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

Ziyi Liu[*], Lifen Jiang[¶], Chaoyi Li[¶], Chengang Li[*], Jingqun Yang[*], Jianjun Yu[*], Renbo Mao[*], and Yi Rao[*,1]

[*]Peking University-Tsinghua University-National Institute of Biological Sciences Joint Graduate Program, School of Life Sciences, PKU-IDG/McGovern Institute for Brain Research, School of Chemistry and Molecular Engineering, School of Pharmaceutical Sciences, Peking University, Beijing 100871, China; Chinese Institute for Brain Research, Beijing; Capital Medical University; Changping Laboratory, Beijing, China

[¶]Institute of Molecular Physiology, Shenzhen Bay Laboratory, Shenzhen, Guangdong, China.

[1]Corresponding author (email: yrao@pku.edu.cn)

Running title: LKB1 and Sleep in Flies and Mice

Keywords: Sleep, LKB1, Flies, Mice

ABSTRACT

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

INTRODUCTION

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

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

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

2014; Hardie et al. 2016; Herzig and Shaw 2018; Lopez and Dieguez 2014). The α, β and γ subunits of AMPK form a heterotrimeric complex (Davies et al. 1994; Michell et al. 1996; Mitchelhill et al. 1994). The catalytic α subunit is regulated by phosphorylation at T172 of AMPKα2 or T183 of AMPKα1 (Hawley et al. 1996).

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

Lkb1-F: 5' -GCCGTCAAGATCCTGACTA-3'

Lkb1-R: 5'-CTCCGCTGGACCAGATG-3'

Rp49-F: 5'-CGACGCTTCAAGGGACAGTATC-3'

Rp49-R: 5'-TCCGACCAGGTTACAAGAACTCTC-3'

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

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

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

Immunoblot analysis: Mouse brains were quickly dissected and washed with phosphate buffer saline (PBS) on ice. Lysis buffer (20mM HEPES, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1mM DTT, freshly supplemented with a protease and phosphatase inhibitors cocktail) were used to homogenize brains by homogenizer (Wiggens, D-500 pro) at 4℃. Brain homogenates were centrifuged at 14,000 revolution per minutes (rpm) for 15 minutes at 4℃. The supernatant was transferred to a new microtube and quantified with bicinchoninic acid assay (Thermo Fisher, 23225). The supernatant was analyzed by SDS-PAGE and proteins were transferred to a nitrocellulose membrane (GE Healthcare, #BA85). Membranes were incubated for 1 h in a blocking solution (Tris-buffered saline (TBS) containing 0.1% Tween-20, 5% milk). Primary antibodies were anti-LKB1 (cell signaling, #3047) and anti-ACTIN (Santa Cruz, sc-8342).

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

The regulatory site T184 in Drosophila AMPK and T196 in Drosophila SIK3 were equivalent to T172 of mammalian AMPK2 and T221 of mammalian SIK3, important for their activities. When the endogenous T184 in fly AMPK was mutated to alanine (A) or glutamic acid (E), flies were lethal. We therefore introduced T184A and T184E mutations into an Ampk transgene whose expression was controlled by UAS. We introduced UAS-Ampk, UAS-Ampk-T184A, and UAS-Ampk-T184E into $lkb1^{T2/T2}$ flies and used a pan-neuronal driver to express them in neurons (Figure 4). Neuronal overexpression of Ampk-T184E (Figure 4A) and UAS-Ampk (Figure 4C) 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

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

DISCUSSION

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

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

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

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

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

368 Ca^{2+}/calmodulin-dependent protein kinase kinase-2 (CaMKK2, also known as
369 CaMKKβ) could phosphorylate AMPKα–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.

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

DATA AVAILABILITY STATEMENT

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

ACKNOWLEDGEMENT

We are grateful to Dr. Bowen Deng for Ampk flies, to Ping-ping Yan, Lan Wang and Yong-hui Zhang for technical assistance, to Drs. Wei Yang and En-xin Zhou for Drosophila video tracing programs, to Dan Wang for mouse rearing, to members of the Rao lab for discussion, to the Bloomington Drosophila Stock Center for flies, to the Jackson Laboratory for mice, to CIBR, Peking-Tsinghua Center for Life Sciences, IDG/McGovern Institute for Brain Research at Peking University and Changping Laboratory for support.

LITERATURE CITED

Alessi DR, Sakamoto K, Bayascas JR. 2006. Lkb1-dependent signaling pathways. Annu Rev Biochem. 75:137-163.

Allada R, Chung BY. 2010. Circadian organization of behavior and physiology in drosophila. Annu Rev Physiol. 72:605-624.

Allada R, Cirelli C, Sehgal A. 2017. Molecular mechanisms of sleep homeostasis in flies and mammals. Cold Spring Harb Perspect Biol. 9(8).

Anderson KA, Ribar TJ, Lin F, Noeldner PK, Green MF, Muehlbauer MJ, Witters LA, Kemp BE, Means AR. 2008. Hypothalamic camkk2 contributes to the regulation of energy balance. Cell Metab. 7(5):377-388.

Bardeesy N, Sinha M, Hezel AF, Signoretti S, Hathaway NA, Sharpless NE, Loda M, Carrasco DR, DePinho RA. 2002. Loss of the lkb1 tumour suppressor provokes intestinal polyposis but resistance to transformation. Nature. 419(6903):162-167.

Barger Z, Frye CG, Liu D, Dan Y, Bouchard KE. 2019. Robust, automated sleep scoring by a compact neural network with distributional shift correction. Plos One. 14(12):e0224642.

Beg ZH, Allmann DW, Gibson DM. 1973. Modulation of 3-hydroxy-3-methylglutaryl coenzyme a reductase activity with camp and wth protein fractions of rat liver cytosol. Biochem Biophys Res Commun. 54(4):1362-1369.

Bjorness TE, Greene RW. 2009. Adenosine and sleep. Curr Neuropharmacol. 7(3):238-245.

Borbely AA. 1982. A two process model of sleep regulation. Hum Neurobiol. 1(3):195-204.

Borbely AA, Baumann F, Brandeis D, Strauch I, Lehmann D. 1981. Sleep deprivation: Effect on sleep stages and eeg power density in man. Electroencephalogr Clin Neurophysiol. 51(5):483-495.

Borbely AA, Daan S, Wirz-Justice A, Deboer T. 2016. The two-process model of sleep regulation: A reappraisal. J Sleep Res. 25(2):131-143.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118(2):401-415.

Carling D, Clarke PR, Zammit VA, Hardie DG. 1989. Purification and characterization of the amp-activated protein-kinase - copurification of acetyl-coa carboxylase kinase and 3-hydroxy-3-methylglutaryl-coa reductase kinase-activities. Eur J Biochem. 186(1-2):129-136.

Carling D, Zammit VA, Hardie DG. 1987. A common bicyclic protein-kinase cascade inactivates the regulatory enzymes of fatty-acid and cholesterol-biosynthesis. Febs Letters. 223(2):217-222.

Carlson CA, Kim KH. 1973. Regulation of hepatic acetyl coenzyme a carboxylase by phosphorylation and dephosphorylation. J Biol Chem. 248(1):378-380.

Carretero J, Medina PP, Pio R, Montuenga LM, Sanchez-Cespedes M. 2004. Novel and natural knockout lung cancer cell lines for the lkb1/stk11 tumor suppressor gene. Oncogene. 23(22):4037-4040.

Chan KY, Jang MJ, Yoo BB, Greenbaum A, Ravi N, Wu WL, Sanchez-Guardado L, Lois C, Mazmanian SK, Deverman BE et al. 2017. Engineered aavs for efficient noninvasive gene delivery to the central and peripheral nervous systems. Nat Neurosci. 20(8):1172-1179.

Chen WF, Maguire S, Sowcik M, Luo W, Koh K, Sehgal A. 2015. A neuron-glia interaction involving gaba transaminase contributes to sleep loss in sleepless mutants. Mol Psychiatry. 20(2):240-251.

447 Chikahisa S, Fujiki N, Kitaoka K, Shimizu N, Sei H. 2009. Central ampk contributes to sleep homeostasis
448 in mice. Neuropharmacology. 57(4):369-374.
449 Choi S, Lim DS, Chung J. 2015. Feeding and fasting signals converge on the lkb1-sik3 pathway to
450 regulate lipid metabolism in drosophila. PLoS Genet. 11(5):e1005263.
451 Chu VT, Graf R, Wirtz T, Weber T, Favret J, Li X, Petsch K, Tran NT, Sieweke MH, Berek C et al. 2016.
452 Efficient crispr-mediated mutagenesis in primary immune cells using crisprgold and a c57bl/6
453 cas9 transgenic mouse line. P Natl Acad Sci USA. 113(44):12514-12519.
454 Chung BY, Kilman VL, Keath JR, Pitman JL, Allada R. 2009. The gaba(a) receptor rdl acts in peptidergic
455 pdf neurons to promote sleep in drosophila. Curr Biol. 19(5):386-390.
456 Cirelli C. 2009. The genetic and molecular regulation of sleep: From fruit flies to humans. Nat Rev
457 Neurosci. 10(8):549-560.
458 Collet TH, van der Klaauw AA, Henning E, Keogh JM, Suddaby D, Dachi SV, Dunbar S, Kelway S, Dickson
459 SL, Farooqi IS et al. 2016. The sleep/wake cycle is directly modulated by changes in energy
460 balance. Sleep. 39(9):1691-1700.
461 Crocker A, Shahidullah M, Levitan IB, Sehgal A. 2010. Identification of a neural circuit that underlies
462 the effects of octopamine on sleep:Wake behavior. Neuron. 65(5):670-681.
463 Daan S, Beersma DG, Borbely AA. 1984. Timing of human sleep: Recovery process gated by a circadian
464 pacemaker. Am J Physiol. 246(2 Pt 2):R161-183.
465 Dai X, Zhou E, Yang W, Mao R, Zhang W, Rao Y. 2021. Molecular resolution of a behavioral paradox:
466 Sleep and arousal are regulated by distinct acetylcholine receptors in different neuronal types
467 in drosophila. Sleep. 44(7).
468 Dai XM, Zhow EX, Yang W, Zhang XH, Zhang WX, Rao Y. 2019. D-serine made by serine racemase in
469 drosophila intestine plays a physiological role in sleep. Nat Commun. 10.
470 Davies SP, Hawley SA, Woods A, Carling D, Haystead TAJ, Hardie DG. 1994. Purification of the
471 amp-activated protein-kinase on atp-gamma-sepharose and analysis of its subunit structure.
472 Eur J Biochem. 223(2):351-357.
473 Deng B, Li Q, Liu X, Cao Y, Li B, Qian Y, Xu R, Mao R, Zhou E, Zhang W et al. 2019. Chemoconnectomics:
474 Mapping chemical transmission in drosophila. Neuron. 101(5):876-893 e874.
475 Dijk DJ, Beersma DG, Daan S. 1987. Eeg power density during nap sleep: Reflection of an hourglass
476 measuring the duration of prior wakefulness. J Biol Rhythms. 2(3):207-219.
477 Donlea JM, Alam MN, Szymusiak R. 2017. Neuronal substrates of sleep homeostasis; lessons from flies,
478 rats and mice. Curr Opin Neurobiol. 44:228-235.
479 Donlea JM, Pimentel D, Miesenbock G. 2014. Neuronal machinery of sleep homeostasis in drosophila.
480 Neuron. 81(4):860-872.
481 Donlea JM, Thimgan MS, Suzuki Y, Gottschalk L, Shaw PJ. 2011. Inducing sleep by remote control
482 facilitates memory consolidation in drosophila. Science. 332(6037):1571-1576.
483 Ferrer A, Caelles C, Massot N, Hegardt FG. 1985. Activation of rat-liver cytosolic
484 3-hydroxy-3-methylglutaryl coenzyme a reductase kinase by adenosine 5'-monophosphate.
485 Biochem Bioph Res Co. 132(2):497-504.
486 Fogarty S, Hawley SA, Green KA, Saner N, Mustard KJ, Hardie DG. 2010. Calmodulin-dependent protein
487 kinase kinase-beta activates ampk without forming a stable complex: Synergistic effects of
488 ca2+ and amp. Biochem J. 426(1):109-118.

Foltenyi K, Greenspan RJ, Newport JW. 2007. Activation of egfr and erk by rhomboid signaling regulates the consolidation and maintenance of sleep in drosophila. Nat Neurosci. 10(9):1160-1167.

Franken P, Chollet D, Tafti M. 2001. The homeostatic regulation of sleep need is under genetic control. J Neurosci. 21(8):2610-2621.

Funato H, Miyoshi C, Fujiyama T, Kanda T, Sato M, Wang Z, Ma J, Nakane S, Tomita J, Ikkyu A et al. 2016. Forward-genetics analysis of sleep in randomly mutagenized mice. Nature. 539(7629):378-383.

Gerstner JR, Vanderheyden WM, Shaw PJ, Landry CF, Yin JCP. 2011. Fatty-acid binding proteins modulate sleep and enhance long-term memory consolidation in drosophila. Plos One. 6(1).

Gill RK, Yang SH, Meerzaman D, Mechanic LE, Bowman ED, Jeon HS, Chowdhuri SR, Shakoori A, Dracheva T, Hong KM et al. 2011. Frequent homozygous deletion of the lkb1/stk11 gene in non-small cell lung cancer. Oncogene. 30(35):3784-3791.

Grubbs JJ, Lopes LE, van der Linden AM, Raizen DM. 2020. A salt-induced kinase is required for the metabolic regulation of sleep. Plos Biology. 18(4).

Guldberg P, thor Straten P, Ahrenkiel V, Seremet T, Kirkin AF, Zeuthen J. 1999. Somatic mutation of the peutz-jeghers syndrome gene, lkb1/stk11, in malignant melanoma. Oncogene. 18(9):1777-1780.

Guo F, Yi W, Zhou MM, Guo AK. 2011. Go signaling in mushroom bodies regulates sleep in drosophila. Sleep. 34(3):273-U173.

Han C, Jan LY, Jan YN. 2011. Enhancer-driven membrane markers for analysis of nonautonomous mechanisms reveal neuron-glia interactions in drosophila. Proc Natl Acad Sci U S A. 108(23):9673-9678.

Hardie DG. 2014. Amp-activated protein kinase: Maintaining energy homeostasis at the cellular and whole-body levels. Annu Rev Nutr. 34:31-55.

Hardie DG, Schaffer BE, Brunet A. 2016. Ampk: An energy-sensing pathway with multiple inputs and outputs. Trends in Cell Biology. 26(3):190-201.

Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, Hardie DG. 2003. Complexes between the lkb1 tumor suppressor, strad alpha/beta and mo25 alpha/beta are upstream kinases in the amp-activated protein kinase cascade. J Biol. 2(4):28.

Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG. 1996. Characterization of the amp-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates amp-activated protein kinase. Journal of Biological Chemistry. 271(44):27879-27887.

Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG. 2005. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for amp-activated protein kinase. Cell Metab. 2(1):9-19.

Hearle N, Schumacher V, Menko FH, Olschwang S, Boardman LA, Gille JJ, Keller JJ, Westerman AM, Scott RJ, Lim W et al. 2006. Frequency and spectrum of cancers in the peutz-jeghers syndrome. Clin Cancer Res. 12(10):3209-3215.

Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Hoglund P et al. 1998. A serine/threonine kinase gene defective in peutz-jeghers syndrome.

Nature. 391(6663):184-187.
Hemminki A, Tomlinson I, Markie D, Jarvinen H, Sistonen P, Bjorkqvist AM, Knuutila S, Salovaara R, Bodmer W, Shibata D et al. 1997. Localization of a susceptibility locus for peutz-jeghers syndrome to 19p using comparative genomic hybridization and targeted linkage analysis. Nat Genet. 15(1):87-90.
Hendricks JC, Finn SM, Panckeri KA, Chavkin J, Williams JA, Sehgal A, Pack AI. 2000. Rest in drosophila is a sleep-like state. Neuron. 25(1):129-138.
Herzig S, Shaw RJ. 2018. Ampk: Guardian of metabolism and mitochondrial homeostasis. Nat Rev Mol Cell Bio. 19(2):121-135.
Honda T, Fujiyama T, Miyoshi C, Ikkyu A, Hotta-Hirashima N, Kanno S, Mizuno S, Sugiyama F, Takahashi S, Funato H et al. 2018. A single phosphorylation site of sik3 regulates daily sleep amounts and sleep need in mice. Proc Natl Acad Sci U S A. 115(41):10458-10463.
Hong SP, Leiper FC, Woods A, Carling D, Carlson M. 2003. Activation of yeast snf1 and mammalian amp-activated protein kinase by upstream kinases. Proc Natl Acad Sci U S A. 100(15):8839-8843.
Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witters LA. 2005. The ca2+/calmodulin-dependent protein kinase kinases are amp-activated protein kinase kinases. J Biol Chem. 280(32):29060-29066.
Ingebritsen TS, Lee HS, Parker RA, Gibson DM. 1978. Reversible modulation of the activities of both liver microsomal hydroxymethylglutaryl coenzyme a reductase and its inactivating enzyme. Evidence for regulation by phosphorylation-dephosphorylation. Biochem Biophys Res Commun. 81(4):1268-1277.
Jan M, O'Hara BF, Franken P. 2020. Recent advances in understanding the genetics of sleep. F1000Res. 9.
Jeghers H, Mc KV, Katz KH. 1949. Generalized intestinal polyposis and melanin spots of the oral mucosa, lips and digits; a syndrome of diagnostic significance. N Engl J Med. 241(26):1031-1036.
Jenne DE, Reimann H, Nezu J, Friedel W, Loff S, Jeschke R, Muller D, Back W, Zimmer M. 1998. Peutz-jeghers syndrome is caused by mutations in a novel serine threonine kinase. Nat Genet. 18(1):38-44.
Ji HB, Ramsey MR, Hayes DN, Fan C, McNamara K, Kozlowski P, Torrice C, Wu MC, Shimamura T, Perera SA et al. 2007. Lkb1 modulates lung cancer differentiation and metastasis. Nature. 448(7155):807-U807.
Jishage K, Nezu J, Kawase Y, Iwata T, Watanabe M, Miyoshi A, Ose A, Habu K, Kake T, Kamada N et al. 2002. Role of lkb1, the causative gene of peutz-jegher's syndrome, in embryogenesis and polyposis. Proc Natl Acad Sci U S A. 99(13):8903-8908.
Joiner WJ, Crocker A, White BH, Sehgal A. 2006. Sleep in drosophila is regulated by adult mushroom bodies. Nature. 441(7094):757-760.
Klarsfeld A, Leloup JC, Rouyer F. 2003. Circadian rhythms of locomotor activity in drosophila. Behav Processes. 64(2):161-175.
Kunst M, Hughes ME, Raccuglia D, Felix M, Li M, Barnett G, Duah J, Nitabach MN. 2014. Calcitonin gene-related peptide neurons mediate sleep-specific circadian output in drosophila. Curr Biol.

24(22):2652-2664.
Liu QL, Liu S, Kodama L, Driscoll MR, Wu MN. 2012. Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in drosophila. Curr Biol. 22(22):2114-2123.
Liu S, Liu QL, Tabuchi M, Wu MN. 2016. Sleep drive is encoded by neural plastic changes in a dedicated circuit. Cell. 165(6):1347-1360.
Liu YX, Wang TV, Cui Y, Gao S, Rao Y. 2022a. Biochemical purification uncovers mammalian sterile 3 (MST3) as a new protein kinase for multifunctional protein kinases AMPK and SIK3. J Biol Chem. 2022.101929.
Liu YX, Wang TV, Cui Y, Li C, Jiang L, Rao Y. 2022b. STE20 phosphorylation of AMPK related kinases revealed by biochemical purifications combined with genetics. J Biol Chem. 2022.101928.
Lizcano JM, Goransson O, Toth R, Deak M, Morrice NA, Boudeau J, Hawley SA, Udd L, Makela TP, Hardie DG et al. 2004. Lkb1 is a master kinase that activates 13 kinases of the ampk subfamily, including mark/par-1. Embo J. 23(4):833-843.
Lopez M, Dieguez C. 2014. Cellular energy sensors: Ampk and beyond. Mol Cell Endocrinol. 397(1-2):1-3.
Martin SG, St Johnston D. 2003. A role for drosophila lkb1 in anterior-posterior axis formation and epithelial polarity. Nature. 421(6921):379-384.
Matsumoto S, Iwakawa R, Takahashi K, Kohno T, Nakanishi Y, Matsuno Y, Suzuki K, Nakamoto M, Shimizu E, Minna JD et al. 2007. Prevalence and specificity of lkb1 genetic alterations in lung cancers. Oncogene. 26(40):5911-5918.
Mehenni H, Gehrig C, Nezu J, Oku A, Shimane M, Rossier C, Guex N, Blouin JL, Scott HS, Antonarakis SE. 1998. Loss of lkb1 kinase activity in peutz-jeghers syndrome, and evidence for allelic and locus heterogeneity. Am J Hum Genet. 63(6):1641-1650.
Michell BJ, Stapleton D, Mitchelhill KI, House CM, Katsis F, Witters LA, Kemp BE. 1996. Isoform-specific purification and substrate specificity of the 5'-amp-activated protein kinase. Journal of Biological Chemistry. 271(45):28445-28450.
Mitchelhill KI, Stapleton D, Gao G, House C, Michell B, Katsis F, Witters LA, Kemp BE. 1994. Mammalian amp-activated protein-kinase shares structural and functional homology with the catalytic domain of yeast snf1 protein-kinase. Journal of Biological Chemistry. 269(4):2361-2364.
Miyoshi H, Nakau M, Ishikawa T, Seldin MF, Oshima M, Taketo MM. 2002. Gastrointestinal hamartomatous polyposis in lkb1 heterozygous knockout mice. Cancer Research. 62(8):2261-2266.
Mohawk JA, Green CB, Takahashi JS. 2012. Central and peripheral circadian clocks in mammals. Annu Rev Neurosci. 35:445-462.
Morton JP, Jamieson NB, Karim SA, Athineos D, Ridgway RA, Nixon C, Mckay CJ, Carter R, Brunton VG, Frame MC et al. 2010. Lkb1 haploinsufficiency cooperates with kras to promote pancreatic cancer through suppression of p21-dependent growth arrest. Gastroenterology. 139(2):586-597.
Munday MR, Carling D, Hardie DG. 1988. Negative interactions between phosphorylation of acetyl-coa carboxylase by the cyclic amp-dependent and amp-activated protein-kinases. Febs Letters. 235(1-2):144-148.
Nagy S, Maurer GW, Hentze JL, Rose M, Werge TM, Rewitz K. 2018. Ampk signaling linked to the

schizophrenia-associated 1q21.1 deletion is required for neuronal and sleep maintenance. Plos Genetics. 14(12).

Nakada D, Saunders TL, Morrison SJ. 2010. Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells. Nature. 468(7324):653-658.

Nitabach MN, Taghert PH. 2008. Organization of the drosophila circadian control circuit. Curr Biol. 18(2):R84-93.

Nixon JP, Mavanji V, Butterick TA, Billington CJ, Kotz CM, Teske JA. 2015. Sleep disorders, obesity, and aging: The role of orexin. Ageing Res Rev. 20:63-73.

Parisky KM, Agosto J, Pulver SR, Shang YH, Kuklin E, Hodge JJL, Kang K, Liu X, Garrity PA, Rosbash M et al. 2008. Pdf cells are a gaba-responsive wake-promoting component of the drosophila sleep circuit. Neuron. 60(4):672-682.

Park M, Miyoshi C, Fujiyama T, Kakizaki M, Ikkyu A, Honda T, Choi J, Asano F, Mizuno S, Takahashi S et al. 2020. Loss of the conserved pka sites of sik1 and sik2 increases sleep need. Sci Rep. 10(1):8676.

Park S, Sonn JY, Oh Y, Lim C, Choe J. 2014. Sifamide and sifamide receptor define a novel neuropeptide signaling to promote sleep in drosophila. Mol Cells. 37(4):295-301.

Peutz JLA. 1921. Very remarkable case of familial polyposis of mucous membrane of intestinal tract and nasopharynx accompanied by peculiar pigmentations of skin and mucous membrane. Nederl Maandschr Geneesk. 10:134-146.

Pimentel D, Donlea JM, Albot CBT, Ong SMS, Hurston AJFT, Miesenbock G. 2016. Operation of a homeostatic sleep switch. Nature. 536(7616):333-+.

Poe AR, Wang B, Sapar ML, Ji H, Li K, Onabajo T, Fazliyeva R, Gibbs M, Qiu Y, Hu Y et al. 2019. Robust crispr/cas9-mediated tissue-specific mutagenesis reveals gene redundancy and perdurance in drosophila. Genetics. 211(2):459-472.

Qian Y, Cao Y, Deng B, Yang G, Li J, Xu R, Zhang D, Huang J, Rao Y. 2017. Sleep homeostasis regulated by 5ht2b receptor in a small subset of neurons in the dorsal fan-shaped body of drosophila. Elife. 6.

Rowan A, Bataille V, MacKie R, Healy E, Bicknell D, Bodmer W, Tomlinson I. 1999. Somatic mutations in the peutz-jeghers (lkb1/stkii) gene in sporadic malignant melanomas. J Invest Dermatol. 112(4):509-511.

Sakamoto K, McCarthy A, Smith D, Green KA, Hardie DG, Ashworth A, Alessi DR. 2005. Deficiency of lkb1 in skeletal muscle prevents ampk activation and glucose uptake during contraction. Embo J. 24(10):1810-1820.

Sanchez-Cespedes M, Parrella P, Esteller M, Nomoto S, Trink B, Engles JM, Westra WH, Herman JG, Sidransky D. 2002. Inactivation of lkb1/stk11 is a common event in adenocarcinomas of the lung. Cancer Research. 62(13):3659-3662.

Saper CB, Fuller PM. 2017. Wake-sleep circuitry: An overview. Current Opinion in Neurobiology. 44:186-192.

Sato F, Muragaki Y, Zhang YP. 2015. Dec1 negatively regulates ampk activity via lkb1. Biochem Bioph Res Co. 467(4):711-716.

Scammell TE, Arrigoni E, Lipton JO. 2017. Neural circuitry of wakefulness and sleep. Neuron. 93(4):747-765.

Sengupta S, Nagalingam A, Muniraj N, Bonner MY, Mistriotis P, Afthinos A, Kuppusamy P, Lanoue D, Cho S, Korangath P et al. 2017. Activation of tumor suppressor lkb1 by honokiol abrogates cancer stem-like phenotype in breast cancer via inhibition of oncogenic stat3. Oncogene. 36(41):5709-5721.

Seugnet L, Suzuki Y, Merlin G, Gottschalk L, Duntley SP, Shaw PJ. 2011. Notch signaling modulates sleep homeostasis and learning after sleep deprivation in drosophila. Curr Biol. 21(10):835-840.

Shackelford DB, Shaw RJ. 2009. The lkb1-ampk pathway: Metabolism and growth control in tumour suppression. Nat Rev Cancer. 9(8):563-575.

Shang YH, Donelson NC, Vecsey CG, Guo F, Rosbash M, Griffith LC. 2013. Short neuropeptide f is a sleep-promoting inhibitory modulator. Neuron. 80(1):171-183.

Shang YH, Griffith LC, Rosbash M. 2008. Light-arousal and circadian photoreception circuits intersect at the large pdf cells of the drosophila brain. P Natl Acad Sci USA. 105(50):19587-19594.

Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. 2000. Correlates of sleep and waking in drosophila melanogaster. Science. 287(5459):1834-1837.

Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC. 2004. The tumor suppressor lkb1 kinase directly activates amp-activated kinase and regulates apoptosis in response to energy stress. P Natl Acad Sci USA. 101(10):3329-3335.

Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, DePinho RA, Montminy M, Cantley LC. 2005. The kinase lkb1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science. 310(5754):1642-1646.

Sheeba V, Fogle KJ, Kaneko M, Rashid S, Chou YT, Sharma VK, Holmes TC. 2008. Large ventral lateral neurons modulate arousal and sleep in drosophila. Curr Biol. 18(20):1537-1545.

Shen Z, Wen XF, Lan F, Shen ZZ, Shao ZM. 2002. The tumor suppressor gene lkb1 is associated with prognosis in human breast carcinoma. Clinical Cancer Research. 8(7):2085-2090.

Skoulidis F, Byers LA, Diao LX, Papadimitrakopoulou VA, Tong P, Izzo J, Behrens C, Kadara H, Parra ER, Canales JR et al. 2015. Co-occurring genomic alterations define major subsets of kras-mutant lung adenocarcinoma with distinct biology, immune profiles, and therapeutic vulnerabilities. Cancer Discovery. 5(8):860-877.

Sutherland CM, Hawley SA, McCartney RR, Leech A, Stark MJR, Schmidt MC, Hardie DG. 2003. Elm1p is one of three upstream kinases for the saccharomyces cerevisiae snf1 complex. Curr Biol. 13(15):1299-1305.

Taheri S, Lin L, Austin D, Young T, Mignot E. 2004. Short sleep duration is associated with reduced leptin, elevated ghrelin, and increased body mass index (bmi). Sleep. 27:146-147.

Tanwar PS, Mohapatra G, Chiang S, Engler DA, Zhang LH, Kaneko-Tarui T, Ohguchi Y, Birrer MJ, Teixeira JM. 2014. Loss of lkb1 and pten tumor suppressor genes in the ovarian surface epithelium induces papillary serous ovarian cancer. Carcinogenesis. 35(3):546-553.

Thimgan MS, Suzuki Y, Seugnet L, Gottschalk L, Shaw PJ. 2010. The perilipin homologue, lipid storage droplet 2, regulates sleep homeostasis and prevents learning impairments following sleep loss. Plos Biology. 8(8).

Tobler I, Borbely AA. 1986. Sleep eeg in the rat as a function of prior waking. Electroen Clin Neuro. 64(1):74-76.

Tomlinson IP, Houlston RS. 1997. Peutz-jeghers syndrome. J Med Genet. 34(12):1007-1011.

699 Ueno T, Tomita J, Tanimoto H, Endo K, Ito K, Kume S, Kume K. 2012. Identification of a dopamine
700 pathway that regulates sleep and arousal in drosophila. Nat Neurosci. 15(11):1516-1523.
701 Wang B, Moya N, Niessen S, Hoover H, Mihaylova MM, Shaw RJ, Yates JR, 3rd, Fischer WH, Thomas JB,
702 Montminy M. 2011. A hormone-dependent module regulating energy balance. Cell.
703 145(4):596-606.
704 Weber F, Dan Y. 2016. Circuit-based interrogation of sleep control. Nature. 538(7623):51-59.
705 Werth E, Dijk DJ, Achermann P, Borbely AA. 1996. Dynamics of the sleep eeg after an early evening
706 nap: Experimental data and simulations. Am J Physiol. 271(3 Pt 2):R501-510.
707 Westerman AM, Entius MM, de Baar E, Boor PP, Koole R, van Velthuysen ML, Offerhaus GJ, Lindhout D,
708 de Rooij FW, Wilson JH. 1999. Peutz-jeghers syndrome: 78-year follow-up of the original
709 family. Lancet. 353(9160):1211-1215.
710 Wingo SN, Gallardo TD, Akbay EA, Liang MC, Contreras CM, Boren T, Shimamura T, Miller DS, Sharpless
711 NE, Bardeesy N et al. 2009. Somatic lkb1 mutations promote cervical cancer progression. Plos
712 One. 4(4):e5137.
713 Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D. 2005.
714 Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of amp-activated
715 protein kinase in mammalian cells. Cell Metab. 2(1):21-33.
716 Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T,
717 Carlson M, Carling D. 2003. Lkb1 is the upstream kinase in the amp-activated protein kinase
718 cascade. Curr Biol. 13(22):2004-2008.
719 Yardeni T, Eckhaus M, Morris HD, Huizing M, Hoogstraten-Miller S. 2011. Retro-orbital injections in
720 mice. Lab Animal. 40(5):155-160.
721 Yeh LA, Kim KH. 1980. Regulation of acetyl-coa carboxylase - properties of coa activation of acetyl-coa
722 carboxylase. P Natl Acad Sci-Biol. 77(6):3351-3355.
723 Yi W, Zhang YP, Tian YJ, Guo J, Li Y, Guo AK. 2013. A subset of cholinergic mushroom body neurons
724 requires go signaling to regulate sleep in drosophila. Sleep. 36(12):1809-1821.
725 Yurgel ME, Kakad P, Zandawala M, Nassel DR, Godenschwege TA, Keene AC. 2019. A single pair of
726 leucokinin neurons are modulated by feeding state and regulate sleep-metabolism
727 interactions. Plos Biology. 17(2).
728 Zhang X, Yan H, Luo Y, Huang Z, Rao Y. 2018. Thermoregulation-independent regulation of sleep by
729 serotonin revealed in mice defective in serotonin synthesis. Mol Pharmacol. 93(6):657-664.
730

FIGURE LEGENDS

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

Figure 2. Rescue of sleep loss in $lkb1^{T2/T2}$ by Lkb1. (A) A diagram of $lkb1^{T2}$-Gal4: a cDNA for the yeast Gal4 gene inserted in the $lkb1^{T2}$ knockdown mutants. (B) Relative Lkb1 mRNA levels in $lkb1^{T2}$-Gal4 (blue), UAS-Lkb1; $lkb1^{T2}$-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),

UAS-Lkb1-sgRNA/57C10-Gal4 (blue, n=41), and 57C10-Gal4/+;+/UAS-Cas9 (black, n=45) flies. (B-E) Statistical analysis of sleep duration, sleep bout number, sleep bout duration and latency to sleep in UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 (red, n=44), UAS-Lkb1-sgRNA/57C10-Gal4 (blue, n=41) and 57C10-Gal4/+;+/UAS-Cas9 (black, n=45) flies. Open bars denote daytime, filled bars nighttime. (B) Sleep duration. Daytime and nighttime sleep duration of UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 was significantly less than those of UAS-Lkb1-sgRNA/57C10-Gal4 and 57C10-Gal4/+;+/UAS-Cas9 flies. (C) Sleep bout number. Daytime and nighttime sleep bout numbers of UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 was not significantly from those of UAS-Lkb1-sgRNA/57C10-Gal4 and 57C10-Gal4/+;+/UAS-Cas9 flies. (D) Sleep bout duration. Nighttime sleep bout duration of UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 was significantly less than that of UAS-Lkb1-sgRNA/57C10-Gal4 and 57C10-Gal4/+;+/UAS-Cas9 flies. (E) Latency to sleep. Latency to sleep after light-off of UAS-Lkb1-sgRNA/57C10-Gal4;+/UAS-Cas9 was longer than that of 57C10-Gal4/+;+/UAS-Cas9 which was not significantly different from that of UAS-Lkb1-sgRNA/57C10-Gal4 flies. One-way ANOVA was used. n.s. denotes $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Error bars represent SEM.

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

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

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

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

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