1 **ppGpp Is Present in and Functions to Regulate Sleep in Drosophila**

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15 SUMMARY

16 Discovery of molecules in living systems and demonstration of their functional roles
 17 are pivotal in furthering our understanding of the molecular basis of biology. ppGpp
 18 (guanosine-5'-diphosphate, 3'-diphosphate) has been detected in prokaryotes for more
 19 than five decades. Here we report that a genetic screen followed by chemical analysis
 20 revealed the presence of ppGpp in *Drosophila*. It can be detected in germ-free
 21 *Drosophila* and its level is controlled by an enzyme encoded by the *mesh1* gene in
 22 *Drosophila*. Loss of function mutations in *mesh1*, which encoded the ppGpp
 23 degrading enzyme led to longer sleep latency and less total sleep. These phenotypes
 24 could be rescued by wild type *mesh1*, but not by the enzymatically defective mutant
 25 Mesh1E66A, functionally implicating ppGpp. Ectopic expression of RelA, the *E. coli*
 26 synthetase for ppGpp, phenocopied *mesh1* knockout mutants, whereas overexpression
 27 of *mesh1* resulted in the opposite phenotypes, supporting that ppGpp is both necessary
 28 and sufficient in sleep regulation. *mesh1* is expressed in a specific population of
 29 neurons, and a chemoconnectomic screen followed by genetic intersection
 30 experiments implicate the pars intercerebralis (PI) as the site of ppGpp function. Our
 31 results have thus revealed that ppGpp is present in animals after long lag since its
 32 discovery in bacteria. Furthermore, we have demonstrated that ppGpp in a specific
 33 subset of neurons plays a physiological role in regulating sleep. We speculate that
 34 ppGpp may play function in mammals.

35

36 INTRODUCTION

37 It was 52 years ago when guanosine-5'-diphosphate, 3'-diphosphate (guanosine
38 tetraphosphate, ppGpp) and guanosine-5'-triphosphate, 3'-diphosphate (guanosine
39 pentaphosphate, pppGpp) were implicated in gene regulation in *Escherichia coli* (*E.*
40 *coli*) (Cashel and Gallant, 1969). Collectively known as (p)ppGpp, they are key
41 players in bacterial stringent response to amino acid starvation (Dalebroux and
42 Swanson, 2012; Field, 2018; Gourse *et al.*, 2018; Hauryliuk *et al.*, 2015; Liu, Nibbittner
43 and Wang, 2015; Magnusson, Farewell and Nyström, 2005; Potrykus *et al.*, 2008;
44 Wang, Sanders and Grossman, 2007). The level of ppGpp is regulated by the
45 RelA/SpoT Homolog (RSH) family (Haseltine and Block, 1973; Hogg *et al.*, 2004).
46 In bacteria, RelA is one of the RSHs (Haseltine and Block, 1973), which contains
47 both a ppGpp synthetase domain (SYNTH) and an inactive ppGpp hydrolase domain
48 (HD) (Hogg *et al.*, 2004). When amino acids are depleted, uncharged tRNAs
49 accumulate (Fangman and Neidhardt, 1964) and activate the ribosome-associated
50 RelA (Yang and Ishiguro, 2001), which increases the production of ppGpp (Cochran
51 and Byrne, 1974). Another typical RSH in bacteria is the ppGpp hydrolase SpoT
52 (Laffler and Gallant, 1974), containing a weak SYNTH (Leung and Yamazaki, 1997)
53 and an active HD (Murry and Bremer, 1996). Upon iron limitation (Vinella *et al.*,
54 2005), carbon starvation (Lesley and Shapiro, 2008), or glucose phosphate stress
55 (Kessler *et al.*, 2017), ppGpp was also increased (Potrykus and Cashel, 2008).

56 ppGpp has also been detected in plants (Boniecka *et al.*, 2017; Ito *et al.*, 2012;
57 Kaisai *et al.*, 2004; Sugliani *et al.*, 2016; Tozawa *et al.*, 2007; van der Biezen *et al.*,

2000; Xiong *et al.*, 2001). More than 30 members of the RSHs have been found in bacteria and plants (Atkinson *et al.*, 2011; Field, 2018).

(p)ppGpp has not been detected in animals (Silverman and Atherly, 1979). Early report of ppGpp in the mouse turned out to be irreproducible (Irr, Kaulenas and Unsworth, 1974; Martini, Irr and Richter, 1977; Silverman and Atherly, 1977). Work in mammalian cell lines also failed to detect ppGpp before or after amino acid deprivation (Dabrowska *et al.*, 2006a; Fan, Fisher and Edlin, 1973; Givens *et al.*, 2004; Kim *et al.*, 2009; Mamont *et al.*, 1972; Rapaport and Bucher, 1976; Sato *et al.*, 2015; Stanners and Thompson, 1974; Smulson, 1970; Takahashi *et al.*, 2004; Thammana, Buerk and Gordon, 1976; Yamada *et al.*, 2004). In *Drosophila*, Mesh1, a member of the RSH family containing only the hydrolase domain for ppGpp has been found (Sun *et al.*, 2010), but ppGpp has not been detected.

Drosophila has served as a model for genetic studies of sleep for two decades (Hendricks *et al.*, 2000, Shaw *et al.*, 2000). Fly sleep is regulated by multiple genes functioning in several brain regions such as the fan-shape bodies (FSBs), the mushroom bodies, and the pars intercerebralis (PI) (e.g., Agosto *et al.*, 2008; Andretic *et al.*, 2005; Bushey *et al.*, 2007; Cirelli *et al.*, 2005; Crocker *et al.*, 2010; Dai *et al.*, 2019; Donlea *et al.*, 2011, 2014; Guo *et al.*, 2016; Hendricks *et al.*, 2003; Koh *et al.*, 2008; Kunst *et al.*, 2014; Liu *et al.*, 2014; Liu *et al.*, 2016; Parisky *et al.*, 2008; Qian *et al.*, 2017; Shang *et al.*, 2011; Sheeba *et al.*, 2008; Sitaraman *et al.*, 2015; Wu *et al.*, 2010; Wu *et al.*, 2014; Yuan *et al.*, 2006; Yurgel *et al.*, 2019).

We have carried out a genetic screen for genes involved in sleep regulation and

discovered that ppGpp is present in flies and regulates sleep. *mesh1* is expressed in a specific population of neurons, and the effects of RelA and Mesh1 overexpression could be detected when they were expressed in neurons, but not in non-neuronal cells. Further dissection narrowed down the functional significance of *mesh1* expressing neurons in the PI. Thus, after 52 years of its discovery, ppGpp has finally been found in animals and shown to play an important physiological role in specific neurons.

RESULTS

Discovery of ppGpp in *Drosophila*

We screened through 1765 P-element insertion lines of *Drosophila* for mutations affecting sleep latency (Eddison *et al.*, 2012) (Figure 1A). An insertion in the *mesh1* gene (*mesh1-ins*) (Figure 1C) was found to have significantly longer sleep latency (Figure 1A and 1D) and less total sleep duration (Figure 1 B).

mesh1 encodes an RSH family member in animals (Sun *et al.*, 2010). Mesh1 protein is predicted to have hydrolase activity, converting ppGpp to GDP (Figure 1E). We expressed the *Drosophila* Mesh1 protein in *E. coli* and purified it. In an in vitro hydrolysis assay with ppGpp, Mesh1 indeed degraded ppGpp (Figure S1A).

ppGpp has not been detected in animals (Hauryliuk *et al.*, 2015; Sun *et al.*, 2010). To measure endogenous ppGpp in *Drosophila*, extracts from more than 2000 flies were made. ppGpp was detected by ultra-performance liquid chromatography and mass spectrometry (UPLC-MS) in wild type (wt) flies (Figure 2A, 2B, 2D, 2E).

We generated a knockout (KO) line of *mesh1* (M1KO) using CRISPR-Cas9 to

102 replace most of its coding sequence following the start codon with 2A-attP (Figure 1C,
103 S1B). More ppGpp was present in M1KO flies than the wt flies (Figure 2D and 2E),
104 consistent with the fact that *mesh1* encoded only a hydrolase domain (Figure S1C).
105 These results also supported that the ppGpp detected by us was regulated by the
106 *Drosophila mesh1* gene.

107 The addition of Mesh1 protein to the extract from M1KO mutant flies also
108 decreased the amount of ppGpp (Figure 2F).

109 Although ppGpp was detected and its level was regulated by the *Drosophila*
110 *Mesh1* gene, which encoded an protein with ppGpp degrading activity, it remained
111 possible that the detected ppGpp was synthesized from bacteria attached to flies. To
112 test this possibility, we generated germ-free lines of wt and M1KO for analysis. We
113 confirmed that the flies were indeed germ-free flies with both bacterial culture (Figure
114 S1D) and 16S rDNA PCR (Figure S1E). ppGpp was detected in both fed and starved
115 germ-free flies, and levels in M1KO were significantly higher in both conditions
116 (Figure 2G, 2H).

117 Taken together, our results indicate that ppGpp exists in *Drosophila*.

118

119 **Sleep Phenotypes of M1KO Mutant Flies**

120 M1KO null *mesh1* mutants allowed us to investigate *mesh1* function in sleep (Figure
121 3A). In the paradigm of 12 hours light and 12 hours darkness (LD), M1KO flies
122 showed decreased nighttime sleep (Figure 3B), decreased total sleep (Figure 3D) and
123 increased sleep latency at night (Figure 3E), but no change in day time sleep level

(Figure 3C) or daytime sleep latency (Figure 3F). Sleep bout number and length either during the night or during the day were not significantly different between wt and M1KO flies (Figure S2A). Circadian rhythm was not significantly different between the wt and M1KO flies (Figure S2J, S2K, S2L and S2M). These results indicate that Mesh1 gene is mainly involved in regulating sleep latency.

We assessed awakening according to a procedure reported recently (Tabuchi *et al.*, 2018). Awakening number at the beginning of nighttime sleep (dusk) (Figure 3G) and awakening number near the end of nighttime sleep (dawn) (Figure 3H) were significantly increased in M1KO mutants.

Under the constant darkness (DD) condition, the same phenotypes in sleep level (Figure S2B, S2D, S2E), latency (Figure S2F, S2G) and awakening (Figure S2H and S2I) were observed. Sleep level in presumptive day is decreased in M1KO flies under DD (Figure S2D), but not under LD condition (Figure 3C).

137

Requirement of the Enzymatic Activity in *mesh1* for Its Rescue of Sleep Phenotypes in M1KO Mutants

We generated another KO line of *mesh1* (M1KOGal4) by replacing its CDS after the start codon with an in-frame fusion of the 2A peptide and the yeast transcription factor Gal4 (Figure 4A and S1B). To examine whether *mesh1* gene functions in sleep through its regulation of ppGpp level, we compared the activities of the wt Mesh1 protein with that of the Mesh1 E66A mutant protein, both in vitro and in vivo.

Mesh1E66A expressed in bacteria could not hydrolyze ppGpp in vitro (Figure

146 S1A). Similar to that in M1KO mutants, the level of ppGpp in flies was also increased
147 in M1KOGal4 (Figure S1G). ppGpp level was reduced to that of the wt flies when the
148 wt *mesh1* gene was expressed in flies under the control of the M1KOGal4 driver
149 (Figure S1G). By contrast, expression of *mesh1E66A* could not reduce the level of
150 ppGpp in M1KOGal4 flies (Figure S1G). These results indicate that wt Mesh1 protein
151 could, but Mesh1 E66A mutant protein could not, hydrolyze ppGpp either in vitro or
152 in vivo.

153 M1KOGal4/M1KO flies were phenotypically similar to M1KO/M1KO in having
154 longer sleep latencies (Figure 4B) and more awakening numbers (Figure 4D).
155 Expression of UAS-*mesh1* driven by M1KOGal4 in the background of
156 M1KOGal4/M1KO rescued all these phenotypes (Figure 4B to 4D). By contrast,
157 UAS-*mesh1E66A* could not rescue the sleep phenotypes in M1KOGal4/M1KO flies
158 (Figure 4B to 4D).

159 Taken together, the in vitro results from bacterially expressed Mesh1 and
160 Mesh1E66A proteins and the in vivo results from genetic rescue experiments strongly
161 support that ppGpp regulates sleep.

162

163 **Pattern of *mesh1* Expression in *Drosophila***

164 To examine the expression pattern of the *mesh1* gene, we crossed M1KOGal4 with
165 each of the following four UAS lines: UAS-mCD8-GFP for neuronal overviews (Lee
166 and Luo, 1999), UAS-redStinger for nuclei (Barolo, Castro and Posakony, 2004),
167 UAS-denmark for dendrites (Nikolai *et al.*, 2010) and UAS-syt.eGFP for axon

168 terminals (Zhang, Rodesch and Broadie, 2002).

169 *mesh1* was found in neurons in the brain and the ventral nerve cord (Figure 5A
170 and 5E). In the brain, *mesh1* expressing neurons were detected in the PI and the
171 suboesophageal ganglia (SOG) (Figure 5A and 5B). The *mesh1* expressing neurons in
172 the PI and their axonal terminals in the SOG (Figure 5A, 5C and 5D) were
173 reminiscent of the insulin-producing cells (IPC) in the PI which were shown
174 previously to regulate sleep (Crocker *et al.*, 2010). Immunostaining with an antibody
175 for the *Drosophila* insulin like peptide 2 (Dilp2) confirmed expression of *mesh1* in
176 IPC neurons positive in the PI (Figure S4C and S4E) (Li and Gong, 2015).

177

178 **Evidence for ppGpp Functioning in Neurons**

179 Because ppGpp regulate bacterial transcription through molecules such as the RNA
180 polymerase ((Cashel and Gallant, 1969; Dalebroux and Swanson, 2012; Gourse *et al.*,
181 2018; Hauryliuk *et al.*, 2015; Magnusson, Farewell and Nyström, 2005; Potrykus *et*
182 *al.*, 2008; Wang, Sanders and Grossman, 2007), it is possible that ppGpp functions in
183 many, even possibly all cells in animals. To test whether ppGpp functions in specific
184 cells, we increased and decreased ppGpp levels in different populations of cells, by
185 using ppGpp synthesizing and hydrolyzing enzymes.

186 The *E. coli* RelA gene encodes a synthetase for ppGpp (Laffler and Gallant, 1974).
187 We confirmed that the RelA from *E. coli* used by us indeed increased ppGpp in vitro
188 (Figure S1A). The biochemical activity of RelA protein is opposite to that of the
189 Mesh1 protein, the effect of RelA overexpression in flies should be similar to *mesh1*

190 knockout mutation. In flies with UAS-RelA driven either by tub-Gal4 for expression
191 in all cells (O'Donnell *et al.*, 1994) or by elav-Gal4 for expression in neurons
192 (Robinow and White, 1991), sleep latency (Figure 6A) and awakening number were
193 increased (Figure 6B). By contrast, UAS-RelA driven by repo-Gal4 for expression in
194 glial cells (Halter *et al.*, 1995) did not affect sleep (Figure 6A and 6B). These results
195 indicate that ppGpp regulation of sleep does not involve glial cells, but specifically
196 neurons.

197 Furthermore, when M1KOGal4 was used to drive UAS-RelA expression, both
198 sleep latency (Figure 6A) and awakening numbers (Figure 6B) were significantly
199 increased, indicating that ppGpp level in *mesh1* positive neurons is sufficient to
200 regulate sleep.

201 To decrease ppGpp level in fly cells, we used UAS-*mesh1*. UAS-*mesh1*
202 overexpression driven by tub-Gal4 in all cells or by elav-Gal4 in neurons significantly
203 decreased sleep latency (Figure 7A) and awakening numbers (Figure 7B). By contrast,
204 UAS-*mesh1* overexpression driven by repo-Gal4 in glial cells did not affect sleep
205 latency or awakening numbers (Figure 7A and 7B). When UAS-*mesh1* was driven by
206 Mesh1 Gal4, sleep latency and awakening number were decreased (Figure 7A and
207 7B).

208 Taken together, data from increasing or decreasing the ppGpp level by RelA and
209 Mesh1 indicate that ppGpp functions in neurons but not in glia to regulate sleep.

210

211 **Dissection of PI Neurons Involved in ppGpp Regulation of Sleep**

212 Our results have shown that neurons, specifically, *mesh1* expressing neurons, are
213 required for ppGpp regulation of sleep (Figure 4B, 4C, 4D, 6A, 6B, 7A and 7B). We
214 have generated a Gal4 library for the chemoconnectome (CCT), which include all the
215 known neurotransmitters, modulators, neuropeptides and their receptors (Deng *et al.*,
216 2019). We carried out a CCT screen by crossing each Gal4 line with UAS-RelA. RelA
217 expression driven by Gal4 lines for Trh, Capa-R, CCHa2-R, LkR, OA2 and CG13229
218 affected sleep (Figure S3A). It was noted that all of these lines drove expression in the
219 PI (Figure S3 B to G). This suggests functional significance of *mesh1* expression in
220 the PI (Figure 5).

221 To further dissect the PI neurons for functional involvement in ppGpp regulation
222 of sleep, we tested five Gal4 lines known to drive expression in PI neurons: a line for
223 PI (c767, Cavanaugh *et al.*, 2014; Donlea *et al.*, 2009), three lines for neuropeptides
224 Dh44 (Cannel *et al.*, 2016; Chen and Dahanukar, 2018), Dilp2 (Crocker *et al.*, 2010;
225 Semaniuk *et al.*, 2018; Yurgel *et al.*, 2019) and SIFa (Martelli *et al.*, 2017; Park *et al.*,
226 2014), and one line for receptor R19G10 (an RYamide receptor) (Collins *et al.*, 2011;
227 Ohno *et al.*, 2017). We crossed each of these PI drivers to a line of knockin flies with
228 in-frame fusion of the flippase gene to the C terminus of the *mesh1* gene (M1KIflp)
229 (Figure S4A), which was recombined with UAS-FRT-stop-FRT-mCD8-GFP as a
230 reporter. Expression of *mesh1* gene in these specific PI neurons were confirmed
231 (Figure S4B, S4C and S4D).

232 Each of these PI drivers was crossed with UAS-RelA to increase ppGpp level in
233 specific neurons. Sleep latency (Figure 6C) and awakening (Figure 6D) were

increased by RelA expression (Figure 6C and 6D) in neurons expressing Dilp2, SIFa, R19G10 or c767. However, RelA expression in Dh44 neurons could affect sleep (Figure 6C and 6D)

Each of these PI drivers was crossed with UAS-Mesh1 to decrease ppGpp level in specific neurons. Overexpression of Mesh1 in Dilp2 neurons decreased sleep latency and awakening (Figure 7D, 7E and 7F). Sleep latency and awakening were decreased by Mesh1 expression in neurons positive for Dilp2, SIFa, R19G10 or c767 (Figure 7D and 7E). However, Mesh1 expression in Dh44 neurons could affect sleep (Figure 7D and 7E).

Taken together, experiments with RelA and Mesh1 expression have provided consistent results indicating that specific subsets of neurons in the PI are involved in ppGpp regulation of sleep.

Role of ppGpp in Starvation Induced Sleep Loss

We investigated whether ppGpp played a role in starvation induced sleep loss (SISL). Similar to humans (MacFadyen, Oswald and Lewis, 1973), flies also show SISL (Keene *et al.*, 2010). Baseline sleep in fed flies was recorded before flies were starved for 24 hours (Yurgel *et al.*, 2019). Sleep during starvation was compared to that before starvation (Figure 8A).

When *mesh1* gene was deleted in M1KO, nighttime SISL in M1KO flies was significantly more than that in wt flies, whereas daytime SISL were similar between the wt and M1KO mutant flies (Figure 8A and 8B). Although nighttime sleep latency

256 during starvation night was still higher in M1KO (Figure S6A and S6B), there was no
257 latency change after starvation during either night time or day time (Figure S6C and
258 S6D). The M1KOGal4 knockin mutants were similar to M1KO flies in having
259 exacerbated SISL (Figure 8C and S5). UAS-*mesh1* but not UAS-*mesh1E66A* could
260 rescue the phenotype of SISL in *mesh1* knockout flies, indicating that the ppGpp
261 hydrolyzing activity is required for Mesh1 involvement in SISL. Expression of the
262 bacterial ppGpp synthesizing enzyme RelA caused a decrease in SISL (Figure 8D),
263 opposite to the SISL enhancing effect of Mesh1 (Figure 9A), indicating that ppGpp
264 level, rather than any other unexpected activities of RelA or Mesh1 were involved.

265 SISL was increased by general or neuronal expression of RelA, but not by glial
266 expression of RelA (Figure 8D, 9A and 9C). SISL was decreased by general or
267 neuronal overexpression of Mesh1, but not by glial overexpression of Mesh1 (Figure
268 9A and 9C).

269 SISL was enhanced by RelA expression driven by Gal4 lines for *c767*, *Dilp2*,
270 *SIFamide* or *R19G10* (Figure 8E). SISL was reduced by Mesh1 overexpression driven
271 by Gal4 lines for *c767*, *Dilp2*, *SIFamide* or *R19G10* (Figure 9B, 9D). By contrast,
272 *Dh44* expressing neurons were not involved in ppGpp regulation of SISL because
273 neither RelA expression nor Mesh1 overexpression in *Dh44* neurons affected SISL
274 (Figure 8E, 9B and 9D).

275 Thus, the same neurons required for ppGpp regulation of sleep latency and
276 awakening are also involved in its regulation of SISL.

277

278 **DISCUSSION**

279 We have discovered a new molecule in animals and provided evidence that it plays a
280 physiological role. These results came from integrated genetic and chemical
281 approaches. We have carried out a genetic screen of P element insertion lines which
282 led to the discovery of a new mutation in the *Drosophila* ppGpp hydrolase *Mesh1*.
283 Our chemical analyses indicate that ppGpp is present in *Drosophila*, not a
284 contaminant of bacteria: germ free flies still had ppGpp and the content of ppGpp is
285 regulated both by an enzyme encoded by a *Drosophila* gene and by starvation of flies.
286 Our chemoconnectomic screen suggests involvement of PI neurons in ppGpp function.
287 Further genetic intersection experiments confirm that ppGpp in specific PI neurons
288 regulate sleep. Our findings indicate that ppGpp is not just a molecule present in
289 prokaryotes and plants, but also exists and functions in animals. It regulates sleep,
290 including sleep latency and SISL.

291

292 **Evidence for ppGpp Presence in *Drosophila***

293 We have shown that ppGpp is present in *Drosophila* and that it is hydrolyzed by
294 *Mesh1* which are expressed in specific neurons in flies. Moreover, ppGpp is detected
295 in germ-free flies, indicating that ppGpp is synthesized in *Drosophila* (Figure 2G, 2H),
296 not a contaminant of bacteria.

297 RSHs with the synthetase domain exist in both bacteria (Cochran and Byrne,
298 1974) and plants (van der Biezen *et al.*, 2000), but not in animals (Atkinson *et al.*,
299 2011; Sun *et al.*, 2010). Our results suggest that the presence of a ppGpp synthetase in

300 animals which does not contain an RSH like domain.

301 Plant RSHs are thought to result from lateral gene transfer events from bacteria
 302 (Ito *et al.*, 2017; Field, 2018). Bioinformatic analyses and biochemical assays indicate
 303 that ppGpp synthetase homologs are distributed widely in plants including:
 304 dicotyledon *A. thaliana* (van der Biezen *et al.*, 2000), monocotyledon *O. sativa*
 305 (Xiong *et al.*, 2001), green algae *C. reinhardtii* (Kasai *et al.*, 2002), *S. japonica*
 306 (Yamada *et al.*, 2004), *N. Tabacum* (Givens *et al.*, 2004), pea plants (Takahashi *et al.*,
 307 2004), *P. nil* (Dabrowska *et al.*, 2006a), *C. annuum* (Kim *et al.*, 2009), and moss *P.*
 308 *patens* (Sato *et al.*, 2015). Presence of the N-terminal chloroplast transit peptide (cTP),
 309 causes most plant RSHs to be located in the chloroplasts (Boniecka *et al.*, 2017; Chen
 310 *et al.*, 2014; Mizusawa *et al.*, 2008; Takahashi *et al.*, 2004; Sugliani *et al.*, 2016; Sato
 311 *et al.*, 2009; Takahashi *et al.*, 2004). It is unclear whether a ppGpp synthetase exists in
 312 *Drosophila* mitochondria.

313

314 **Evidence for ppGpp Function in *Drosophila***

315 We obtain evidence for ppGpp function from four lines of experiments. First, three
 316 mutations in *mesh1* caused sleep phenotypes in *Drosophila*. The first mutation is a
 317 P-element insertion (Eddison *et al.*, 2012), whose phenotype (Figure 1A and 1B) and
 318 molecular nature we have characterized as *mesh1-ins* (Figure 1C). The second
 319 mutation is a knockout generated by us as M1KO (Figure 4A top panel). The third
 320 mutation is a knockin generated by us as M1KOGal4 (Figure 4A bottom panel). All
 321 three lines have the same phenotypes (Figure 1, 3 and 4).

Second, we have shown that the hydrolyzing activity of Mesh1 is required to rescue the *mesh1* knockout mutant phenotype: if a point mutation was introduced to amino acid residue 66 by converting it from E to A, then it was enzymatically inactive in vitro (Figure S1C and S1D) and unable to rescue the sleep phenotype in vivo (Figure 4B, 4C, 4D and 8C).

Third, expression of RelA, a bacterial ppGpp synthetase, in *Drosophila* phenocopied *mesh1* knockout mutants (Figure 6, 8D and 8E).

Fourth, the phenotypes of Mesh1 overexpression are opposite to those of RelA expression (Figure 7 and 9).

Function of ppGpp in Neurons

Does ppGpp function in all cells or only in some cells? Results obtained here support that ppGpp functions in specific neurons in the PI to regulate sleep.

Mesh1 gene has been found to be expressed in specific neurons (Figure 5). Because Mesh1 is a ppGpp hydrolase, its expression can show the location where ppGpp is hydrolyzed, but not necessarily where it is synthesized or where it functions. When we used Gal4 lines from CCT to drive the expression of RelA, sleep phenotypes were observed in 6 lines which were all expressed in the PI (Figure 7). When Gal4 lines for PI neurons were used to drive RelA or Mesh1 expression, several of them could indeed cause sleep phenotypes (Figure 6, 7, 8 and 9). Expression of RelA or Mesh1 in glial cells did not affect sleep (Figure 6A, 6B, 7A and 7B).

Because it is difficult to imagine that both synthetase and hydrolase could cause

the same multiple phenotypes in the same neurons if these neurons are not where
ppGpp functions, our results are most consistent with the idea that ppGpp functions in
these PI neurons.

Molecular Targets of ppGpp

In bacteria, the best-known direct target of ppGpp is RNA polymerase (RNAP) (e.g.,
Artsimovitch *et al.*, 2004; Barker *et al.*, 2001; Kajitani and Ishihama, 1984; Kingston
et al., 1981; Lindahl and Nomura, 1976). ppGpp interaction with the RNA
polymerase leads to blockage of transcription initiation (Artsimovitch *et al.*, 2004)
and elongation (Kingston *et al.*, 1981). There are also other targets for ppGpp (e.g.,
Paul *et al.*, 2005); Gourse *et al.*, 2018; Maciag *et al.*, 2010; Nomura *et al.*, 2014;
Sherlock *et al.*, 2018; Corrigan *et al.*, 2016; Pao and Dyess, 1981; Dalebroux and
Swanson, 2012; Zhang *et al.*, 2019). A recent study utilizing a ppGpp-coupled bait
uncovered new ppGpp target proteins in bacteria, including a large group of GTPase
and metabolism-related enzymes (Wang *et al.*, 2019). Future studies are required to
identify the molecule(s) directly mediating the sleep regulating function of ppGpp in
Drosophila.

361 **SUPPLEMENTAL INFORMATION**

362 Supplemental information includes 6 Figure.

363

364 **ACKNOWLEDGEMENTS**

365 We are grateful to Dr. U. Heberlein for sharing P-element insertion mutant library, Drs.

366 J. Ni and G. Gao for providing us with CRISPR/Cas9 plasmids and flies, to Dr. Z.-F.

367 Gong for sharing antibodies, to the Beijing Commission of Science and Technology

368 and the National Natural Science Foundation of China (Project 31421003 to Y. R.) for

369 grant support.

370

371 **AUTHOR CONTRIBUTIONS**

372 Y.R supervised and initiated the project. W. Y. performed the majority of experiments

373 and data analysis. X. H. W. carried our experiments of UPLC-MS. W. Y., E. Z. and H.

374 D. X. carried out experiments of sleep screen on P-element insertion mutants. W. Y.

375 implemented the fly tracing program, and E. Z. developed software for sleep analyses.

376 W. Y. and Y. R. wrote the manuscript.

377

378

379 **DECLARATION OF INTERESTS**

380

381 The authors declare no competing interests.

382

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FIGURE LEGENDS

Figure 1. Mesh1 Gene and ppGpp Level in Drosophila

- (A) Results of a screen of 1765 P-element insertion lines. The y axis shows sleep latency in minutes and the x axis shows P element insertion lines. Grey shadows showed the standard error of the mean (SEM) from multiple flies of each line, and blue dots and rectangles showed the range of 3-fold of standard deviation from the value of wt. *mesh1-ins* had longer sleep latency than the wt.
- (B) Results of a screen of 1765 P-element insertion lines. The y axis shows total sleep levels in hours and the x axis shows P element insertion lines. Grey shadows showed SEM from multiple flies of each line, and blue dots and rectangles showed the range of 3-fold of standard deviation from the value of wt. *mesh1-ins* had less total sleep than the wt.
- (C) Diagrams of *mesh1-ins* and M1KO. In *mesh1-ins*, the P-element was found to be inserted into the CDS of the 1st exon. In M1KO, the entire CDS except the start codon was replaced with stop-2A-attP and 3Px3-RFP. Introns were labeled as straight lines, CDS as unfilled rectangles and untranslated regions (UTRs) as filled rectangles.
- (D) Statistical analysis of sleep latency. Compared to the wt, *mesh1-ins* exhibited significantly longer sleep latency (Student's t-test, **** denotes $p < 0.0001$).
- (E) ppGpp metabolism. In *E. coli*, GDP is converted to ppGpp by its synthetase RelA, whereas ppGpp is converted to GDP by the hydrolase SpoT. In *D. melanogaster*, only the hydrolase Mesh1 has been discovered but the synthetase is unknown.

705 **Figure 2. Identification and Quantitative Analysis of ppGpp in *Drosophila***

706 (A-C) UPLC-MS profiles of standard ppGpp (A), wt fly extracts (B), and M1KO fly
707 extracts (C). Horizontal axis is time (minutes), and vertical axis is intensity of MS
708 signal at $m/z = 601.95$, which corresponds to standard ppGpp (A).

709 (D-E) Statistical analyses of ppGpp measurements under fed (D) or starved (E)
710 conditions in *Drosophila*. For each genotype, approximately 2000 male flies were
711 sacrificed for UPLC-MS measurements. In both fed and starved conditions, compared
712 to that in wt, ppGpp level was significantly increased in M1KO (Student's t-test, ****
713 denoting $p < 0.0001$).

714 (F) Mesh1 protein expressed in *E.coli* was purified and added into extracts from
715 M1KO flies. ppGpp in the extracts was reduced by Mesh1.

716 (G-H) Statistical analyses of ppGpp measurements under fed (D) or starved (E)
717 conditions in germ-free *Drosophila*. Germ-free flies were generated and amplified
718 (see method in details), and in each condition 2000 male flies were sacrificed for
719 measurement by UPLC-MS.

720

721 **Figure 3. Sleep Phenotypes in M1KO Mutant Flies**

722 (A) Profiles of sleep and awakening in M1KO Flies. Rest time for every 30 min was
723 plotted (with the SEM as the error bars). Compared to the wt flies, M1KO flies began
724 sleep later and wake up earlier. Dusk (ZT 0-3) and dawn (ZT 9-12) of night time sleep
725 were denoted with green bars.

726 (B-D) Statistical analyses of sleep level during night time (B), day time (C) or in total

(D). Compared to the wt, M1KO flies slept significantly less at night (Student's t-test, **, **** denoting $p < 0.01$, and $p < 0.0001$ respectively).

(E-F) Statistical analyses of sleep latency during night time (E), or day time (F). Compared to the wt, M1KO flies exhibited a significant increase in night sleep latency (Student's t-test, **** denoting $p < 0.0001$).

(G-H) Statistical analyses of summed awakening numbers at dusk (G, ZT0-3) and dawn (H, ZT9-12) shown in (A). Awakening numbers of both dusk and dawn were significantly increased in M1KO flies (Student's t-test, **** denoting $p < 0.0001$).

735

Figure 4. Significance of the Hydrolysis Activity of Mesh1 in Drosophila Sleep

(A) Diagrams of constructs used to generate M1KO and M1KOGal4 flies. *mesh1* CDS except the start codon was deleted in both M1KO and M1KOGal4, and the only difference is that a T2A-fused Gal4 (with a stop codon) were placed in M1KOGal4.

(B) Statistical analyses of sleep latency at night. Columns 1-3: wt, M1KOGal4/+ and M1KOGal4/M1KOGal4 flies, with the sleep latency significantly increased in M1KOGal4/M1KOGal4 (Column 3). Columns 4-5: M1KOGal4/M1KO and M1KOGal4/+. Columns 6-8: M1KOGal4/M1KO > UAS-Mesh1/+, M1KO/M1KO, UAS-Mesh1/+, M1KOGal4/+>UAS-Mesh1/+. M1KOGal4/M1KO>UAS-Mesh1 (Column 6) could fully rescue the sleep latency phenotype (Columns 7-8). Columns 9-11: M1KOGal4/M1KO>UAS-Mesh1E66A/+, M1KO/M1KO, UAS-Mesh1E66A/+, M1KOGal4/+>UAS-Mesh1E66A/+. The hydrolysis-deficient line UAS-Mesh1E66A/+ driven by M1KOGal4/M1KO (Column 9) failed to rescue sleep

latency (Columns 10-11). Comparison was based on two-way ANOVA and Bonferroni post-tests with **** denotes $p < 0.0001$.

(C) Statistical analyses of awakening number at dusk. Columns 1-3: awakening number significantly increased in M1KO (Column 3). Columns 4-5: M1KOGal4/M1KO (Column 4) had more awakening number than the wt. Columns 6-8: M1KOGal4/M1KO > UAS-Mesh1 (Column 6) could rescue the awakening number deficiency (Columns 7-8). Columns 9-11: the hydrolysis-deficient UAS-Mesh1E66A/+ driven by M1KOGal4/M1KO (Column 9) failed to rescue the awakening number deficiency (Columns 10-11).

(D) Statistical analyses of awakening number at dawn.

Figure 5. The Expression Pattern of M1KOGal4

(A) Neurons expressing mCD8-GFP driven by M1KOGal4 in the brain (left) and the VNC (right). Magenta showed immunostaining by the nc82 monoclonal antibody specific for neurons.

(B) Nuclei expressing red-stinger driven by M1KOGal4 in the brain (left) and the VNC (right). Redstinger is presented in green.

(C) Dendrites revealed by M1KOGal4>UAS-denmark in the brain (left) and the VNC (right). The dendritic marker denmark is presented in green.

(D) Synaptic terminals revealed by M1KOGal4>UAS-Syt.eGFP in the brain (left) and the VNC (right). Syt.eGFP is presented in green.

(E) A diagrammatic summary of neurons revealed by M1KOGal4. A: anterior sections.

771 P: posterior sections.

772

773 **Figure 6. Effects of RelA Expression on Drosophila Sleep**

774 (A) Statistical analyses of sleep latency when RelA was expressed in all cells with
775 (tub-Gal4), neurons (elav-Gal4), glia (repo-Gal4) or mesh1 positive cells
776 (mesh1Gal4). (1) wt, (2) M1KO, (3) UAS-RelA/+, (4) M1KOGal4/+>UAS-RelA/+,
777 (5) M1KOGal4/+, (6) elav-Gal4/+> UAS-RelA/+, (7) elav-Gal4/+, (8)
778 repo-Gal4/+>UAS-RelA/+, (9) repo-Gal4/+, (10) tub-Gal4/+>UAS-RelA/+, (11)
779 tub-Gal4/+. Comparisons were based on two-way ANOVA and Bonferroni post-tests
780 with **** denoting $p < 0.0001$.

781 (B) Statistical analyses of awakening number at dusk when RelA was expressed in all
782 cells (10), neuron (6), glia (8) or *mesh1* positive cells (4).

783 (C) Statistical analyses of sleep latency with RelA expression in different subsets of
784 PI neurons. (1) wt, (2) M1KO, (3) UAS-RelA/+, (4) c767/+>UAS-RelA/+, (5)
785 c767/+, (6) Dh44-Gal4/+>UAS-RelA/+, (7) Dh44-Gal4/+, (8) Dilp2-Gal4/+>
786 UAS-RelA/+, (9) Dilp2-Gal4/+, (10) SIFa2-Gal4/+>UAS-RelA/+, (11) SIFa2-Gal4/+,
787 (12) R19610-Gal4/+>UAS-RelA/+, (13) UAS-RelA/+. Comparisons were based on
788 two-way ANOVA and Bonferroni post-tests, with **, ***, **** denoting $p < 0.01$, p
789 < 0.001 , $p < 0.0001$ respectively.

790 (D) Statistical analyses of awakening number with RelA expression in different
791 subsets of PI neurons. Comparisons were based on two-way ANOVA and Bonferroni
792 post-tests with **, ***, **** denoting $p < 0.01$, $p < 0.001$, $p < 0.0001$ respectively.

793

794 **Figure 7. Effects of Mesh1 Overexpression on Drosophila Sleep**

795 (A) Statistical analyses of sleep latency when Mesh1 was overexpressed in all cells,
796 neurons, glia or *mesh1* positive cells. (1) wt, (2) M1KO, (3) UAS-Mesh1/+, (4)
797 M1KOGal4/+ > UAS- Mesh1/+, (5) M1KOGal4/+, (6) elav-Gal4/+ > UAS- Mesh1/+,
798 (7) elav-Gal4/+, (8) repo-Gal4/+>UAS-Mesh1/+, (9) repo-Gal4/+, (10) tub-Gal4/+,
799 UAS-Mesh1/+, (11) tub-Gal4/+. Comparisons were based on two-way ANOVA and
800 Bonferroni post-tests with **, *** denoting $p < 0.01$ and $p < 0.001$ respectively.

801 (B) Statistical analyses of awakening number when Mesh1 was overexpressed in all
802 cells, neurons, glia or *mesh1* positive cells. Comparisons were based on two-way
803 ANOVA and Bonferroni post-tests with *** and **** denoting $p < 0.001$ and $p <$
804 0.0001 respectively.

805 (C) Representative sleep profiles for lines in (A) and (B). Red arrow denotes sleep
806 latency at dusk. UAS-Mesh1/+ in the top and bottom panels was the shared control,
807 with the top panel showing that repo-Gal4>UAS Mesh1 did not affect sleep latency
808 whereas the bottom panel showing that elav-Gal4>UAS Mesh1 shortened sleep
809 latency.

810 (D) Statistical analyses of sleep latency with Mesh1 overexpression in different PI
811 subsets. (1) wt, (2) M1KO, (3) UAS-Mesh1/+, (4) c767-Gal4/+ and UAS-Mesh1/+, (5)
812 c767-Gal4/+, (6) Dh44-Gal4/+ and UAS-Mesh1/+, (7) Dh44-Gal4/+, (8)
813 Dilp2-Gal4/+ and UAS-Mesh1/+, (9) Dilp2-Gal4/+, (10) SIFa2-Gal4/+ and
814 UAS-Mesh1/+, (11) SIFa2-Gal4/+, (12) R19G10-Gal4/+ and UAS-Mesh1/+, (13)

815 R19G10-Gal4/+. Comparisons were based on two-way ANOVA and Bonferroni
816 post-tests with **, ***, **** denoting $p < 0.01$, $p < 0.001$ and $p < 0.0001$
817 respectively.

818 (E) Statistical analyses of awakening number with Mesh1 overexpression in different
819 PI subsets. Comparisons were based on two-way ANOVA and Bonferroni post-tests
820 with *, **, *** denoting $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

821 (F) Representative sleep profiles for lines in (D) and (E). Red arrow denotes sleep
822 latency at dusk. Note that the UAS-Mesh1/+ profile in the top and bottom panels was
823 the shared control, with the top panel showing that Dh44-Gal4>UAS Mesh1 did not
824 affect sleep latency whereas the bottom panel showing that Dilp2-Gal4>UAS Mesh1
825 shortened sleep latency.

826

827 **Figure 8. Effects of Mesh1 Knockout Mutations or RelA Expression on SISL**

828 (A) SISL in M1KO. Baseline sleep was recorded on food for 3 days (recording at day
829 2 and day 3), before flies were transferred to 1% agar at the end of the 3rd day,
830 followed by sleep recording while starvation of 24hr.

831 (B) Statistical analysis of SISL in (A). Sleep was lost ~10% at night and ~50% at day,
832 compared to the baseline in wt. This was significantly exacerbated in M1KO at night
833 (Student's t-test with *** denoting $p < 0.001$).

834 (C) Statistical analyses of SISL with Mesh1 and Mesh1E66A rescue experiments. (1)
835 wt, (2) M1KO/+, (3) M1KO/M1KO, (4) M1KOGal4/M1KO, (5) M1KOGal4/+, (6)
836 M1KOGal4/M1KO and UAS-Mesh1/+, (7) M1KO/M1KO and UAS-Mesh1/+, (8)

837 M1KOGal4/+ and UAS-Mesh1/+, (9) M1KOGal4/M1KO and UAS-Mesh1E66A/+,
838 (10) M1KO/M1KO and UAS-Mesh1E66A/+, (11) M1KOGal4/+ and
839 UAS-Mesh1E66A/+. Comparison was based on two-way ANOVA and Bonferroni
840 post-tests with **, *** denoting $p < 0.01$, $p < 0.001$ respectively.

841 (D) Statistical analyses of SISL with RelA expression in all cells, neurons, glia or
842 *mesh1* positive cells. (1) wt, (2) M1KO, (3) UAS-RelA/+, (4) M1KOGal4/+>
843 UAS-RelA/+, (5) M1KOGal4/+, (6) elav-Gal4/+>UAS-RelA/+, (7) elav-Gal4/+, (8)
844 repo-Gal4/+>UAS-RelA/+, (9) repo-Gal4/+, (10) tub-Gal4/+>UAS-RelA/+, (11)
845 tub-Gal4/+. Comparisons were based on two-way ANOVA and Bonferroni post-tests
846 with **** denoting $p < 0.0001$.

847 (E) Statistical analyses of SISL with RelA expressing in different subsets of PI. (1)
848 wt, (2) M1KO, (3) UAS-RelA /+, (4) c767-Gal4/+ and UAS-RelA /+, (5)
849 c767-Gal4/+ (6) Dh44-Gal4/+ and UAS-RelA /+, (7) Dh44-Gal4/+, (8) Dilp2-Gal4/+
850 and UAS-RelA/+, (9) Dilp2-Gal4/+, (10) SIFa2-Gal4/+ and UAS-RelA/+, (11)
851 SIFa2-Gal4/+, (12) R19G10-Gal4/+ and UAS-RelA/+, (13) R19G10-Gal4/+.
852 Comparison was based on t-test with *** denoting $p < 0.001$.

853

854 **Figure 9. Effect of Mesh1 Overexpression of on SISL**

855 (A) Statistical analyses of SISL with Mesh1 overexpression in neurons or other cells.
856 (1) wt, (2) M1KO, (3) UAS-Mesh1/+, (4) M1KOGal4/+>UAS-Mesh1/+, (5)
857 M1KOGal4/+, (6) elav-Gal4/+>UAS-Mesh1/+, (7) elav-Gal4/+, (8)
858 repo-Gal4/+>UAS- Mesh1/+, (9) repo-Gal4/+, (10) tub-Gal4/+>UAS-Mesh1/+

859 tub-Gal4/+ (11). Comparisons were based on two-way ANOVA and Bonferroni
860 post-tests with ***, **** denoting $p < 0.001$, $p < 0.0001$ respectively.

861 (B) Statistical analyses of SISL with Mesh1 overexpression in different subsets of PI.

862 (1) wt, (2) M1KO, (3) UAS-Mesh1/+, (4) c767-Gal4/+>UAS-Mesh1/+, (5)

863 c767-Gal4/+, (6) Dh44-Gal4/+>UAS-Mesh1/+, (7) Dh44-Gal4/+, (8) Dilp2-Gal4/+>

864 UAS-Mesh1/+, (9) Dilp2-Gal4/+, (10) SIFa2-Gal4/+>UAS-Mesh1/+, (11)

865 SIFa2-Gal4/+, (12) R19G10-Gal4/+>UAS-Mesh1/+, (13) R19G10-Gal4/+.

866 Comparison was based on t-test with *** denoting $p < 0.001$.

867 (C) Representative sleep profiles of SISL in (A). Baseline sleep in black, and sleep

868 during starvation in blue. Black arrows indicate that night SISL is not significantly

869 different from wt; red arrows indicate SISL phenotype similar to that in M1KO.

870 (D) Representative sleep profiles of SISL in (B).

871

872 **Figure S1. Quantifications: Mesh1 RNA and ppGpp**

873 (A) Examination of germ-free status by growing bacteria on LB plate. 6 conditions

874 were examined: (1) blank: direct incubation of LB plate; (2) water germ-: strike LB

875 plate with germ-free water; (3) wt germ-: extract germ-free wt flies with germ-free

876 water, and strike LB plate with the extract; (4) M1KO germ-: extract germ-free

877 M1KO flies with germ-free water, and strike LB plate with the extract; (5) wt germ+:

878 extract normal wt flies with germ-free water, and strike LB plate with the extract; (6)

879 M1KO germ+: extract normal M1KO flies with germ-free water, and strike LB plate

880 with the extract. Among these conditions, bacteria only appear on plates of wt germ+

881 and M1KO germ+.

882 (B) Examination of germ-free status by 16S rDNA PCR. 5 pairs of 16S PCR primers

883 (denoted as pm) were used, including (1) 16S-27F/16S-519R; (2)

884 16S-357F/16S-907R; (3) 16S-530F/16S-1110R; (4) 16S-926F/16S-1492R; (5)

885 16S-1114F/16S-1525R. The upper and lower bands at marker lane (Trans2K)

886 correspond to 500bp and 250bp. PCR signals by pm1, pm3 and pm5 were detected in

887 both controls (wt germ+ and M1KO germ+), while no signals were detected in all

888 three germ-free groups (water germ-, wt germ-, M1KO germ-).

889 (C) Measurement of Mesh1 RNA by quantitative polymerase chain reaction (qPCR).

890 Log₁₀(RQ) is the log form of relative quantity of Mesh1 mRNA in Drosophila. (1)

891 M1KO/+, (2) *mesh1*-ins, (3) M1KO, (4) M1KOGal4/M1KO, (5) M1KOGal4/M1KO

892 and UAS-Mesh1/+, (6) M1KO/M1KO and UAS-Mesh1/+, (7) M1KOGal4/+,

893 UAS-Mesh1/+, (8) M1KOGal4/+ and UAS-Mesh1/UAS-Mesh1, (9) M1KOGal4/+.

894 (D) Diagrams of the ppGpp synthetase domain (SD) and the hydrolase domain (HD)

895 in RSH proteins from E. coli, D. melanogaster, and H. sapiens. The mammalian hddc3

896 and the Drosophila Mesh1 proteins contain only HD, the bacterial RelA protein

897 contains a weak HD, an active SD and a regulatory domain (RD). Bacterial SpoT

898 contains an active HD, a weak SD and a RD.

899 (E) Standard curve of MS peak area to ppGpp concentration. x-axis is log₂ value of

900 standard ppGpp concentration (nM), and y-axis is log₂ value of peak area of MS

901 signal.

902 (F) Measurement of ppGpp in vitro. Blank is the mixture of reaction buffer, GDP and

ATP. Standard ppGpp is a commercial sample. RelA+GDP is the addition of purified RelA to the mixture of reaction buffer, GDP and ATP. Mesh1+ppGpp is the standard ppGpp plus purified Mesh1. Mesh1E66A+ppGpp is the standard ppGpp plus the mutant protein Mesh1E66A expressed in *E. coli* and purified.

(G) Measurement of ppGpp level in vivo. (1) M1KO/+, (2) M1KO, (3) M1KOGal4/+, (4) M1KOGal4/M1KO and UAS-Mesh1/+, (5) M1KOGal4/+ and UAS-Mesh1/+, (6) M1KOGal4/M1KO and UAS-Mesh1E66A/+, (7) M1KOGal4/+ and UAS-Mesh1E66A/+.

911

Figure S2. Phenotypic Analyses of Sleep and Circadian Rhythm

(A) Sleep analysis under LD condition. Nighttime bout number and bout length (in filled boxes on the left) and daytime bout number and bout length (unfilled boxes on the right) were not significantly different between the wt and the M1KO flies.

(B) Sleep and awakening number profiles under DD condition.

(C-I) Sleep analyses under DD conditions. Sleep level at presumptive night (C), at presumptive day (D), total sleep level (E), awakening number at dusk (H), at dawn (I), sleep latency at presumptive night (F), at presumptive day (G).

(K-L) Periodic length (K) and rhythmicity strength Qp (L) of wt and M1KO flies.

(L) Circadian profiles of wt flies under the LD and DD conditions.

(M) Circadian profiles of M1KO flies under the LD and DD conditions.

923

Figure S3. A Functional Screen of CCT-Gal4 Lines

925 (A) A pilot screen of sleep latency with 102 CCT-Gal4 lines driving RelA expression.
 926 Candidates above blue line showing three standard deviations away from the mean. 6
 927 Gal lines were found to be able to increased sleep latency significantly: Capa-R,
 928 CCHa2-R, LkR, OA2, CG13229 and Trh.
 929 (B-G) mCD8-GFP expression driven by each of the CCT Gal4 lines: Capa-R (B),
 930 OA2 (C), CCHa2-R (D), CG13229 (E), LkR (F) and Trh (G). PI neurons were
 931 observed in all these lines.

932

933 **Figure S4. Expression Examined by Intersection with Mesh1**

934 (A) Diagram of M1KIflp. The CDS after the start codon of *mesh1* gene was replaced
 935 by 2A-flp-3Px3-RFP. This is similar to Mesh1Gal4 but with Flp in place of Gal4. This
 936 line was used to intersect with Gal4 lines from the CCT screen (Capa-R, CCHa2-R,
 937 LkR, OA2, CG13229 and Trh). Each was indeed expressed in Mesh1 positive PI
 938 neurons. Shown from B to D are examples.

939 (B-D) The UAS-FRT-stop-FRT-mCD8-GFP expression patterns of M1KIflp and
 940 PI-Gal4 intersection lines. Insets show higher magnification views of the PI.

941 (E) An anti-Dilp2 antibody was used in immunostaining in
 942 M1KOGal4>UAS-mCD8-GFP flies and showed Mesh1 positive neurons to be also
 943 positive for Dilp2.

944

945 **Figure S5. Sleep profiles of rescue lines in SISL**

946 (A-K) baseline sleep is shown in black, and sleep during starvation shown in blue;

black arrows indicate that night SISL is not significantly changed compared to wt or corresponding parental controls; red arrows indicate that a specific genotype phenocopied M1KO in SISL.

Figure S6. Sleep Latency in SISL

Sleep latency (A, C) at night or day (B, D). A and B are before starvation, whereas C and D are during starvation.

Supplementary Table S1. Primers for PCR, qPCR and Genotyping

Supplementary Table S2. Standard Curve Data of MS peak area to ppGpp concentration.

EXPERIMENTAL PROCEDURES

Fly Stocks and Rearing Conditions

All flies were reared on standard corn meal at 25°C and 60% humidity, under 12hr:12hr LD cycle (unless specified otherwise). Before behavioral assays, stocks were backcrossed into the background of an isogenized Canton S wt line in the lab for 7 generations (Zhou, Rao and Rao, 2008).

Lines ordered from the Bloomington Stock Center included: 47887 (R19G10-Gal4), 30848 (c767-Gal4), 51987 (Dh44-Gal4), nos-phiC31, 37516 (Dilp2-Gal4), 5137 (UAS-mCD8-GFP). SIFa-Gal4 was a gift from Dr. J. Veenstra (University of Bordeaux). P-element insertion lines were a gift from Dr. U Heberlein, and CCT-Gal4 library was a collection previously generated in our lab (Deng *et al.*, 2019). isoCS and w¹¹¹⁸ were wild-type and white-eye wild-type lines.

970

971 **Reagents and Plasmids**

972 PCR was performed with Phanta-Max Super-Fidelity DNA Polymerase (Vazyme).
 973 Genotyping PCR was performed with 2x Taq PCR StarMix with Loading Dye
 974 (GenStar). Restriction enzymes KpnI-HF, SacII, NotI-HF, XbaI, XhoI, BamHI-HF,
 975 DpnI, EcoRI-HF and XbaI were from New England BioLabs. Total RNA was
 976 extracted from flies with RNAprep pure Tissue Kit (TIANGEN). Reverse
 977 transcription for cDNA cloning was performed with PrimeScript™ II 1st Strand cDNA
 978 Synthesis kit (Takara), Gibson assembly was performed with NEBuilder HiFi DNA
 979 Assembly Master Mix. Transformation for cloning was performed with Trans-5 α
 980 (TransGen), and transformation for expression was performed with Transetta
 981 (TransGen). BL21 (TransGen) was used as the bacterial gene template. Reverse
 982 transcription for quantitative PCR (qPCR) analyses was performed with PrimeScript
 983 RT Master Mix kit (Takara). qPCR was performed with TransStart Top Green qPCR
 984 SuperMix kit (TransGen).

985 pACU2 was a gift from Drs. Lily and YN Jan. The plasmids previously used in
 986 our lab included: pBSK, pET28a+. Templates of STOP-attP-3Px3-RFP,
 987 T2A-Gal4-3Px3-RFP, and T2A-flp-3Px3-RFP were previously generated in the lab
 988 (Deng *et al.*, 2019).

989

990 **Molecular Cloning and Generation of Genetically Modified Flies**

991 Generation of all KO and KI lines was based on the CRISPR-Cas9 system with

992 homologous recombination, according to previous procedures described (Ren *et al.*,
993 2013). U6b vector was used for the transcription of sgRNA (Ren *et al.*, 2013) and
994 construction of targeting vectors were based on previous procedures in our lab (Deng
995 *et al.*, 2019).

996 To generate KO lines, a mixture of two U6b-sgRNA plasmids and one targeting
997 vector was injected into *Drosophila* embryos. To generate U6b-sgRNA, two sgRNAs
998 were selected on website (<https://www.flyrnai.org/crispr>), and designed into a pair of
999 primers (M1KOSgRNA-F and M1KOSgRNA-R) without PAM sequence. The other
1000 pair of primers (U6b-laczrv and U6b-primer1) were designed on the backbone of U6b
1001 vector, so that they could generate PCR products containing two parts of U6b (shorter
1002 fragment by M1KO sgRNA-F and U6b-laczrv, longer fragment by M1KO sgRNA-R
1003 and U6b-primer1), which shared overlapping sequences in both ends. U6b-sgRNA
1004 plasmid was built by Gibson-assembly of the PCR products. To generate the targeting
1005 vector, two fragments (2kbps) flanking the entire CDS of gene *mesh1* except start
1006 codon were cloned as 5'Arm (PCR with primers M1KO5F and M1KO5R) and 3'Arm
1007 (PCR with primers M1KO3F and M1KO3R). Vector pBSK was digested with *KpnI*
1008 and *SacII*, and the PCR products were introduced into the digested pBSK by
1009 Gibson-assembly. New restriction sites (*NotI* and *XhoI*) were introduced between the
1010 two arms for further use. To generate a targeting vector for M1KO, the fragment
1011 STOP-attP-3Px3-RFP was cloned with primers attP2M1KOF and attP2M1KOR, and
1012 inserted between *NotI* and *XhoI* by Gibson-assembly; and for M1KOGal4,
1013 T2A-Gal4-3Px3-RFP was inserted at same location. A mixture of U6b-sgRNA and

the targeting vector was injected into embryos of *nano*-Cas9 or *vasa*-Cas9 (Ren *et al.*, 2013). F1 individuals of RFP+ eyes were collected after being crossed with w¹¹¹⁸ flies.

To generate KI line M1KIflp, similar procedures were performed. For U6b-sgRNA, primers for shorter fragment were M1KISgRNA-F and U6b-laczrv, while primers for longer fragment were M1KISgRNA-R and U6b-primer1. For the targeting vector, PCR for 5'Arm was performed with M1KI5F and M1KI5R, and 3'Arm with M1KI3F and M1KI3R. The site flanked by two arms was selected near the very end of *mesh1* CDS, so that the stop codon was removed and the CDS was fused with T2A-flp-3Px3-RFP with primers flp2M1KIF and flp2M1KIR.

Generation of transgenic UAS lines was based on vector pACU2 (Han, Jan and Jan, 2011). *Drosophila* cDNA was generated by reverse transcription from total RNA. By using the cDNA as template, *mesh1* CDS was amplified with primers (M1CDSF and M1CDSR), and inserted into digested pBSK (by *EcoRI* and *KpnI*). Point mutation of *mesh1*E66A was generated on this plasmid with primers M1E66AF and M1E66AR. Both *mesh1* CDS and Mesh1E66A were cloned with primers M1ACU2F and M1ACU2R. RelA sequence was cloned from BL21 bacteria with primers RAACU2F and RAACU2R. All the above were inserted into digested pACU2 (*EcoRI* and *XbaI*) by Gibson assembly. pACU2 constructs were inserted into attP2 by nos-phiC31 during embryo injection.

All fly stocks were confirmed by PCR and qPCR, and primers were shown in Supplementary Table S1.

1036

1037 **Molecular Cloning and Inducible Expression in Bacteria**

1038 pET28a+ was used for bacterial expression of Mesh1 and RelA proteins. *mesh1* CDS
1039 and *mesh1E66A* were generated with primers M1ET28F and M1ET28R. RelA was
1040 cloned from BL21 bacteria with primers RAET28F and RAET28R. All the above
1041 were inserted into pET28a+ by Gibson assembly. Competent cells transformed with
1042 the above constructs were amplified for inducible expression. To induce expression, a
1043 colony was inoculated into 1ml kanamycin containing Luria broth (LB) media, and
1044 incubated at 37°C for 2 hr. 800μl was transferred into a flask with 800ml kanamycin+
1045 LB media and incubated for ~3hr at 37°C 220rpm, until OD₆₀₀ was 0.5 to 0.6. 800μl
1046 1M isopropyl β-D-1 thiogalactopyranoside (IPTG) was added into the flask. For RelA,
1047 the induction was at 37°C for 3hr; for Mesh1 and Mesh1E66A, it was at 16°C for 16
1048 hr. Bacteria was harvested by centrifugation followed by lysis with ultrasonication
1049 (Power: 30%, lysis 2s, wait 2s, 30 cycles) and centrifugation. Supernatants were
1050 passed through nickle columns, followed by two times of wash with 10ml binding
1051 buffer (20mM Tris-HCl pH 7.4, 0.5M NaCl, 5mM imidazole), and elution with 5ml
1052 elution buffer (20mM Tris-HCl pH7.4, 0.5M NaCl, 500mM imidazole). Each step
1053 was monitored on 10% SDS-PAGE to check protein expression. Eluted protein was
1054 enriched in Millipore Amicon Ultra-15, and re-suspended in 1ml of protein storage
1055 buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 0.3% CHAPS, 1mM DTT).

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1057 **Generation of Germ-Free Flies**

1058 Replace the sponge of bottle containing standard fly medium with autoclavable PP
1059 bag, and autoclave twice. Evaporate water of food surface in laminar hood and under
1060 UV light for 1 hour.

1061 According to previous report (Kietz, Pollari and Meinander 2018), prepare a simple
1062 device for *Drosophila* egg collection and filter for egg wash, and use the device to
1063 collect fly eggs for 5 hours, while filters were soaked in 75% EtOH overnight.

1064 Prepare 5x 50ml centrifuge tubes, filled with the following solutions: A. 3.3% Walsh
1065 hand-sanitizer; B. 75% EtOH; C. 1% active chlorine; D. 0.1% TritonX-100 + 1xPBS;
1066 E. sterilized ddH₂O.

1067 Use cell scraper to collect eggs onto filters, and wash filters from A to E, and disperse
1068 the resuspended eggs onto autoclaved fly medium.

1069 Keep rearing at clean incubator for 20 days, and amplify the generated germ-free
1070 flies.

1071 **Extraction and Measurement of ppGpp**

1072 To extract ppGpp from *Drosophila*, 24ml formic acid was added into about every
1073 2000 flies. After grinding 10,000 rpm for 15 seconds, 3ml 30% tri-chloric acetic acid
1074 was added to precipitate proteins. The above procedures were repeated to collect
1075 extracts if necessary. Supernatants were enriched with SPE column (Waters Oasis 6cc
1076 WAX), and effluent was titrated and lyophilized overnight, and powders were
1077 re-suspended with 200µl water.

1078 To measure the level of ppGpp, ZORBAX Eclipse XDB-C18 2.1x100mm
1079 column was used in UPLC-MS (Thermo ULtiMate 3000, and Thermo QE HF-X),

1080 according to a modified version of previous method (Ihara, Ohta and Masuda, 2015).
 1081 The mobile phase included: Buffer A (8mM *N,N*-dimethylhexylamine, 160μl acetic
 1082 acid, 500ml water) and Buffer B (Acetonitrile). The UPLC program was: at 0min,
 1083 A:B = 100%:0%; at 10min, A:B = 40%:60%, with linear increment; at 10.01-14min,
 1084 keep A:B = 0%:100%; at 14.01-18min, keep A:B=100%:0%. The m/z of ppGpp
 1085 should be 601.95, and fly number was used for normalization.

1086

1087 **Behavioral Assays**

1088 To analyze baseline sleep under 12hr:12hr LD cycles, approximately 48 flies of each
 1089 genotype were loaded into glass tubes for video tracing (fps=1), which was analyzed
 1090 by an in-house software as described previously (Qian *et al.*, 2017; Dai *et al.*, 2019;
 1091 Deng *et al.*, 2019). Continuous immobility of >5min was defined as a sleep bout
 1092 (Hendricks *et al.*, 2000, Shaw *et al.*, 2000). Sleep latency at dusk was defined as the
 1093 that from light-off to the point when the 1st sleep bout appeared.

1094 To analyze the circadian rhythm, activities under 12hr:12hr LD cycles were
 1095 recorded for 7 days, which was then computed by ActogramJ (Schmid *et al.*, 2011).
 1096 Period was calculated by periodogram of Chi-square, and rhythmicity was assessed
 1097 with Qp.

1098 To analyze awakening numbers, video tracing data was converted to data of
 1099 simulated cross-beam. In the simulation, the middle line for each tube was set as the
 1100 virtual beam. According to a previous study (Tabuchi *et al.*, 2018), brief awakening
 1101 was defined as 1 cross per min, and the awakening number was the sum of such

1102 events in every 30 min. Awakening number of dusk was the sum of brief awakenings
1103 at ZT0-3, and awakening number of dawn was the sum of brief awakenings at
1104 ZT9-12.

1105 To test starvation-induced sleep loss (SISL), sleep was recorded for the first 3
1106 days (3rd day defined as baseline), and flies were quickly transferred to 1% agar at the
1107 end of the light phase of 3rd day, followed by a 24 hr recording of sleep during
1108 starvation. SISL ratio was defined as (starvation sleep-baseline sleep)/ baseline sleep.

1109

1110 **Immunohistochemistry and Imaging**

1111 To prepare flies for imaging, 5 flies per genotype were dissected in
1112 phosphate-buffered saline (PBS). Dissected tissues were transferred to a tube of 400μl
1113 2% PFA, and fixed for 55 min. Tissues were washed 3 times with 400μl brain wash
1114 buffer (PBS containing 1% TritonX-100, 3% m/V NaCl), and then transferred to
1115 400μl blocking buffer (PBS containing 2% TritonX-100, 10% normal goat serum),
1116 followed by incubation at 4°C overnight. Tissues were transferred to dilution buffer
1117 (0.25% TritonX-100, 1% NGS, 1x PBS) and added with primary antibodies including,
1118 1:1000 chick anti-GFP (Abcam) and 1:40 mouse anti-nc82 (DSHB). Tissues were
1119 stained at 4°C overnight, followed by 3 washes with 400μl brain wash buffer.
1120 Samples were transferred to fresh dilution buffer containing secondary antibodies
1121 including, 1:200 Alexa Fluor goat anti-chick 488 (Invitrogen) and 1:200 Alexa Fluor
1122 goat anti-mouse 633 (Invitrogen), followed by 3 washes with 400μl brain wash buffer.

1123 To prepare for imaging, samples were placed into a drop of Focus Clear (Cell

1124 Explorer Labs, FC-101), which was restricted by a piece of paper circle on a glass
1125 slide. Samples were visualized on Zeiss LSM 710 confocal microscope.

1126

1127 **CONTACT FOR REAGENT AND RESOURCE SHARING**

1128 Further information and requests for resources and reagents should be directed to and
1129 will be fulfilled by the Lead Contact, Yi Rao (yrao@pku.edu.cn).

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