The regulation of circadian rhythm by insulin signaling in *Drosophila*

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Author Contributions

SY, JT, and KK designed the experiments. SY conducted all experiments and data analysis. SY, JT, and KK wrote the manuscript.

Competing Interests

The authors declare no conflict of interests.

Abstract

Circadian rhythm is well conserved across species and relates to numerous biological functions. Circadian misalignment impairs metabolic function. Insulin signaling is a key modulator of metabolism in the fruit fly as well as mammals and its defects cause metabolic disease. Daily diet timing affects both circadian rhythmicities of behavior and metabolism. However, the relationship between circadian clock and insulin signaling is still elusive. Here, we report that insulin signaling regulates circadian rhythm in *Drosophila melanogaster*. We found the insulin receptor substrate mutant, *chico*^{1}, showed a shorter free-running circadian period. The knockdown of *insulin receptor* (*InR*), or another signaling molecule downstream of *InR*, *dp110*, or the expression of a dominant-negative form of InR resulted in the shortening of the circadian period and diminished its amplitude. The impairment of insulin signaling both in all neurons and restricted circadian clock neurons altered circadian period length, indicating the insulin signaling plays a role in the regulation of circadian rhythm in clock cells. Among 3 insulin-like ligands expressed in the brain, *dilp5* showed the largest effect on circadian phenotype when deleted. These results suggested that insulin signaling contributes to the robustness of the circadian oscillation and coordinates metabolism and behavior.

Highlights

- Insulin receptor substrate mutant, *chico*^{1}, displayed circadian rhythm phenotype.
- Pan-neuronal inhibition of insulin receptor signaling shortened circadian cycle.
- Inhibition of insulin signaling only in clock neurons altered circadian cycle.
- Dilp5 is a major insulin receptor ligand for circadian effects.

Introduction

Intrinsic circadian rhythms exist in many species and are involved in a variety of physiological phenomena (Dunlap, 1999; Green et al., 2008; Roenneberg and Merrow, 2005). In humans, circadian misalignment has been shown to cause changes in gene expression as well as affect metabolic functions such as plasma insulin and glucose (Archer et al., 2014; Scheer et al., 2009). It has been suggested that such changes due to disruption of circadian rhythm increase the risk of obesity and diabetes (Depner et al., 2014; Noh, 2020).

The fruit fly, *Drosophila melanogaster*, has been used as a genetic model organism to reveal genes regulating circadian rhythm and sleep (Bedont et al., 2021; Dubowy and Sehgal, 2017; Toda et al., 2019; Tomita et al., 2017). We pioneered its sleep regulation (Kume et al., 2005; Tomita et al., 2015, 2011; Ueno et al., 2012; Ueno and Kume, 2014). To date, we examined the relationship between sleep and metabolism or feeding behavior and found that c-Jun N terminal kinase, Sik3, and nutrition or taste of the food function to regulate sleep (Funato et al., 2016; Hasegawa et al., 2017; Takahama et al., 2012; Yamazaki et al., 2012).

Metabolism and feeding behavior tightly relate to not only sleep but also circadian rhythm. For example, null mutation of clock genes disrupts glucose homeostasis and lipid metabolism as in mammals (Rudic et al., 2004; Seay and Thummel, 2011; Turek et al., 2005). Furthermore, feeding with a high-fat diet or high sucrose diet affected clock gene expression or daily activity profile (Lee et al., 2021; Nayak and Mishra, 2021). However, the molecular mechanisms of how metabolism affects circadian rhythm are still elusive.

Insulin signaling, a critical regulator of metabolism in mammals, is well conserved in flies (Nässel et al., 2015; Nässel and Broeck, 2016). Flies have eight mammalian insulin homologs, *drosophila insulin-like peptides* (*dilps*),

one *insulin receptor* (*InR*), and one mammalian insulin receptor substrate homolog, *chico*. These molecules have been shown to regulate not only metabolism (Rajan and Perrimon, 2012; Ugrankar et al., 2015) and development (Böhni et al., 1999; Brogiolo et al., 2001; Fernandez et al., 1995; Sano et al., 2015) but also behaviors, such as memory, feeding behavior, courtship, and sleep (Barber et al., 2021; Metaxakis et al., 2014; Naganos et al., 2016; Tanabe et al., 2017; Watanabe and Sakai, 2016). In addition to these reports, previous research has shown that *Akt* and *target of rapamycin* (*TOR*), downstream components of insulin signaling, affect circadian rhythm (Zheng and Sehgal, 2010). Although the result suggested that insulin signaling regulates circadian rhythm, it is not clear whether *InR* and *chico* in the central nervous system regulate circadian rhythm. In this study, we aimed to clarify the regulation of circadian rhythm by neuronal insulin signaling.

Material & Methods

Fly strains and culture conditions

Flies (*Drosophila melanogaster*) were reared with conventional food, which contained yeast, glucose, cornmeal, wheat germ, and agar, at 24.5˚C under a 12-h:12-h light: dark (LD) cycle as described before (Kume et al., 2005). The following stocks were obtained from the Bloomington Stock Center, Indiana University: *elav*-Gal4 (Stock number: 458), *tim*-Gal4 (7126), UAS-*InR* DN (8252), $chico^{1}$ (10738), *dilp2* KO (30881), *dilp3* KO (30882), *dilp5* KO (30884), *dilp2*, *3*, and *5* KO (30889), y^{1} v^{1}; *P{CaryP}attP2* (36303), y^{1} v^{1}; *P{CaryP}attP40* (36304), UAS-*chico*

60 RNAi (36665), UAS-*InR* RNAi (51518), and UAS-*dp110* RNAi (61182). *DvPdf*-Gal4 (Bahn et al., 2009) and *Pdf*-

61 Gal4 was provided by Dr. Taishi Yoshii. *Pdf*-GS was gifted from Dr. Takaomi Sakai. UAS-*dilp5* RNAi (VDRC

62 49520), UAS-*Dicer-2* (60008), and w^{1118} (60000) which is the genetic background of the UAS-*dilp5* RNAi line were

63 purchased from Vienna Drosophila RNAi Center (VDRC) (Dietzl et al., 2007). *elav*-Gal4, *tim*-Gal4, *dilp2* KO, *dilp3*

64 KO, *dilp5* KO, and UAS-*InR* DN were backcrossed to the control strain (w^{1118}) at least five times.

65

66 **Circadian behavioral analysis**

67 The two- to five-days-old male flies were loaded into a glass tube (length, 65 mm; inside diameter, 3 mm) with

68 sucrose food (1% agar supplemented with 5% sucrose) at one end and were entrained for at least 3 days under LD

69 cycle conditions before being transferred to constant dark (DD) conditions. In the experiment with *Pdf*-GS, the two-

70 to five-days-old male flies were loaded into a glass tube with sucrose food containing vehicle (EtOH, - RU486) or

71 100 μM RU486 (+ RU486) at one end and transferred to DD conditions. Data were collected in 1 min bin for 10 days

72 under DD conditions using the *Drosophila* Activity Monitor system (Trikinetics). The period length and amplitude

73 of circadian rhythm were calculated using locomotor activity data for 9 or 10 days by Fourier transform (FT) with a

74 custom-made R code. Flies with the maximal value of the power spectrum calculated by the FT less than 0.04 were

75 considered arrhythmic and were removed from the calculation of the mean of circadian period length. The data from

76 the experiments of the *InR* DN expression with *elav*-Gal4, *tim*-Gal4, and *DvPdf*-Gal4, or the progenies from crosses

77 between *dilps* KO and TKO were also used in the published paper (Yamaguchi et al., 2021). However, the analysis

78 methods are completely different between this study and the published paper.

79

80 **mRNA analysis with quantitative RT-PCR**

81 mRNA levels were examined by quantitative RT-PCR. The 2- to 5-days-old male flies were collected at

82 zeitgeber time 6. Fifteen to thirty heads were divided into three and used for total RNA extraction with RNAiso

83 Plus (TAKARA) according to the manufacturer's instruction. cDNA was synthesized from the total RNA using

84 ReverTra Ace qPCR Master Mix with gDNA Remover (Toyobo), then used for quantitative RT-PCR using

85 THUNDERBIRD SYBR qPCR Master Mix (Toyobo). *GAPDH2* expression levels were used as an internal control.

86 The primers were 5'-AGGAAGGAAAAGCGGAAAAG-3' and 5'-GGGGAGGATAAACGAGGTGT-3' for

87 *GAPDH2*, 5'-GCTTCGCTGCATTTCAACTT-3' and 5'-AGTGTCCCAGGCGCTTATTA-3' for *chico*, 5'-

88 GGACAAGGAGCGAATCAAAC-3' and 5'-TGCAGTTCTCCAGCTGATTG-3' for *InR*, 5'-

89 CCATCCGGTGCTTACACTTT-3' and 5'-AGATCGCTTTCCAGGTACGA-3' for *dp110*, 5'-

90 CCAGGAAGGTTGTCATCTCG-3' and 5'-GAAAGCCCGCTCGTAGATAAG-3' for *4E-BP*, 5'-

91 TGAGTATGGTGTGCGAGGAG-3' and 5'-ACAAACTGCAGGGGATTGAG-3' for *dilp2*, 5'-

92 AGAGAACTTTGGACCCCGTGAA-3' and 5'-TGAACCGAACTATCACTCAACAGTCT-3' for *dilp3*, 5'-

93 GAGGCACCTTGGGCCTATTC-3' and 5'-CATGTGGTGAGATTCGGAGCTA-3' for *dilp5*. The primer

94 sequences for *dilp3* and *dilp5* are referred from a previous report (Broughton et al., 2005). Each mRNA expression

95 level was normalized to GAPDH2, then normalized to the average of three independent control samples.

96

97 **Statistical analysis**

98 Data were analyzed as described in the figure legend using Microsoft Excel and the freely available statistical

99 software package R 3.5.3 (https://www.r-project.org/).

100

101

102 **Results**

103

104 ***chico*^{1} mutation caused a shorter circadian period.**

105 To investigate the relationship between insulin signaling and circadian rhythm, we used the genetic mutant

106 related to insulin signaling. Because homozygous *InR* null mutant is lethal, we used *chico*^{1}, *chico* mutant line which

107 has P element insertion at 80 bp downstream of translation start site and this insertion cause complete loss of chico

108 protein to examine the effect of InR signal on circadian rhythm (Böhni et al., 1999; Naganos et al., 2012). To remove

109 the genetic background effect, we outcrossed *chico*^{1} for five generations into w^{1118}, but the homozygous outcrossed

110 *chico*^{1} mutant was lethal. Therefore, we measured the activity of the heterozygous mutants of outcrossed or original

111 *chico*^{1} with w^{1118} (*chico*^{1} / + (BC5), *chico*^{1} / + (F1)) in addition to the homozygous mutants of original *chico*^{1} to

112 investigate the effect of *chico*^{1} mutation. *chico*^{1} showed a shorter circadian period length (mean ± SEM; 1395 ± 4.78)

113 than the other groups (Fig. 1A, B), although the results were not directly comparable due to the difference in genetic

114 background. Nonetheless, there was little difference in circadian period length between the *chico*^{1} / + (BC5) (1421 ±

115 4.05) and *chico*^{1} / + (F1) (1420 ± 2.01), implying the little genetic background effect on circadian period length.

116 Moreover, *chico*^{1} / + (BC5) tended to have a shorter circadian period length than w^{1118} (+, 1433 ± 4.95). These results

117 suggested that *chico* relates to the regulation of circadian rhythm and its defect leads to a shorter circadian period.

118

119 **Insulin signaling in neurons, especially in the clock neurons, regulates circadian rhythm.**

120 To determine whether the neuronal insulin signaling regulates circadian rhythm, we inhibited the insulin
121 signaling in specific regions using Gal4 / upstream activation sequence (UAS) system (Brand and Perrimon, 1993).
122 First, we used pan-neuronal Gal4 driver, *elav*-Gal4, and UAS-*chico* RNAi to obtain the flies with pan-neuronal
123 knockdown of *chico*. The results showed that the circadian period length was significantly shortened in flies with
124 pan-neuronal knockdown of *chico* (Fig. 2A, Sup. Fig. 1A). Also, we conducted the expression of a dominant-negative
125 form of *InR* (*InR* DN) and knockdown of *InR* or *dp110* (*Phosphatidylinositol 3-kinase 92E*), one of the downstream
126 of insulin signaling, to validate the effect of insulin signaling inhibition on circadian rhythm. These manipulations
127 with *elav*-Gal4 caused shorter circadian period length as well as pan-neuronal knockdown of *chico* (Fig. 2A, Sup.
128 Fig. 1B-D). In addition, pan-neuronal knockdown of *InR* caused decreased amplitude of circadian rhythm (Fig. 2B).
129 To confirm the efficiency of RNAi, we quantified the mRNA level of each gene and *4E-BP*, the expression of which
130 is negatively regulated by insulin signaling (Jünger et al., 2003; Puig et al., 2003). Although *4E-BP* mRNA level was
131 significantly increased only by the pan-neuronal knockdown of *chico*, the mRNA level of *chico* or *dp110* was
132 significantly declined in flies with pan-neuronal knockdown of *chico* or *dp110*, respectively (Sup. Fig. 2A, C).
133 Although we couldn't detect any change of *InR* mRNA level in flies with pan-neuronal knockdown of *InR*, there is a
134 significant increase of *4E-BP* mRNA level in these flies (Sup. Fig. 2B, D), suggesting the inhibition of insulin
135 signaling. Taken together, insulin signaling in the brain could regulate circadian rhythm.

136 Since inhibition of neuronal insulin signaling affected behavioral rhythm, we focused on the clock neurons,
137 which express clock genes and are essential for the generation of circadian rhythms. (Dubowy and Sehgal, 2017). To
138 manipulate the insulin signaling in the clock neurons, we employed *tim*-Gal4 which expresses GAL4 under the
139 promoter of *timeless*, one of the clock genes in *Drosophila* (Myers et al., 1995; Sehgal et al., 1995). Inhibition of
140 insulin signaling with *tim*-Gal4 shortened circadian period length (Fig. 2C, Sup. Fig. 3A-D). Moreover, the expression
141 of *InR* DN or knockdown of *InR* declined the amplitudes of the circadian rhythm (Fig. 2D). These results indicated
142 that insulin signaling in clock neurons regulates circadian rhythm.

143 Among the clock neurons, ventral and dorsal lateral neurons (LNvs and LNds) are recently demonstrated to be
144 the two key components of the clock neuron network (Delventhal et al., 2019; Schlichting et al., 2019). These studies
145 showed that the lack of a clock gene both in LNvs and LNds caused complete loss of behavioral rhythmicity and the
146 molecular clock either in LNvs or in LNds is sufficient to generate the behavioral circadian rhythm. On the other
147 hand, LNvs themselves are known to be necessary to synchronize the clock neuron network via the release of a
148 principal circadian messenger, pigment dispersing factor (PDF) (Renn et al., 1999). To validate whether insulin
149 signaling both in LNvs and LNds or only in LNvs relates to circadian rhythm regulation, inhibition of insulin
150 signaling was conducted by *DvPdf*-Gal4 and *Pdf*-Gal4. As a result, knockdown of *chico*, *InR* or *dp110* by *DvPdf*-
151 Gal4 shortened circadian period length (Fig. 3A, Sup. Fig. 4A-D). Although expression of *InR* DN with *DvPdf*-Gal4
152 did not significantly change period length, it caused arrhythmic-like behavior after four to five days of DD conditions
153 (Sup. Fig. 4B). Furthermore, all these manipulations with *DvPdf*-Gal4 decreased the amplitudes of the circadian
154 rhythm (Fig. 3B). In addition, the knockdown of *InR* or *dp110* with *Pdf*-Gal4 shortened circadian period length (Fig.

155 3C, Sup. Fig. 5A-D). The *InR* knockdown by *Pdf*-Gal4 weakened the circadian rhythm amplitude (Fig. 3D). On the

156 other hand, both the knockdown of *chico* and the expression of *InR* DN with *Pdf*-Gal4 caused a longer circadian

157 period length (Fig. 3C). These results indicated that insulin signaling in LNvs contribute to maintain the proper

158 behavioral rhythm.

159 Since insulin signaling relates to development and growth (Naganos et al., 2012; Song et al., 2003), constitutive

160 inhibition of insulin signaling could cause behavioral changes via some developmental defects. To avoid inhibition

161 of insulin signaling during development and growth stages, we used a drug-inducible gene expression system, Gene-

162 Switch (GS) system (McGuire et al., 2004). Knockdown of *chico* and expression of *InR* DN specifically at the adult

163 stage were achieved by using *Pdf*-GS. Without RU486, flies with UAS-*chico* RNAi or UAS-*InR* DN did not show

164 significant changes in circadian period length (Fig. 4A, B, Sup. Fig. 6A, B). With RU486, flies with *chico* knockdown

165 or expression of *InR* DN by *Pdf*-GS showed significantly longer circadian period length (Fig. 4A, B). Since the

166 amplitudes from flies with UAS-*chico* RNAi or UAS-*InR* DN are different from each control without RU486, there

167 might be some genetical background effects. These results indicated that insulin signaling in LNvs relates to circadian

168 rhythm regulation, at least in part, independently of its developmental function.

169

170 ***dilp5* contributes to the regulation of circadian rhythm via insulin signaling**

171 Next, we focused on *dilp2*, *dilp3*, and *dilp5*, which are expressed in the brain, among the eight *dilps* (Nässel et

172 al., 2015; Nässel and Broeck, 2016). To inhibit the compensational expression of other *dilps* in a knockout mutant of

173 one *dilp* (Grönke et al., 2010), we crossed every single knockout mutant of *dilp2* (*dilp2* KO), *dilp3* (*dilp3* KO), or

174 *dilp5* (dilp5 KO) with a triple knockout mutant of *dilp2*, *dilp3*, and *dilp5* (TKO) and analyzed the flies of one *dilp*

175 homozygous KO with two heterozygous KO. Among these mutants, only *dilp5* KO (TKO x *dilp5* KO) showed a

176 significantly shorter circadian period length than control (TKO crossed with w^{1118}, TKO x w^{1118}) (Fig. 5A, Sup. Fig.

177 7). As we previously reported, each heterozygous mutant expressed an excess amount of *dilp* other than

178 systematically knockout one (Yamaguchi et al., 2021). To elucidate the effect of systematically *dilps* knockout on

179 insulin signaling, the mRNA level of *4E-BP* was quantified. *4E-BP* expression was significantly increased only in

180 TKO x *dilp5* KO, suggesting that insulin signaling is inhibited in TKO x *dilp5* KO (Fig. 4B). On the other hand, *4E-*

181 *BP* mRNA level did not change in the heterozygous mutant of TKO and *dilp2* KO (TKO x *dilp2* KO) or *dilp3* KO

182 (TKO x *dilp3* KO), despite their mutation in *dilps*. To confirm the function of *dilp5* in the regulation of circadian

183 rhythm, we knocked down *dilp5* with *elav*-Gal4. Accordingly, flies with pan-neuronal knockdown of *dilp5* showed

184 a significant reduction of circadian period length without any changes of mRNA level of *dilp2* or *dilp3* (Fig. 5C, Sup.

185 Fig. 8). These results indicated the regulation of circadian rhythm by *dilp5*.

186

187

188 **Discussion**

189 In this study, we investigated the relationship between circadian rhythm and insulin signaling. To clarify the

190 relationship, we conducted inhibition of insulin signaling in the targeted region. We also analyzed the activity of the

191 *dilps* mutants to specify the ligand involved in the regulation of circadian rhythm. Through these experiments, we

192 showed that *InR* and its downstream molecules in the clock neurons regulate circadian rhythm. In addition, we

193 discovered that *dilp5* contributes to the regulation of circadian rhythm. These findings support the relationship

194 between circadian rhythm and metabolism in *Drosophila melanogaster*.

195 Specifically, we examined the effect of inhibition of insulin signaling on circadian rhythm by the expression of

196 a dominant-negative form of *InR*, *InR* DN, and by the knockdown of *chico*, *InR*, and *dp110*. All of these manipulations

197 with *elav*-Gal4 and *tim*-Gal4 drivers shortened circadian period length (Fig. 2A, Fig. 2C), although we couldn't detect

198 a significant change of *InR* expression in flies with pan-neuronal knockdown of *InR*. It may be because *InR* is

199 expressed in various tissues, including the neurons, glia, and fat body. Knockdown of the three genes with *DvPdf*-

200 Gal4 also caused the same phenotype in period length (Fig. 3A). Also, knockdown of *InR* or *dp110* by *Pdf*-Gal4

201 shortened circadian period length. On the other hand, knockdown of *chico* or expression of *InR* DN by *Pdf*-Gal4

202 lengthened period length (Fig. 3C). In previous research, flies with silencing of *TOR* in the cells expressing *period*,

203 one of the core clock genes, showed the altered circadian period length (Kijak and Pyza, 2017). Other paper reported

204 that activation of *Akt* and *TOR* signaling in LNvs lengthened circadian period length(Zheng and Sehgal, 2010).

205 Although the detailed distribution of *InR* is poorly understood to date, cell-specific RNA sequencing of each clock

206 cell detected mRNA expression of the insulin signaling molecules in the clock neurons (Abruzzi et al., 2017; Ma et

207 al., 2021). These results support the idea that neuronal insulin signaling, especially in the clock neurons, regulates

208 circadian rhythm.

209 In our experiment, most of the neuronal insulin signaling inhibition altered circadian period length. Although

210 only the expression of *InR* DN with *DvPdf*-Gal4 did not cause significant changes in the circadian period length (Fig.

211 3A), these flies seem to have a shorter circadian period length than control according to the activity in the first 4-

212 days of DD condition (Sup. Fig. 4B). Also, these flies showed arrhythmic-like behavior after the 4-5 days of DD
213 condition. Such phenotype might cause a loss of accuracy for quantification of circadian period length. Contrary to
214 the shortened circadian period length in the knockdown of *InR* or *dp110* by *Pdf*-Gal4, the knockdown of *chico* and
215 the expression of *InR* DN with *Pdf*-Gal4 lengthened circadian period length (Fig. 3C). There are some possible causes
216 of these contradictions, such as the genetic background, the strength of insulin signaling inhibition, or the
217 developmental defects. Although there were some contradictions, we speculated that the insulin signaling in the clock
218 neurons contribute to maintain the proper behavioral rhythm.

219 Recently, Schlichting et al. reported that knockout of *period* in all or subset of lateral neurons differently affected
220 circadian period length. In addition, Schlichting et al. and Delventhal et al. demonstrated that the clock neurons act
221 not in a hierarchy but as a distributed network to regulate circadian activity (Delventhal et al., 2019; Schlichting et
222 al., 2019). In these papers, cell-specific knockout of clock genes revealed that the clock in each clock neuron,
223 especially in LNvs and LNds, collaborate to achieve the close to 24-hr period length. Inhibition of insulin signaling
224 in the lateral neurons might diminish such communication between the lateral neurons. Although inhibition of insulin
225 signaling by *elav*-Gal4 and *tim*-Gal4 did not cause severe defects in circadian behavior as much as *DvPdf*-Gal4 did,
226 it might be due to the difference of Gal4 expression levels in LNvs and LNds. Furthermore, *shaggy* (*sgg*, homolog
227 of *GSK3*), which is negatively regulated by insulin signaling (Papadopoulou et al., 2004), is known to phosphorylate
228 clock protein and manipulate the intrinsic speed of the molecular clock (Martinek et al., 2001). Additionally, Zhang
229 and Sehgal reported that *Akt* and *TOR* modulate the timing of nuclear expression of TIM in the clock neurons (Zheng
230 and Sehgal, 2010). Although we did not assess whether our manipulation of insulin signaling changes the core clock

231 gene behavior or neural communication, it is possible that insulin signaling modulated the molecular clock via the
232 regulation of *sgg* activity as previous research reported. Further experiments are needed to clarify the effect of insulin
233 signaling on the clock gene expression rhythm or the clock neuron activity.

234 Since insulin signaling is important for development, the inhibition of insulin signaling with constitutive Gal4
235 might induce some developmental defects (Böhni et al., 1999; Naganos et al., 2012; Song et al., 2003). However, we
236 demonstrated that the adult stage-specific insulin signaling inhibition in LNvs by *Pdf*-GS altered behavioral rhythm
237 (Fig. 4A, B). These results suggested that insulin signaling, at least in LNvs, regulates circadian rhythm in a
238 development-independent manner.

239 We demonstrated that *dilp5* alone among three insulin ligands tested showed a significant effect on the circadian
240 period length when only one of the *dilps* was systemically knocked out or knocked down in neurons. An earlier study
241 reported that *dilp* single mutation caused compensational expression of other *dilps* and little change of *4E-BP*
242 expression (Grönke et al., 2010). We previously reported the similar compensational effects in the progenies from
243 crosses between TKO and single KO mutants (Yamaguchi et al., 2021). Especially, *dilp2* mRNA level in the mutants
244 of *dilp5* was significantly increased. *dilp2* mRNA increase was also observed in the mutants of *dilp3* which did not
245 show any changes in circadian period length. In addition, pan-neuronal knockdown of *dilp5* shortened circadian
246 period length without an increase of other *dilps* mRNA (Fig. 5F, Sup. Fig. 8B). According to these results, we
247 conjectured that increase of *dilp2* mRNA did not cause the changes of circadian period length. However, it is still
248 possible that *dilp2* or *dilp3* plays a role in the regulation of circadian rhythm. It should also be noted that *dilps*,
249 especially *dilp2*, *dilp3*, and *dilp5*, are thought to have a functional redundancy (Nässel et al., 2015). Nevertheless, we

250 demonstrated that *dilp5* has a significant effect on circadian rhythm.

251 In conclusion, our results suggest that neuronal insulin signaling contributes to the regulation of robust circadian
252 rhythm in flies. In mammals, previous studies showed the relationship between insulin signaling and circadian rhythm
253 (Boden et al., 1996; Marcheva et al., 2010; Sato et al., 2014). Recently, Crosby et al. demonstrated that insulin and
254 insulin-like growth factor 1 are important for the entrainment of behavioral rhythms to feeding time and have some
255 activity in the suprachiasmatic nucleus, the master clock of mammals (Crosby et al., 2019). Taken together, our results
256 shed light on the conserved role of insulin and insulin signaling in the regulation of circadian rhythm from flies to
257 mammals.

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260 **References**

261 Abruzzi, K.C., Zadina, A., Luo, W., Wiyanto, E., Rahman, R., Guo, F., Shafer, O., Rosbash, M., 2017. RNA-seq
262 analysis of Drosophila clock and non-clock neurons reveals neuron-specific cycling and novel candidate
263 neuropeptides. PLoS Genetics 13, e1006613. https://doi.org/10.1371/journal.pgen.1006613

264 Archer, S.N., Laing, E.E., Möller-Levet, C.S., van der Veen, D.R., Bucca, G., Lazar, A.S., Santhi, N., Slak, A., Kabiljo,
265 R., von Schantz, M., Smith, C.P., Dijk, D.J., 2014. Mistimed sleep disrupts circadian regulation of the human
266 transcriptome. Proc Natl Acad Sci U S A 111. https://doi.org/10.1073/pnas.1316335111

267 Bahn, J.H., Lee, G., Park, J.H., 2009. Comparative analysis of Pdf-mediated circadian behaviors between Drosophila
268 melanogaster and D. virilis. Genetics 181, 965–975. https://doi.org/10.1534/genetics.108.099069

269 Barber, A.F., Fong, S.Y., Kolesnik, A., Fetchko, M., Sehgal, A., 2021. Drosophila clock cells use multiple
270 mechanisms to transmit time-of-day signals in the brain. Proc Natl Acad Sci U S A 118, 1–8.
271 https://doi.org/10.1073/pnas.2019826118
272 Bedont, J.L., Toda, H., Shi, M., Park, C.H., Quake, C., Stein, C., Kolesnik, A., Sehgal, A., 2021. Short and long
273 sleeping mutants reveal links between sleep and macroautophagy. Elife 10, 1–27.
274 https://doi.org/10.7554/eLife.64140
275 Boden, G., Ruiz, J., Urbain, J.L., Chen, X., 1996. Evidence for a circadian rhythm of insulin secretion. American
276 Journal of Physiology-Endocrinology and Metabolism 271, E246–E252.
277 https://doi.org/10.1152/ajpendo.1996.271.2.E246
278 Böhni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., Hafen, E.,
279 1999. Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell
280 97, 865–875. https://doi.org/10.1016/S0092-8674(00)80799-0
281 Brand, a H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant
282 phenotypes. Development 118, 401–15.
283 Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., Hafen, E., 2001. An evolutionarily conserved
284 function of the drosophila insulin receptor and insulin-like peptides in growth control. Current Biology 11, 213–
285 221. https://doi.org/10.1016/S0960-9822(01)00068-9
286 Broughton, S.J., Piper, M.D.W., Ikeya, T., Bass, T.M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D.J.,
287 Leevers, S.J., Partridge, L., 2005. Longer lifespan, altered metabolism, and stress resistance in Drosophila from

288 ablation of cells making insulin-like ligands. Proc Natl Acad Sci U S A 102, 3105–3110.
289 https://doi.org/10.1073/pnas.0405775102
290 Crosby, P., Hamnett, R., Putker, M., Hoyle, N.P., Reed, M., Karam, C.J., Maywood, E.S., Stangherlin, A., Chesham,
291 J.E., Hayter, E.A., Rosenbrier-Ribeiro, L., Newham, P., Clevers, H., Bechtold, D.A., O'Neill, J.S., 2019.
292 Insulin/IGF-1 Drives PERIOD Synthesis to Entrain Circadian Rhythms with Feeding Time. Cell 177, 896-
293 909.e20. https://doi.org/10.1016/j.cell.2019.02.017
294 Delventhal, R., O'Connor, R.M., Pantalia, M.M., Ulgherait, M., Kim, H.X., Basturk, M.K., Canman, J.C., Shirasu-
295 Hiza, M., 2019. Dissection of central clock function in Drosophila through cell-specific CRISPR-mediated
296 clock gene disruption. Elife 8, 1–14. https://doi.org/10.7554/eLife.48308
297 Depner, C.M., Stothard, E.R., Wright, K.P., 2014. Metabolic consequences of sleep and circadian disorders. Current
298 Diabetes Reports 14, 1–15. https://doi.org/10.1007/s11892-014-0507-z
299 Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer,
300 S., Couto, A., Marra, V., Keleman, K., Dickson, B.J., 2007. A genome-wide transgenic RNAi library for
301 conditional gene inactivation in Drosophila. Nature 448, 151–156. https://doi.org/10.1038/nature05954
302 Dubowy, C., Sehgal, A., 2017. Circadian rhythms and sleep in Drosophila melanogaster. Genetics 205, 1373–1397.
303 https://doi.org/10.1534/genetics.115.185157
304 Dunlap, J.C., 1999. Molecular bases for circadian clocks. Cell 96, 271–90. https://doi.org/10.1016/s0092-
305 8674(00)80566-8
306 Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., Schlessinger, J., 1995. The Drosophila insulin receptor

307 homolog: A gene essential for embryonic development encodes two receptor isoforms with different signaling
308 potential. EMBO Journal 14, 3373–3384. https://doi.org/10.1002/j.1460-2075.1995.tb07343.x
309 Funato, H., Miyoshi, C., Fujiyama, T., Kanda, T., Sato, M., Wang, Z., Ma, J., Nakane, S., Tomita, J., Ikkyu, A.,
310 Kakizaki, M., Hotta-Hirashima, N., Kanno, S., Komiya, H., Asano, F., Honda, T., Kim, S.J., Harano, K.,
311 Muramoto, H., Yonezawa, T., Mizuno, S., Miyazaki, S., Connor, L., Kumar, V., Miura, I., Suzuki, T., Watanabe,
312 A., Abe, M., Sugiyama, F., Takahashi, S., Sakimura, K., Hayashi, Y., Liu, Q., Kume, K., Wakana, S., Takahashi,
313 J.S., Yanagisawa, M., 2016. Forward-genetics analysis of sleep in randomly mutagenized mice. Nature 539,
314 378–383. https://doi.org/10.1038/nature20142
315 Green, C.B., Takahashi, J.S., Bass, J., 2008. The Meter of Metabolism. Cell 134, 728–742.
316 https://doi.org/10.1016/j.cell.2008.08.022
317 Grönke, S., Clarke, D.-F., Broughton, S., Andrews, T.D., Partridge, L., 2010. Molecular evolution and functional
318 characterization of Drosophila insulin-like peptides. PLoS Genetics 6, e1000857.
319 https://doi.org/10.1371/journal.pgen.1000857
320 Hasegawa, T., Tomita, J., Hashimoto, R., Ueno, T., Kume, S., Kume, K., 2017. Sweetness induces sleep through
321 gustatory signalling independent of nutritional value in a starved fruit fly. Scientific Reports 7, 14355.
322 https://doi.org/10.1038/s41598-017-14608-1
323 Jünger, M.A., Rintelen, F., Stocker, H., Wasserman, J.D., Végh, M., Radimerski, T., Greenberg, M.E., Hafen, E.,
324 2003. The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated
325 with reduced insulin signaling. Journal of Biology 2, 20. https://doi.org/10.1186/1475-4924-2-20

326 Kijak, E., Pyza, E., 2017. TOR signaling pathway and autophagy are involved in the regulation of circadian rhythms
327 in behavior and plasticity of L2 interneurons in the brain of Drosophila melanogaster. PLoS ONE 12, e0171848.
328 https://doi.org/10.1371/journal.pone.0171848
329 Kume, K., Kume, S., Park, S.K., Hirsh, J., Jackson, F.R., 2005. Dopamine is a regulator of arousal in the fruit fly.
330 Journal of Neuroscience 25, 7377–7384. https://doi.org/10.1523/JNEUROSCI.2048-05.2005
331 Lee, S.H., Cho, E., Yoon, S.E., Kim, Y., Kim, E.Y., 2021. Metabolic control of daily locomotor activity mediated by
332 tachykinin in Drosophila. Communications Biology 4. https://doi.org/10.1038/s42003-021-02219-6
333 Ma, D., Przybylski, D., Abruzzi, K.C., Schlichting, M., Li, Q., Long, X., Rosbash, M., 2021. A transcriptomic
334 taxonomy of Drosophila circadian neurons around the clock. Elife 10, e63056.
335 https://doi.org/10.7554/eLife.63056
336 Marcheva, B., Ramsey, K.M., Buhr, E.D., Kobayashi, Y., Su, H., Ko, C.H., Ivanova, G., Omura, C., Mo, S., Vitaterna,
337 M.H., Lopez, J.P., Philipson, L.H., Bradfield, C.A., Crosby, S.D., Jebailey, L., Wang, X., Takahashi, J.S., Bass,
338 J., 2010. Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes.
339 Nature 466, 627–631. https://doi.org/10.1038/nature09253
340 Martinek, S., Inonog, S., Manoukian, A.S., Young, M.W., 2001. A Role for the Segment Polarity Gene shaggy/GSK-
341 3 in the Drosophila Circadian Clock. Cell 105, 769–779. https://doi.org/10.1016/S0092-8674(01)00383-X
342 McGuire, S.E., Mao, Z., Davis, R.L., 2004. Spatiotemporal Gene Expression Targeting with the TARGET and Gene-
343 Switch Systems in Drosophila. Science's STKE 2004, pl6–pl6. https://doi.org/10.1126/stke.2202004pl6
344 Metaxakis, A., Tain, L.S., Grönke, S., Hendrich, O., Hinze, Y., Birras, U., Partridge, L., 2014. Lowered Insulin

Signalling Ameliorates Age-Related Sleep Fragmentation in Drosophila. PLoS Biology 12, e1001824. https://doi.org/10.1371/journal.pbio.1001824

Myers, M.P., Wager-Smith, K., Wesley, C.S., Young, M.W., Sehgal, A., 1995. Positional Cloning and Sequence Analysis of the Drosophila Clock Gene, timeless. Science (1979) 270, 805–808. https://doi.org/10.1126/science.270.5237.805

Naganos, S., Horiuchi, J., Saitoe, M., 2012. Mutations in the Drosophila insulin receptor substrate, CHICO, impair olfactory associative learning. Neuroscience Research 73, 49–55. https://doi.org/10.1016/j.neures.2012.02.001

Naganos, S., Ueno, K., Horiuchi, J., Saitoe, M., 2016. Learning defects in Drosophila growth restricted chico mutants are caused by attenuated adenylyl cyclase activity. Molecular Brain 9, 37. https://doi.org/10.1186/s13041-016-0217-3

Nässel, D.R., Broeck, J. vanden, 2016. Insulin/IGF signaling in Drosophila and other insects: Factors that regulate production, release and post-release action of the insulin-like peptides. Cellular and Molecular Life Sciences 73, 271–290. https://doi.org/10.1007/s00018-015-2063-3

Nässel, D.R., Liu, Y., Luo, J., 2015. Insulin/IGF signaling and its regulation in Drosophila. General and Comparative Endocrinology 221, 255–266. https://doi.org/10.1016/j.ygcen.2014.11.021

Nayak, N., Mishra, M., 2021. High fat diet induced abnormalities in metabolism, growth, behavior, and circadian clock in Drosophila melanogaster. Life Sciences 281, 119758. https://doi.org/10.1016/j.lfs.2021.119758

Noh, J., 2020. The effect of circadian and sleep disruptions on obesity risk. Journal of Obesity and Metabolic Syndrome 27, 78–83. https://doi.org/10.7570/JOMES.2018.27.2.78

364 Papadopoulou, D., Bianchi, M.W., Bourouis, M., 2004. Functional Studies of Shaggy/Glycogen Synthase Kinase 3
365 Phosphorylation Sites in Drosophila melanogaster . Molecular and Cellular Biology 24, 4909–4919.
366 https://doi.org/10.1128/mcb.24.11.4909-4919.2004
367 Puig, O., Marr, M.T., Ruhf, M.L., Tjian, R., 2003. Control of cell number by Drosophila FOXO: Downstream and
368 feedback regulation of the insulin receptor pathway. Genes and Development 17, 2006–2020.
369 https://doi.org/10.1101/gad.1098703
370 Rajan, A., Perrimon, N., 2012. Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely
371 controlling insulin secretion. Cell 151, 123–137. https://doi.org/10.1016/j.cell.2012.08.019
372 Renn, S.C.P., Park, J.H., Rosbash, M., Hall, J.C., Taghert, P.H., 1999. A pdf Neuropeptide Gene Mutation and
373 Ablation of PDF Neurons Each Cause Severe Abnormalities of Behavioral Circadian Rhythms in Drosophila.
374 Cell 99, 791–802. https://doi.org/10.1016/S0092-8674(00)81676-1
375 Roenneberg, T., Merrow, M., 2005. Circadian clocks — the fall and rise of physiology. Nature Reviews Molecular
376 Cell Biology 6, 965–971. https://doi.org/10.1038/nrm1766
377 Rudic, R.D., McNamara, P., Curtis, A.M., Boston, R.C., Panda, S., Hogenesch, J.B., FitzGerald, G.A., 2004. BMAL1
378 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS
379 Biology 2. https://doi.org/10.1371/journal.pbio.0020377
380 Sano, H., Nakamura, A., Texada, M.J., Truman, J.W., Ishimoto, H., Kamikouchi, A., Nibu, Y., Kume, K., Ida, T.,
381 Kojima, M., 2015. The Nutrient-Responsive Hormone CCHamide-2 Controls Growth by Regulating Insulin-
382 like Peptides in the Brain of Drosophila melanogaster. PLoS Genetics 11, e1005209.

https://doi.org/10.1371/journal.pgen.1005209

Sato, M., Murakami, M., Node, K., Matsumura, R., Akashi, M., 2014. The Role of the Endocrine System in Feeding-Induced Tissue-Specific Circadian Entrainment. Cell Reports 8, 393–401. https://doi.org/10.1016/j.celrep.2014.06.015

Scheer, F.A.J.L., Hilton, M.F., Mantzoros, C.S., Shea, S.A., 2009. Adverse metabolic and cardiovascular consequences of circadian misalignment. Proceedings of the National Academy of Sciences 106, 4453–4458. https://doi.org/10.1073/pnas.0808180106

Schlichting, M., Díaz, M.M., Xin, J., Rosbash, M., 2019. Neuron-specific knockouts indicate the importance of network communication to drosophila rhythmicity. Elife 8, e48301. https://doi.org/10.7554/eLife.48301

Seay, D.J., Thummel, C.S., 2011. The Circadian Clock, Light, and Cryptochrome Regulate Feeding and Metabolism in Drosophila. Journal of Biological Rhythms 26, 497–506. https://doi.org/10.1177/0748730411420080

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P., Young, M.W., 1995. Rhythmic Expression of *timeless* : A Basis for Promoting Circadian Cycles in *period* Gene Autoregulation. Science (1979) 270, 808–810. https://doi.org/10.1126/science.270.5237.808

Song, J., Wu, L., Chen, Z., Kohanski, R.A., Pick, L., 2003. Axons guided by insulin receptor in Drosophila visual system. Science 300, 502–5. https://doi.org/10.1126/science.1081203

Takahama, K., Tomita, J., Ueno, T., Yamazaki, M., Kume, S., Kume, K., 2012. Pan-neuronal knockdown of the c-Jun N-terminal Kinase (JNK) results in a reduction in sleep and longevity in Drosophila. Biochemical and Biophysical Research Communications 417, 807–811. https://doi.org/10.1016/j.bbrc.2011.12.040

402 Tanabe, K., Itoh, M., Tonoki, A., 2017. Age-Related Changes in Insulin-like Signaling Lead to Intermediate-Term
403 Memory Impairment in Drosophila. Cell Reports 18, 1598–1605. https://doi.org/10.1016/j.celrep.2017.01.053
404 Toda, H., Williams, J.A., Gulledge, M., Sehgal, A., 2019. A sleep-inducing gene, nemuri , links sleep and immune
405 function in Drosophila. Science (1979) 363, 509–515. https://doi.org/10.1126/science.aat1650
406 Tomita, J., Ban, G., Kume, K., 2017. Genes and neural circuits for sleep of the fruit fly. Neuroscience Research 118,
407 82–91. https://doi.org/10.1016/j.neures.2017.04.010
408 Tomita, J., Mitsuyoshi, M., Ueno, T., Aso, Y., Tanimoto, H., Nakai, Y., Aigaki, T., Kume, S., Kume, K., 2011. Pan-
409 neuronal knockdown of calcineurin reduces sleep in the fruit fly, Drosophila melanogaster. J Neurosci 31,
410 13137–13146. https://doi.org/10.1523/JNEUROSCI.5860-10.2011
411 Tomita, J., Ueno, T., Mitsuyoshi, M., Kume, S., Kume, K., 2015. The NMDA Receptor Promotes Sleep in the Fruit
412 Fly, Drosophila melanogaster. PLoS ONE 10, e0128101. https://doi.org/10.1371/journal.pone.0128101
413 Turek, F.W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A.,
414 Jensen, D.R., Eckel, R.H., Takahashi, J.S., Bass, J., 2005. Obesity and metabolic syndrome in circadian Clock
415 mutant nice. Science (1979) 308, 1043–1045. https://doi.org/10.1126/science.1108750
416 Ueno, T., Kume, K., 2014. Functional characterization of dopamine transporter in vivo using Drosophila
417 melanogaster behavioral assays. Frontiers in Behavioral Neuroscience 8, 1–11.
418 https://doi.org/10.3389/fnbeh.2014.00303
419 Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., Kume, K., 2012. Identification of a dopamine pathway
420 that regulates sleep and arousal in Drosophila. Nature Neuroscience 15, 1516–1523.

https://doi.org/10.1038/nn.3238

Ugrankar, R., Berglund, E., Akdemir, F., Tran, C., Kim, M.S., Noh, J., Schneider, R., Ebert, B., Graff, J.M., 2015. Drosophila glucome screening identifies Ck1alpha as a regulator of mammalian glucose metabolism. Nature Communications 6, 7102. https://doi.org/10.1038/ncomms8102

Watanabe, K., Sakai, T., 2016. Knockout mutations of insulin-like peptide genes enhance sexual receptivity in Drosophila virgin females. Genes and Genetic Systems 90, 237–241. https://doi.org/10.1266/ggs.15-00025

Yamaguchi, S.T., Tomita, J., Kume, K., 2021. Insulin signaling in clock neurons regulates sleep in Drosophila. Biochemical and Biophysical Research Communications 591, 44–49. https://doi.org/10.1016/j.bbrc.2021.12.100

Yamazaki, M., Tomita, J., Takahama, K., Ueno, T., Mitsuyoshi, M., Sakamoto, E., Kume, S., Kume, K., 2012. High calorie diet augments age-associats sleep impairment in Drosophila. Biochemical and Biophysical Research Communications 417, 812–816. https://doi.org/10.1016/j.bbrc.2011.12.041

Zheng, X., Sehgal, A., 2010. AKT and TOR Signaling Set the Pace of the Circadian Pacemaker. Current Biology 20, 1203–1208. https://doi.org/10.1016/j.cub.2010.05.027

437 **Figure Legends**

438

439 **Figure 1. *chico*^{1} mutation caused a shorter circadian period length.**

440 (A) Average activity from wild type (+, n = 14), backcrossed heterozygous (*chico*^{1} / + (BC5), n = 15), original

441 heterozygous (*chico*^{1} / + (F1), n = 31), and original homozygous (*chico*^{1}, n = 30) *chico* mutant flies. Each fly activity

442 was recorded for 10 days in constant dark (DD) conditions. Activity counts over 7 days in DD condition are shown.

443 Subjective day and night are depicted by the gray and black bars, respectively. (B) Circadian period lengths for +,

444 *chico*^{1} / + (BC5), *chico*^{1} / + (F1), and *chico*^{1} flies. Circadian period lengths were calculated using locomotor activity

445 data for 10 days by FT. (C) Amplitudes of circadian rhythm for +, *chico*^{1} / + (BC5), *chico*^{1} / + (F1), and *chico*^{1}.

446 Amplitudes were calculated using locomotor activity data for 10 days by FT. *** $p < 0.001$; *t*-test.

447

448 **Figure 2. Inhibition of insulin signaling in clock neurons resulted in shorter circadian period length.**

449 (A, B) Circadian period lengths (A) and amplitudes of circadian rhythm (B), which were calculated using locomotor

450 activity data for 10 days by FT, for each genotype denoted at bottom of the panels. w^{1118}, the genetical background

451 of UAS-*InR* DN, was used as a control for UAS-*InR* DN. y^{1} v^{1}; *P{CaryP}attP2* was used as ctrl for UAS-*chico* RNAi.

452 y^{1} v^{1}; *P{CaryP}attP40* was used as ctrl for UAS-*InR* RNAi and UAS-*dp110* RNAi. Since each experiment was

453 conducted independently, the control group for UAS-*InR* RNAi and UAS-*dp110* RNAi showed different circadian

454 period lengths and amplitudes, although they are the same genotype. The numbers of flies of each bar were 31, 31,

455 29, 32, 16, 16, 16, and 11 from the left. (C, D) Circadian period lengths (C) and amplitudes of circadian rhythm (D),

456 which were calculated using locomotor activity data for 10 days by FT, for each genotype denoted at bottom of the

457 panels. The numbers of flies of each bar were 32, 27, 32, 32, 32, 32, 32, and 32 from the left. * $p < 0.05$, *** $p <$

458 0.001; *t*-test.

459

460 **Figure 3. Insulin signaling in the lateral neurons regulates circadian rhythm.**

461 (A, B) Circadian period lengths (A) and amplitudes of circadian rhythm (B), which were calculated using locomotor

462 activity data for 10 days by FT, for each genotype denoted at bottom of the panels. w^{1118}, the genetical background

463 of UAS-*InR* DN, was used as a control for UAS-*InR* DN. y^1 v^1; *P{CaryP}attP2* was used as ctrl for UAS-*chico* RNAi.

464 y^1 v^1; *P{CaryP}attP40* was used as ctrl for UAS-*InR* RNAi and UAS-*dp110* RNAi. Since each experiment was

465 conducted independently, the control group for UAS-*InR* RNAi and UAS-*dp110* RNAi showed different circadian

466 period lengths and amplitudes, although they are the same genotype. The numbers of flies of each bar were 32, 31,

467 31, 30, 32, 32, 32, and 32 from the left. (C, D) Circadian period lengths (C) and amplitudes of circadian rhythm (D),

468 which were calculated using locomotor activity data for 10 days by FT, for each genotype denoted at bottom of the

469 panels. The numbers of flies of each bar were 32, 32, 32, 31, 32, 32, 29, and 28 from the left. Error bars indicate

470 SEM. *** $p < 0.001$; *t*-test.

471

472 **Figure 4. Insulin signaling in LNvs regulates circadian rhythm in a development-independent manner.**

473 (A) Circadian period lengths and amplitudes of circadian rhythm, which were calculated using locomotor activity

474 data for 10 days by FT, for control (*Pdf*-GS x ctrl, white bar) and flies with *chico* knockdown by *Pdf*-GS (*Pdf*-GS x

475 UAS-*chico* RNAi, black bar) without RU486 (- RU486) or with RU486 (+ RU486). y^1 v^1; *P{CaryP}attP2* was used

476 as ctrl for UAS-*chico* RNAi. The numbers of flies of each bar were 24, 28, 18, and 20 from the left. Error bars indica

477 te SEM. (B) Circadian period lengths and amplitudes of circadian rhythm, which were calculated using locomotor

478 activity data for 10 days by FT, for control (*Pdf*-GS x w^{1118}, white bar) and flies with *InR* DN expression by *Pdf*-GS

479 (*Pdf*-GS x UAS-*InR* DN, black bar) without RU486 (- RU486) or with RU486 (+ RU486). The numbers of flies of

480 each bar were 26, 25, 27, and 26 from the left. Error bars indicate SEM. w^{1118}, the genetical background of UAS-*InR*

481 DN, was used as a control for UAS-*InR* DN. n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Tukey Kramer

482 method.

483

484 **Figure 5. *dilp5* relates to the regulation of circadian rhythm by insulin signaling.**

485 (A) Circadian period lengths and amplitudes of circadian rhythm, which were calculated using locomotor activity

486 data for 10 days by FT, for each genotype denoted at bottom of the panels: TKO x w^{1118} (n = 15), TKO x *dilp2* KO

487 (n = 16), TKO x *dilp3* KO (n = 16), TKO x *dilp5* KO (n = 13). (B) Relative expression of *4E-BP* for each genotype

488 denoted at bottom of each panel. (C) Circadian period lengths and amplitudes of circadian rhythm, which were

489 calculated using locomotor activity data for 10 days by FT, for *elav*-Gal4; UAS-*Dicer-2* x ctrl (n = 14) and *elav*-

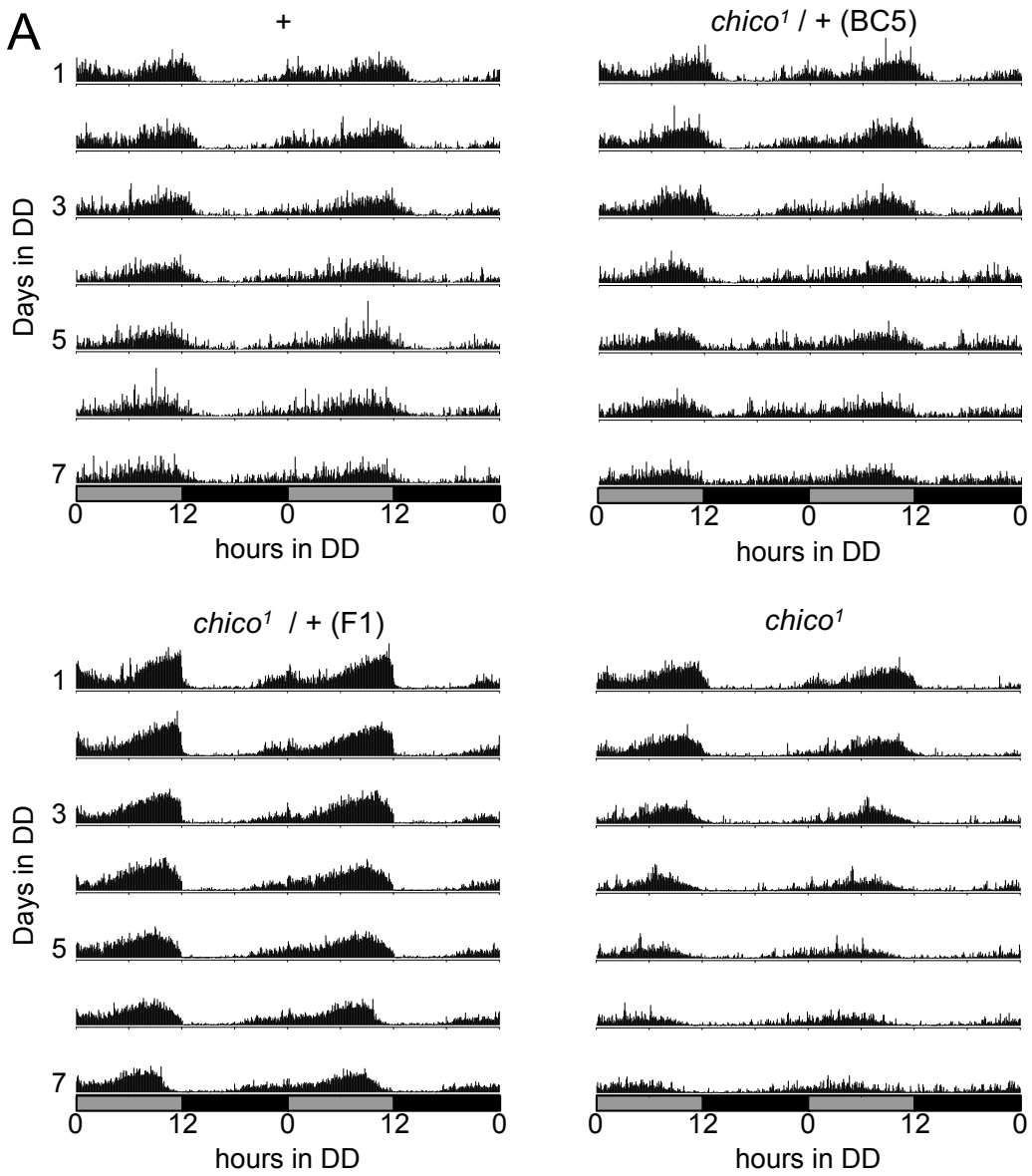
490 Gal4; UAS-*Dicer-2* x UAS-*dilp5* RNAi (n = 11). w^{1118}(VDRC stock number: 60000) was used as ctrl for UAS-*dilp5*

491 RNAi. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$; vs. TKO x w^{1118}; Tukey Kramer method (A, B). ** $p < 0.01$;

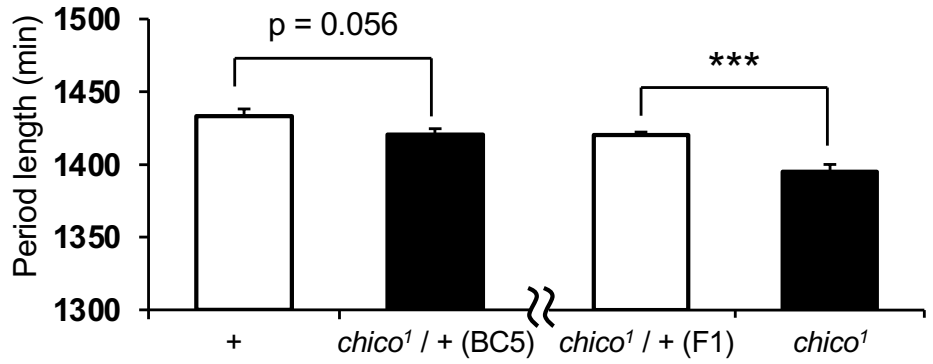
492 *t*-test (C)

Figure 1

A


+
chico¹ / + (BC5)
chico¹ / + (F1)
chico¹
Days in DD
1
3
5
7
0
12
0
12
0
hours in DD

B


Period length (min)
1500
1450
1400
1350
1300
p = 0.056
***
+
chico¹ / + (BC5)
chico¹ / + (F1)
chico¹

C

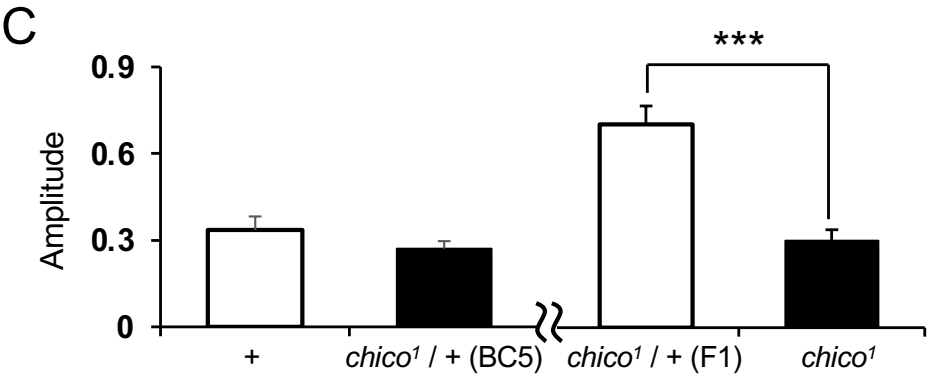

Amplitude
0.9
0.6
0.3
0
***
+
chico¹ / + (BC5)
chico¹ / + (F1)
chico¹

Figure 2

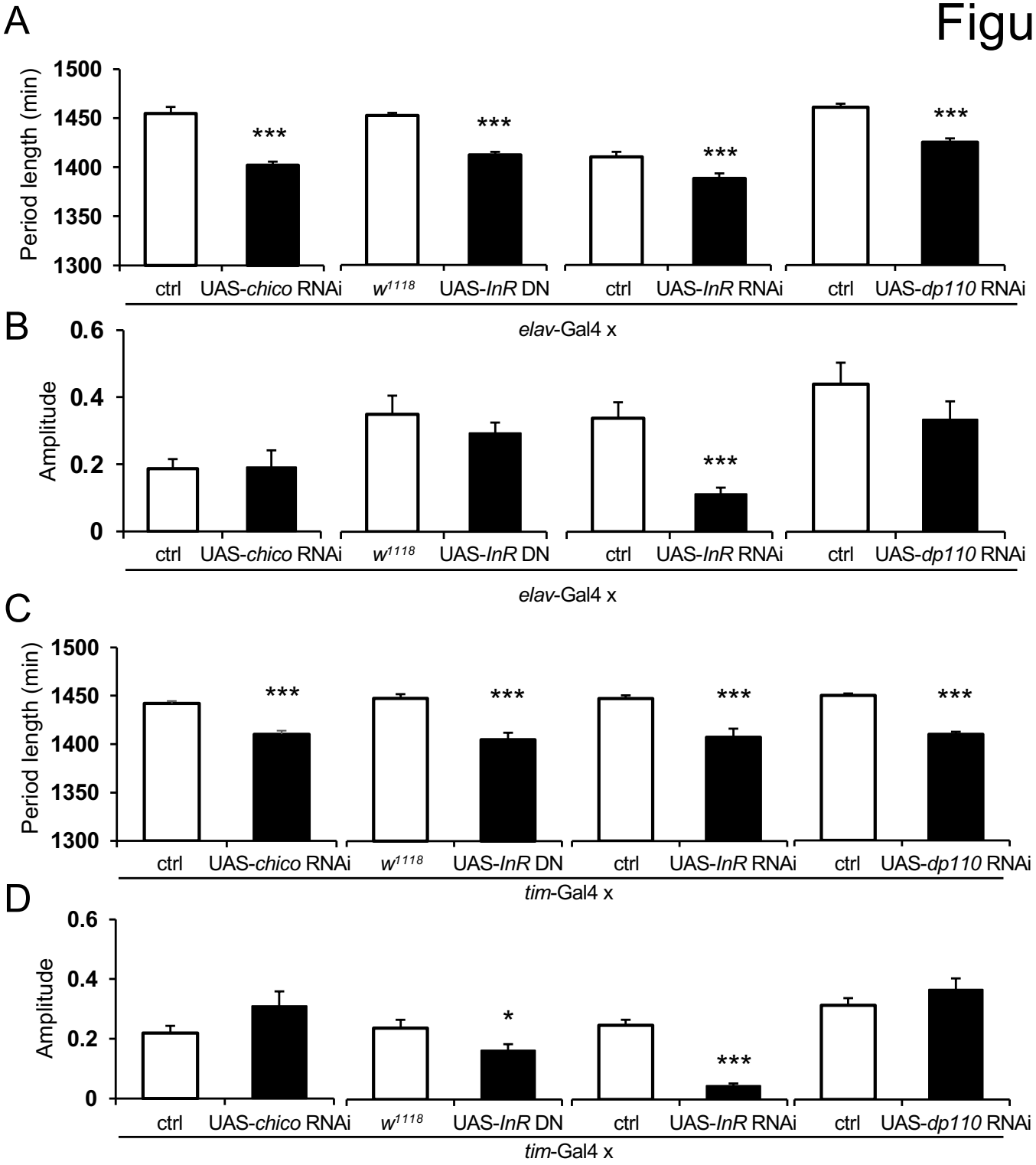
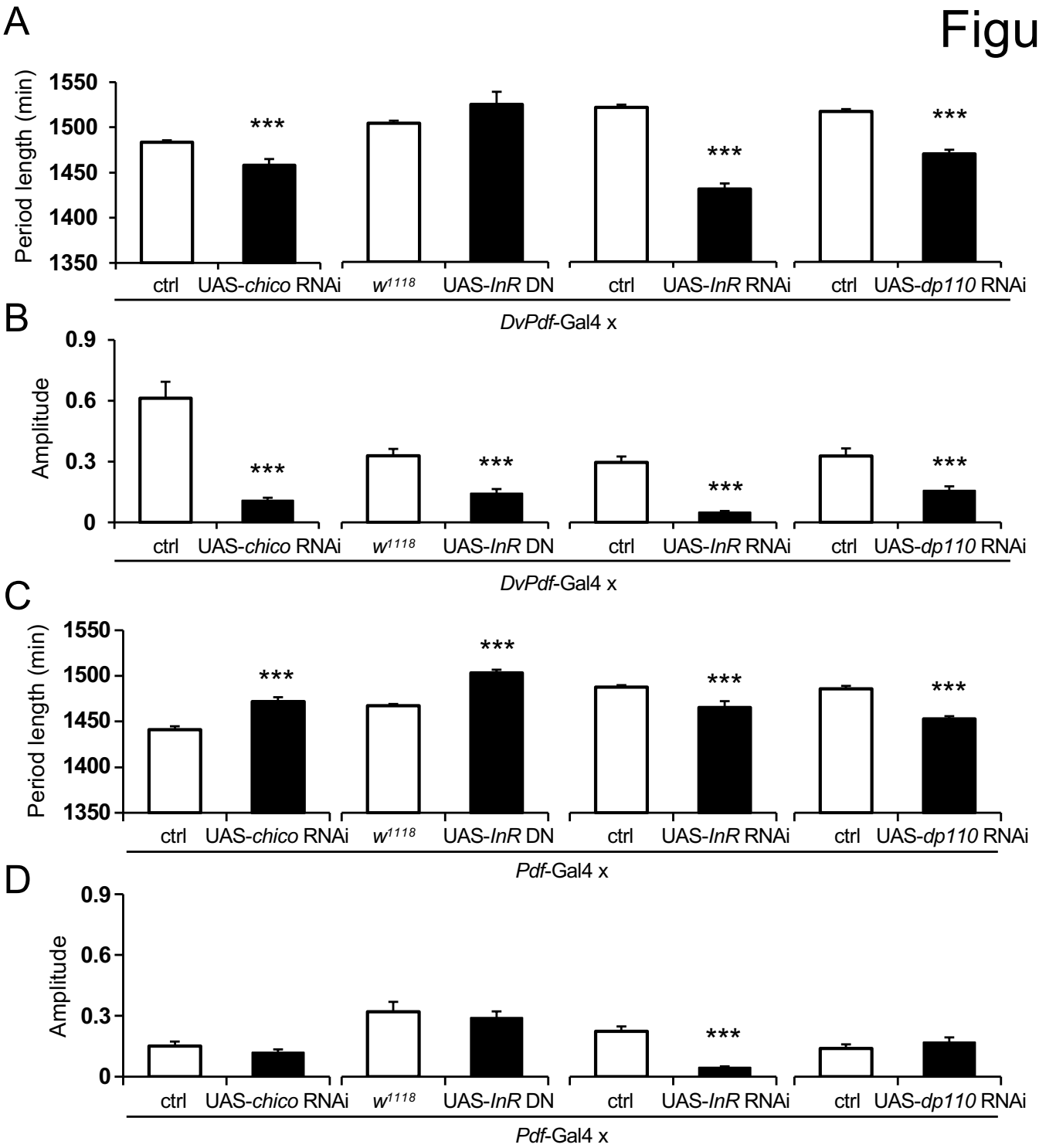

A
Period length (min)
1500
1450
1400
1350
1300
***
***
***
***
ctrl
UAS-chico RNAi
w1118
UAS-InR DN
ctrl
UAS-InR RNAi
ctrl
UAS-dp110 RNAi
elav-Gal4 x
B
Amplitude
0.6
0.4
0.2
0
***
ctrl
UAS-chico RNAi
w1118
UAS-InR DN
ctrl
UAS-InR RNAi
ctrl
UAS-dp110 RNAi
elav-Gal4 x
C
Period length (min)
1500
1450
1400
1350
1300
***
***
***
***
ctrl
UAS-chico RNAi
w1118
UAS-InR DN
ctrl
UAS-InR RNAi
ctrl
UAS-dp110 RNAi
tim-Gal4 x
D
Amplitude
0.6
0.4
0.2
0
*
***
ctrl
UAS-chico RNAi
w1118
UAS-InR DN
ctrl
UAS-InR RNAi
ctrl
UAS-dp110 RNAi
tim-Gal4 x

Figure 3


A
Period length (min)
1550
1500
1450
1400
1350
***
***
***
ctrl
UAS-chico RNAi
w1118
UAS-InR DN
ctrl
UAS-InR RNAi
ctrl
UAS-dp110 RNAi
DvPdf-Gal4 x
B
Amplitude
0.9
0.6
0.3
0
***
***
***
***
ctrl
UAS-chico RNAi
w1118
UAS-InR DN
ctrl
UAS-InR RNAi
ctrl
UAS-dp110 RNAi
DvPdf-Gal4 x
C
Period length (min)
1550
1500
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ctrl
UAS-chico RNAi
w1118
UAS-InR DN
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UAS-InR RNAi
ctrl
UAS-dp110 RNAi
Pdf-Gal4 x
D
Amplitude
0.9
0.6
0.3
0
***
ctrl
UAS-chico RNAi
w1118
UAS-InR DN
ctrl
UAS-InR RNAi
ctrl
UAS-dp110 RNAi
Pdf-Gal4 x

Figure 4

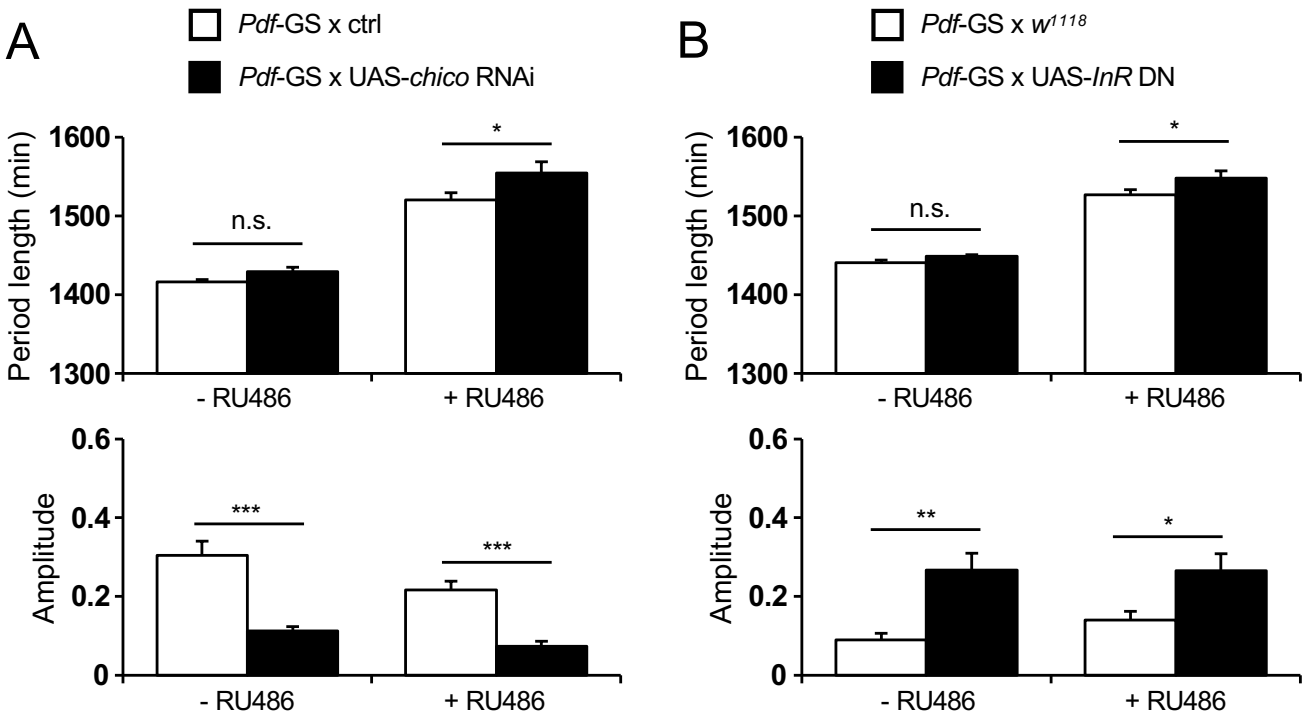
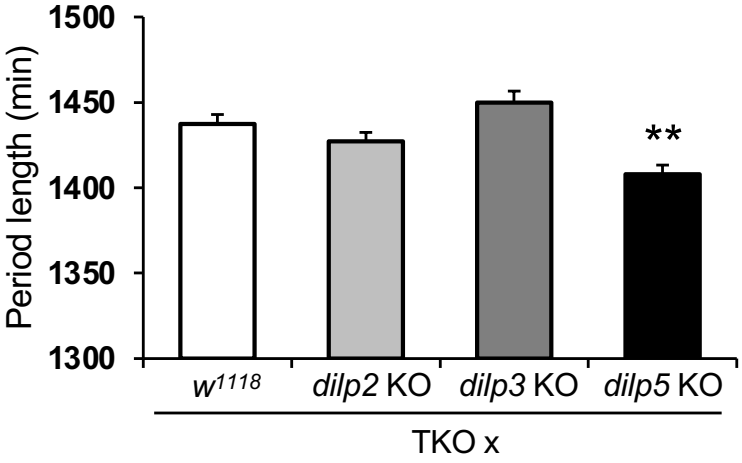
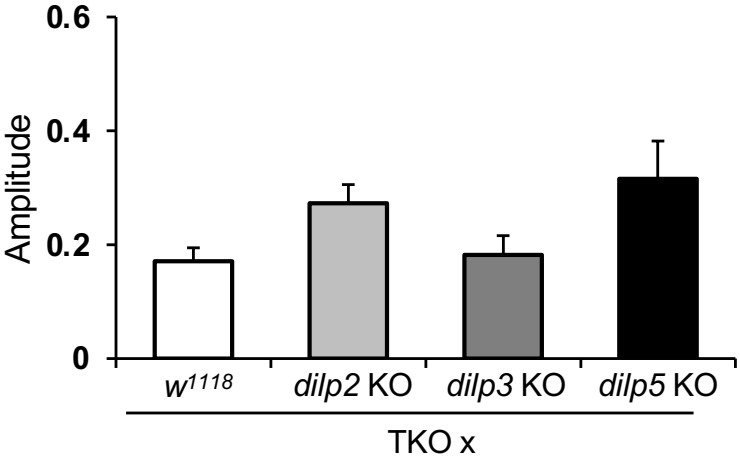

A
Pdf-GS x ctrl
Pdf-GS x UAS-chico RNAi
Period length (min)
1600
1500
1400
1300
n.s.
*
- RU486
+ RU486
Amplitude
0.6
0.4
0.2
0
***
***
- RU486
+ RU486
B
Pdf-GS x w1118
Pdf-GS x UAS-InR DN
Period length (min)
1600
1500
1400
1300
n.s.
*
- RU486
+ RU486
Amplitude
0.6
0.4
0.2
0
**
*
- RU486
+ RU486

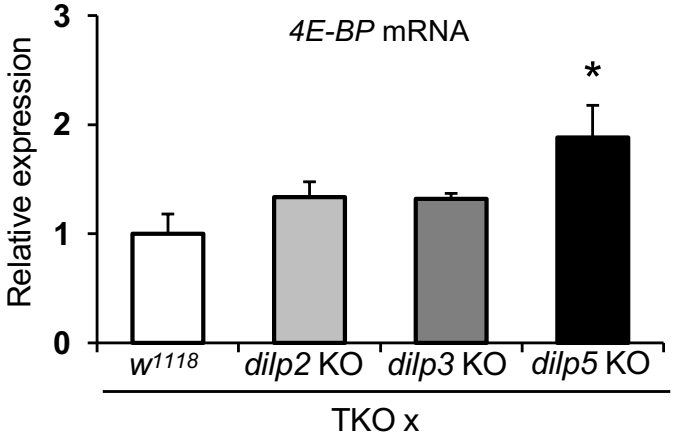
Figure 5

A

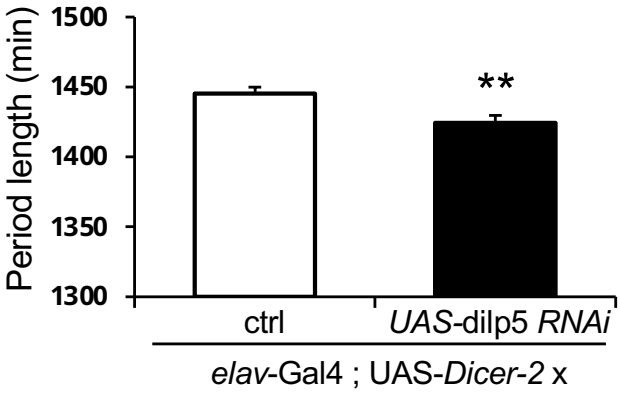

Period length (min)
1500
1450
1400
1350
1300
**
w1118
dilp2 KO
dilp3 KO
dilp5 KO
TKO x

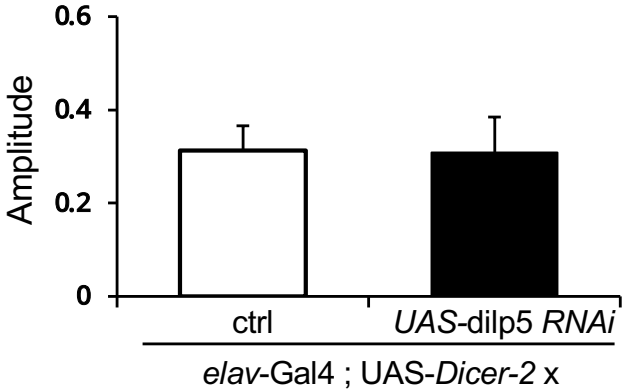

Amplitude
0.6
0.4
0.2
0
w1118
dilp2 KO
dilp3 KO
dilp5 KO
TKO x

B


4E-BP mRNA
Relative expression
3
2
1
0
*
w1118
dilp2 KO
dilp3 KO
dilp5 KO
TKO x

C


Period length (min)
1500
1450
1400
1350
1300
**
ctrl
UAS-dilp5 RNAi
elav-Gal4 ; UAS-Dicer-2 x


Amplitude
0.6
0.4
0.2
0
ctrl
UAS-dilp5 RNAi
elav-Gal4 ; UAS-Dicer-2 x