

1 **LKB1 Is Physiologically Required for Sleep from *Drosophila melanogaster* to the**
2 ***Mus musculus***

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20 **ABSTRACT**

21 Liver Kinase B1 (LKB1) is known as a master kinase for 14 kinases related to the
22 adenosine monophosphate (AMP)-activated protein kinase (AMPK). Two of them salt
23 inducible kinase 3 (SIK3) and AMPK α have previously been implicated in sleep
24 regulation. We generated loss-of-function (LOF) mutants for Lkb1 in both Drosophila
25 and mice. Sleep, but not circadian rhythms, was reduced in Lkb1-mutant flies and in
26 flies with neuronal deletion of Lkb1. Genetic interactions between Lkb1 and
27 Threonine to Alanine mutation at residue 184 of AMPK in Drosophila sleep or those
28 between Lkb1 and Threonine to Glutamic Acid mutation at residue 196 of SIK3 in
29 Drosophila viability have been observed. Sleep was reduced in mice after virally
30 mediated reduction of Lkb1 in the brain. Electroencephalography (EEG) analysis
31 showed that non-rapid eye movement (NREM) sleep and sleep need were both reduced
32 in Lkb1-mutant mice. These results indicate that LKB1 plays a physiological role in
33 sleep regulation conserved from flies to mice.

34

35 INTRODUCTION

36 Human Peutz-Jeghers syndrome (PJS) (Jeghers et al. 1949; Peutz 1921) is an autosomal
37 dominant disorder with gastrointestinal (GI) polyps and increased cancer risk of
38 multiple tissues (Tomlinson and Houlston 1997; Westerman et al. 1999). The gene
39 mutated in, and responsible for, PJS encodes the liver kinase B1 (LKB1, also known as
40 STK11) (Hemminki et al. 1998; Hemminki et al. 1997; Jenne et al. 1998). *Lkb1* is thus
41 a tumor suppressor gene, mutated in multiple cancers, especially the GI (Bardeesy et al.
42 2002; Hearle et al. 2006; Jishage et al. 2002; Mehenni et al. 1998; Miyoshi et al. 2002)
43 and lung adenocarcinoma (Carretero et al. 2004; Gill et al. 2011; Ji et al. 2007;
44 Matsumoto et al. 2007; Sanchez-Cespedes et al. 2002; Skoulidis et al. 2015), cervical
45 cancer (Wingo et al. 2009), ovarian cancer (Tanwar et al. 2014), breast cancer (Hearle
46 et al. 2006; Sengupta et al. 2017; Shen et al. 2002), pancreatic cancer (Morton et al.
47 2010) and melanoma (Guldberg et al. 1999; Rowan et al. 1999).

48 LKB1 phosphorylates threonine 172 (T172) of the α subunit of adenosine
49 monophosphate (AMP)-activated protein kinase (AMPK α) (Hawley et al. 2003; Hong
50 et al. 2003; Lizcano et al. 2004; Sakamoto et al. 2005; Shaw et al. 2004; Shaw et al.
51 2005; Sutherland et al. 2003; Woods et al. 2003), and positively regulates the activity of
52 AMPK.

53 AMPK is a well-known kinase (Beg et al. 1973; Carling et al. 1989; Carling et al.
54 1987; Carlson and Kim 1973; Ferrer et al. 1985; Ingebritsen et al. 1978; Munday et al.
55 1988; Yeh and Kim 1980) with important physiological and pathological roles (Hardie

56 2014; Hardie et al. 2016; Herzig and Shaw 2018; Lopez and Dieguez 2014). The α , β
57 and γ subunits of AMPK form a heterotrimeric complex (Davies et al. 1994; Michell et
58 al. 1996; Mitchelhill et al. 1994). The catalytic α subunit is regulated by
59 phosphorylation at T172 of AMPK α 2 or T183 of AMPK α 1 (Hawley et al. 1996).

60 There are 12 additional mammalian AMPK-related kinases (ARKs), similar to the
61 α subunit of AMPK, all regulated at the site equivalent to AMPK-T172 (Lizcano et al.
62 2004). LKB1 and its associated proteins STE20-related adaptor (STRAD) and mouse
63 protein 25 (MO25) have been reported to phosphorylate all 14 ARKs (Lizcano et al.
64 2004), making LKB1 a master kinase for ARKs (Alessi et al. 2006; Lizcano et al. 2004;
65 Shackelford and Shaw 2009). We have recently found that more than 20 kinases in the
66 STE20 family of mammalian serine-threonine kinases could phosphorylate ARKs in
67 vitro, though the physiological roles of STE20 kinases in ARK phosphorylation remain
68 unknown (Liu et al., 2022a, 2022b).

69 Some ARKs have been reported to regulate sleep. In mice, inhibitors of AMPK
70 were found to decrease sleep, whereas activators of AMPK were found to increase
71 sleep (Chikahisa et al. 2009). In flies, knockdown of AMPK β in neurons decreased the
72 total amount of sleep and resulted in fragmented sleep (Nagy et al. 2018). Knockdown
73 of AMPK α in a specific pair of neurons suppressed sleep (Yurgel et al. 2019).

74 Studies in mice have shown that sleep was increased in gain-of-function (GOF)
75 mutations in the salt inducible kinase (SIK) 3 (Funato et al. 2016), and sleep need was
76 reduced in GOF mutants of SIK 1, 2 and 3 (Funato et al. 2016; Honda et al. 2018; Park

77 et al. 2020). Sleep was also decreased when SIK3 was downregulated in flies (Funato et
78 al. 2016). Here we investigated the functional role of LKB1 in regulating sleep in flies
79 and mice.

80

81 **MATERIALS AND METHODS**

82 **Fly lines and rearing conditions:** Flies were reared on standard corn meal at 25°C,
83 60% humidity and kept in 12 hours (h) light/12 h dark (LD) conditions. 57C10-Gal4,
84 nos-phiC31, hs-Cre on X were from the Bloomington Stock Center. vas-Cas9 was a
85 gift from Dr. J. Ni (Tsinghua University, Beijing). UAS-Cas9, UAS-Ampk,
86 UAS-Ampk-T184A, UAS-Ampk-T184E, Sik3-flag, Sik3--T196A-flag,
87 Sik3-T196E-flag flies were from our laboratory.

88 Flies used in behavioral assays were backcrossed into our isogenized Canton S
89 background for 7 generations. All results of sleep analysis in this paper were obtained
90 from female flies.

91 **Generation of KO, KI and transgenic lines:** Total RNA was extracted from isoCS
92 by TRIzol reagent (Invitrogen). Using the PrimeScript II 1st Strand cDNA Synthesis
93 Kit (Takara), we reverse-transcribed the extracted mRNA into cDNA. The UAS-Lkb1
94 flies was constructed by inserting the coding sequence of CG9374 amplified from
95 cDNA into the pACU2 plasmid (a gift from the Jan Lab at UCSF) (Han et al. 2011)
96 before being inserted into the attP40 site.

97 The UAS-Lkb1-sgRNA construct was designed by inserting the sgRNAs into
98 pMt:sgRNA^{3XEF} vectors based on pACU2, with rice tRNA separating the different

99 sgRNAs. CRISPR-Gold website was used to design 3 sgRNAs of Lkb1 (Figure S3)
100 (Chu et al. 2016; Poe et al. 2019). The construct was inserted into the attP40 site.

101 KO and KI lines were generated as described previously (Deng et al. 2019).
102 Knockout flies were generated with the CRISPR/Cas9 system. Two different sgRNAs
103 were constructed with U6b-sgRNA plasmids. The 5' homologous arm and the 3'
104 homologous arm of ~2kb amplified from the wt fly genome were inserted into a
105 pBSK plasmid for homologous recombination repair. The cassette of attP-3P3-RFP
106 was introduced in the middle. sgRNA plasmids and the donor plasmids were injected
107 into vas-Cas9 embryos to introduce attP-3P3-RFP into the genome at the region of
108 interest and replaced it by homologous recombination. 3P3-RFP served as a marker to
109 screen for the correct flies. Primers across the homologous arms were designed to
110 verify the sequences by PCR and DNA sequencing. attP site was introduced into the
111 genome with 3P3-RFP-LoxP. For KI files, the nos-phiC31 virgin females were first
112 crossed with knock-out males and the pBSK plasmid inserted with
113 attB-T2A-Gal4-miniwhite-LoxP cassette was injected into the female embryos.
114 Miniwhite serves as a marker to screen for the correct flies, which could be excised by
115 the Cre/LoxP system. Primers were designed to verify the sequence by PCR and DNA
116 sequencing.

117 **Quantitative PCR:** Total RNA was extracted from 30 flies of 5-7 days old by TRIzol
118 reagent (Invitrogen). The genomic template was removed using DNase (Takara).
119 cDNA was reverse-transcribed using Takara's PrimeScript II 1st Strand cDNA

120 synthesis kit (Takara). Quantitative PCR was carried out with TransStart Top Green
121 qPCR SuperMix kit (TransGen) in the Bio-Rad PCR system (CFX96 Touch Deep
122 Well). The sequences of primers used to detect Lkb1 and RP49(endogenous control)
123 mRNA are:

124 Lkb1-F: 5' -GCCGTCAAGATCCTGACTA-3'

125 Lkb1-R: 5'-CTCCGCTGGACCAGATG-3'

126 Rp49-F: 5'-CGACGCTTCAAGGGACAGTATC-3'

127 Rp49-R: 5'-TCCGACCAGGTTACAAGAACTCTC-3'

128 **Drosophila sleep analysis:** Drosophila sleep analysis was performed as described
129 previously (Dai et al. 2019; Qian et al. 2017). 5-7 days old flies were placed in a
130 65mm x 5mm clear glass tube with one end containing food and the other end
131 plugging with cotton. All flies were recorded by video-cameras. Before sleep
132 measurement, flies were entrained to an LD cycle at 25°C, 60% humidity for at least
133 two days, and infrared LED light was used to ensure constant illumination when lights
134 off. Immobility longer than 5 minutes was defined as one sleep event (Hendricks et al.
135 2000; Shaw et al. 2000). Information of fly location was tracked and sleep parameters
136 were analyzed using Matlab (Mathworks), from which dead flies were removed.
137 Sleep duration, sleep bout duration, sleep bout number and sleep latency for each LD
138 were analyzed. Each experiment was repeated at least three times.

139 **Drosophila circadian analysis:** Flies were reared and recorded in the same condition
140 as sleep assay as described in papers from our lab (Dai et al. 2021; Qian et al. 2017),

141 except that the condition was constant darkness. 6-8 days activity was measured and
142 calculated in ActogramJ (Klarsfeld et al. 2003). Rhythmic strength, power and period
143 were calculated by Chi-square method.

144 **Immunoblot analysis:** Mouse brains were quickly dissected and washed with
145 phosphate buffer saline (PBS) on ice. Lysis buffer (20mM HEPES, 10mM KCl,
146 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM DTT, freshly supplemented with a
147 protease and phosphatase inhibitors cocktail) were used to homogenize brains by
148 homogenizer (Wiggins, D-500 pro) at 4°C. Brain homogenates were centrifuged at
149 14,000 revolution per minutes (rpm) for 15 minutes at 4°C. The supernatant was
150 transferred to a new microtube and quantified with bicinchoninic acid assay (Thermo
151 Fisher, 23225). The supernatant was analyzed by SDS-PAGE and proteins were
152 transferred to a nitrocellulose membrane (GE Healthcare, #BA85). Membranes were
153 incubated for 1 h in a blocking solution (Tris-buffered saline (TBS) containing 0.1%
154 Tween-20, 5% milk). Primary antibodies were anti-LKB1 (cell signaling, #3047) and
155 anti-ACTIN (Santa Cruz, sc-8342).

156 **Retro-orbital injection in mice:** Mice were reared at controlled temperature and
157 humidity conditions with 12 h light/ 12 h dark cycle. Food and water were provided
158 ad libitum. Lkb1^{fl/fl} mice were from the Jackson Laboratory (JAX #014143). They
159 contained loxP sites flanking exons 3-6 of Lkb1 gene (Nakada et al. 2010).
160 AAV-PHP.eB-hSyn-Cre-GFP and AAV-PHP.eB-hSyn-GFP virus were from Chinese

161 Institute for Brain Research, Beijing. All results of sleep analysis in this paper were
162 obtained from female mice.

163 0.2 ml/10 g Avertin was injected intraperitoneally into the mice for
164 anesthetization. Rodent eyes were protruded by gentle downward pressure to the skin
165 on the dorsal and ventral sides of the eye. The operator inserted the needle beveled
166 downward into the retro-orbital sinus at the medial corner of the eye (Yardeni et al.
167 2011). The AAV-PHP.eB virus was injected for whole brain infection (Chan et al.
168 2017).

169 **Mouse sleep analysis:** Mouse sleep analysis was described in a previous article from
170 our laboratory (Zhang et al. 2018). Eight-week-old mice were selected for
171 retro-orbital injection. One week after viral injection, EEG and EMG electrode
172 implantation procedures were performed. Mice were allowed to recover for more than
173 5 days individually and placed in a recording cage and tethered to an omni-directional
174 arm (RWD Life Science Inc.) with connection cable for 2 days of habituation before
175 recording. EEG and EMG data were recorded with custom software at a sampling
176 frequency of 200 Hz for 2 consecutive days to analyze sleep/wake behavior under
177 baseline conditions. The recording chamber was maintained at 12 h LD cycle and
178 controlled temperature (24-25°C). EEG/EMG data were initially processed by
179 Accusleep (Barger et al. 2019) before manual correction in SleepSignTM to improve
180 accuracy. WAKE was scored as high amplitude and variable EMG and fast and low
181 amplitude EEG. NREM was scored as high amplitude δ (1-4 Hz) frequency EEG and

182 low EMG tonus. REM was scored as a complete silent of EMG signals and low
183 amplitude high frequency θ (6-9 Hz)-dominated EEG signal.

184 For power spectrum analysis, EEG was subjected to fast Fourier transform (FFT)
185 analysis with a Hamming window method by SleepSignTM, yielding power spectra
186 between 0-25 Hz with a 0.39Hz bin resolution. Epochs containing movement artifacts
187 were marked, included in sleep duration analysis but excluded from the power spectra
188 analysis. Power spectra for each vigilance state represents the mean power distribution
189 of this state during a 24-h baseline recording. The δ -power density of NREMS per hour
190 represents the average of δ -power density as a percentage of δ -band power (1-4 Hz) to
191 total power (0-25 Hz) for each NREM epoch contained in an hour.

192

193 **Statistics:** All statistical analyses were performed with Prism 7 (GraphPad Software).
194 Differences in means between samples larger than two groups were analyzed using
195 ordinary One-way ANOVA. Unpaired t test was used for two groups comparison.
196 Power spectrum between different lines was compared by two-way ANOVA followed
197 by Turkey's multiple comparisons test. Ns denotes $p > 0.05$, * denotes $p < 0.05$, **
198 denotes $p < 0.01$ and *** denotes $p < 0.001$ for all statistical results in this paper.

199

200 **RESULTS**

201 **Sleep phenotypes of *Drosophila* Lkb1 mutants:** Null mutants for Lkb1 are lethal in
202 *Drosophila* (Martin and St Johnston 2003). We had generated a Lkb1 knockout

203 (“lkb1^{T1}”) line (Figure S1A, S3B) and found that lkb1^{T1/T1} mutation was lethal in the
204 pupa stage. The level of Lkb1 mRNA was reduced in the heterozygous lkb1^{T1/+} flies
205 (Figure S1B). We then tested whether the heterozygous lkb1^{T1} had any phenotype in
206 sleep using flies kept in 12 hours (h) light/ 12 h dark (LD) cycles (Figure S1C). While
207 lkb1^{T1/+} flies were not significantly different from the wild type (wt) flies in sleep bout
208 numbers (Figure S1E), or daytime sleep duration (Figure S1D), daytime sleep bout
209 duration (Figure S1F), lkb1^{T1/+} flies showed significantly lower nighttime sleep
210 duration (Figure S1D), nighttime sleep bout duration (Figure S1F) and longer latency to
211 sleep (Figure S1G). Thus, there was dosage-sensitive physiological requirement of
212 Lkb1 in nighttime sleep.

213 We tried to, and succeeded in, generating lkb1^{T2}, a hypomorphic mutation for Lkb1
214 in flies (Figure 1A, S3A and S3B). Lkb1 mRNA was significantly reduced in lkb1^{T2/+}
215 and lkb1^{T2/T2} flies (Figure 1B). During the day, lkb1^{T2/T2} flies were not significantly
216 different from the lkb1^{T2/+} and wt flies in sleep duration (Figure 1C, 1D), sleep bout
217 number (Figure 1E), sleep bout duration (Figure 1F) or latency to sleep (Figure 1G).
218 During the night, not only lkb1^{T2/T2} flies showed significantly reduced sleep duration
219 (Figure 1C, 1D), highly reduced sleep bout duration (Figure 1F) and highly increased
220 latency (Figure 1G) than the wt flies, but also the heterozygous lkb1^{T2/+} flies were
221 significantly different from the wt flies in all these parameters (Figure 1C to 1G),
222 indicating a dosage sensitive requirement for Lkb1.

223 We examined the phenotypes of $lkb1^{T1/T2}$. Consistent with the $lkb1^{T2/T2}$, the
224 mRNA levels of *Lkb1* were significantly reduced in $lkb1^{T1/T2}$ compared to wt, $lkb1^{T1/+}$
225 and $lkb1^{T2/+}$, and even lower than that in $lkb1^{T2/T2}$ (Figure S2A).

226 The sleep phenotype in $lkb1^{T1/T2}$ flies was also consistent with $lkb1^{T2/T2}$, with
227 highly reduced nighttime sleep duration (Figure S2B, S2C), highly reduced sleep bout
228 duration (Figure S2E) and highly increased latency to sleep (Figure S2F), when
229 compared with wt, $lkb1^{T1/+}$ and $lkb1^{T2/+}$ flies.

230 Results of sleep analysis of $lkb1^{T1/+}$, $lkb1^{T2/+}$, $lkb1^{T2/T2}$, and $lkb1^{T1/T2}$ mutant flies
231 all consistently support that *Lkb1* plays a physiological role in promoting sleep.

232 **Rescue of sleep phenotypes by *Lkb1* in flies:** We inserted the sequence of the yeast
233 transcription factor Gal4 into the $lkb1^{T2}$ mutant flies, flanking the *lkb1* promoter, and
234 obtained $lkb1^{T2}$ -Gal4 flies (Figure 2A). We also generated UAS-*Lkb1* flies in which
235 the *Lkb1* coding sequence (CDS) was expressed under the control of the upstream
236 activation sequence (UAS) (Brand and Perrimon 1993). Because Gal4 protein binds to
237 the UAS, the expression of *Lkb1* in flies resulting from the crosses between
238 $lkb1^{T2}$ -Gal4 flies and UAS-*Lkb1* flies would be under the control of the endogenous
239 *Lkb1* promoter. Indeed, expression of *Lkb1* mRNA was restored when $lkb1^{T2}$ -Gal4 and
240 UAS-*Lkb1* were present in the same flies (Figure 2B), whereas *Lkb1* mRNA was less
241 in wt flies. UAS-*Lkb1*; $lkb1^{T2/T2}$ mutant flies, and $lkb1^{T2}$ -Gal4/ $lkb1^{T2}$ -Gal4 flies than
242 that in the wt. UAS-*Lkb1* alone could not restore *Lkb1* mRNA expression level to that
243 in wt flies (Figure 2B).

244 Both daytime and nighttime sleep durations were less in $lkb1^{T2}\text{-Gal4}/lkb1^{T2}\text{-Gal4}$
245 flies than those in wt flies (Figure 2C). Introduction of UAS-Lkb1 in $lkb1^{T2/T2}$ flies or
246 $lkb1^{T2}\text{-Gal4}$ alone could not restore sleep. When both $lkb1^{T2}\text{-Gal4}$ and UAS-Lkb1 were
247 present, nighttime sleep durations were restored (Figure 2D). Nighttime sleep bout
248 number, nighttime sleep bout duration and nighttime latency were restored when both
249 $lkb1^{T2}\text{-Gal4}$ and UAS-Lkb1 were present, but not when $lkb1^{T2}\text{-Gal4}$ or UAS-Lkb1
250 alone was present (Figure 2E, 2F and 2G).

251 These results support that the sleep phenotypes of $lkb1^{T2/T2}$ were attributable to the
252 reduction of Lkb1 mRNA expression in these flies.

253 **Sleep phenotypes of flies carrying neuronal deletion of the Lkb1 gene:** To
254 determine whether Lkb1 functions in neurons, we used the CRISPR-Cas9 system to
255 delete Lkb1 from neurons specifically (Figure S4). A pan-neuronal Gal4 driver
256 (57C10-Gal4) was used to control the expression of small guide RNA (sgRNA)
257 targeting Lkb1 in neurons. Compared to 57C10-Gal4>UAS-Cas9 alone or
258 57C10-Gal4>UAS-Lkb1-sgRNA alone, when both UAS-Cas9 and UAS-Lkb1-sgRNA
259 were present in flies, nighttime sleep duration (Figure 3B) and nighttime sleep bout
260 duration (Figure 3D) were significantly reduced and nighttime sleep latency
261 significantly lengthened (Figure 3E). Daytime sleep duration, bout number, bout
262 duration and latency were not significantly affected by neuronal gene targeting of Lkb1
263 (Figure 3B, 3C, 3D and 3E).

264 We also investigated any potential effect that overexpression of Lkb1 in neurons
265 might cause (Figure S5A). We detected no phenotype resulting from neuronal
266 overexpression of Lkb1 on daytime and nighttime sleep duration, sleep bout number,
267 sleep bout duration or latency (Figure S5B, S5C, S5D, S5E and S5F).

268 In all three series of experiments (Figures 1, 2 and 3), nighttime sleep phenotypes
269 were more obvious than daytime sleep phenotypes. These results strongly indicate that
270 Lkb1 expression in neurons are required physiologically for sleep, especially nighttime
271 sleep.

272 **Genetic interactions between Lkb1 and Ampk or Sik3 in flies:** To examine
273 potential genetic interactions of Lkb1 with either Ampk or Sik3, we combined the
274 LOF Lkb1 mutation $lkb1^{T2}$ with specific point mutations in either Ampk or Sik3.

275 The regulatory site T184 in *Drosophila* AMPK and T196 in *Drosophila* SIK3
276 were equivalent to T172 of mammalian AMPK2 and T221 of mammalian SIK3,
277 important for their activities. When the endogenous T184 in fly AMPK was mutated
278 to alanine (A) or glutamic acid (E), flies were lethal. We therefore introduced T184A
279 and T184E mutations into an Ampk transgene whose expression was controlled by
280 UAS. We introduced UAS-Ampk, UAS-Ampk-T184A, and UAS-Ampk-T184E into
281 $lkb1^{T2/T2}$ flies and used a pan-neuronal driver to express them in neurons (Figure 4).
282 Neuronal overexpression of Ampk-T184E (Figure 4A) and UAS-Ampk (Figure 4C)
283 in $lkb1^{T2}$ flies did not significantly change the sleep phenotypes of $lkb1^{T2/T2}$ flies, but

284 neuronal overexpression of UAS-Ampk-T184A (Figure 4B) in *lkb1*^{T2/T2} flies further
285 decreased nighttime sleep duration.

286 Point mutations of Sik3-flag, Sik3-T196A-flag and Sik3-T196E-flag were
287 constructed in *Drosophila*. When the endogenous T196 in Sik3 was mutated to A or E,
288 we could get homozygous flies. Upon crossing to *lkb1*^{T2/T2}, Sik3-T196A-flag;*lkb1*^{T2/T2}
289 were homozygous lethal. The cross of Sik3-T196E-flag into the *lkb1*^{T2/T2} background
290 generated viable flies, with no detectable change in sleep (Figure 5).

291 Allele-specific genetic interactions between *Lkb1* and *Ampk* in sleep, or between
292 *Lkb1* and *Sik3* in viability, suggest, but do not prove, regulatory relationships between
293 *Lkb1* and *Ampk* in sleep or *Sik3* in viability.

294 **Circadian rhythm in *Lkb1* mutant flies:** The transcription factor differentiated
295 embryo-chondrocyte 1 (DEC1) regulates circadian rhythm and can negatively regulate
296 the transcription of *Lkb1* and subsequently reduce AMPK activity (Sato et al. 2015).

297 We tested whether the circadian rhythm was affected in *Lkb1* mutant flies. *Lkb1*
298 mutant flies were not different from wt flies in period length (Figure S7B). Relative
299 rhythmic power was increased in *lkb1*^{T2/+} and *lkb1*^{T2/T2} mutants than wt flies. (Figure
300 S7).

301 **Sleep phenotypes in *Lkb1* conditional knockout mice:** To investigate potential
302 involvement of *Lkb1* in regulating sleep of mammalian animals, we obtained *Lkb1*^{fl/fl}
303 mice in which the loxP sites flanked exons 3 to 6 of the *Lkb1* gene (Nakada et al. 2010).
304 To delete the *Lkb1* gene from these mice, we injected adeno-associated viral (AAV)

305 constructs expressing either the Cre recombinase together with the green fluorescent
306 protein (GFP) or GFP alone to infect the mouse brain. Cre-GFP or GFP was under the
307 control of a neuronal specific promoter human Synapsin I (hSyn) (in
308 AAV-PHP.eB-hSyn-Cre-GFP or AAV-PHP.eB-hSyn-GFP).

309 We analyzed the expression of LKB1 protein in mice (Figure 6A, 6B). Injection of
310 Cre-GFP expressing virus into wt or $Lkb1^{fl/+}$ mice did not reduce LKB1 protein
311 expression in the brain. Neither did injection of only GFP expressing virus into $Lkb1^{fl/fl}$
312 mice. This conclusion was reached by examination of either several mouse brains
313 combined (Figure 6A), or individual mouse brains (Figure 6B).

314 Functionally, only when the Cre-GFP expressing virus was injected into $Lkb1^{fl/fl}$
315 mice, wake duration was significantly increased during daytime (Figure 6C, S6A),
316 non-rapid eye movement (NREM) sleep duration was significantly decreased during
317 daytime (Figure 6E, S6B). Controls (Cre-GFP injection into wt or $Lkb1^{fl/+}$ mice, GFP
318 injection into $Lkb1^{fl/fl}$ mice) did not significantly changed any sleep phenotypes.

319 Rapid eye movement (REM) sleep duration was not significantly affected by
320 Cre-GFP injection into $Lkb1^{fl/fl}$ mice (Figure 6G, S6C).

321 Power density in the 1-4 Hz range (δ power density) of NREM is a commonly
322 accepted indicator of sleep need (Borbely 1982; Borbely et al. 1981; Daan et al. 1984;
323 Dijk et al. 1987; Franken et al. 2001; Tobler and Borbely 1986; Werth et al. 1996). We
324 found that NREM δ power density was significantly reduced when the Cre-GFP
325 expressing virus was injected into $Lkb1^{fl/fl}$ mice (Figure 6F). Analysis over 24 h

326 indicated that significant reduction was observed over most of the daily cycle (Figure
327 6I).

328

329 **DISCUSSION**

330 Our results indicate that LKB1 is required for sleep regulation: it plays an important
331 and conserved role by promoting sleep in both flies and mice. LKB1 plays this role in
332 neurons in both species because gene targeting of *Lkb1* in neurons led to reduction of
333 sleep. In mice, with the additional advantage of EEG analysis, we find that LKB1
334 regulates sleep need as indicated by reduced NREM δ power density in *Lkb1*
335 knockdown mutant mice.

336 Sleep is important for animals. Sleep regulation is accomplished through two
337 processes: circadian and sleep homeostatic (Borbely 1982; Borbely et al. 2016). The
338 circadian clock regulates the timing of sleep, and homeostatic process regulates the
339 sleep drive. Molecular mechanisms of the circadian clock have been revealed through
340 research in *Drosophila* and other organisms (Hendricks et al. 2000; Shaw et al. 2000;
341 Allada and Chung 2010; Mohawk et al. 2012; Nitabach and Taghert 2008). Although
342 many sleep-related genes have been identified in sleep regulation (Allada et al. 2017;
343 Cirelli 2009; Jan et al. 2020), our understanding of the mechanisms underlying sleep
344 homeostatic regulation remains limited (Allada et al. 2017; Donlea et al. 2017).

345 Multiple regions in *Drosophila* and mouse brains have been implicated in sleep
346 regulation. In flies, sleep is regulated by several regions including: the ILNv and DN1

347 clock neurons which are important for circadian control of sleep (Chung et al. 2009;
348 Kunst et al. 2014; Parisky et al. 2008; Shang et al. 2013; Shang et al. 2008; Sheeba et
349 al. 2008). And the mushroom bodies (MBs), the dorsal of fan-shaped body (dFB), the
350 ellipsoid body (EB), the pars intercerebralis (PI) and glia. (Chen et al. 2015; Crocker
351 et al. 2010; Donlea et al. 2014; Donlea et al. 2011; Foltenyi et al. 2007; Guo et al.
352 2011; Joiner et al. 2006; Liu et al. 2012; Liu et al. 2016; Park et al. 2014; Pimentel et
353 al. 2016; Seugnet et al. 2011; Ueno et al. 2012; Yi et al. 2013). In mammals, sleep is
354 regulated by monoaminergic, cholinergic, glutamatergic, and GABAergic neurons
355 that are distributed in multiple regions including the brain stem, the preoptic
356 hypothalamus, the lateral hypothalamus and the basal forebrain (Saper and Fuller
357 2017; Scammell et al. 2017; Weber and Dan 2016). It will be interesting to investigate
358 whether Lkb1 functions in all or a limited subset of neurons to regulate sleep.

359 Lkb1 as a master kinase can regulate the activities of ARKs by phosphorylating
360 the site in the active T loop equivalent to AMPK-T172 (Lizcano et al. 2004). Because
361 both AMPK and SIK3 are involved in sleep regulation, it will be interesting to
362 investigate downstream kinases mediating the function of Lkb1 in sleep regulation. Is
363 it SIK3, AMPK, or other members of the AMPK related kinases? Our findings of
364 allele-specific genetic interactions between Lkb1 and Ampk suggest that they could be
365 upstream and downstream of each other in regulating sleep. Because of the lethality of
366 double mutation combination of Sik3 and lkb1, we can not rule out that Sik3 may also
367 be downstream of Lkb1 in regulating Drosophila sleep. The

368 Ca^{2+} /calmodulin-dependent protein kinase kinase-2 (CaMKK2, also known as
369 CaMKK β) could phosphorylate AMPK α -T172 (Anderson et al. 2008; Hawley et al.
370 2005; Hurley et al. 2005; Woods et al. 2005), but CaMKK2 could not phosphorylate the
371 equivalent sites in the other AMPK related kinases, including SIK3 (Fogarty et al.
372 2010). It will be interesting to investigate whether and how CaMKK2 regulates sleep.

373 In *Drosophila*, LKB1 functions through SIK3 which phosphorylates histone
374 deacetylase 4 (HDAC4) to regulate lipid storage (Choi et al. 2015). It will be interesting
375 to investigate whether HDAC4 is downstream of LKB1 in sleep regulation.

376 More importantly, an important question for further studies is whether Lkb1
377 regulation of sleep is related to its regulation of metabolism. Changes in energy
378 homeostasis directly and reversibly influence the sleep/wake cycle (Collet et al. 2016).
379 Some molecules involved in metabolism regulate sleep (Bjorness and Greene 2009;
380 Gerstner et al. 2011; Grubbs et al. 2020; Nixon et al. 2015; Taheri et al. 2004; Thimgan
381 et al. 2010). In *Drosophila*, starvation suppresses sleep without building up sleep drive
382 (Thimgan et al. 2010). Lkb1 and its downstream components are involved in regulating
383 metabolism, with examples such as LKB1-AMPK signaling in the liver regulating
384 glucose homeostasis (Shaw et al. 2005), SIK3-HDAC4 regulating energy balance in
385 *Drosophila* (Wang et al. 2011). Either LKB1 has two independent roles in sleep and
386 metabolism or that its two roles are related.

387 Our recent in vitro biochemical discoveries of STE20 phosphorylation of AMPK
388 and SIK3 (and other ARKs) raise more questions about physiological significance of
389 any STE20 or any other ARK in sleep (Liu et al., 2022a, 2022b).

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391

392 **DATA AVAILABILITY STATEMENT**

393 Strains and plasmids are available upon request. All data necessary for confirming the
394 conclusions of the article are present within the article and its supplementary data.

395

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404

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730

731 **FIGURE LEGENDS**

732

733 **Figure 1.** Sleep phenotypes of *Lkb1* knock-down mutant flies. (A) A diagram
734 illustrating the *Lkb1* insertion mutant *lkb1*^{T2}. (B) Relative *Lkb1* mRNA levels in
735 *lkb1*^{T2/T2} (red), *lkb1*^{T2/+} (blue) and wt (black) flies. (C) Sleep profiles of *lkb1*^{T2/T2} (red,
736 n=42), *lkb1*^{T2/+} (blue, n=44), and wt (black, n=44) flies in a 12 h light/12 h dark (LD)
737 cycle. (D-G) Statistical analysis of sleep duration, sleep bout number, sleep bout
738 duration and latency to sleep in *lkb1*^{T2/T2} (red, n=42), *lkb1*^{T2/+} (blue, n=44) and wt
739 (black, n=44) flies. Open bars denote daytime, filled bars denote nighttime. (D) Sleep
740 duration. Nighttime sleep durations of *lkb1*^{T2/T2} mutants was significantly less than
741 those in *lkb1*^{T2/+} and wt flies. (E) Sleep bout number. Daytime sleep bout number of
742 *lkb1*^{T2/T2} mutants was less than that of wt flies. (F) Sleep bout duration. Nighttime
743 sleep bout duration of *lkb1*^{T2/T2} mutants was significantly less than those of *lkb1*^{T2/+}
744 and wt flies. (G) Latency to sleep. Latency to sleep after light-off of *lkb1*^{T2/T2} mutants
745 was significantly prolonged than *lkb1*^{T2/+} and wt flies. One-way ANOVA was used.
746 n.s. denotes p>0.05, *p<0.05, **p<0.01, ***p<0.001. Error bars represent standard
747 error of the mean (SEM).

748

749 **Figure 2.** Rescue of sleep loss in *lkb1*^{T2/T2} by *Lkb1*. (A) A diagram of
750 *lkb1*^{T2}-Gal4: a cDNA for the yeast Gal4 gene inserted in the *lkb1*^{T2} knockdown
751 mutants. (B) Relative *Lkb1* mRNA levels in *lkb1*^{T2}-Gal4 (blue), UAS-*Lkb1*;
752 *lkb1*^{T2}-Gal4 (red), UAS-*Lkb1*; *lkb1*^{T2} (yellow) and wt (black) flies. (C-G) In

753 $lkb1^{T2}$ -Gal4 homozygous flies, UAS-Lkb1 cDNA driven by Gal4 to rescue sleep
754 phenotypes of $lkb1$ knockdown mutants. (C) Sleep profiles of UAS-Lkb1;
755 $lkb1^{T2}$ -Gal4 (red, n=45), UAS-Lkb1; $lkb1^{T2}$ (yellow, n=47), $lkb1^{T2}$ -Gal4 (blue,
756 n=46) and wt (black, n=36) flies. (D-G) Statistical analysis of sleep duration,
757 sleep bout number, sleep bout duration and latency to sleep in UAS-Lkb1;
758 $lkb1^{T2}$ -Gal4 (red, n=45), UAS-Lkb1; $lkb1^{T2}$ (yellow, n=47), $lkb1^{T2}$ -Gal4 (blue,
759 n=46) and wt (black, n=36) flies. Open bars denote daytime, filled bars nighttime.
760 (D) Sleep duration. Nighttime sleep duration of UAS-Lkb1; $lkb1^{T2}$ -Gal4 was
761 similar to that of wt mutants, both significantly higher than UAS-Lkb1; $lkb1^{T2}$
762 and $lkb1^{T2/T2}$ -Gal4 flies. (E) Sleep bout number. Nighttime sleep bout number of
763 UAS-Lkb1; $lkb1^{T2}$ -Gal4 was similar to the wt but significantly higher than
764 UAS-Lkb1; $lkb1^{T2}$ and $lkb1^{T2}$ -Gal4 flies. (F) Sleep bout duration. Nighttime
765 sleep bout duration of UAS-Lkb1; $lkb1^{T2}$ -Gal4 was similar to the wt but
766 significantly higher than UAS-Lkb1; $lkb1^{T2}$ and $lkb1^{T2}$ -Gal4 flies. (G) Latency to
767 sleep. Latency after light-off of UAS-Lkb1; $lkb1^{T2}$ -Gal4 was similar to the wt but
768 significantly shorter than UAS-Lkb1; $lkb1^{T2}$ and $lkb1^{T2}$ -Gal4 flies. One-way
769 ANOVA was used. n.s. denotes $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error
770 bars represent SEM.

771

772 **Figure 3.** Sleep phenotypes of mutants from whose neurons Lkb1 was targeted.

773 (A) Sleep profiles of UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 (red, n=44),

774 UAS-Lkb1-sgRNA/57C10-Gal4 (blue, n=41), and 57C10-Gal4/+;+/UAS-Cas9
775 (black, n=45) flies. (B-E) Statistical analysis of sleep duration, sleep bout number,
776 sleep bout duration and latency to sleep in
777 UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 (red, n=44),
778 UAS-Lkb1-sgRNA/57C10-Gal4 (blue, n=41) and 57C10-Gal4/+;+/UAS-Cas9
779 (black, n=45) flies. Open bars denote daytime, filled bars nighttime. (B) Sleep
780 duration. Daytime and nighttime sleep duration of
781 UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 was significantly less than those of
782 UAS-Lkb1-sgRNA/57C10-Gal4 and 57C10-Gal4/+;+/UAS-Cas9 flies. (C) Sleep
783 bout number. Daytime and nighttime sleep bout numbers of
784 UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 was not significantly from those of
785 UAS-Lkb1-sgRNA/57C10-Gal4 and 57C10-Gal4/+;+/UAS-Cas9 flies. (D) Sleep
786 bout duration. Nighttime sleep bout duration of
787 UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 was significantly less than that of
788 UAS-Lkb1-sgRNA/57C10-Gal4 and 57C10-Gal4/+;+/UAS-Cas9 flies. (E)
789 Latency to sleep. Latency to sleep after light-off of
790 UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 was longer than that of
791 57C10-Gal4/+;+/UAS-Cas9 which was not significantly different from that of
792 UAS-Lkb1-sgRNA/57C10-Gal4 flies. One-way ANOVA was used. n.s. denotes
793 $p>0.05$, $*p<0.05$, $**p<0.01$, $***p<0.001$. Error bars represent SEM.
794

795 **Figure 4.** Genetic interactions between *lkb1* and *ampk*. (A) Sleep profiles of
796 UAS-*Ampk*-T184E/57C10;*lkb1*^{T2} (red, n=19), 57C10;*lkb1*^{T2} (yellow, n=24),
797 UAS-*Ampk*-T184E;*lkb1*^{T2} (blue, n=22) and wt (black, n=24) flies. (B) Sleep
798 profiles of UAS-*Ampk*-T184A/57C10;*lkb1*^{T2} (red, n=24), 57C10;*lkb1*^{T2} (yellow,
799 n=24), UAS-*Ampk*-T184A;*lkb1*^{T2} (blue, n=23) and wt (black, n=24) flies. (C)
800 Sleep profiles of UAS-*Ampk* /57C10;*lkb1*^{T2} (red, n=24), 57C10;*lkb1*^{T2} (yellow,
801 n=24), UAS-*Ampk*;*lkb1*^{T2} (blue, n=11) and wt (black, n=24) flies. (D-G)
802 Statistical analysis of sleep duration, sleep bout number, sleep bout duration and
803 latency to sleep in wt (black, n=24), 57C10;*lkb1*^{T2} (yellow, n=24),
804 UAS-*Ampk*-T184E;*lkb1*^{T2} (blue, n=22), UAS-*Ampk*-T184E/57C10;*lkb1*^{T2} (red,
805 n=19), UAS-*Ampk*-T184A;*lkb1*^{T2} (blue, n=23),
806 UAS-*Ampk*-T184A/57C10;*lkb1*^{T2} (red, n=24), UAS-*Ampk*;*lkb1*^{T2} (blue, n=11)
807 and UAS-*Ampk* /57C10;*lkb1*^{T2} (red, n=24) flies. Open bars denote daytime, filled
808 bars nighttime. N.s. not shown. One-way ANOVA was used. n.s. denotes $p>0.05$,
809 * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars represent SEM.

810

811 **Figure 5.** Genetic interactions between *lkb1* and *sik3*. (A) Sleep profiles of
812 *lkb1*^{T2} (red, n=45), *Sik3*-T196E-flag;*lkb1*^{T2} (dark red, n=46), *Sik3*-flag;*lkb1*^{T2}
813 (orange, n=48), *Sik3*-T196E-flag (blue, n=45), *Sik3*-flag (dark blue, n=44) and
814 wt (black, n=46) flies. (D-G) Statistical analysis of sleep duration, sleep bout
815 number, sleep bout duration and latency to sleep in wt (black, n=46), *Sik3*-flag

816 (dark blue, n=44), Sik3-T196E-flag (blue, n=45), Sik3-flag;lkb1^{T2} (orange, n=48),
817 Sik3-T196E-flag;lkb1^{T2} (dark red, n=46) and lkb1^{T2} (red, n=45) flies. Open bars
818 denote daytime, filled bars nighttime. Statistics for groups Sik3-flag;lkb1^{T2} and
819 Sik3-T196E-flag;lkb1^{T2} were presented. One-way ANOVA was used. n.s.
820 denotes p>0.05, *p<0.05, **p<0.01, ***p<0.001. Error bars represent SEM.

821

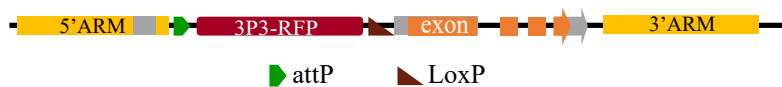
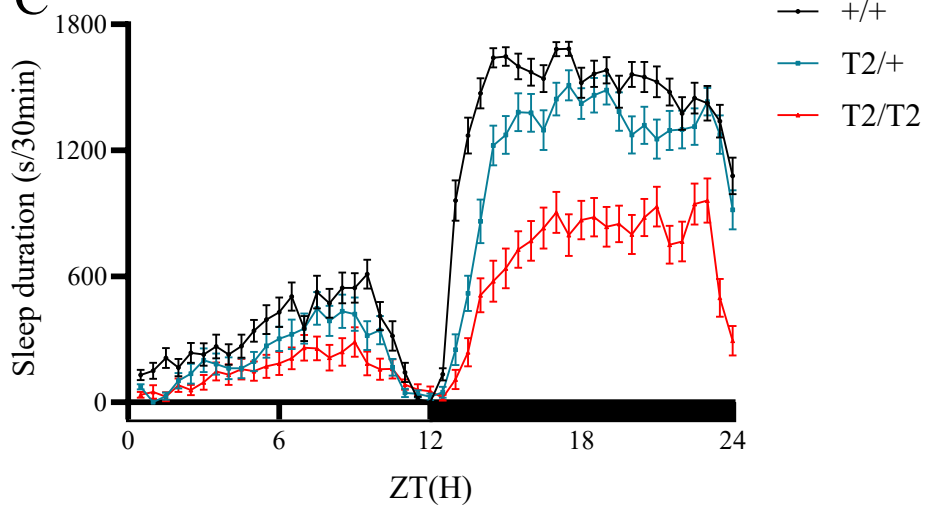
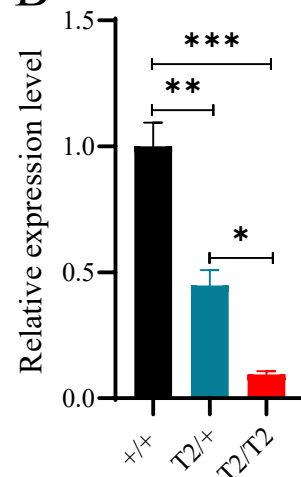
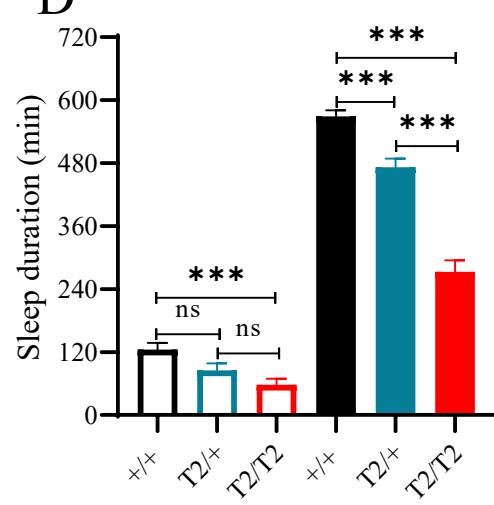
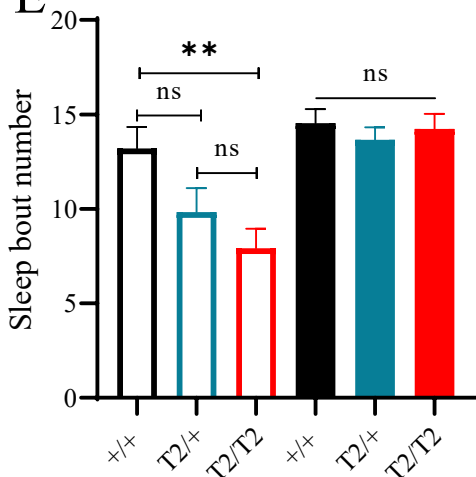
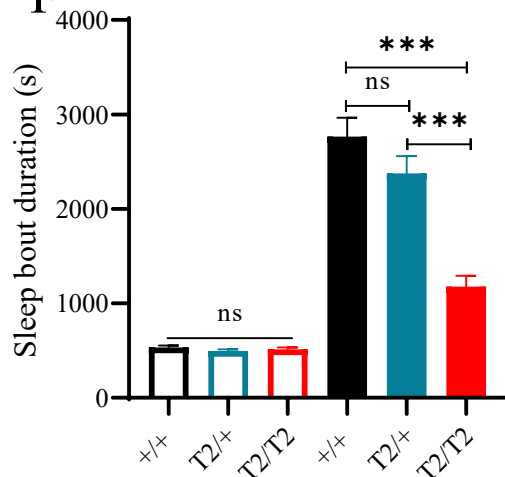
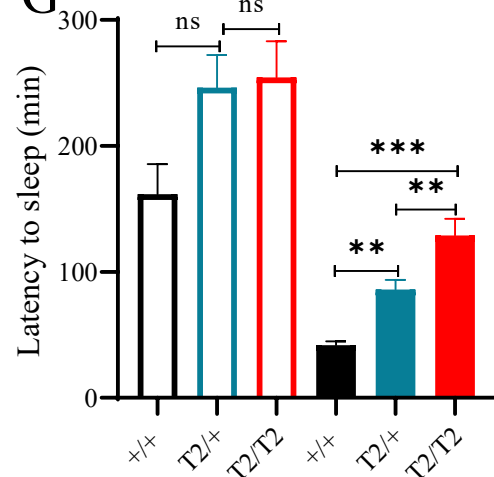
822 **Figure 6.** Sleep phenotypes of lkb1 conditional knockout mice. (A) Levels of LKB1
823 protein from Lkb1^{fl/fl} mice injected with AAV-hSyn-Cre-GFP virus (Cre⁺ Lkb1^{fl/fl},
824 n=4), Lkb1^{fl/fl} mice injected with AAV-hSyn-GFP virus (GFP⁺ Lkb1^{fl/fl}, n=3), Lkb1^{fl/+}
825 mice injected with AAV-hSyn-Cre-GFP virus (Cre⁺ Lkb1^{fl/+}, n=2) and Lkb1^{+/+} mice
826 injected with AAV-hSyn-Cre-GFP virus (Cre⁺ Lkb1^{+/+}, n=2). These mice were among
827 those used for EEG recording and analysis. (B) Levels of LKB1 protein in individual
828 mice (genotypes labelled: Cre⁺ Lkb1^{fl/fl}, GFP⁺ Lkb1^{fl/fl}, Cre⁺ Lkb1^{fl/+} and Cre⁺ Lkb1^{+/+}.
829 These mice were the same mice as those in (A) but presented individually. (C, E, G)
830 Statistical analysis of wake duration, NREM duration and REM duration in Cre⁺
831 Lkb1^{fl/fl} (red, n=10), GFP⁺ Lkb1^{fl/fl} (yellow, n=5), Cre⁺ Lkb1^{fl/+} (blue, n=7) and Cre⁺
832 Lkb1^{+/+} (black, n=4) mice in a 12:12 LD cycle. White background denotes daytime,
833 gray background nighttime. (C) Wake duration. Daytime wake duration of Cre⁺
834 Lkb1^{fl/fl} mice was higher than those of GFP⁺ Lkb1^{fl/fl}, Cre⁺ Lkb1^{fl/+} or Cre⁺ Lkb1^{+/+}
835 mice. Nighttime wake duration of Cre⁺ Lkb1^{fl/fl} mice was higher than that of GFP⁺
836 Lkb1^{fl/fl} mice. (E) NREM duration. Daytime NREM duration of Cre⁺ Lkb1^{fl/fl} mice

837 was lower than those of GFP⁺ Lkb1^{fl/fl}, Cre⁺ Lkb1^{fl/+} and Cre⁺ Lkb1^{+/+} mice.
838 Nighttime NREM duration of Cre⁺ Lkb1^{fl/fl} mice was lower than that of GFP⁺ Lkb1^{fl/fl}
839 mice. (G) REM duration. Daytime and nighttime REM durations of Cre⁺ Lkb1^{fl/fl} mice
840 was not significantly different from those of GFP⁺ Lkb1^{fl/fl}, Cre⁺ Lkb1^{fl/+} and Cre⁺
841 Lkb1^{+/+} mice. (D, F, H) EEG power spectrum of (D) WAKE, (F) NREM and (H)
842 REM states in Cre⁺ Lkb1^{fl/fl} (red, n=8), GFP⁺ Lkb1^{fl/fl} (yellow, n=5), Cre⁺ Lkb1^{fl/+}
843 (blue, n=6) and Cre⁺ Lkb1^{+/+} (black, n=4) mice. (I) NREM δ -power density of Cre⁺
844 Lkb1^{fl/fl} (red, n=8), GFP⁺ Lkb1^{fl/fl} (yellow, n=5), Cre⁺ Lkb1^{fl/+} (blue, n=6) and Cre⁺
845 Lkb1^{+/+} (black, n=4) mice. One-way ANOVA was used in C, E, G for comparison of
846 Cre⁺ Lkb1^{fl/fl}, Cre⁺ Lkb1^{fl/+} and Cre⁺ Lkb1^{+/+} mice. Unpaired t test was used in C, E,
847 G for comparison of Cre⁺ Lkb1^{fl/fl} and GFP⁺ Lkb1^{fl/fl} mice. Two-way ANOVA
848 followed by Turkey's multiple comparisons test was used in D, F, H, I. n.s. denotes
849 $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars represent SEM.

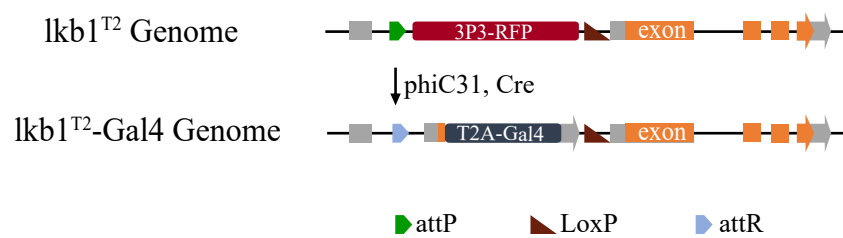
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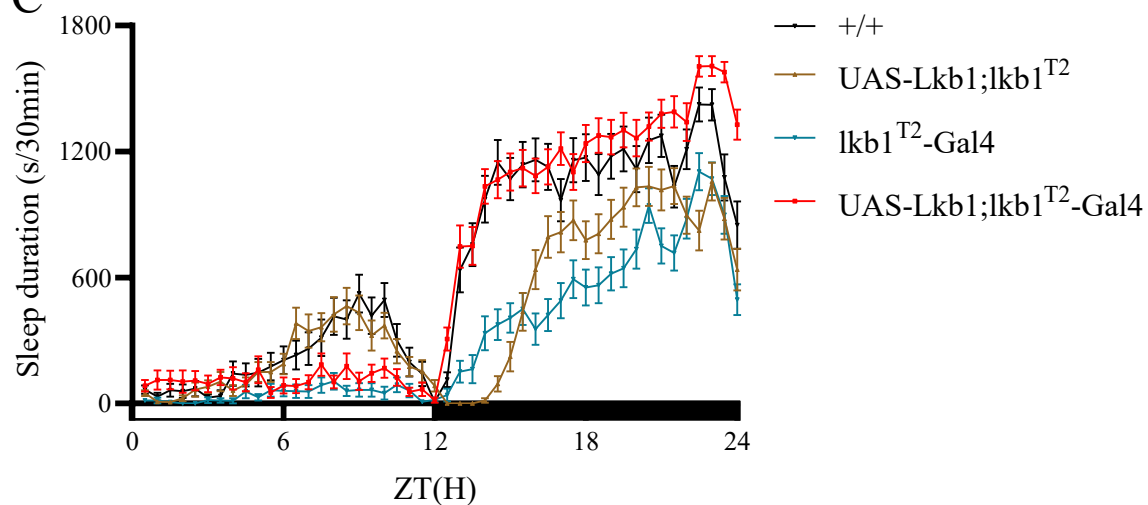
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Alklb1^{T2} Genome**C****B****D****E****F****G**

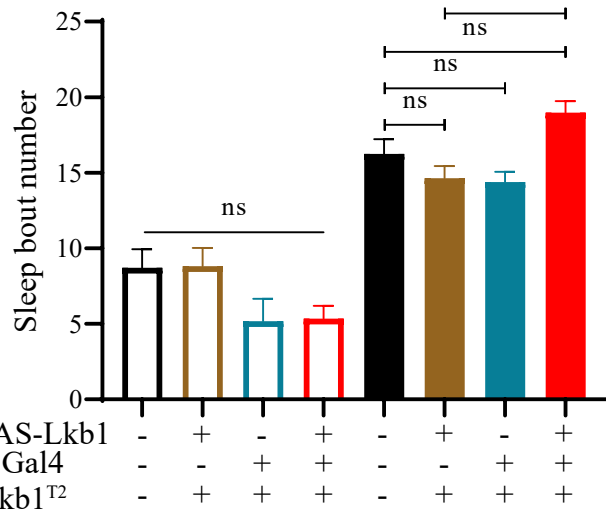
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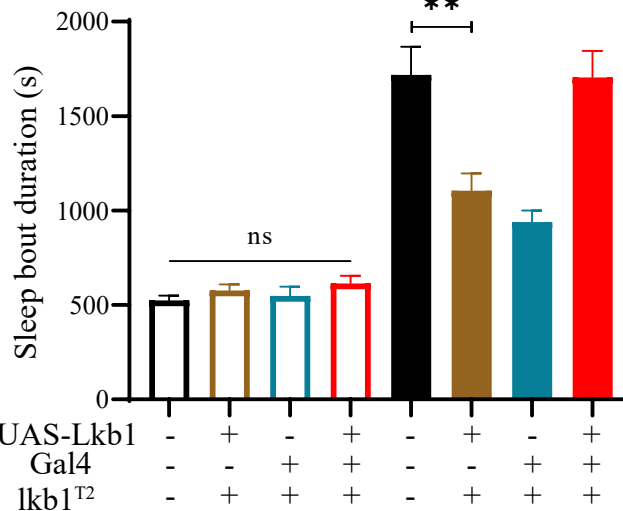
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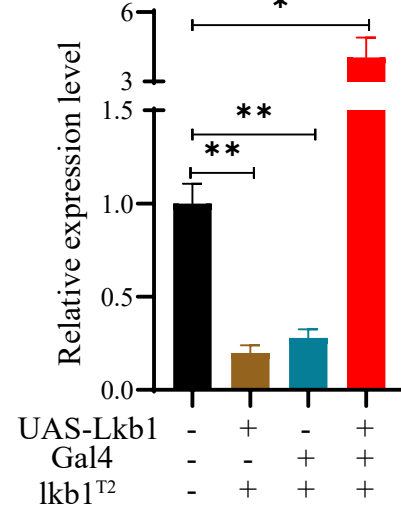
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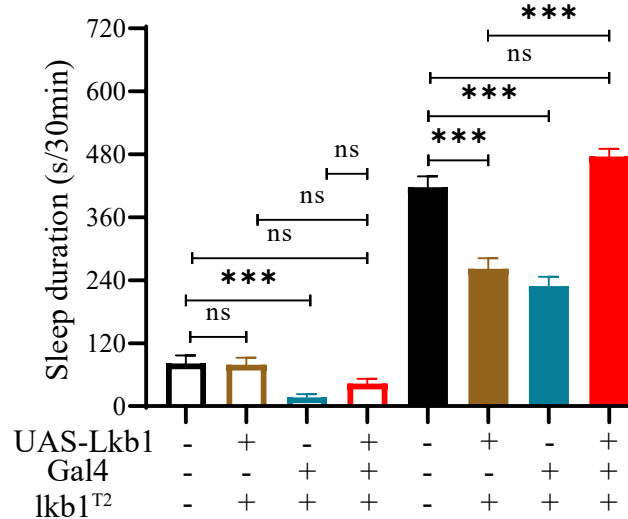
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