0.0184
Relative expression (Hand-Gal4>)
Hand/+
ATPSynC
Bruce
Lsn
Imp
E
% Survival
Age (Days)
Hand/+
ATPSynC
Bruce
Lsn
Imp

Figure 4. Cardiac-specific suppression of CVD- and insomnia-related genes leads to myofibrillar disorganization, cardiac fibrosis, inflammation, and shortened lifespan. Representative images showing actin-containing myofibrils (A) and pericardin (B) from 1-week-old male flies with cardiac RNAi knockdown of CVD- and insomnia-related genes with *Hand-Gal4.* Each data point is a fly. Quantification of pericardin signal (C). Inflammatory markers UPD3 (D) transcript levels in male hearts (n=10-12 per data point per group. Lifespan assay (E) for male flies with cardiac RNAi knockdown of CVD- and insomnia-related genes with cardiac-specific *Hand-Gal4* driver resulted in significant decrease in lifespan ($p<0.0001$) of ATPSynC, Lsn and Imp, and a significant increase in lifespan of Bruce ($p=0.0057$). Graph plots % survival ($n>100$ for each group) vs. time post-eclosion. Statistics were calculated by 1-way ANOVA for C-D and a Kaplan-Meier test was performed for G.

Figure 5

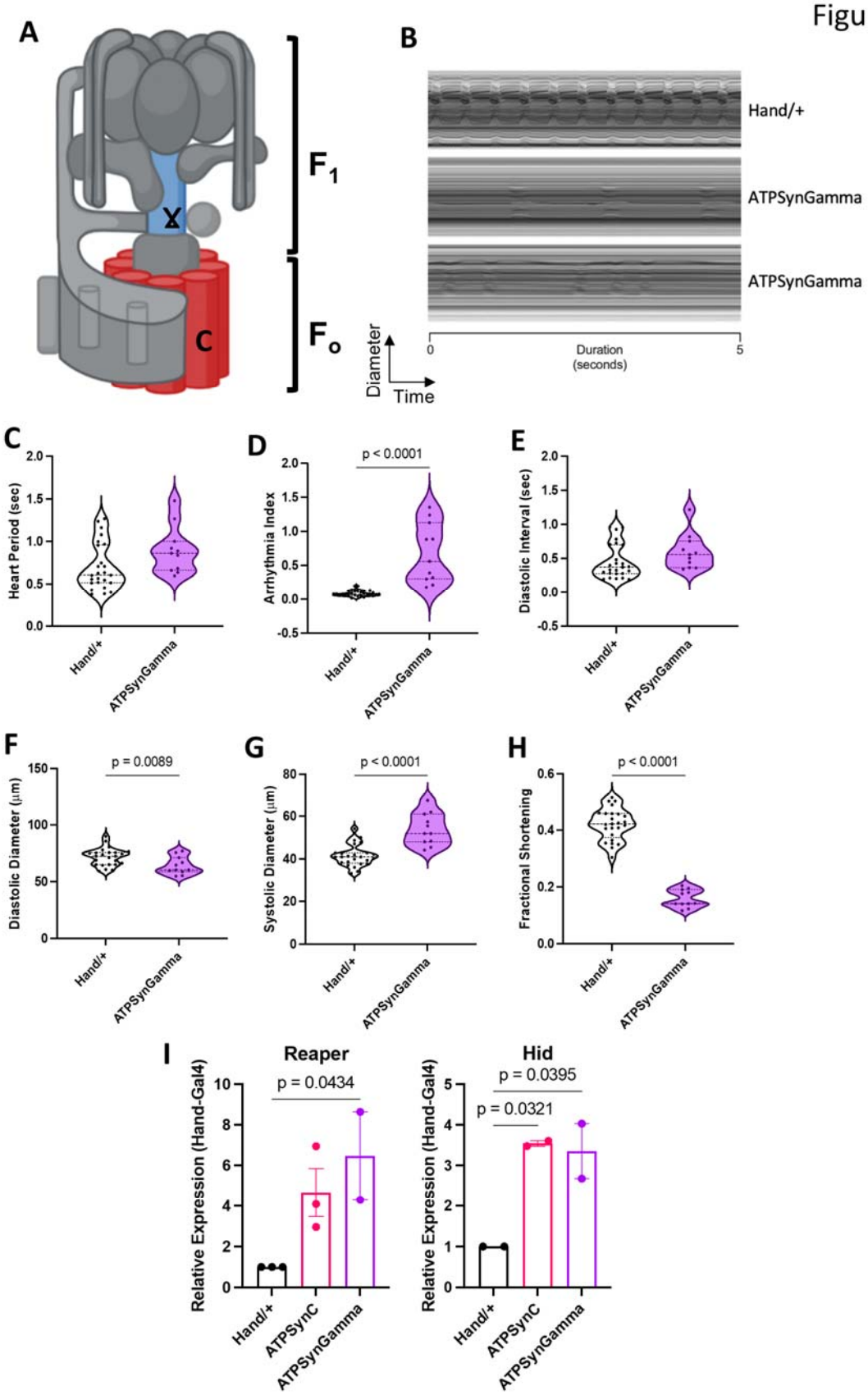
A
F1
Fo
γ
C
B
Hand/+
ATPSynGamma
ATPSynGamma
Diameter
Time
0
Duration
(seconds)
5
C
Heart Period (sec)
Hand/+
ATPSynGamma
D
p < 0.0001
Arrhythmia Index
Hand/+
ATPSynGamma
E
Diastolic Interval (sec)
Hand/+
ATPSynGamma
F
p = 0.0089
Diastolic Diameter (μm)
Hand/+
ATPSynGamma
G
p < 0.0001
Systolic Diameter (μm)
Hand/+
ATPSynGamma
H
p < 0.0001
Fractional Shortening
Hand/+
ATPSynGamma
I
Reaper
p = 0.0434
Relative Expression (Hand-Gal4)
Hand/+
ATPSynC
ATPSynGamma
Hid
p = 0.0395
p = 0.0321
Relative Expression (Hand-Gal4)
Hand/+
ATPSynC
ATPSynGamma

Figure 5. Knockdown of *ATPSynC* leads to disruption of ATP Synthase. Graphical scheme showing ATP Synthase complex (red is *ATPSynC*, blue is *ATPSynGamma*) (A). Representative 5-second mechanical-modes showing beating pattern observed in flies with cardiac-specific knockdown of *ATPSynGamma* (B) using *Hand-Gal4*. Violin plots for cardiac physiological parameters from 1-week-old male flies with cardiac-specific knockdown of *ATPSynGamma* (C-H). N=11-25 per group. Each data point represents one fly. Transcript levels of Upd3 in hearts of 1-week-old flies with cardiac-specific knockdown of these genes (N= 7-10 hearts per data point per group) (F). Statistics were calculated by unpaired t-test for C-H, and 1-way ANOVA for I.

Figure 6

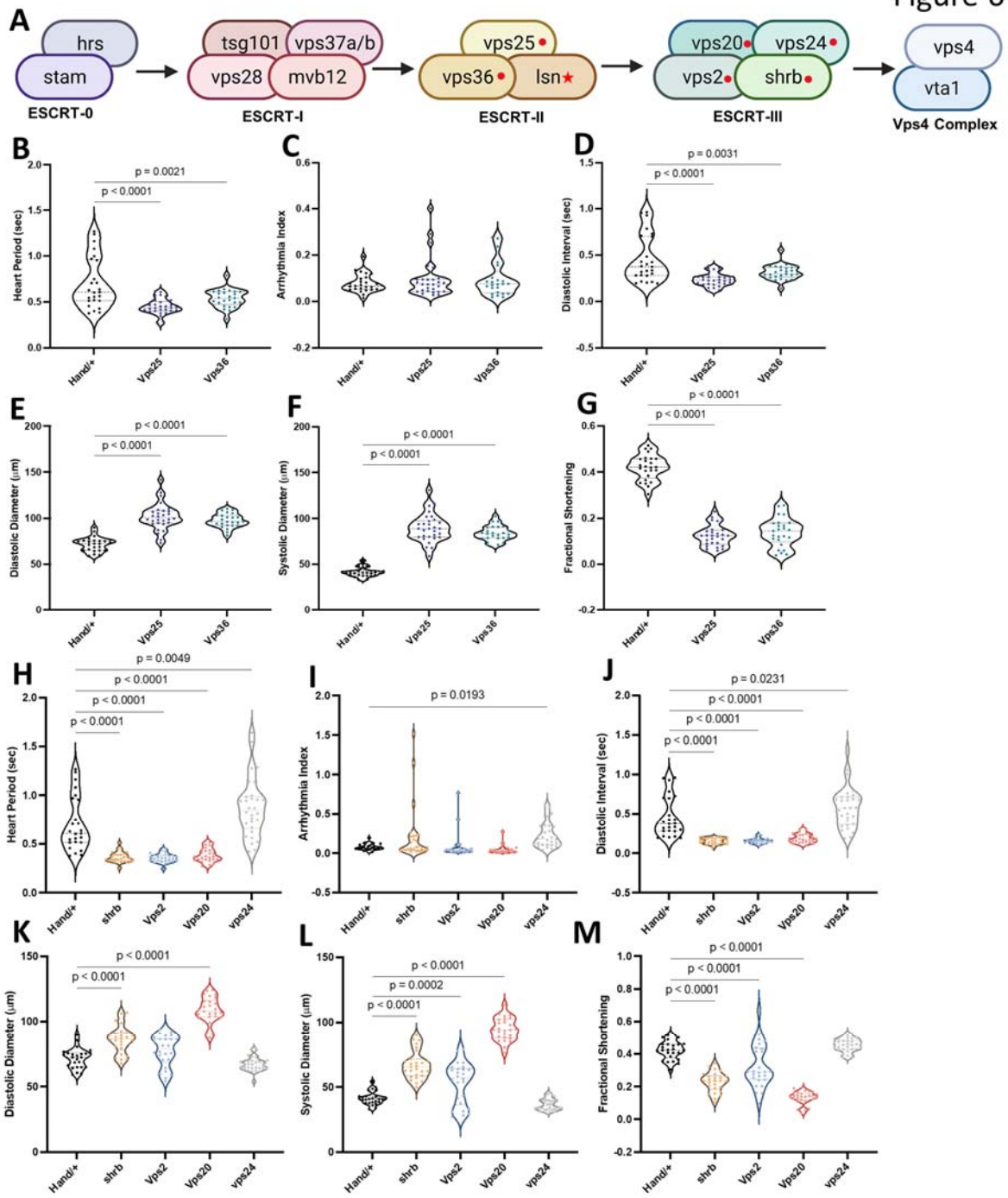
A
hrs
stam
ESCRT-0
tsg101
vps37a/b
vps28
mvb12
ESCRT-I
vps25
vps36
lsn
ESCRT-II
vps20
vps24
vps2
shrb
ESCRT-III
vps4
vta1
Vps4 Complex
B
Heart Period (sec)
p = 0.0021
p < 0.0001
C
Arrhythmia Index
D
Diastolic Interval (sec)
p = 0.0031
p < 0.0001
E
Diastolic Diameter (µm)
p < 0.0001
p < 0.0001
F
Systolic Diameter (µm)
p < 0.0001
p < 0.0001
G
Fractional Shortening
p < 0.0001
p < 0.0001
H
Heart Period (sec)
p = 0.0049
p < 0.0001
p < 0.0001
p < 0.0001
I
Arrhythmia Index
p = 0.0193
J
Diastolic Interval (sec)
p = 0.0231
p < 0.0001
p < 0.0001
p < 0.0001
K
Diastolic Diameter (µm)
p < 0.0001
p < 0.0001
L
Systolic Diameter (µm)
p < 0.0001
p = 0.0002
p < 0.0001
M
Fractional Shortening
p < 0.0001
p < 0.0001
p < 0.0001
Hand/+
Vps25
Vps36
shrb
Vps2
Vps20
vps24

Figure 6. Knockdown of *Lsn* leads to the disruption of the ESCRT pathway. Graphical scheme showing ESCRT pathway (A). Violin plots for cardiac physiological parameters, heart period (B), arrythmia index (C), diastolic interval (D), diastolic diameter (E), systolic diameter (F), fractional shortening (G) from 1-week-old male flies with cardiac-specific knockdown of ESCRT-II genes (B-G) and ESCRT-III genes (H-M) using *Hand-Gal4*. N= 25-30 per group. Each data point represents one fly. Statistics were calculated by 1-way ANOVA.

Figure 7

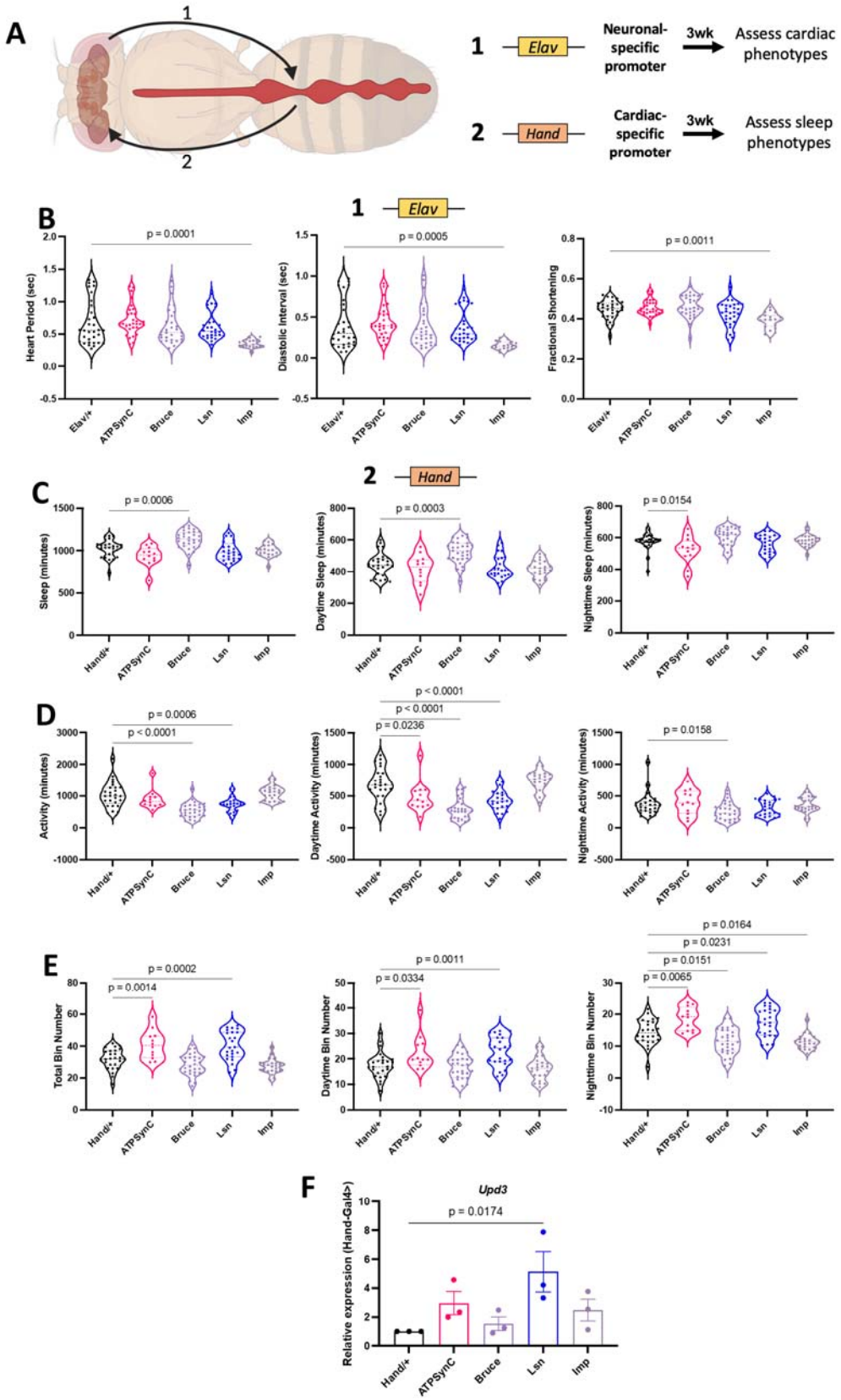
A
1
2
1 Elav Neuronal-specific promoter 3wk Assess cardiac phenotypes
2 Hand Cardiac-specific promoter 3wk Assess sleep phenotypes
B
1 Elav
Heart Period (sec)
p = 0.0001
Diastolic Interval (sec)
p = 0.0005
Fractional Shortening
p = 0.0011
Elav/+
ATPSynC
Bruce
Lsn
Imp
C
2 Hand
Sleep (minutes)
p = 0.0006
Daytime Sleep (minutes)
p = 0.0003
Nighttime Sleep (minutes)
p = 0.0154
Hand/+
D
Activity (minutes)
p = 0.0006
p < 0.0001
Daytime Activity (minutes)
p < 0.0001
p < 0.0001
p = 0.0236
Nighttime Activity (minutes)
p = 0.0158
E
Total Bin Number
p = 0.0002
p = 0.0014
Daytime Bin Number
p = 0.0011
p = 0.0334
Nighttime Bin Number
p = 0.0164
p = 0.0231
p = 0.0151
p = 0.0065
F
Upd3
Relative expression (Hand-Gal4>)
p = 0.0174

Figure 7. Non-cell-autonomous mechanisms linking CVD with insomnia. Graphical scheme showing experimental layout (A). Violin plots for cardiac physiological parameters, heart period, diastolic interval and fractional shortening from 3-week-old male flies with neuronal-specific knockdown of CVD- and insomnia-related genes (N= 19-32 per group) (B). Violin plots for quantitative sleep parameters; sleep amount (C), locomotor activity (D), and bin number (E) from 3-week-old male *Drosophila* with cardiac-specific knockdown of CVD- and insomnia-related genes. N= 12-30 per group. Each data point represents one fly. Transcript levels of Upd3 in heads of 3-week-old flies with cardiac-specific knockdown of these genes (N= 7-10 heads per data point per group) (F). Statistics were calculated by 1-way ANOVA.