

1 **Thermosensitive alternative splicing senses and mediates**
2 **temperature adaptation in *Drosophila***

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4 Naveh Evantal^{1,*}, Ane Martin Anduaga^{2,*}, Osnat Bartok¹, Ines Lucía Patop², Ron Weiss¹ and
5 Sebastian Kadener^{1,2,#,+}

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7 ¹ Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, 91904,
8 Israel.

9 ² Biology Department, Brandeis University, Waltham, MA, 02454, USA.

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18 * These authors contributed equally to this work

19 # To whom correspondence should be addressed

20 Sebastian Kadener skadener@brandeis.edu

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23 + Lead Contact

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25 **SUMMARY**

26 Circadian rhythms are generated by the cyclic transcription, translation and degradation of
27 clock genes, including *timeless* (*tim*). Currently, little is known about the mechanisms by which
28 the circadian clock senses and adapts to temperature changes. Here we show that
29 temperature dramatically changes the splicing pattern of *tim*. We found that at 18°C TIM
30 protein levels are diminished due to the induction of two cold-specific splicing isoforms (*tim-*
31 *cold* and *tim-short&cold*). At 29°C, another isoform, *tim-Medium* is strongly upregulated. We
32 found that this isoform switching mechanism allows flies to regulate the levels and activity of
33 TIM by setting miRNA-dependent thresholds for expression as well as by expressing isoforms
34 with specific functions. Flies in which the production of *tim-short&cold* is abrogated display
35 altered patterns of locomotor activity and altered *tim* expression. Interestingly, the introns of
36 *tim* carry the information for the temperature sensitivity, suggesting that *tim* splicing *per se* is
37 the temperature sensor.

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40 **KEYWORDS:** circadian, *timeless*, RNA, splicing, temperature

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42 **INTRODUCTION**

43 Circadian rhythms organize most physiological and behavioral processes to 24 hours cycles
44 (Allada and Chung, 2010; Pilorz et al., 2018). The current model postulates that circadian
45 clocks keep time through a complex transcriptional-translational negative feedback loop that
46 takes place in the so-called “clock cells” (Helfrich-Forster, 2003; Shafer et al., 2006). Each one
47 of these clock cells has been proposed to function autonomously (each cell is its own
48 oscillator) (Dissel et al., 2014; Yoshii et al., 2012). In *Drosophila*, the master regulators CLOCK
49 (CLK) and CYCLE (CYC) activate the circadian system by promoting rhythmic transcription of
50 several key clock genes. Products of three of these genes, PERIOD (PER), TIMELESS (TIM),
51 and CLOCKWORK ORANGE (CWO) repress CLK-CYC mediated transcription in an
52 oscillatory manner (Hardin and Panda, 2013; Zhou J, 2016). These cycles of transcriptional
53 activation and repression lead to 24-hour molecular oscillations, which ultimately generate
54 behavioral rhythms. In addition to transcriptional control, post-transcriptional and post-
55 translational regulatory processes play essential roles in circadian timekeeping (Hardin and
56 Panda, 2013; Harms et al., 2004; Kojima et al., 2011; Lim and Allada, 2013). The
57 transcriptional repressors PER and TIM are post-translationally modified, and the modification
58 status as well as the rates with which these modifications take place have a significant
59 influence on their degradation rates (Hardin and Panda, 2013; Ozkaya and Rosato, 2012).
60 Modification of PER may additionally influence its transcriptional repressor activity or the
61 timing of its activity (Hardin and Panda, 2013; Ozkaya and Rosato, 2012).
62 Circadian clocks are extraordinarily robust systems; they are able to keep time accurately
63 without timing cues. In addition, and despite their biochemical nature, they are resilient to large
64 variations in environmental conditions. The robustness of the circadian system is likely the
65 result of multiple layers of regulation that assure accurate timekeeping and buffering of
66 stochastic changes into the molecular clockwork. These levels of regulation are physically
67 and/or functionally interconnected and, importantly, extend even beyond the single-cell level.
68 Circadian neurons in the brain are organized in a network that is believed to synchronize the
69 individual neuronal oscillators thereby contributing to a coherent and robust behavioral output

70 (Allada and Chung, 2010; Mezan et al., 2016; Peng et al., 2003; Schlichting et al., 2016;
71 Stoleru et al., 2004; Weiss et al., 2014). The PDF neuropeptide, main neuromodulator of the
72 circadian neuronal network, is expressed in a small subset of these neurons. PDF is essential
73 for normal circadian activity patterns in light:dark (LD) cycles and for persistent circadian
74 rhythms in constant darkness (DD) (Im et al., 2011; Peng et al., 2003; Taghert and Shafer,
75 2006). Circadian clocks are also remarkably plastic systems. For example, organisms can
76 quickly adjust to different light and temperature regimes (Roessingh et al., 2015; Wolfgang et
77 al., 2013). The plasticity of the circadian clock results from the existence of very efficient input
78 pathways that can convey the external signals into the core oscillator machinery (Bartok et al.,
79 2013; Ogueta et al., 2018).

80 *Timeless* is at the crossroad of both the robustness and plasticity of the circadian clock. *tim* is
81 a core circadian oscillator component and mutations in *tim* coding sequence lead to flies with
82 short, long, or no circadian rhythms (Myers et al., 1995; Rothenfluh et al., 2000; Sehgal et al.,
83 1994). TIM stabilizes PER, which is also essential for circadian rhythmicity, and this complex
84 represses CLK-mediated transcription in an oscillatory manner (Hall, 2003). Importantly, the
85 stoichiometric relationship between PER, CLK and TIM is tightly controlled and probably the
86 major regulator of circadian period (Fathallah-Shaykh et al., 2009; Hardin, 2011; Kadener et
87 al., 2008; Yu et al., 2006) . In addition, TIM is the key factor for communicating external
88 information to the central core oscillator. For example, upon light stimulus, the protein encoded
89 by *cryptochrome*, CRY, binds to TIM and promotes TIM degradation through the ubiquitin
90 ligase *jetlag*, which results in phase advances or delays of the circadian oscillator (Emery et
91 al., 1998; Koh et al., 2006; Lin et al., 2001). Last but not least, PDF signalling has been
92 proposed to synchronize the circadian clock by regulating TIM degradation (Li et al., 2014;
93 Seluzicki et al., 2014), suggesting that TIM is the key pathway for conveying external
94 information to the circadian system at the molecular level.

95 Temperature has diverse effects on the *Drosophila* circadian system (Afik et al., 2017; Kidd et
96 al., 2015). Flies show temperature-dependent changes in the distribution of daily locomotor
97 activity, named seasonal adaptation (Low et al., 2008; Majercak et al., 1999). They are also

98 entrained/synchronized by daily temperature cycles (Roessingh et al., 2015), are phase-
99 shifted by temperature pulses or steps (Glaser and Stanewsky, 2005), and can keep 24-hour
100 periods in a wide range of temperatures (temperature compensation (Kidd et al., 2015)).
101 Adaptation of *Drosophila* to different environmental conditions relies on the presence of at
102 least two neuronal circuits: one that controls the morning peak of locomotor activity (M) and
103 another that controls the evening (E) burst of activity (Grima et al., 2004; Stoleru et al., 2004;
104 Stoleru et al., 2005). The environment fine-tunes the activity pattern by altering the timing of
105 these oscillators. In the laboratory, mimicking summer by subjecting the flies to hot
106 temperatures and long photoperiods shift their M activity to pre-dawn and their E activity into
107 the early night (Majercak et al., 1999). In contrast, under shorter day lengths and cooler
108 temperatures, mimicking autumn, the M and E activity components fall closer together and
109 occur around the middle of the day, enabling maximal activity at the warmest temperatures.
110 Work from the Edery lab in 1999 first linked the splicing of *per* 3' untranslated region (UTR) to
111 seasonal adaptation (Majercak et al., 1999). The evening advance in locomotor activity at
112 lower temperatures correlates with an advance in the phase of oscillation of *tim* and *per*
113 mRNAs. The authors postulated that this shift of the clock is driven by the splicing of an
114 alternative exon located in *per* 3' UTR. Further studies revealed that the efficiency of *per*
115 mRNA splicing is regulated not only by temperature but also by the photoperiod (Collins et al.,
116 2004). The effect of light on *per* splicing does not require a functional clock and phospholipase
117 C likely plays a physiological, non-photic role in downregulating the production of the spliced
118 transcripts (Collins et al., 2004). Moreover, in 2008, a new work examined the splicing pattern
119 of *per* in the tropical species *Drosophila yakuba*, which faces no significant seasonal variation
120 in day length or temperature (Ko et al., 2003; Low et al., 2008; Russo et al., 1995). Although
121 the *per* gene of *D. yakuba* has a 3'-terminal intron, this intron is removed over a wide range of
122 temperatures and photoperiods, consistent with the marginal effect of temperature on this
123 tropical species. Despite these findings, it is still not clear how the regulation of the 3' UTR
124 splicing impacts PER levels or if other transcriptional and/or post-transcriptional events are
125 also responsible for the regulation of activity in response to temperature changes.

126 Post-transcriptional control by miRNAs and RNA binding proteins has been shown to be
127 important for circadian timekeeping in flies and other organisms (Chen and Rosbash, 2016;
128 Kadener et al., 2009; Lerner et al., 2015; Lim and Allada, 2013; Xue and Zhang, 2018). On
129 the other hand, only more recently a few reports show a role of alternative splicing on circadian
130 timekeeping (Bartok et al., 2013; Petrillo et al., 2011; Sanchez et al., 2010; Wang et al., 2018).
131 This is surprising, given the prevalence of alternative splicing in the brain and the importance
132 of this process in regulating the amount and type of mRNAs generated from a given locus
133 (Baralle and Giudice, 2017; Sanchez et al., 2011). Alternative mRNA processing is co-
134 transcriptional and usually happens due to the presence of suboptimal processing signals (i.e.
135 splice or cleavage and polyadenylation sites). Many factors can influence alternative splicing
136 including changes in RNA structure, the presence of RNA binding proteins and the elongation
137 rate of RNA Polymerase II (Caceres and Kornblihtt, 2002; de la Mata et al., 2003).
138 Here we show that temperature dramatically and specifically changes the splicing pattern of
139 *tim*. We found that the lower levels of the canonical TIM protein at 18°C are due to the induction
140 of two cold-specific splicing isoforms (*tim-cold* and *tim-short&cold*). *Tim-cold* encodes a
141 canonical TIM protein but it is under strong miRNA-mediated control as showed by AGO1 IP
142 and *in vivo* luciferase reporters. Moreover, *tim-short&cold* (*tim-sc*) encodes a short TIM
143 isoform which can advance the phase of the circadian clock when overexpressed.
144 Interestingly, most of the changes in *tim* splicing patterns are conserved across *Drosophila*
145 species and correlate well with the capacity of the species to adapt their activity to temperature
146 changes. We follow by using CRISPR to generate flies in which the production of *tim-sc* is
147 abrogated. These flies display altered patterns of locomotor activity at 18 and 25°C as well as
148 altered expression of the remaining *tim* isoforms, demonstrating the importance of *tim-sc*
149 production. Moreover, we showed that the temperature-dependent changes in *tim* alternative
150 splicing are independent of the circadian clock. Furthermore, we could reproduce them in
151 *Drosophila* S2 cells and utilizing splicing minigenes. The latter results strongly suggest that
152 the intronic sequences of *tim* themselves are the temperature sensor for these changes in
153 splicing.

154 **RESULTS**

155 ***Temperature remodels the circadian transcriptome***

156 To determine the effect temperature has on general and circadian gene expression we
157 evaluated gene expression genome-wide of heads of flies at 18°C, 25°C or 29°C. Briefly, we
158 performed 3' sequencing at 6 different timepoints in flies entrained to 12:12 Light:Dark (LD)
159 cycles at these three temperatures. We identified hundreds of genes displaying oscillating
160 expression over the day (Figure 1A and Table S1). Interestingly, most of the cycling RNAs
161 were temperature specific (Figure 1B, Table S1), with only a few RNAs consistently displaying
162 mRNA oscillations at all the assayed temperatures. The set of oscillating mRNAs was enriched
163 for different Gene Ontology (GO) terms, demonstrating that the circadian program is
164 temperature dependent (Table S2). Interestingly, RNAs oscillating at 18°C display earlier
165 phases than those cycling at 25 or 29°C (Figure 1A and Table S1). This phase advance was
166 even more noticeable on the RNAs oscillating at more than one temperature including the core
167 circadian components (Figure 1C), suggesting that the circadian clock gene expression
168 program is advanced at 18°C. These phase advance mirrors the advance of the evening peak
169 of activity observed at 18°C. Interestingly and despite the clear behavioral differences, we
170 observed smaller phase changes in the flies entrained at 29°C compared to those at 25°C
171 (Figure 1A, S1 and Table S1). Interestingly, our data revealed no major changes in the overall
172 levels of most circadian components when entrained at different temperatures (Figure 1D). As
173 stated above (and previously described (Majercak et al., 1999)), *tim* and *per* mRNA profiles
174 were advanced at 18°C. This phase advance is transcriptional, as can be observed by
175 assessing *tim* and *per* RNAs from nascent (chromatin-bound) RNA (Figure S2). This phase
176 advance in the mRNA level provokes a phase advance notable in TIM and PER protein levels
177 (Majercak et al., 1999).

178 To complement these data, we analyzed gene expression at the three temperatures utilizing
179 oligonucleotide microarrays from samples that were collected at 6 timepoints but pooled
180 before the gene expression assessment. Pooling the timepoints allows a more precise
181 comparison of average gene expression, avoiding big variations due to timepoints. When

182 examining the Microarray data we were able to identify hundreds of mRNA transcripts that
183 change their expression in response to adaptation to different temperatures. We found
184 changes in both directions, i.e higher or lower at high/low temperatures (Figure S3A). We
185 found a number of Gene Ontology (GO) categories enriched among the differentially
186 expressed genes, spanning a wide range of biological functions (Figure S3B). These results
187 are consistent with previously reported data (Boothroyd et al., 2007a). For example, we
188 observed an increase in expression of ribosomal proteins, genes involved in protein folding
189 and metabolic processes at 29°C. At 18°C we saw increased expression of proteins involved
190 in cuticle development, which is consistent with previous reports showing that cuticle
191 deposition is temperature dependent (Boothroyd et al., 2007a; Ito et al., 2011). These results
192 demonstrate the dramatic effect that temperature has on mRNA steady-state levels and on
193 various cellular and metabolic processes. Overall, the data above demonstrates the eminent
194 role of temperature in global gene expression, and specifically on the circadian molecular and
195 transcriptional network.

196

197 ***Temperature modulates tim alternative splicing***

198 3'-seq datasets are a powerful tool for examining changes in gene expression. However, they
199 lack the ability to capture many processing events such as alternative splicing and alternative
200 promoter usage. Therefore, we generated whole-transcript polyA⁺ RNA-seq datasets from
201 heads of flies entrained at 18°C, 25°C or 29°C. We found that temperature changes greatly
202 impact the pattern of alternative splicing of *tim* mRNA but not any other clock gene (Figure 2A
203 and data not shown). We identified four major isoforms generated from the *tim* locus (Figure
204 2B). Except for the canonical transcript, which is generated from an already characterized
205 transcript (referred to from now on as *tim-L*), all other three isoforms changed their expression
206 in a temperature dependent manner. Two of those were more abundant at 18°C compared to
207 25°C. The first one was previously described as being induced by cold temperatures (*tim-cold*)
208 (Boothroyd et al., 2007a; Wijnen et al., 2006). It includes the last intron of *tim* and has a stop
209 codon at the beginning of the intron, generating a slightly smaller protein than the canonical

210 *tim-L* isoform, but with a longer 3' UTR. The second cold-enriched isoform, although
211 annotated, was not previously described, and we named it *tim-short&cold* (abbreviated *tim-*
212 *sc*), as putatively encodes a much smaller protein. In contrast to the other isoforms which are
213 generated by intron retention, *tim-sc* mRNA is generated by the usage of an alternative
214 cleavage and polyadenylation site located with the intron 10 of *tim* which can only be used
215 before this intron is spliced out. The third isoform we identified potentially encodes for a
216 medium sized protein (smaller than those encoded by *tim-L* and *tim-cold* and larger than *tim-*
217 *sc*) that we named *tim-medium* (abbreviated *tim-M*). This isoform was recently described and
218 named *tim-tiny* (Shakhmantir et al., 2018). However, to avoid confusion with *tim-sc* (which
219 encodes an even smaller isoform), we will call this isoform *tim-M*. This isoform, is generated
220 by the inclusion of an intron containing a stop codon and shares all downstream exons with
221 *tim-L*, hence presenting the longest 3' UTR of all isoforms. We found that this isoform is highly
222 expressed in flies entrained to 25°C or 29°C compared to flies kept at 18°C.
223 Since three of these isoforms (*tim-L*, *tim-cold* and *tim-M*) share the same 3' end, they are
224 undistinguishable in the 3'-seq datasets. In contrast, we could easily detect a significant
225 increase in the levels of *tim-sc* mRNA at 18°C in this dataset (Figure S4). We also could
226 observe the effect in the whole-transcript datasets, although the differences were more difficult
227 to appreciate due to the underrepresentation of 3' ends in whole-transcript sequencing. We
228 confirmed the changes detailed above by qRT-PCR using isoform specific primers (Figure
229 S5A). Importantly, the levels of *tim-L* cannot be unequivocally assessed by qPCR as its
230 sequence overlaps with *tim-M*. Temperature-dependent changes in the splicing patterns of *tim*
231 are, at least, as impressive as the previously described changes in the splicing of a small
232 intron in the 3' UTR of *per* (Majercak et al., 1999).
233 We next utilized the whole RNA-seq datasets to quantify the relative amount of each of the
234 four isoforms (Figure 2C). We found the change in the distribution of the four isoforms between
235 the different temperatures to be clearly dramatic, from a fairly even distribution at 18°C to no
236 expression of *tim-sc* and *tim-cold* and significant elevation of *tim-M* at 29°C. Surprisingly, we
237 found that *tim-M* constitutes about fifty percent of *tim* mRNAs at 25°C, having a similar, if not

238 higher, expression than the canonical isoform *tim-L*. This suggests the existence of previously
239 unknown aspects of *tim* regulation even in canonical conditions. In comparison to the big
240 changes driven by temperature observed in *tim* alternative splicing, the well-characterized
241 changes in *per* alternative splicing are very modest (Low et al., 2008) (Figure2D). To assess
242 if the observed changes in *tim* RNA processing upon temperature changes also happen in the
243 fly brain, we dissected the brains of flies entrained at 18, 25 and 29°C and measured the levels
244 of each *tim* isoform by qPCR. As observed when utilizing fly heads, entrainment of the flies to
245 lower temperatures (18°C) resulted in a strong increase of *tim-s* and *tim-cold* levels while
246 higher temperatures (29°C) promoted *tim-M* expression (Figure S5B).

247

248 ***Temperature-specific alternative splicing is conserved across Drosophilae and***
249 ***correlates with temperature adaptation***

250 If changes in the splicing pattern of *tim* are necessary for temperature adaptation, it should be
251 conserved among other *Drosophila* species that adapt to temperature changes. Therefore, we
252 determined both the behavioral and splicing patterns of three additional species, belonging to
253 the *Drosophila* genus at 18, 25 and 29°C. Two of those species (*D.simulans* and *D.Yakuba*)
254 belong, like *D.melanogaster*, to the *Sophophora* subgenus, while the third (*D.virilis*) belongs
255 to the *Drosophila* subgenus. Furthermore, even if they share the same tropic origin,
256 *D.melanogaster* and *D.simulans* have become cosmopolitan, while the distribution of
257 *D.Yakuba* is restricted to Africa (Kuntz and Eisen, 2014; Markow and O'Grady, 2007). *D.virilis*,
258 a Holarctic species, is also geographically temperate. Although all species displayed some
259 degree of behavioral adaptation to temperature, we could detect differences between species
260 when analyzing the ratio between light and dark activity in all three temperatures (Figure 3A).
261 As expected, *D.melanogaster* displays a temperature-dependent gradient in the day/night
262 activity ratio, with highest ratios at 18°C (Figure 3A, left). *D.simulans* showed the same trend
263 of significant decrease in light/dark activity ratios in elevating temperatures, suggesting
264 behavioral adaptation to high and low temperatures (Figure 3A, middle-left ; Figure S6A upper
265 left panel and data not shown). In contrast, while adjusting to high temperatures, *D.Yakuba*

266 did not show any significant difference between the ratios of 25°C and 18°C, suggesting
267 reduced adaptational capacity to lower temperatures (Figure 3A, middle-right; Figure S6A
268 middle left panel and data not shown). It should be mentioned that while the ratio at 29°C in
269 *D.virilis* is the same as in the other species, its activity was affected in the opposite trend than
270 the others, decreasing during the dark (Figure 3A, right ; Figure S6A lower left panel and data
271 not shown).

272 We followed by generating whole-transcript polyA⁺ RNA-seq datasets from the heads of all
273 three species entrained at the 3 different temperatures. When examining the splicing pattern
274 of *tim*, we could see that all species displayed dynamic changes in *tim-cold* and *tim-M* in
275 response to temperature changes, with *tim-cold* increasing and *tim-M* decreasing at lower
276 temperatures (Figure 3B and Figure S6). This suggests that these changes might be important
277 for the behavioral changes shared by all species. However, we only detected *tim-sc* in
278 *D.simulans* and *D.melanogaster*, which had very similar temperature-dependent alternative
279 splicing profiles (Figure 3B and Figure S6). As mentioned above, this isoform appears only at
280 18°C, strongly suggesting that this isoform is responsible for the adaptation to cold
281 temperatures, which is shared between *D.simulans* and *D.melanogaster*. The results
282 displayed above showed a strong correlation among the different fly species between the
283 changing on *tim* mRNA isoforms and the capacity to adapt to colder temperatures. We also
284 looked at changes in *per* 3' UTR splicing in the different species between temperatures.
285 *D.virilis* displayed no changes in *per* splicing, while the changes in *D.Yakuba* were mild, in
286 both cases the spliced isoform dominated (Figure 3B and Figure S6B).

287

288 ***Cold temperature decreases TIM-L levels by two independent mechanisms***

289 As previously described (Majercak et al., 1999), we found that the levels of TIM-L protein are
290 significant downregulated at 18°C compared to 25°C or 29°C. We could not clearly detect or
291 differentiate the other isoforms by Western Blot (data not shown). Nevertheless, TIM-COLD
292 has been reported to be expressed by Boothroyd et al. (2007a). *tim-M* mRNA is very abundant
293 but we could not see a protein of the predicted size in the western blots, which strongly

294 suggests that that this isoform is poorly and/or not translated. Hence, expression of this
295 isoform could serve as a way to regulate the amount of *tim-L* production. This agrees with a
296 recent publication that identified this mRNA isoform as non-coding (Shakhmantir et al., 2018).
297 We also could not univocally detect TIM-SC. While we observed bands of TIM-SC expected
298 size in the TIM immunoblot, these bands are present at all temperatures (data not shown). We
299 believe that these bands might represent canonical TIM degradation products of similar size
300 than TIM-SC (see below). In addition, the utilized TIM antibody was raised against the whole
301 protein and detects TIM-SC with low efficiency (see below).
302 Interestingly, the lower levels of TIM-L at 18°C are not due to changes in total *tim* mRNA, as
303 observed in our datasets (Figure S4) and reported by others (Boothroyd et al., 2007b). This
304 suggests that the lower TIM levels are due to post-transcriptional and/or post-translational
305 regulation. miRNA-mediated repression is one of the most common mechanisms of post-
306 transcriptional control. Moreover, recent work demonstrated that *tim* mRNA is regulated by
307 miR-276 (Chen and Rosbash, 2016). We then decided to determine whether *tim* is regulated
308 by miRNAs in a temperature dependent way. To do so, we tested the genome-wide
309 association of mRNAs with the ARGONAUTE 1 (AGO1, the only miRNA-RISC effector protein
310 in *Drosophila* (Förstemann, Horwich, Wee, Tomari, & Zamore, 2007) at 18°C, 25°C and 29°C.
311 Briefly, we performed AGO1 immunoprecipitation (IP) from fly heads followed by hybridization
312 to oligonucleotide microarrays. In these datasets, we can only assess overall *tim* levels (as
313 these microarrays cannot distinguish between *tim-L*, *tim-cold* and *tim-M*). We first confirmed
314 that the overall distribution of AGO1-associated mRNAs (and hence miRNA-mediated
315 regulation) is similar at all three temperatures (Figure S7). Interestingly, we observe changes
316 in the association of *tim* to AGO1 (Figure 4A). Binding of *tim* to AGO1 is very strong at 18°C
317 while there is very little or no association at 25 or 29°C. This pattern is *tim*-specific and, when
318 examining other core circadian clock components, we could not see such temperature-driven
319 AGO1 association changes. Some are bound at all temperatures (like *Clk* or *vri*) and some do
320 not bind at all (like *cry*, *cyc* and *per*; Figure 4A). Importantly, the probes in the oligonucleotide
321 microarray do not allow distinguishing between *tim-L*, *tim-cold* and *tim-M*. To determine

322 whether the association of the different *tim* transcripts to the miRNA-effector machinery is
323 temperature dependent, we performed isoform-specific qPCRs from newly generated AGO1-
324 IP samples. While the mRNA encoding the transcription factor CBT (a known miRNA-
325 regulated RNA (Kadener et al., 2009; Lerner et al., 2015)) was strongly bound to AGO1 in all
326 the assayed temperatures, we discovered that the association of *tim-L/M*, *tim-cold* and *tim-M*
327 to AGO1 was significantly higher at 18°C (Figure 4B).

328 To determine the consequences of AGO1 binding on TIM expression, we generated flies
329 carrying luciferase reporters fused to the different 3' UTRs of *tim*. All transgenes were targeted
330 and inserted in the same genomic location, and we expressed these UAS-transgenes using
331 the *tim-ga4* driver. To minimize the effects of temperature on the GAL4 transgene, we
332 performed the experiment at 25°C. The Luciferase levels of the transgenes carrying the 3'
333 UTRs of *tim-L* and *tim-sc* were high, suggesting little (or no) post-transcriptional control, at
334 least at 25°C. Interestingly, the reporter carrying the 3' UTR of *tim-cold* showed a strong
335 decrease of Luciferase levels compared to the rest of the reporters, suggesting that the
336 presence of this 3' UTR strongly diminishes the translation potential of this isoform either by
337 affecting the stability of the mRNA or, directly, by preventing its translation (Figure 4C).
338 Similarly, the *tim-M* 3' UTR reporter displayed lower luciferase levels, although the effect was
339 milder than the one observed with *tim-cold* 3' UTR.

340 To further understand the post-transcriptional regulation of *tim*, we analyzed the different 3'
341 UTRs for potential miRNA binding sites using Target Scan (Agarwal, Bell, Nam, & Bartel,
342 2015). Three of the isoforms, *tim-L*, *tim-cold* and *tim-M* contain a large number of miRNA
343 binding sites, many of which are evolutionary conserved and highly expressed in fly heads
344 (Table S3). *Tim-sc* contains only 5 miRNA binding sites and was not bound to AGO1 at any
345 temperature demonstrating it is not regulated by miRNAs (Figure 4D).

346 The miRNAs responsible for the association of *tim* isoforms with AGO1 could target only a
347 temperature-specific isoform, more than one isoform, be expressed in a temperature-
348 dependent way or a combination of these. We therefore sequenced AGO1-associated
349 miRNAs from heads of flies entrained to 12:12 LD cycles at 18, 25 and 29°C. While the

350 expression of most miRNAs was not affected by temperature, a few of them were (Figure 4E,
351 Table S4). We first focus on the miRNAs targeting the 3' UTR region common to *tim-L*, *tim-*
352 *cold* and *tim-M*. Of the 17 miRNAs predicted to target this region, only two change with
353 temperature. One of them (miR-33) is modestly increased at 29°C in comparison with 18C.
354 Notably miR-969, which contains a strong binding site in this 3' UTR, is strongly (~3 fold)
355 upregulated at 18°C, with intermediate expression at 25°C and low at 29°C (Figure 4F).
356 Changes in the abundance of this miRNA might explain the temperature dependent
357 association of *tim-L* to AGO1. However, *tim-M* and *tim-cold* contain additional 3' UTR regions
358 and these regions are predicted to be targeted by other miRNAs that are strongly regulated
359 by temperature. For each of these *tim* isoform, half of the miRNAs predicted to bind them are
360 highly expressed at 18°C and half highly expressed at 29°C (Figure 4F and Tables S3 and
361 S4). This strongly suggests the existence of a mechanism for downregulating TIM-COLD and
362 TIM-M at all temperatures. The fact that TIM-COLD is indeed present at 18°C (Boothroyd et
363 al., 2007a; Wijnen et al., 2006), suggest that the main function of the miRNA-mediated
364 regulation of this isoform is to impose a threshold for avoiding expression of this protein at
365 temperatures other than 18°C.
366 In sum, these results, strongly suggest that, at 18°C, changes in the canonical TIM protein are
367 due to the deviation of *timeless* transcription towards the production of a short isoform (*tim-*
368 *sc*) and another isoform under strong post-transcriptional regulation (*tim-cold*). In addition, at
369 this temperature even *tim-L* is subjected to a stronger post-transcriptional control by miRNAs.
370 Last, *tim-M* is also regulated post-transcriptionally at all temperatures (similar to *tim-cold*).
371

372 ***Overexpression of the different TIM isoforms alters circadian behavior differently***

373 We next sought to determine the functionality of the different TIM isoforms. To do so we
374 generated plasmids expressing the different TIM protein isoforms fused to a C-terminal FLAG
375 tag and the same 3' UTR under the control of the UAS-transgene (Figure S8A). To verify the
376 expression of TIM from these plasmids we co-transfected each one of them into *Drosophila*
377 S2 cells (which do not express *tim*) together with a GAL4 expressing plasmid. As expected,

378 using an anti-FLAG antibody we observed the bands of predicted size for TIM-SC, TIM-M,
379 TIM-COLD and TIM-L (Figure S8B left). Interestingly, anti-TIM antibodies can efficiently detect
380 TIM-L and TIM-COLD but barely detected TIM-SC (Figure S8B right). Moreover, we observed
381 a degradation product that overlaps with TIM-SC, demonstrating that it is very difficult (if not
382 impossible) to detect this isoform univocally by western blot. We followed by generating flies
383 containing these plasmids by targeting them into the same *attB* insertion site, in order to obtain
384 flies that can express the proteins at similar levels. We then over-expressed each of these
385 TIM protein isoforms using the *tim-gal4* driver and determined the locomotor activity patterns
386 of these flies at 25°C. When kept under a 12:12 LD cycle, we observed a clear advance in the
387 start of the evening activity peak in the *tim-sc* overexpression flies compared to the *tim-Gal4*
388 control (Figure 5A, B). This, together with the fact that in DD overexpression of TIM-SC led to
389 a one hour shortening of the period compared to the control (Figure 5C), suggests a role for
390 *tim-sc* mRNA and protein in the behavioral advance observed in *wild type* flies at 18°C.
391 Additionally and as previously described by Yang and Sehgal (2001), overexpression of TIM-
392 L at 25°C resulted in a high percentage of flies displaying long, weak or no rhythms in DD
393 conditions (Figure 5C). Overexpression of TIM-COLD also resulted in weak and/or longer
394 rhythms although at a lower degree than TIM-L, which suggests that the two proteins are not
395 fully equivalent (although both are functional). Surprisingly, overexpression of TIM-M did not
396 significantly change the locomotor activity pattern neither in LD nor in DD, suggesting that this
397 protein is non-functional (Figure 5).

398

399 ***Elimination of tim-sc results in changes in tim processing and locomotor activity***

400 To definitively establish whether *tim-sc* is functional, we generated flies in which the cleavage
401 and polyadenylation site used for the generation of *tim-sc* mRNA is mutated by CRISPR (40A
402 flies, Figure 6A). As expected, these flies do not express *tim-sc* at any temperature (see qPCR
403 in Figure 6B). We followed by assessing the locomotor behavior of 40A mutants and their
404 isogenic controls. Control flies presented two different responses when transferred to 18°C:
405 an advance of the evening peak as well as a decreased night activity in comparison to the

406 flies maintained at 25°C (Figure 6C, 6D and 6E). In addition, the activity patterns of the 40A
407 flies are strikingly similar at the different temperatures. At 18 and 25°C, 40A mutants display
408 lower activity than control flies only during the night while at 29°C, these 40A flies were less
409 active both in the light and dark periods (Figures 6D and S9). Even more importantly, and
410 opposite to TIM-SC overexpression, 40A mutants display a significant delay in the time of
411 evening activity onset specially at 18°C but also more mildly at 25°C (Figures 6C and 6E).
412 Finally, we measured the levels of the other *tim* isoforms in control and 40A flies at 18, 25 and
413 29°C in order to assess the molecular consequences of the disruption of *tim-s* production.
414 *Tim-sc* mutant flies do not show changes in the overall levels of *tim* mRNA (as assessed by
415 determining the levels of the constitutive exon 5 and 6; Figure 6F left). However, elimination
416 of *tim-sc* results in an increase in the levels of *tim-cold* both 18 and 25°C at the maximum
417 expression timepoint (ZT15; Figure 6E). *tim-L/M* levels also are increased in the mutant, but
418 only at 25°C.
419 These results strongly suggest that both TIM-SC protein and *tim-sc* production can regulate
420 the daily pattern of locomotor activity and the response to temperature changes. Importantly,
421 we still observe some degree of temperature adaptation in 40A mutants, which we postulate
422 are mediated by the increased production of *tim-cold*.

423

424 ***Tim alternative splicing is regulated directly by temperature in-vitro and in-vivo***

425 To get insights into the mechanism by which temperature regulates *tim* alternative splicing,
426 we determined if *tim*⁰¹ and *per*⁰¹ mutants also change *tim* alternative splicing in a temperature-
427 dependent way. Hence, we entrained control and both mutant lines in LD conditions at 18°C
428 and at 29°C, collected flies every four hours, extracted RNA from fly heads from a mix of all
429 time points and performed qPCR. We observed a similar trend in all three fly lines, namely:
430 an increase in *tim-cold* and *tim-sc* and a decrease in *tim-M* at 18°C (Figure 7A). This
431 demonstrates that, as previously shown for *per* temperature-dependent splicing, *tim*
432 thermosensitive splicing events are independent of the circadian clock.

433 The splicing sites flanking alternatively spliced exons are usually weaker and temperature is
434 known, at least *in vitro*, to strongly influence the efficiency of splicing. Hence, we determined
435 the strengths of the different splice sites in *tim* using a publicly available software (Reese et
436 al., 1997). Almost all *tim* constitutive introns exhibited strong 5' and 3' splice site strengths
437 (above 0.6 scores, Figure S10). Interestingly, the introns retained in *tim-M* and in *tim-cold* have
438 strong 5' splice sites and a weak 3' splice site. This suggests that at least part of the
439 mechanism underlying the production of these isoforms is due to the presence of weak 3'
440 splice sites. However, the splice sites of the intron associated with *tim-sc* bear strong 5' and
441 3' splice site (**Figure S10**), suggesting that the increase of *tim-short* at lower temperatures
442 might be the result of a more efficient cleavage and polyadenylation rather than changes on
443 the splicing efficiency. In addition, we observed that *D.melanogaster*, *D.simulans* and
444 *D.Yakuba* possess strong 5' splice sites and weak 3' splice sites of the introns retained in *tim-*
445 *cold* and *tim-M* whose alternative splicing is temperature sensitive in these species (Figure
446 S10). Interestingly both *tim-cold* and *tim-M* respond in opposite ways to changes in
447 temperatures (with *tim-cold* strongly retained at 18°C and *tim-M* more skipped at this
448 temperature).

449 *Tim* was one of the few genes in which we observed temperature dependent changes in
450 alternative splicing (data not shown). Hence, it is possible that *tim* alternative splicing *per se*
451 could be thermosensitive. To test this possibility, we utilized the embryonic cell line Schneider
452 2 (S2 cells). These cells do not express most circadian components including *Clk*, *per* and *tim*.
453 However, *tim* expression can be induced by expression of CLK (McDonald & Rosbash, 2001).
454 So, we transfected these cells with a plasmid driving expression of *Clk*. We used an inducible
455 vector in order to bypass any transfection variation that might arise due to different
456 temperature conditions (Lerner et al., 2015). After the induction of *Clk*, we cultured the cells
457 either at 25°C or 18°C for 24 hours. We then collected the cells, extracted RNA and
458 characterized the splicing pattern of *tim* by qPCR. For each of the alternative isoforms, we
459 measured the ratio between the unspliced and spliced variants and compared these ratios
460 between the two temperatures. Indeed, we completely reproduce the results obtained *in vivo*,

461 namely: *tim-cold* and *tim-sc* were higher at 18°C, while *tim-M* showed the opposite trend
462 (Figure 7B). However, we did not observe any temperature-induced changes of a constitutive
463 *tim* exon (Control, Figure 7B) demonstrating that this effect is specific for the thermosensitive
464 introns.

465 One intriguing possibility is *tim* splicing *per se* functioning as a thermometer. This could be
466 accomplished if temperature could, for example, expose binding sites for a specific splicing
467 factor or if the splicing of these introns is temperature dependent. To test this possibility, we
468 generated three different *tim* minigene reporters consisting of the exons and intron involved in
469 each event of *tim* alternative splicing (Figure 7C). As observed *in vivo* and in the context of
470 the whole *tim* gene in S2 cells, we observed a temperature-dependent effect on the splicing
471 of the three different *tim* minigenes. We did not observe any temperature dependence in the
472 splicing of a minigene generated from a constitutive exon (Figure 7D), demonstrating a direct
473 effect of temperature on *tim* alternative splicing.

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493 **DISCUSSION**

494 In this study we show that temperature dramatically and specifically changes the splicing
495 pattern of the core circadian component *timeless*. We found that the lower levels of the
496 canonical TIM (TIM-L) protein at 18°C are due to the induction of two cold-specific splicing
497 isoforms (*tim-cold* and *tim-short&cold*). *Tim-cold* encodes a protein very similar to TIM-L but it
498 is under strong post-transcriptional control, as showed by AGO1-IP and *in vivo* luciferase
499 reporters. Moreover, *tim-sc* encodes a short TIM isoform which results in an advanced phase
500 of the circadian clock when overexpressed. Interestingly, these changes in *tim* splicing
501 patterns are conserved across several *Drosophila* species and correlate well with the capacity
502 of the species to adapt their activity to temperature changes. We then generated flies in which
503 the production of *tim-sc* is abrogated. These flies display altered patterns of locomotor activity
504 at 18°C and 25°C as well as altered expression of the remaining *tim* isoforms demonstrating
505 the importance of *tim-sc* production. Last, we showed that the temperature-dependent
506 changes in *tim* alternative splicing are independent of the circadian clock. Moreover, we could
507 reproduce them in *Drosophila* S2 cells either promoting endogenous *tim* expression using the
508 p-Act-C/k plasmid or utilizing splicing minigenes. The latter results strongly suggest that *tim*
509 intronic sequences themselves are the temperature sensor for these splicing changes.
510 The fact that despite being the most abundant RNA isoform, we could not detect TIM-M
511 suggests that this RNA is poorly (or not) translated. Alternatively, the protein could be quickly
512 degraded. However, the protein is fairly stable as we could detect large amounts of TIM-M
513 upon overexpression (Figure S8). Moreover, our luciferase reporter experiments suggest that
514 little protein is produced from this RNA, likely by regulation by miRNAs. It is also possible that
515 this RNA isoform is not even exported from the nucleus and nuclear/cytoplasmic fractionation
516 experiments could be useful to test this possibility. Regarding *tim-cold*, this isoform is strongly
517 regulated by miRNAs at all temperatures and was reported (Montelli et al., 2015) to display
518 weaker binding to CRY in yeast. Our results with the *tim-sc* null mutant provide support for a
519 specific (and even dominant) function of TIM-COLD. We observed that *tim-sc* mutants have
520 increased levels of *tim-cold* mRNA at 25°C. At this temperature these flies behave as if they

521 were at lower temperature (low daytime activity and advanced evening peak of activity).
522 Similar to *tim-cold*, *tim-sc* seems to work both at the RNA and protein levels. On one hand,
523 the increase in the production of *tim-sc* at 18°C helps regulate the amount of *tim-L* and *tim-*
524 *cold* produced at this temperature (Figure S8). In addition, overexpression of TIM-SC
525 advances the phase and shortens the period of the circadian clock, suggesting that expression
526 of this isoform might mediate some of the changes seen upon introduction of flies at 18°C. An
527 accompanying manuscript (Foley et al., Submitted) shows that the splicing factor *psi* regulates
528 *tim* alternative splicing in the same direction as when transferring the flies to colder
529 temperatures. However, *psi* expression and activity are not modulated by temperature,
530 suggesting that the temperature is sensed by a different system.

531 While *tim-cold* and *tim-M* are strongly post-transcriptionally regulated at all temperatures, the
532 RISC binds also stronger to *tim-L* at 18°C. Indeed, *tim* has been reported to be regulated by
533 miRNAs276a (Chen and Rosbash, 2016). Our miRNA-seq experiments suggest that miR-969
534 might be responsible for this temperature-specific regulation. However, it is possible that other
535 miRNAs are involved and/or that other RNA binding proteins enhance the use of some
536 miRNAs in a temperature-dependent way. CRISPR experiments targeting the miR-969 site
537 could help distinguish between these possibilities. The miRNA profiling and AGO-IP
538 experiments also suggest that other mRNAs might be regulated by miRNAs in a temperature-
539 dependent way. It will be really interesting to match those datasets in order to understand how
540 the transcriptional and post-transcriptional expression programs are regulated by temperature.
541 Despite the large number of RNAs and miRNAs that are regulated in a temperature-dependent
542 manner, the impact of temperature in alternative splicing seems to be quite restricted.
543 Although we performed only a superficial analysis of the data, only *tim*, *per* and *Hsf* show
544 evident temperature-dependent regulation of splicing (data not shown). Splicing is strongly
545 temperature dependent, at least *in vitro*. Flies might have mechanisms that make the outcome
546 of splicing temperature independent. This could be achieved by compensatory changes in
547 chromatin structure, RNA pol II elongation rate or RNA editing which is known to be altered
548 by temperature (Buchumenski et al., 2017). In addition, the specificity of the splicing strongly

549 suggests that the changes in *tim* splicing are unlikely to be due to the expression or activation
550 of a specific splicing factor. This possibility is supported by the findings reported in the
551 accompanying manuscript by Foley et al. showing that while *psi* regulates *tim* splicing, this
552 factor on its own cannot explain the temperature sensitivity of these alternative splicing events.
553 Importantly, our results demonstrate that the introns in isolation are still able to sense and
554 respond to the temperature changes (Figure 7). These results suggest that RNA structure
555 likely plays a key role in making this splicing events temperature sensitive. For example,
556 temperature could change the accessibility of *psi* binding sites in a temperature dependent
557 fashion. Interestingly, each temperature-driven splicing event might operate differently. For
558 example, production of *tim-sc* results from the competition between splicing and a cleavage
559 and polyadenylation event. On the other hand, production of *tim-M* and *tim-cold* depends on
560 an intron retention event. Interestingly, both introns have weak 3' splice sites but display
561 opposite temperature sensitivities, which suggest that their regulation depends on specific
562 structures and/or sequences and are not the results of general effects of temperature on
563 splicing.

564 We observed that flies that cannot generate *tim-sc* re-route *timeless* transcription towards the
565 other isoforms, mainly *tim-cold* (at both 18 and 25°C). These mutant flies also display altered
566 behavior. But what are the molecular actors responsible for these defects? *Tim-sc* mutant flies
567 display higher levels of *tim-cold* and *tim-L* at 25°C. It is difficult to rationalize how increased
568 levels of *tim-L* could trigger behavioral patterns observed in control flies at 18°C. We indeed
569 favor the possibility that the behavior observed in these flies at 25°C is due to increased
570 expression of *tim-cold* mRNA and protein. Previous work showed that TIM-COLD might act
571 differently than TIM-L (Montelli et al., 2015). Importantly, the increase in TIM-COLD protein in
572 the mutant flies might be significantly higher than the one on its RNA levels. This could happen
573 if for example, the increase in *tim-L* mRNA indirectly increases TIM-COLD levels by titrating
574 miR-969 or other miRNA targeting both to *tim-L* and *tim-cold* mRNAs. Additional CRISPR
575 mutants (i.e. abrogating *tim-cold* expression or making it constitutive) could help to completely
576 understand the mechanism behind this regulation.

577 Based on these and previous results we propose the following model in which miRNA-
578 mediated control imposes temperature-dependent thresholds for protein expression for the
579 different *tim* isoforms (Figure 8). At 25°C, *tim-L* and *tim-M* are produced. Both are moderately
580 regulated by miRNAs, due to sequences present in their last exon. *Tim-cold* production is low
581 at 25°C and the miRNA-mediated control is enough to abolish most (or all) protein expression
582 from this isoform at this temperature. In addition, *tim-M* is target of many additional miRNAs,
583 some of which are not differentially regulated by temperature, while others are higher at 29°C
584 or 18°C. Therefore, we predict that no TIM-M is produced at any temperature, and the
585 production of this isoform has a regulatory role (as recently suggested by Shakhmantir et al.
586 (2018)). When the temperature is decreased, the strong increase in *tim-cold* overcomes
587 miRNA-mediated repression and some TIM-COLD is produced. In addition, *tim-sc* RNA and
588 protein are produced. We hypothesize that both TIM-COLD and TIM-SC contribute to the
589 phase advance and lower night activity observed at 18°C. TIM-SC lacks the cytoplasmic
590 retention domain and increased levels of this protein might lead to the phase advance
591 observed at 18°C. At 29°C, *Tim-M* is increased but TIM amounts are maintained constant
592 likely due to higher production rates of TIM (due to a more modest association of *tim-L* with
593 AGO1). In this way, *tim* co and post-transcriptional regulation can modulate the amounts and
594 type of TIM proteins to facilitate temperature adaptation. It is possible that these changes in
595 alternative splicing could regulate other situations in which the circadian clock requires
596 adjustments of TIM levels and/or activity independently of CLK-driven transcription. These
597 could include entrainment by temperature or light as well as cell to cell synchronization.
598 Moreover, as *tim* is being alternatively spliced, it is even possible that some of these protein
599 isoforms could be used by the cell as temperature sensors.
600 In sum, in this study we determine that *tim* is extensively regulated by alternative splicing in a
601 temperature dependent way. Moreover, we show that this regulation is, at least partially,
602 responsible for temperature adaptation. We propose that this complex regulation of alternative
603 splicing and miRNA-mediated control provides a mechanism for altering the relationship
604 between TIM and PER proteins. This would allow the modulation of the circadian phase

605 without affecting the period (which is strongly dependent on CLK and PER protein levels and
606 activity). Last but not least, our data suggest that *tim* alternative splicing might act as a
607 thermometer for the cell and might regulate temperature responses not related to the circadian
608 clock.

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637 and Budgeting Committee in Israel (1796/12). All the RNA-seq data has been submitted to
638 GEO (entries GSE124134, 124135, 124141, 123142, 124200 and 124201).

639

640 **AUTHOR CONTRIBUTIONS:**

641 NE generated the plasmids, performed the cell culture experiments and generate the CRISPR
642 flies. AMA performed the behavioral analysis as well as the qRT-PCRs, analyzed the data,
643 generated the figures and wrote the manuscript. OB generated the RNA-seq libraries, AGO-
644 IP experiments and western blots. ILP performed the computational analysis. RW performed
645 the behavioral experiments in the non-melanogaster *Drosophila* species. SK designed the
646 experiments and wrote the manuscript.

647

648 **DECLARATION OF INTEREST:**

649 The authors declare no competing interests.

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661 **Materials and Methods**

662 **Fly husbandry**

663 *Drosophila yakuba*, *Drosophila simulans* and *Drosophila virilis* were obtained from the
664 Drosophila Species Stock Center (DSSC) at the University of California, San Diego. CantonS
665 flies were used as wild type strain for *Drosophila melanogaster*. Additionally, *tim*⁰ and *per*⁰¹
666 strains have been described previously in (Konopka and Benzer, 1971; Sehgal et al., 1994).
667 Transgenic lines for the overexpression of different FLAG-tagged *tim* isoforms or luciferase
668 fused to their 3'UTRs were generated by injecting the plasmids (described below) in a site-
669 specific manner into the pattP2 site using the PhiC31 integrase-mediated transgenesis system
670 (Best Gene Drosophila Embryo Injection Services). These transgenic flies were crossed to
671 *tim-Gal4* driver (Blau and Young, 1999; Kaneko and Hall, 2000).

672 All crosses were performed and raised at 25°C. Newborn adults were either maintained at
673 25°C or switched to colder (18°C) or warmer (29°C) temperatures as described in the text.

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675 **Generation of *tim-sc* polyadenylation and cleavage signal mutants by CRISPR**

676 The *tim-sc* mutant flies were generated following the protocol from Ge et al. (2016) with some
677 modifications. pCFD5 plasmid (Addgene, plasmid #73914) was modified to exclude *Vermilion*
678 and *attB* (pCFD5d). 3 gRNAs (one targeting *w* gene and two, *tim*) were generated from PCR
679 templates and cloned into pCFD5d (pCFD5dw/*tim*-1,2) as described in (Fu et al., 2014; Port
680 and Bullock, 2016; Port et al., 2014). The donor template for homologous recombination
681 contained a point mutation in an intronic sequence as well as a silent point mutation (one in
682 each site targeted by the gRNAs). This fragment was then cloned into pUC57-*white*[coffee]
683 (Addgene, plasmid #84006) between the *SacI* and *HindIII* sites (pUC57-*timShort*).

684 pCFD5dw/*tim*-1,2, pUC57-*white*[coffee] and pUC57-*timShort* were injected into *vas-Cas9* (y^1 ,
685 *M*(Ferguson et al.)ZH-2A) flies (Ge et al., 2016) by Rainbow Transgenic Flies, Inc. (Camarillo,
686 CA). G0 flies were crossed to second chromosome balancers and individual G1 Cyo flies from
687 non-red-eyed populations were again mated to the 2nd chr balancers. Individual G1 flies were

688 subsequently genotyped to verify the deletion and the possibility of random integration either
689 in the genome or at CAS9 cutting sites of the plasmid was assessed as described in (Ge et
690 al., 2016). G2 *cyo* male and female flies from positive crosses were crossed to obtain non *cyo*
691 homozygous stocks. Finally, the entire *tim* locus was sequenced in the *tim-sc* mutants and
692 their isogenic controls to verify that there were no additional mutations in the *tim* locus.

693

694 **Plasmids for S2 cells transfection and/or injection into *Drosophila***

695 pMT-C/*k* was previously described in (Weiss et al., 2014) and p-Act-Gal4 (Addgene, plasmid
696 #24344).

697 **C-terminus FLAG-tagged UAS-*tim* overexpression plasmids**

698 cDNA from Canton-S heads of flies reared at 18 (for *tim-sc* and *tim-cold*), 25 (*tim-L*) or 29°C
699 (*tim-M*) were used to amplify each isoform. NotI and KpnI restriction sites and a FLAG-tag
700 sequence (at the 3' of the coding DNA but before the stop codon) were added by PCR. Each
701 isoform was finally cloned into the pUASTattB vector between the NotI and KpnI sites.

702 **UAS-luciferase-3'UTR of each *tim* isoform plasmids**

703 A PCR product containing the *Firefly Luciferase* gene was cloned into pUASTattB (pUAST-
704 *luc*-attB). The 3' UTRs of all four isoforms were amplified from fly head cDNA and cloned into
705 the pUAST-*luc*-attB vector between NotI and Xhol sites.

706 ***tim* exon-intron-exon minigenes**

707 A plasmid containing exon5-intron5-exon6 (*tim-control*), exon10-intron10-exon11 (*tim-sc*),
708 exon13-intron13-exon14 (*tim-M*) and exon16-intron16-exon17 (*tim-cold*) fragments defined by
709 KpnI and Xhol restriction sites was ordered from Syntezza Bioscience Ltd. This plasmid was
710 then cut with KpnI and Xhol. The four fragments were extracted and cloned into pMT-V5
711 (Invitrogen).

712

713 **Cell culture and transfections**

714 *Drosophila* Schneider-2 (S2) cells were cultured in 10% fetal bovine serum (Invitrogen) insect
715 tissue culture medium (Biological industries) as in (Weiss et al., 2014). The experiments were
716 performed at 18°C or 25°C, as described in the text.

717

718 **Luciferase activity assay**

719 Luciferase activity from S2 cells was measured using the Dual Luciferase Assay Kit (Promega)
720 following the manufacturer's instructions as in (Krishnan et al., 2001; Weiss et al., 2014).
721 Luciferase measurements from fly wings was performed as previously described (Plautz and
722 Kay, 1999).

723

724 **RNA libraries preparation for RNA-seq**

725 **DGE (3'end-seq)**

726 Flies were entrained for at least 3 days at 18°C, 25°C or 29°C and collected at 6 different
727 timepoints (ZT3, ZT7, ZT11, ZT15, ZT19 and ZT23). RNA from the fly heads was extracted
728 using TRIzol reagent (SIGMA). 3'-seq libraries were prepared as previously described (Shishki
729 et al., Afik et al., 2017). These data have been deposited at NCBI GEO as XXX.

730 **Total RNA-seq**

731 RNA from fly heads collected at two or three different timepoints (ZT 3, 15 and 21 for 18C and
732 29C and ZT3 and 15 for 25C) was extracted using Trizol and used to generate polyA+ RNA-
733 seq libraries. The library preparation procedure was modified from (Engreitz et al., 2013) as
734 follows: 0.5µg of total RNA was polyA+ selected (using Oligo(dT) beads, Invitrogen),
735 fragmented in FastAP buffer (Thermo Scientific) for 3min at 940 C, then dephosphorylated
736 with FastAP, cleaned (using SPRI beads, Agencourt) and ligated to a linker1
737 (5Phos/XXXXXXXXXAGATCGGAAGAGCGTCGTGTAG/3ddC/, where XXXXXXXX is an
738 internal barcode specific for each sample), using T4 RNA ligase I (NEB). Ligated RNA was
739 cleaned-up with Silane beads (Dynabeads MyOne, Life Technologies) and pooled into a single
740 tube. RT was then performed for the pooled sample, with a specific primer (5'-
741 CCTACACGACGCTCTTCC-3') using AffinityScript Multiple Temperature cDNA Synthesis Kit

742 (Agilent Technologies). Then, RNA-DNA hybrids were degraded by incubating the RT mixture
743 with 10% 1M NaOH (e.g. 2ul to 20ul of RT mixture) at 700 C for 12 minutes. pH was then
744 normalized by addition of corresponding amount of 0.5M AcOH (e.g. 4ul for 22 ul of NaOH+RT
745 mixture). The reaction mixture was cleaned up using Silane beads and second ligation was
746 performed, where 3'end of cDNA was ligated to linker2
747 (5Phos/AGATCGGAAGAGCACACGTCTG/3ddC/) using T4 RNA ligase I. The sequences of
748 linker1 and linker2 are partially complementary to the standard Illumina read1 and
749 read2/barcode adapters, respectively. Reaction Mixture was cleaned up (Silane beads) and
750 PCR enrichment was set up using enrichment primers 1 and 2:
751 (5'AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGA
752 TCT-3', 5'-CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGTTCAGAC
753 GTGTGCTCTCCGATCT-3', where XXXXXX is barcode sequence) and Phusion HF
754 MasterMix (NEB). 12 cycles of enrichment were performed. Libraries were cleaned with 0.7X
755 volume of SPRI beads. Libraries were characterization by Tapestation. RNA was sequenced
756 as paired-end samples, in a NextSeq 500 sequencer (Illumina).

757

758 **AGO1-IP followed by small RNA sequencing**

759 The samples were collected at 18°C, 25°C or 29°C at four different timepoints (ZT3, ZT9, ZT15
760 and ZT21). AGO1 immunoprecipitation was performed as previously described (Kadener et
761 al., 2009; Lerner et al., 2015). The small RNA libraries were constructed using NEBNext®
762 Small RNA Library Prep Set for Illumina.

763

764 **AGO1-IP followed by Oligonucleotide microarrays**

765 The samples were collected at 18°C, 25°C or 29°C. AGO1 immunoprecipitation was
766 performed as previously described (Kadener et al., 2009; Lerner et al., 2015). INPUT and
767 AGO IP Total RNA was extracted from fly heads using TRI reagent (Sigma) according to
768 manufacturer's protocol. cDNA synthesis was carried out as described in the Expression
769 Analysis Technical Manual (Affymetrix). The cRNA reactions were carried out using the IVT

770 kit (Affymetrix). Affymetrix high-density arrays for *Drosophila melanogaster* version 2.0 were
771 probed, hybridized, stained, and washed according to the manufacturer's protocol.

772

773 **Microarray Analysis:**

774 The R Bioconductor affy package (<http://www.bioconductor.org>) was used to normalize and
775 calculate summary values from Affymetrix CEL files using gcRMA (Bioconductor). Gene
776 ontology enrichment analysis was performed as described in Mezan et al. (2013). To
777 determine AGO-IP enrichment ranking, we ranked the residuals from a linear model was
778 between the normalized reads in the IP and the INPUT.

779

780 **RNA seq analysis:**

781 The total RNA sequencing reads were aligned to the *Drosophila melanogaster* genome
782 version dm3. For the different species, alternative splicing proportion was calculated manually
783 searching the exon-exon junction.

784 For the small RNA sequencing, the reads were processed using miRExpress pipeline (Wang
785 et al., 2009) using miRBase 21 version. The different circadian timepoints were considered
786 independent replicates (n=4) and the differential gene expression analysis was done using a
787 negative binomial model using DeSeq2 package on R. A miRNA with p adjusted value less
788 than 0.05 and an absolute log2(fold change) more than 1 was considered differentially
789 expressed.

790 For the DGE data, the circadian analysis was performed using the package MetaCycle (Wu
791 et al., 2016). For each temperature and circadian timepoint, two replicates were analyzed
792 (n=2). To normalize over different library preparation, after normalizing by library size the
793 counts were divided by the maximum in each replicate. Genes with more than two zero counts
794 in any timepoint was discarded from further analysis. The amplitude for each replicate was
795 then calculated as the maximum divided the minimum for each gene. JTK algorithm was used
796 for the circadian analysis. A gene was considered as cycling if the JTK pvalue was less than

797 0.05 and the amplitude was more than 1.5. For these genes, the phase shift was calculated
798 as the phase at 25°C minus the phase at 18°C or 29°C.

799

800 **TargetScan analysis of putative miRNA binding to each *tim* 3'UTR**

801 As not all of the 3'UTRs for the different *tim* isoforms are annotated, TargetScanFly version
802 6.1 was run locally (which allows manual entry of the sequences of interest). UTR sequences
803 were downloaded from UCSC dm6 27way conservation. This way we identified several
804 putative miRNAs binding to each of the isoforms. We continued by manually looking at the
805 abundance of these miRNAs (from the AGO1-IP followed by smallRNA sequencing) in order to
806 determine which of those putative miRNAs are being expressed in the fly heads. Additionally,
807 we defined as miRNAs that change in a temperature-dependent manner those miRNAs that
808 had more than 2-fold change between 18 and 29°C and in which this difference was
809 statistically significant (pval<0.05).

810

811 **Chromatin-bound (nascent) RNA**

812 Performed as described in (Lerner et al., 2015).

813

814 **Real Time PCR analysis**

815 Total RNA was extracted from adult fly heads (or brains) at the mentioned timepoints using
816 TRI Reagent (Sigma) and treated with DNase I (NEB) following the manufacturer's protocol.
817 cDNA was synthesized from this RNA (using iScript and oligodT primers, Bio-Rad) and diluted
818 1:60 prior to performing the quantitative real-time PCR using SYBR green (Bio-Rad) in a
819 C1000 Thermal Cycler Bio-Rad. Primers used for amplifying each isoform were: *tim*-sc (5'-
820 AACACAACCAGGAGCATAC -3' and 5'- ATGGTCCACAAATGTTAAAA -3'), *tim*-M (5'-
821 GGAGACAATGTACGGACTC -3' and 5'- ATTCACACAGAGAGAGAGC -3'), *tim*-cold (5'-
822 GCATCTGTGTACGAAAAGGA -3' and 5'- ATGTAACCTATGTGCGACTC -3'), *tim*-L and *tim*-
823 M (5'- CTCCATGAAGTCCTCGTTC -3' and 5'- TGTCGCTGTTAATTCCCTTC -3') and

824 junction between exons 5-6 which are present in all isoforms (5'-
825 AAAAGCAGCCTCATCAACAT -3' and 5'- AGATAGCTGTAACCCTTGAG -3').
826 The PCR mixture was subjected to 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s,
827 55 °C for 10 s and 72 °C for 30 s followed by a melting curve analysis. Fluorescence intensities
828 were plotted versus the number of cycles by using an algorithm provided by the manufacturer
829 (CFX Maestro Software, Bio-Rad). The results were normalized against *rp49* (5'-
830 TACAGGCCAAGATCGTGAA -3' and 5'- CCATTGTGCGACAGCTTAG -3') and *tub* (5'-
831 TGCTCACGAAAAGCTCTCCT -3' and 5'- CACACACGCCTATCAGCAA -3') levels.

832

833 **Western blotting**

834 Protein was extracted from fly heads and WB performed as in (Weiss et al., 2014). Antibodies
835 used: rat anti-TIM (1:30,000, a kind gift from Michael Rosbash), mouse anti-FLAG-M2
836 (1:20,000; Sigma F3165) and mouse anti-Tub (1:20,000, DM1A, SIGMA). Quantifications
837 were done utilizing Image J software.

838

839 **Assessment of the locomotor behavior**

840 Male flies were placed into a glass tube containing 2% agarose and 5% sucrose food and their
841 activity was monitored using Trikinetics *Drosophila* Activity Monitors (Waltham, MA, USA).
842 Flies were maintained for 4 days in 12:12 Light: Dark cycles (LD) and 5 days in constant
843 darkness (DD). The experiments were performed at 18, 25 or 29°C, as indicated. Analyses
844 were performed with a signal processing toolbox (Levine et al., 2002).

845 All the activity assessments were done in LD. For the calculation of the beginning of the
846 evening peak, the activity of flies were analyzed individually on 30-minutes intervals. The
847 criteria were: (1) have a trend of increasing activity, (2) no more than 1/3 of the bins can be
848 outliers and (3) a reduction of less than 10% of the activity was not counted as an outlier. Only
849 flies in which an evening peak was clearly distinguishable were included in this analysis.

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1097 **Figure Legends**

1098 **Figure 1. Temperature remodels the circadian transcriptome.** **A.** Heat maps of normalized
1099 gene expression for genes that display 24hs cycling expression at different temperatures. Flies
1100 were entrained at the indicated temperatures for 3 days in 12:12 Light: Dark (LD) conditions.
1101 Fly heads were collected every 4 hours in 12 independent timepoints. Cycling expression was
1102 assessed as indicated in material and methods. **B.** Venn diagrams indicating the number of
1103 oscillating genes at different temperatures. **C.** Density plot showing the phases of core clock
1104 genes cycling at both 18°C and 25°C. The blue and grey shades indicate the phase of these
1105 genes at 18°C and 25°C respectively. **D.** Total expression of the indicated clock genes in the
1106 microarray for each temperature. Only *per* shows a significant difference between 18°C and
1107 29°C.

1108

1109 **Figure 2. Temperature modulates *tim* alternative splicing.** **A.** IGV snapshot of the *timeless*
1110 locus region indicating the expression of this gene at 18°C (blue), 25°C (black) and 29°C (red).
1111 The presented data includes the aggregated data from the 3'-seq datasets presented in Figure
1112 1 (upper traces) as well as full transcript polyA⁺ RNA seq datasets (lower traces). The latter
1113 includes 2 timepoints at 25°C and three timepoints at 18°C and 29°C (see text for details). The
1114 arrows indicate the alternative splicing events that are regulated by temperature. **B.** A scheme
1115 of the alternatively spliced *tim* isoforms. In grey are constitutive exons, in red sequences found
1116 mainly at high temperatures and in blue, sequences found mainly at low temperatures. A zoom
1117 on the exons surrounding each non-canonical isoform is represented in the rectangle.
1118 Alternative stop codons (STOP) and cleavage and polyadenylation sites (PAS) in these
1119 isoforms are also indicated. **C.** Quantification of the relative amount of *tim-sc* (dark blue), *tim-*
1120 *cold* (light blue), *tim-M* (red) and *tim-L* (grey) isoforms at the three assayed temperatures. To
1121 quantify the different isoforms we counted the relative number of spliced junctions specific for
1122 each isoform. **D.** Quantification of the relative amount of *per-spliced* (blue) and *per-unspliced*
1123 (red) RNAs at the three assayed temperatures performed as in C.

1124

1125 **Figure 3. Temperature-specific alternative splicing is conserved across different**
1126 **Drosophila species.** **A.** Quantification of the ratio between day and night activity counts of
1127 locomotor activity for *D. melanogaster*, *D. simulans*, *D. yakuba* and *D. virillis* at 18°C (blue),
1128 25°C (grey) or 29°C (red). Each bar represents the ratio between the mean locomotor activities
1129 (light and dark) of all four days (N~32). Significance was calculated performing multiple t-test
1130 analysis using the Holm-Sidak method. (*, pval<0.05; **, pval<0.01 ***, pval<0.001; ****,
1131 pval<0.0001). **B.** Quantification of the relative amount of *tim-sc* (dark blue), *tim-cold* (light
1132 blue), *tim-M* (red) and *tim-L* (grey) from whole transcriptome (polyA⁺) mRNA-seq in the species
1133 at the indicated temperatures. The ratios between the isoforms were calculated as indicated
1134 in Figure 2 and explained in the methods section.

1135

1136 **Figure 4. Alternatively spliced isoforms of *timeless* are post-transcriptionally regulated.**
1137 **A.** *Tim* mRNA is bound to AGO-1 in a temperature dependent way. The graph represents the
1138 ranking of enrichment of RNAs in AGO-1 immunoprecipitates isolated from flies entrained at
1139 18°C (blue), 25°C (grey) or 29°C (red). Briefly, we ranked RNAs according to their binding
1140 enrichment (lower ranking value corresponds to higher binding to AGO-1). To assess the
1141 ranking, we averaged the residuals in a linear model: signal in AGO1-
1142 immunoprecipitate/signal in input (n=2 for each temperature). **B.** *tim* isoform-specific qPCR of
1143 AGO1-bound mRNAs at 18°C (blue), 25°C (grey) or 29°C (red). Each bar represents an
1144 average of all time points normalized to a negative control (cyc). Blue starts represent
1145 statistical significance to 18°C of the indicated isoform and temperature determined
1146 performing multiple t-test analysis using the Holm-Sidak method. (*, pval<0.05; **,
1147 pval<0.001; ****, pval<0.0001). Four different timepoints in each temperature were used as
1148 biological replicas. **C.** Luciferase assay of the wings of flies expressing *luciferase* fused to
1149 different *tim* 3'UTRs. We utilized the GAL4-UAS system to express the luciferase reporter. In
1150 all cases we utilized the *tim-gal* driver. All the UAS-Luciferase lines were inserted in the same
1151 genomic location using the Attb system. Blue and grey starts represent statistical significance
1152 to *tim-sc* or *tim-L*, respectively, determined by performing multiple t-test analysis using the

1153 Holm-Sidak method. (*, pval<0.05; **, pval<0.001; ****, pval<0.0001. n=3). **D.** Summary table
1154 of the number of miRNAs binding identified in the different *tim* isoforms using TargetScan (Left
1155 column). **E.** Volcano plot showing differentially expressed miRNAs between 18°C and 29°C
1156 (n = 4 for each temperature). The color code represents the significance for each miRNA
1157 differentiating all the combinations for fold change > 2 (FC>2) and padj < 0.05. miRNAs with
1158 predicted binding sites present in any of the *tim* isoforms 3'UTR were identified using
1159 TargetScanFly.6.2 and are labelled in the graph (blue, grey or red for being putative targets
1160 for *tim-cold*, common sequence or *tim-M* 3'UTRs, respectively). miRNA abundance was
1161 assessed by small-RNAseq from AGO1 immunoprecipitates. **F.** Relative abundance of
1162 miRNAs that are differentially expressed (FC>2 and padj < 0.05) at different temperatures and
1163 that are predicted to target the 3' UTR of the different isoforms. For each miRNA, the values
1164 have been normalized to the minimum and their relative expression (RE) is represented.
1165 miRNA abundance was assessed from small-RNAseq from AGO1 immunoprecipitations.

1166

1167 **Figure 5. Over-expression of *tim* isoforms at 25°C results in various behavioral defects.**

1168 **A.** Actograms of flies of the indicated genotypes in LD12:12 at 25°C. In all cases we over-
1169 expressed the indicated isoform utilizing the *tim-gal4* driver. Light phase is represented in
1170 white and dark-phase in grey. **B.** Quantification of the start of the evening peak in the last 3
1171 days of LD. Stars represent statistical significance to *tim-Gal4* control calculated by one-way
1172 ANOVA. (*, pval<0.05; **, pval<0.01 ***, pval<0.001; ****, pval<0.0001. n=27-31). **C.** Summary
1173 table of the behavior in DD indicating % of rhythmicity, period length, power and their SEM.

1174

1175 **Figure 6. *Tim-sc* null flies present behavioral and molecular defects. A.** Schematic
1176 representation of the mutation performed by CRISPR used to generate *tim-sc* null flies (40A
1177 flies). The black and green arrow represent the location of the forward and reverse primers
1178 used for the verification of *tim-sc* depletion. **B.** Levels of *tim-sc* after the genetic manipulation
1179 depicted in A. The displayed qPCR result was obtained using RNA obtained from heads of
1180 flies entrained at 18°C. (n=5). **C.** Average actograms of control (left) and 40A (right) flies at

1181 18°C (blue), 25°C (grey) and 29°C (red) in LD12:12 cycles. (N=23-32). **D.** Ratio between total
1182 day and night-time activity at 18°C (blue), 25°C (grey) and 29°C (red) in 12:12LD. Blue and
1183 red stars represent statistical significance between flies kept at 18°C or 29°C and those at
1184 25°C, while black ones represent differences to the control flies. Multiple t-test analysis using
1185 the Holm-Sidak method. (**, pval<0.01 ***, pval<0.001. n=16-31). **E.** Quantification of the start
1186 of the evening peak in day 3 of LD. Blue and red stars represent statistical significance
1187 between flies kept at 18°C or 29°C and those at 25°C, while black ones represent differences
1188 to the control flies. Multiple t-test analysis using the Holm-Sidak method. (**, pval<0.01 ***,
1189 pval<0.001. n=16-31). **F.** Assessment by qPCR of exons5-6 (common in all *tim* isoforms,
1190 left) *tim-cold* (middle left), *tim-L* + *tim-M* (middle right) and *tim-M* transcription (right) at 18°C
1191 (blue), 25°C (grey) and 29°C (red) at ZT15 in 12:12LD. Multiple t-test analysis using the Holm-
1192 Sidak method. (**, pval<0.01 ***, pval<0.001. n=5).

1193

1194 **Figure 7. Splicing of *timeless* is temperature dependent and clock independent. A.**
1195 qPCR results from entrained WT as well as *tim*⁰¹ and *per*⁰¹ clock mutant flies at 18°C or at
1196 29°C. Each bar represents the ratio between the unspliced and splice variant of *tim-sc* (dark
1197 blue), *tim-cold* (light blue), *tim-m* (red) and *tim* control (grey). **B.** *Drosophila* S2 cells
1198 transfected with pMT-Clk display the same *tim* alternative splicing patterns observed in living
1199 flies at 18°C (blue) or at 25°C (grey). Each bar represents the ratio between the unspliced and
1200 splice variant of *tim-sc*, *tim-cold*, *tim-m* and *tim* control. Multiple t-test analysis using the Holm-
1201 Sidak method. (**, pval<0.01 ***, pval<0.001. n=3). **C.** Scheme of the different *tim* isoform
1202 minigenes utilized in (D) and location of the primers used to measure the spliced (top) and
1203 unspliced (bottom) of each intron. **D.** Unspliced/spliced ratios for the indicated minigenes at
1204 18°C (blue) or at 25°C (grey). Multiple t-test analysis using the Holm-Sidak method. (**,
1205 pval<0.01 ***, pval<0.001. n=3).

1206

1207 **Figure 8. Model of the effects of thermosensitive alternative splicing of *timeless* in**
1208 **sensing and mediating temperature adaptation. A.** Temperature affects differently each

1209 one of the four *tim* isoforms (*tim-sc*, *tim-cold*, *tim-M* and *tim-L*, represented in dark and light
1210 blue, red and grey respectively). While *tim-sc* and *tim-cold* are higher in colder temperatures,
1211 *tim-M* is only expressed at the highest temperatures. Nevertheless, transcription levels of the
1212 canonical isoform, *tim-L*, do not change. **B.** Temperature regulates expression of miRNAs,
1213 which set a threshold to the amount of mRNA that gets translated from each isoform. *tim-sc*
1214 has the lowest and *tim-M* the highest thresholds in all temperatures. *tim-cold*'s has the biggest
1215 temperature-dependent variation. **C.** These differences between the transcript levels and their
1216 thresholds results in changes in TIM expression as well as **D.** behavioral changes in the phase
1217 and day/night ratios that are linked to temperature adaptation.

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1237 **Supplementary Figure Legends:**

1238 **Figure S1. Density plot showing the phases of core clock genes cycling at both 29°C**
1239 **and 25°C.** The red and grey shades indicate the phase of these genes at 29°C and 25°C
1240 respectively.

1241

1242 **Figure S2. *Tim* transcription is advanced at 18°C compared to 25°C and 29°C. A.** q-RT
1243 PCR results for *tim* mRNA, top, or chromatin-bound (nascent RNA) *tim*, bottom, levels on
1244 entrained WT fly heads at ZT3, ZT7, ZT11, ZT15, ZT19 and ZT23 (consecutive bars) at 18°C
1245 (blue), 25°C (grey) or 29°C (red). **B.** Same as in (A) but for *per*.

1246

1247 **Figure S3. Temperature regulates gene expression. A.** Heat map showing an
1248 unsupervised clustering of differentially expressed genes from a Microarray experiment
1249 performed on RNA extracted from entrained fly heads at three temperatures. Each lane
1250 represents a mix of all time points at a given temperature. **B.** A pie chart demonstrating
1251 enriched GO terms of the differentially expressed genes.

1252

1253 **Figure S4. Relative expression of *timRA* and *timRC* in the two DGE described in Figure 1.**
1254 *tim-RA* reports expression levels for *tim-L*, *tim-cold* and *tim-M*, as they share the 3' end. For
1255 Each DGE dataset, we averaged the expression of the different *tim* isoforms over the day at
1256 each temperature. Results are shown normalized to 25°C.

1257

1258 **Figure S5. *Tim* expression is regulated by temperature co and post-transcriptionally.**
1259 **A.** Isoform-specific q-PCR of *timeless* in the heads of flies entrained at 18°C (blue) and 29°C
1260 (red). Each bar represents a different time point (ZT3, ZT9, ZT15 and ZT21). Values have
1261 been normalized to the maximum of both temperatures. **B.** Quantification of the different *tim*
1262 isoforms in the adult brain at ZT11 after entraining the flies at 18°C (blue), 25°C (grey) and
1263 29°C (red). Black stars represent statistical significance between flies kept at 18°C and those

1264 at 25°C. Multiple t-test analysis using the Holm-Sidak method. **, pval<0.01 ***, pval<0.001.

1265 n=16-31. n=3.

1266

1267 **Figure S6. Temperature-specific alternative splicing is conserved across different**

1268 **Drosophila species.** **A.** IGV snapshot of whole-transcript RNA-seq showing the *tim* locus in
1269 three additional species (*D. simulans*, *D. Yakuba* and *D. virilis*, top to bottom) at different
1270 temperatures. The upper panel (blue) corresponds to 18°C, the middle one (black) to 25°C
1271 and the lower one to 29°C. Arrows indicate the different areas unique to the different isoforms:
1272 *tim-sc* (light blue), *tim-M* (red) and *tim-cold* (blue). Green arrow below the IGV snapshots
1273 represent the direction of *tim* transcription. **B.** Quantification of the relative amount of per-
1274 spliced (light blue), *per*-unspliced (red) and *tim-L* (grey) from whole transcriptome (polyA⁺)
1275 mRNA-seq in all species at the indicated temperatures. The ratios between the isoforms were
1276 calculated as indicated in Figure 2 and explained in the methods section.

1277

1278 **Figure S7. Density plot representing 4000 genes, selected as ‘AGO1-
1279 enriched/associated’.** The distributions represent the frequency of each fold enrichment for
1280 a particular temperature.

1281

1282 **Figure S8. Overexpression of different *tim* isoforms with FLAG-tagged C-terminus. A.**
1283 Schematic representation of TIM protein. The starts mark the location of the different FLAG-
1284 tags for each isoform. NLS: Nuclear Localization Signal. CLD: Cytoplasmic Localization
1285 Domain. **B.** Western blot membranes showing anti-FLAG and anti-TIM staining of the over-
1286 expression of the four 3' FLAG-tagged UAS-*tim* isoforms in S2 cells. Arrows mark a
1287 degradation product ~ 60kDa smaller than the original TIM isoform.

1288

1289 **Figure S9. Total activity counts in control and 40A mutant flies at 18°C (blue), 25°C
1290 (grey) and 29°C(red) in LD12:12 during the day (left) and night (right).** Stars represent

1291 significant differences between the control and mutants from the same temperature. Multiple
1292 t-test analysis using the Holm-Sidak method. **, pval<0.01 ***, pval<0.001. N=23-32.

1293

1294 **Figure S10. Summary table of the 3' and 5' splicing strengths of each *tim* intron for**
1295 ***Drosophila melanogaster*, *Drosophila simulans* and *Drosophila virilis*.**

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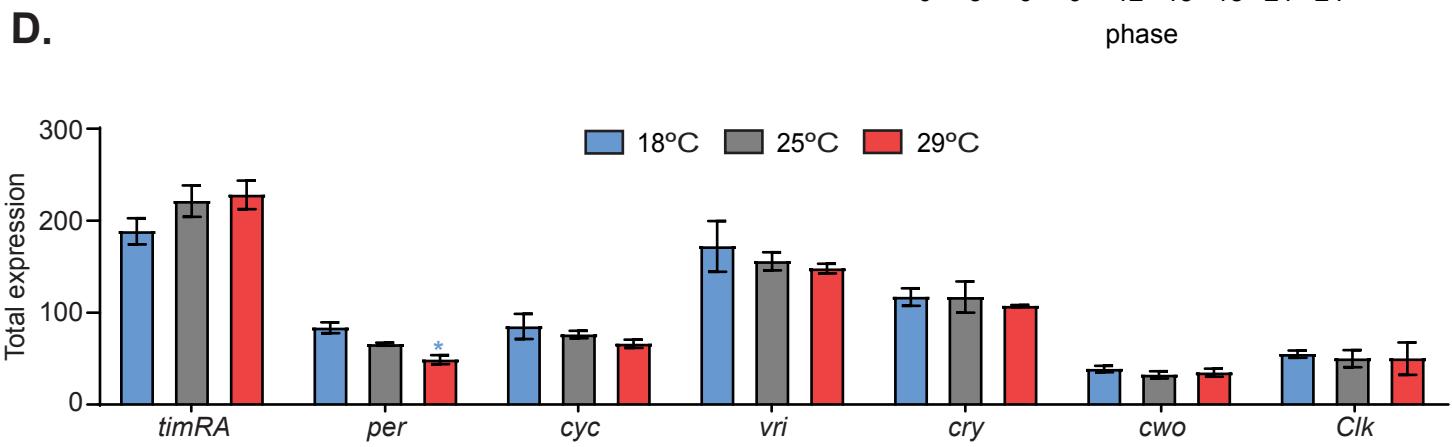
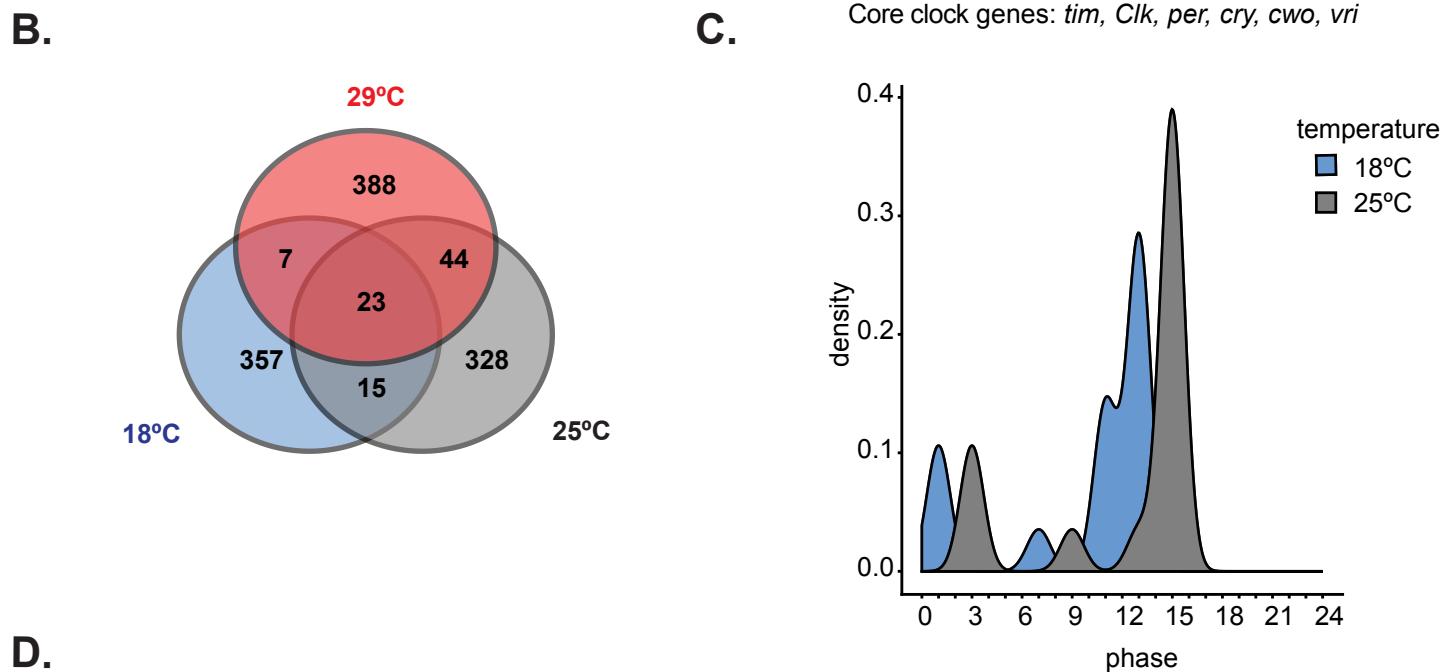
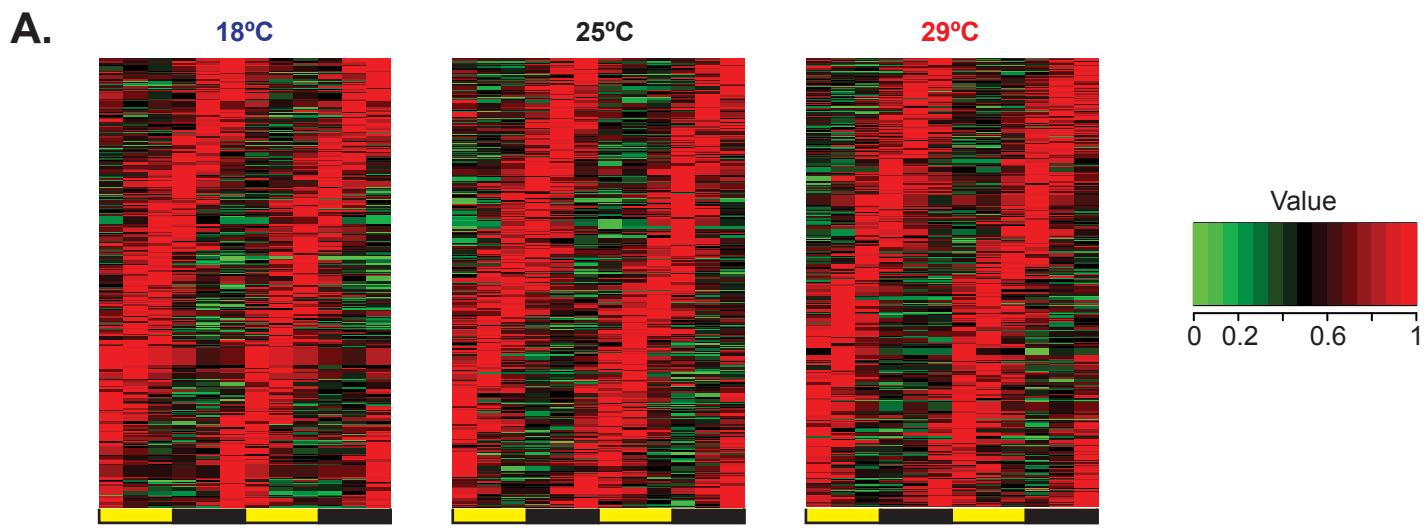


Figure 1

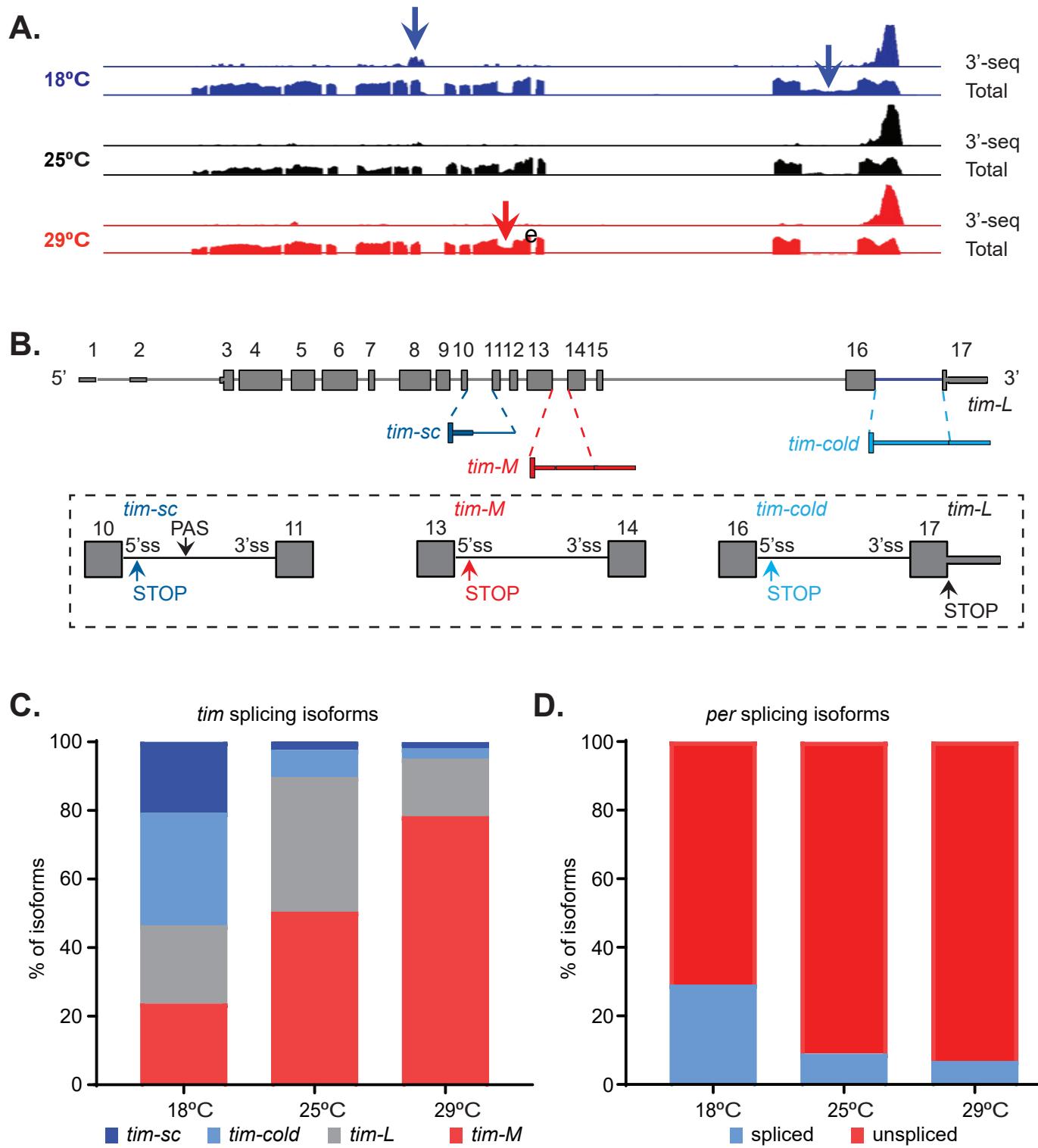
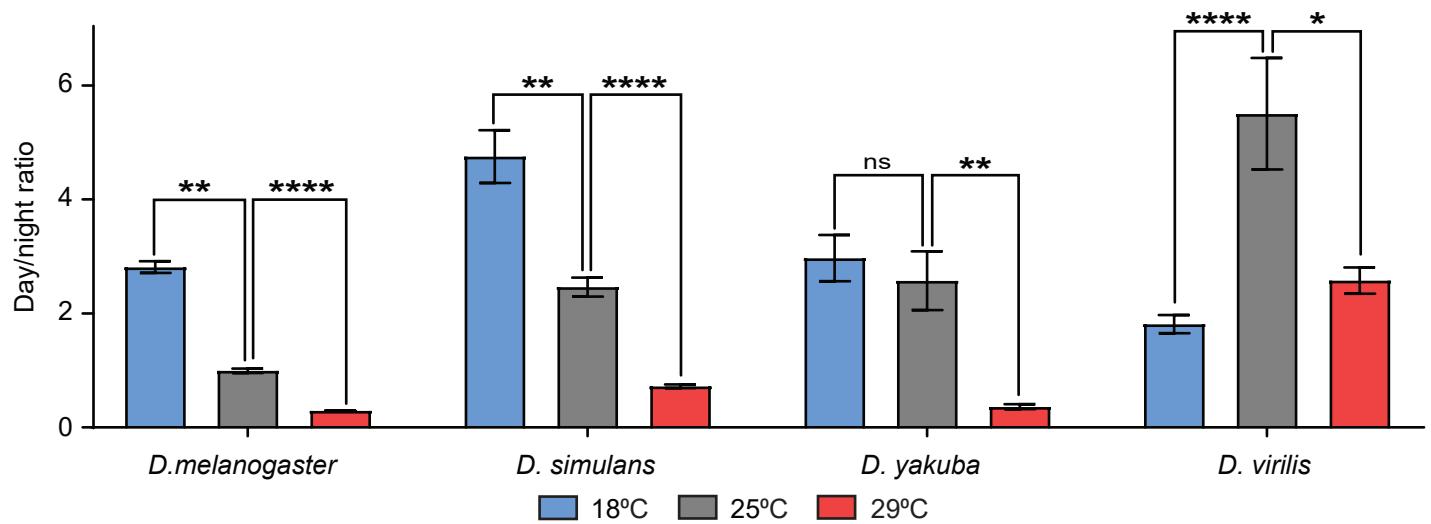
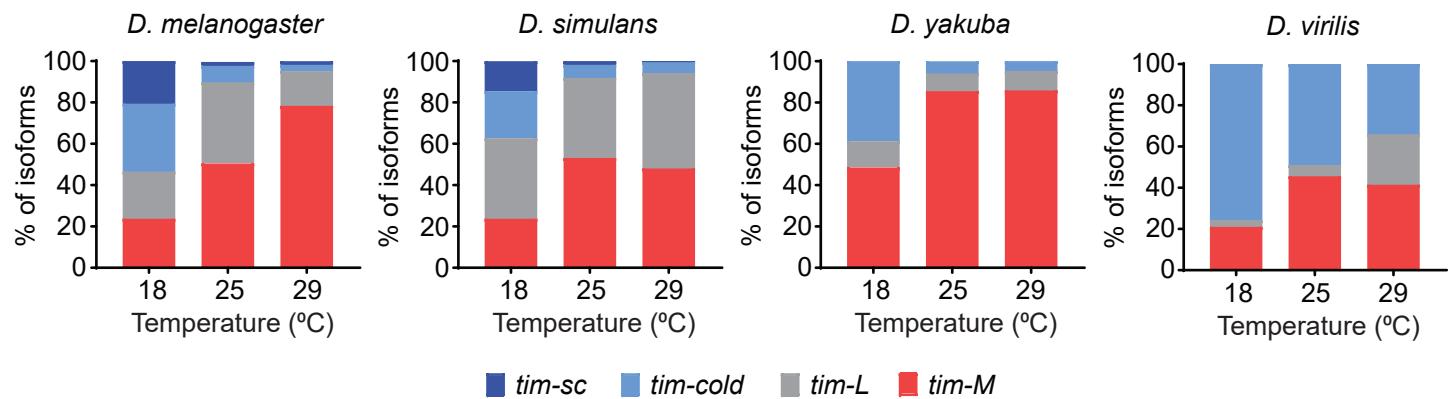
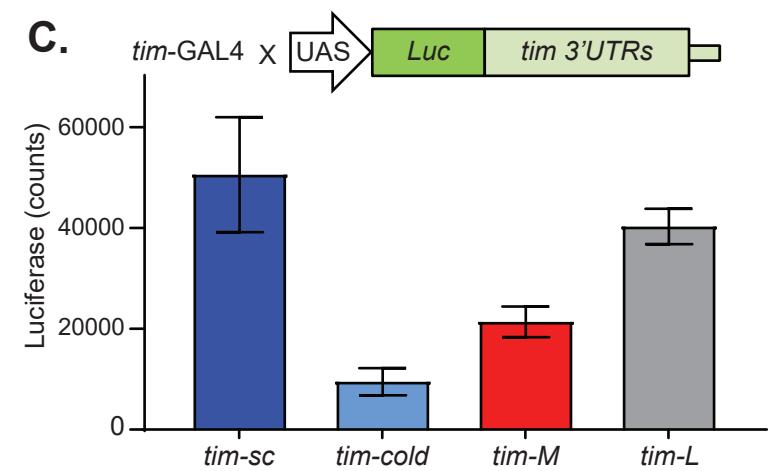
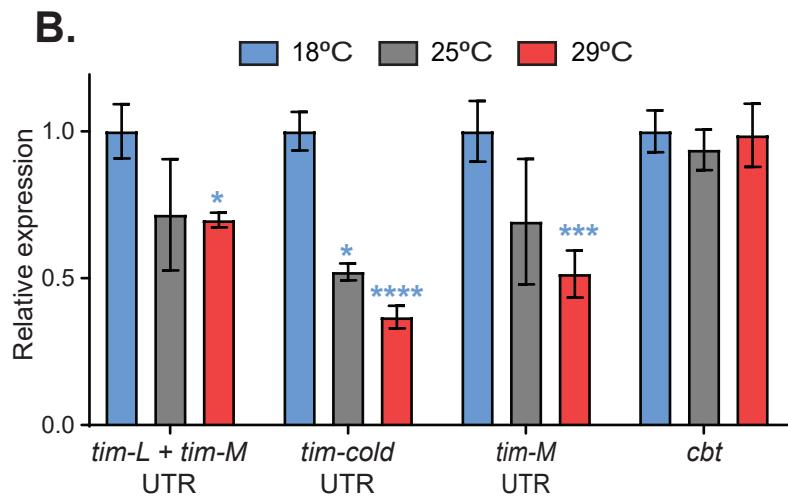
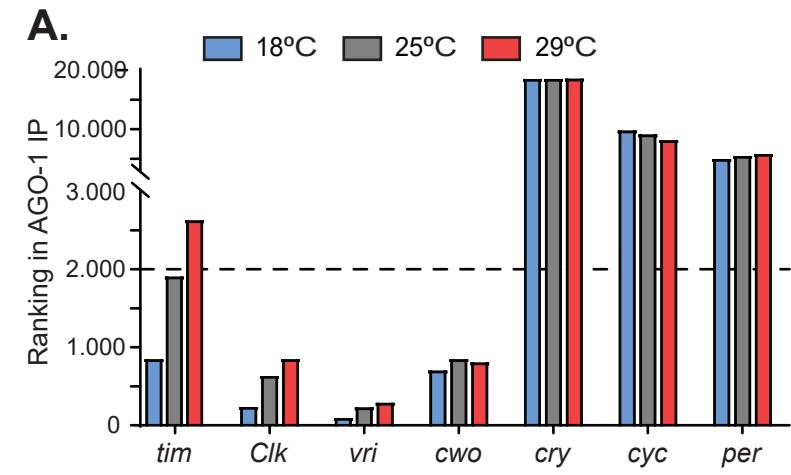


Figure 2

A.**B.****Figure 3**



D.

	# Predicted miRNAs (binding sites)
Common to <i>tim-M</i> , <i>tim-L</i> and <i>tim-cold</i>	17 (17)
common to <i>tim-M</i> and <i>tim-cold</i>	1 (1)
Unique to <i>tim-M</i>	14 (13)
Unique to <i>tim-cold</i>	23 (28)
Unique to <i>tim-sc</i>	3 (5)

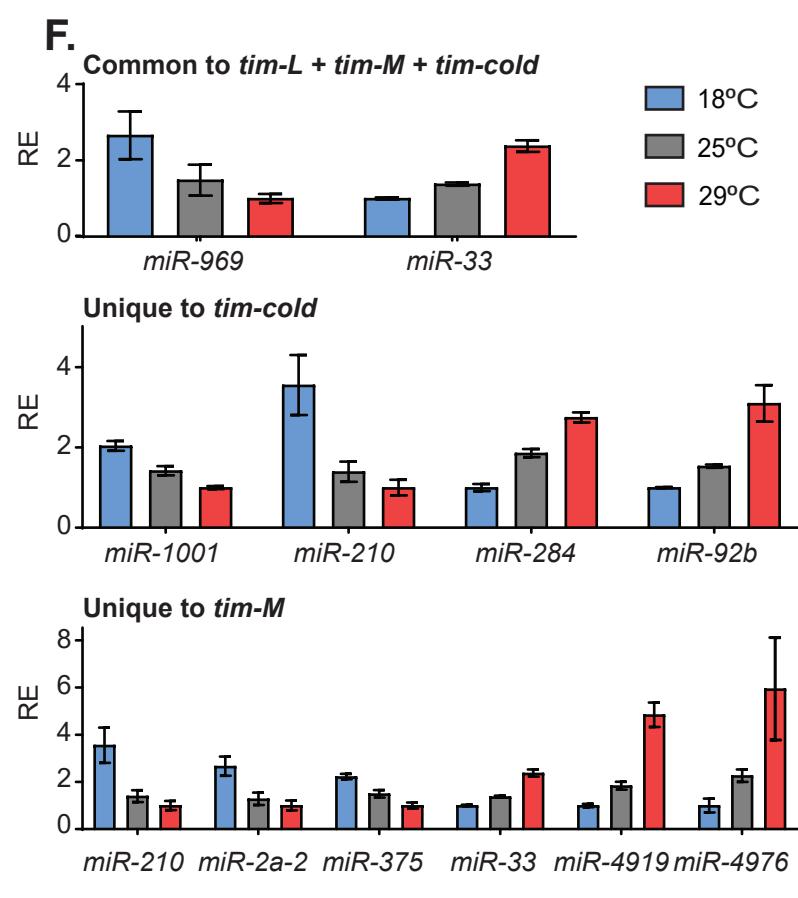
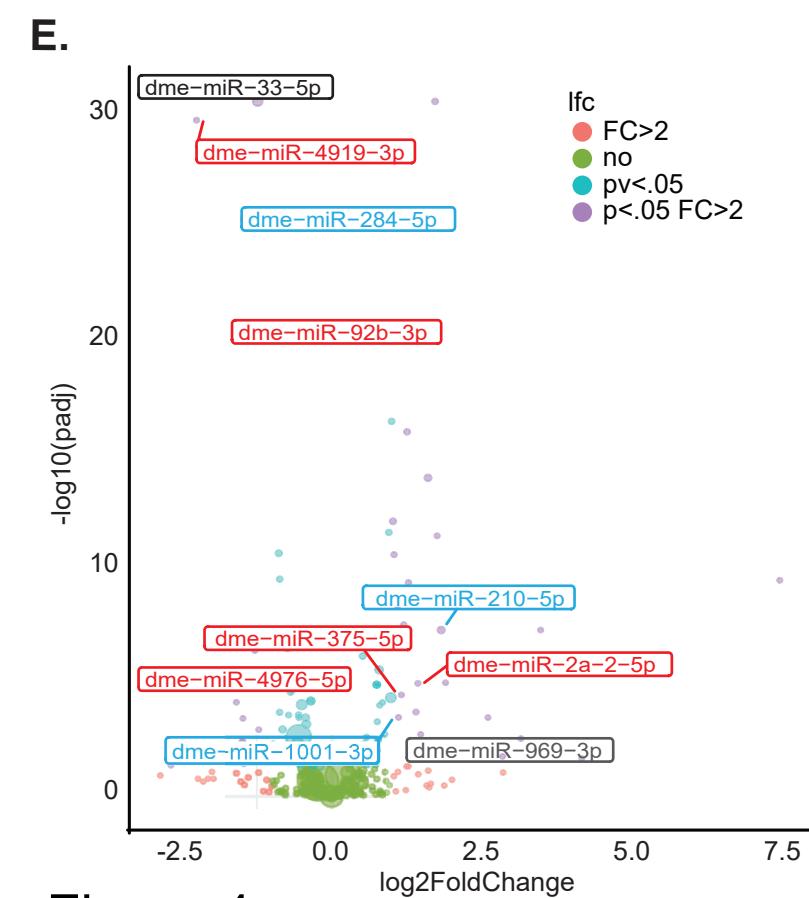


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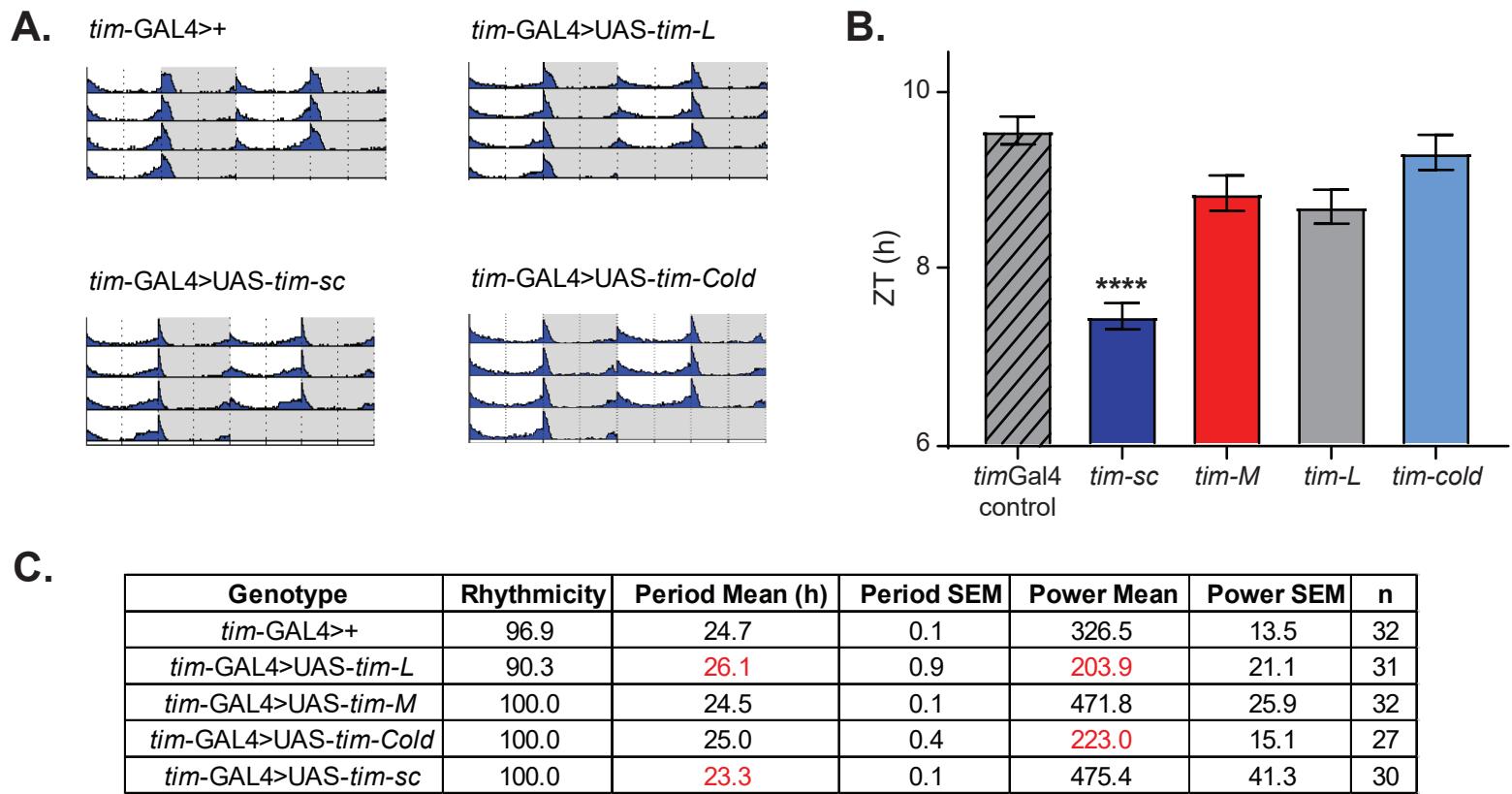


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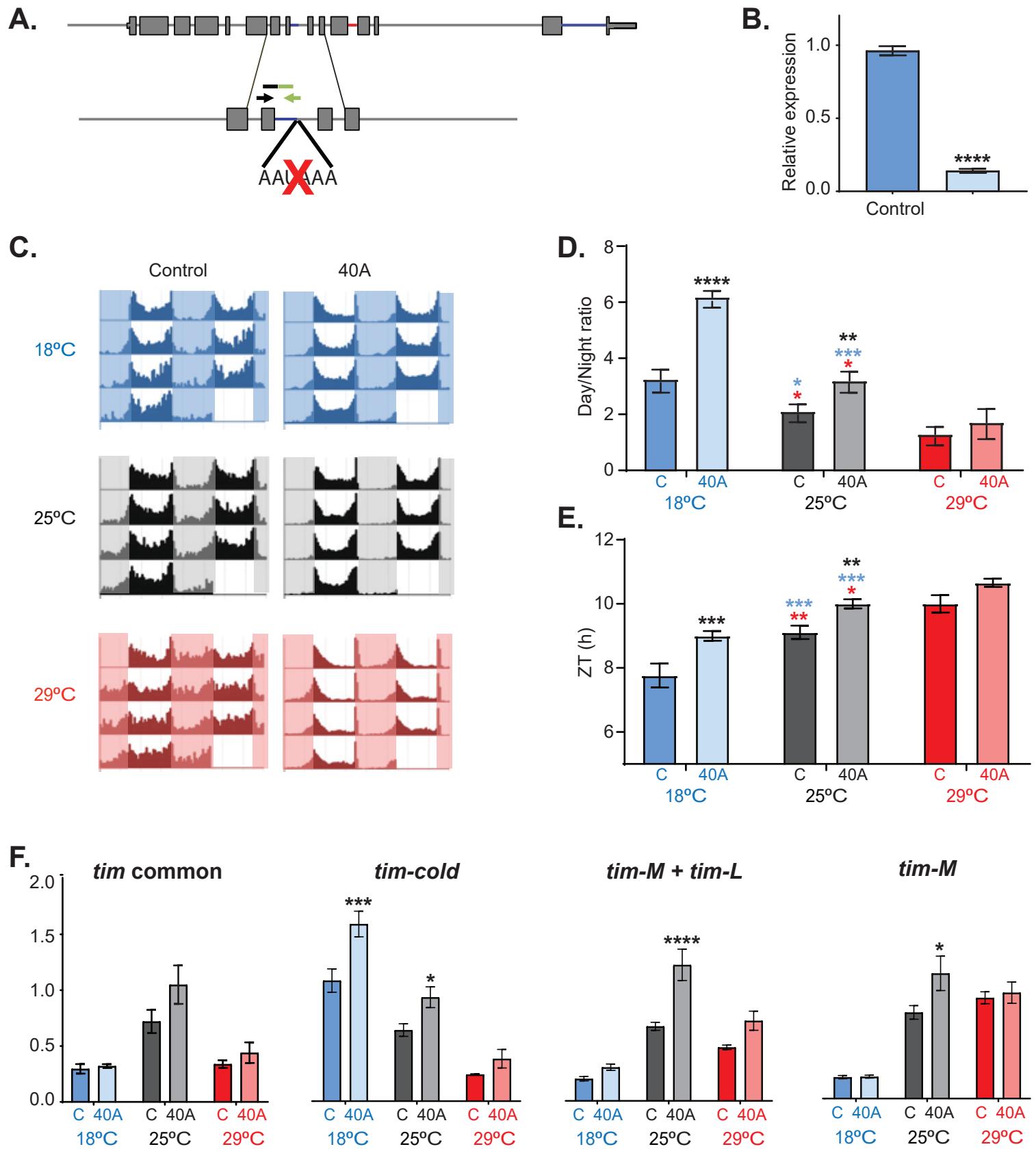


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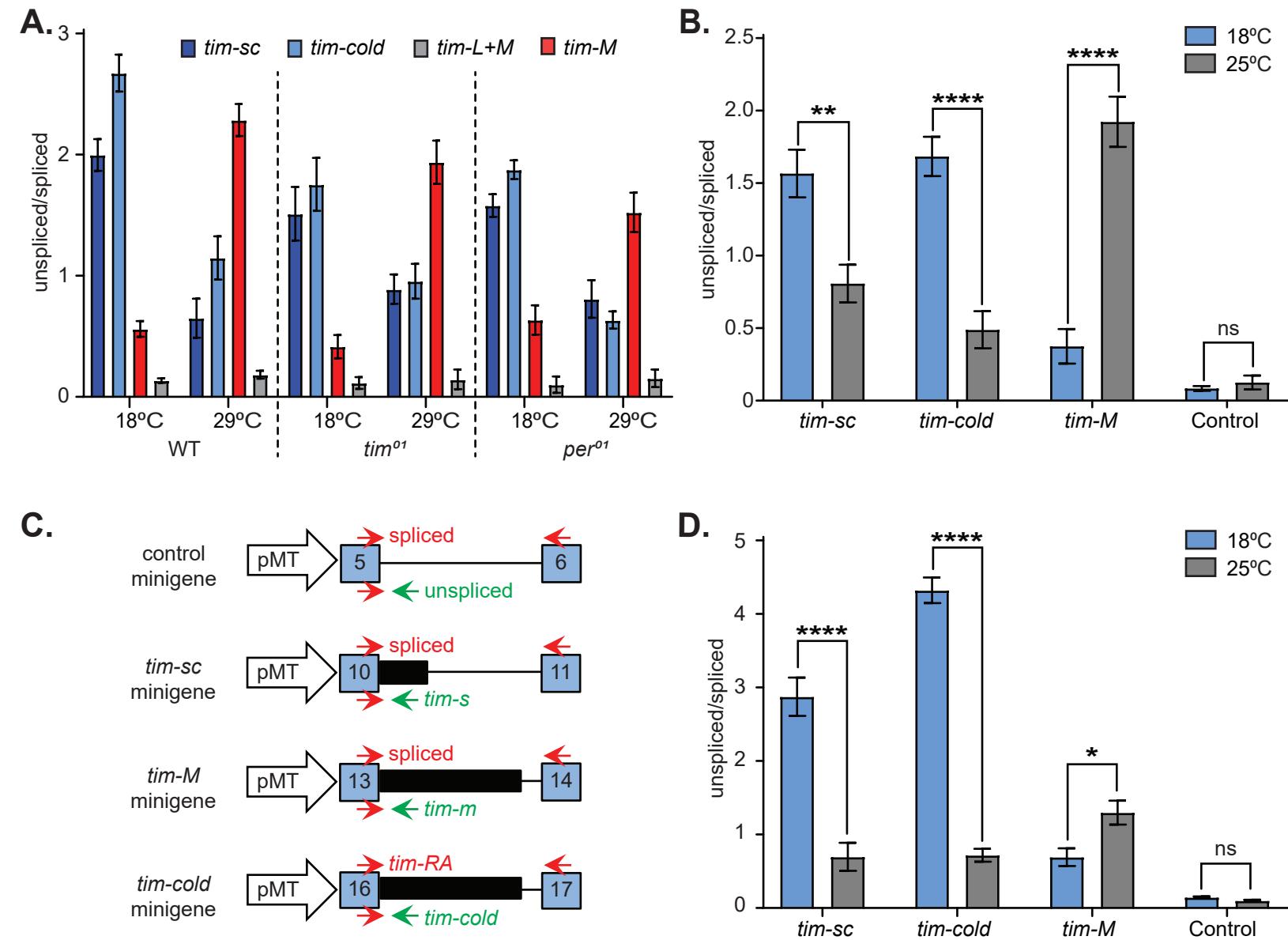


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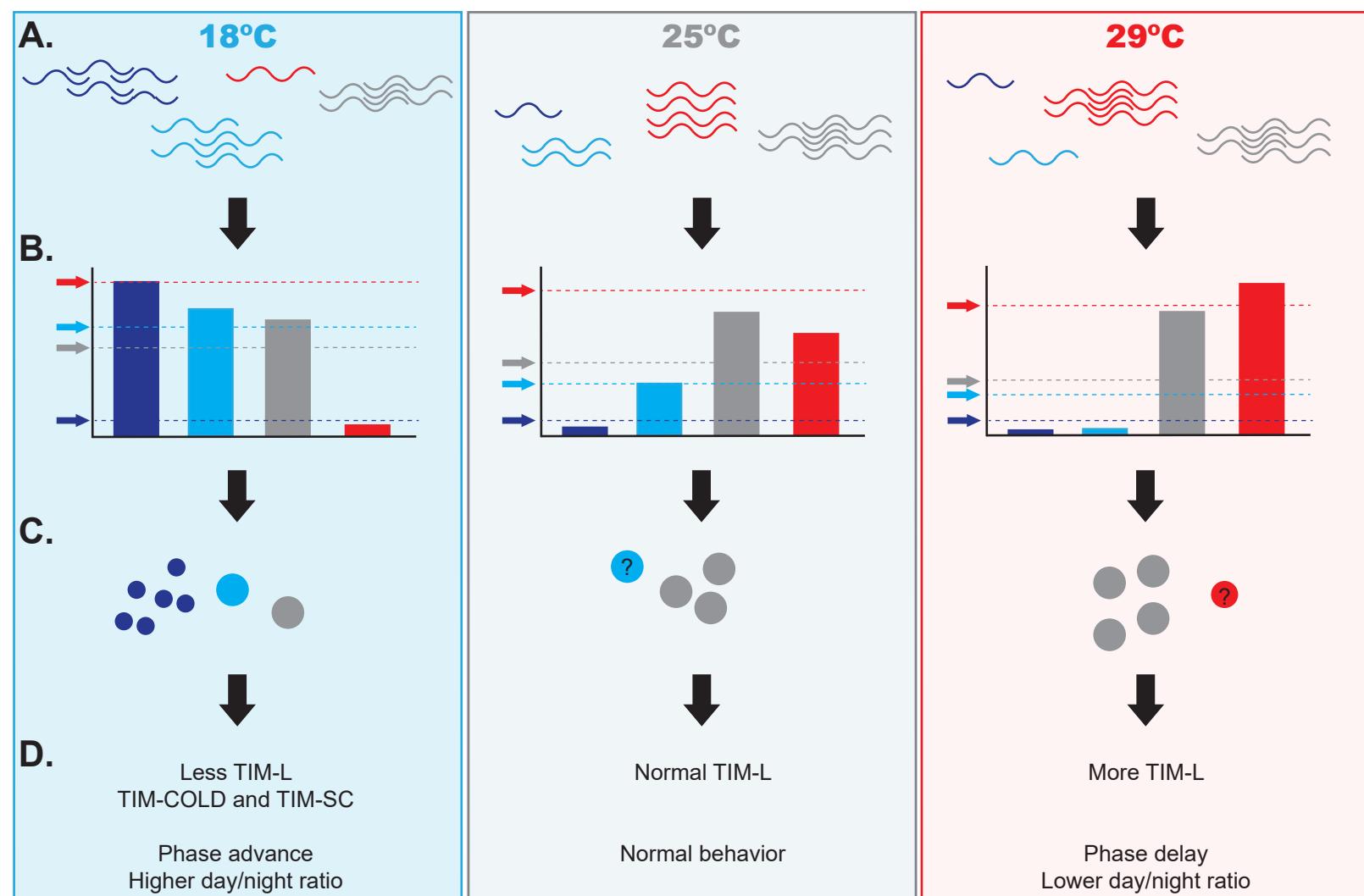
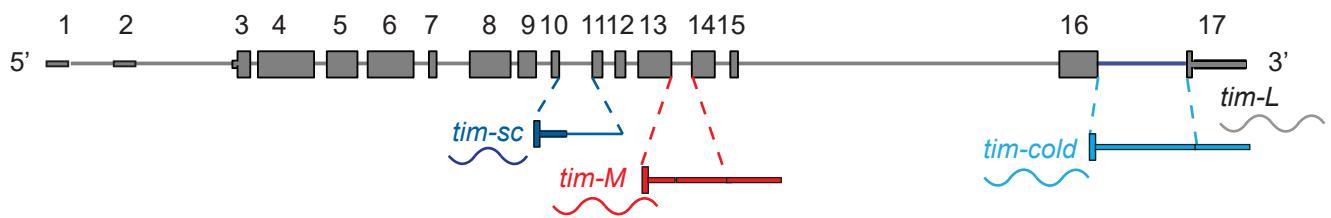


Figure 8

clk genes: *tim*, *Clk*, *per*, *cry*, *cwo*

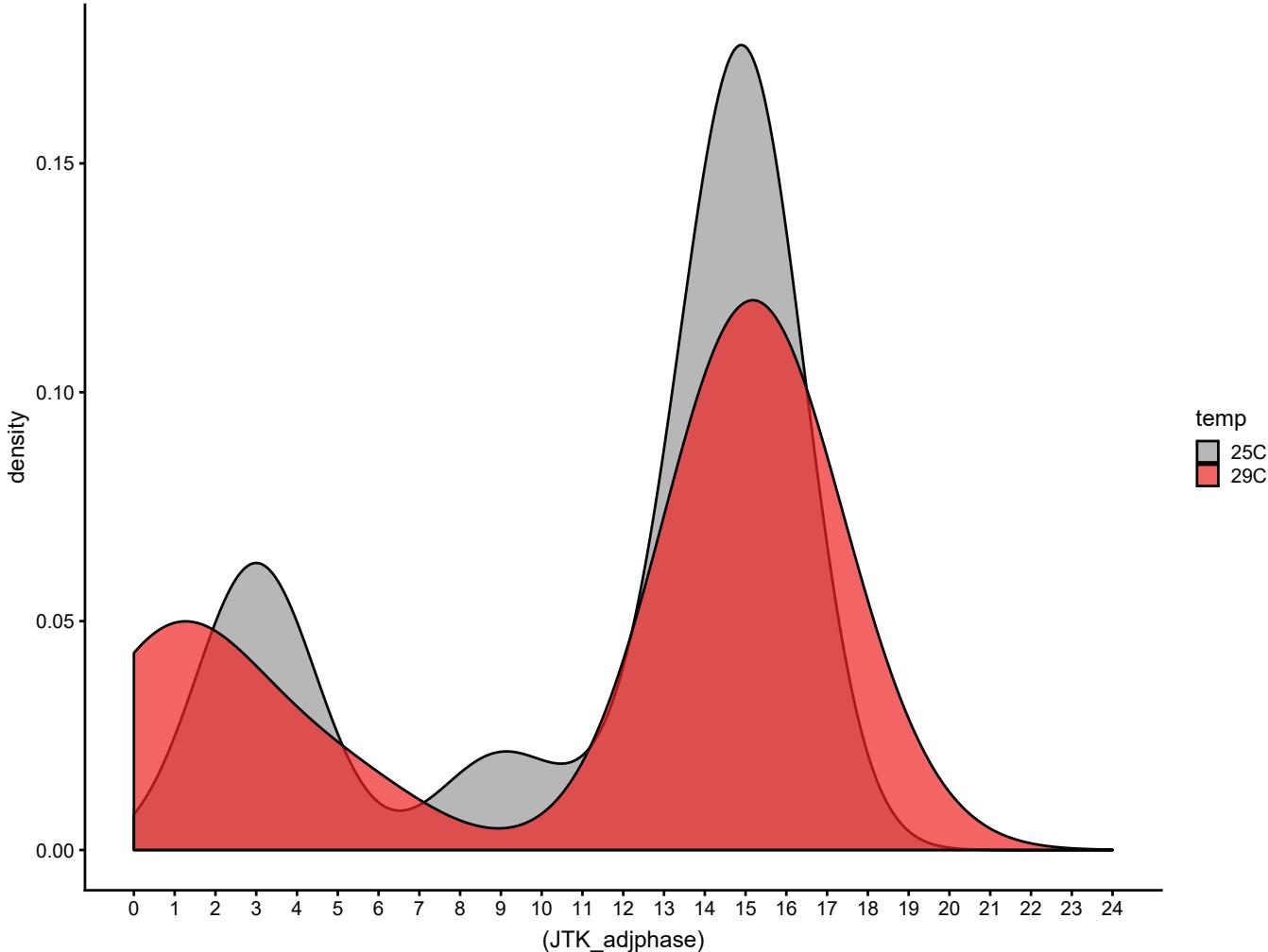
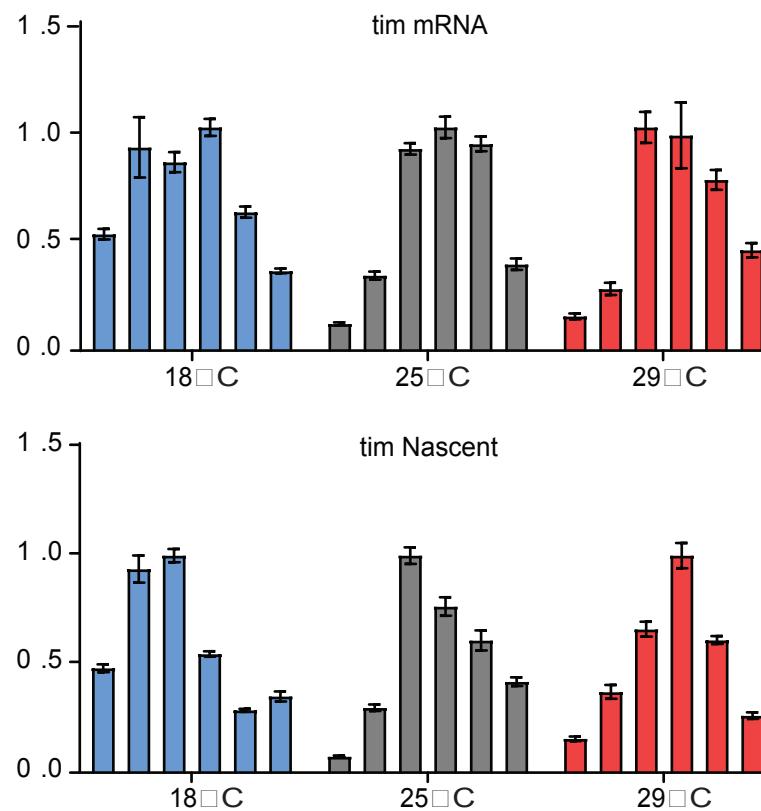
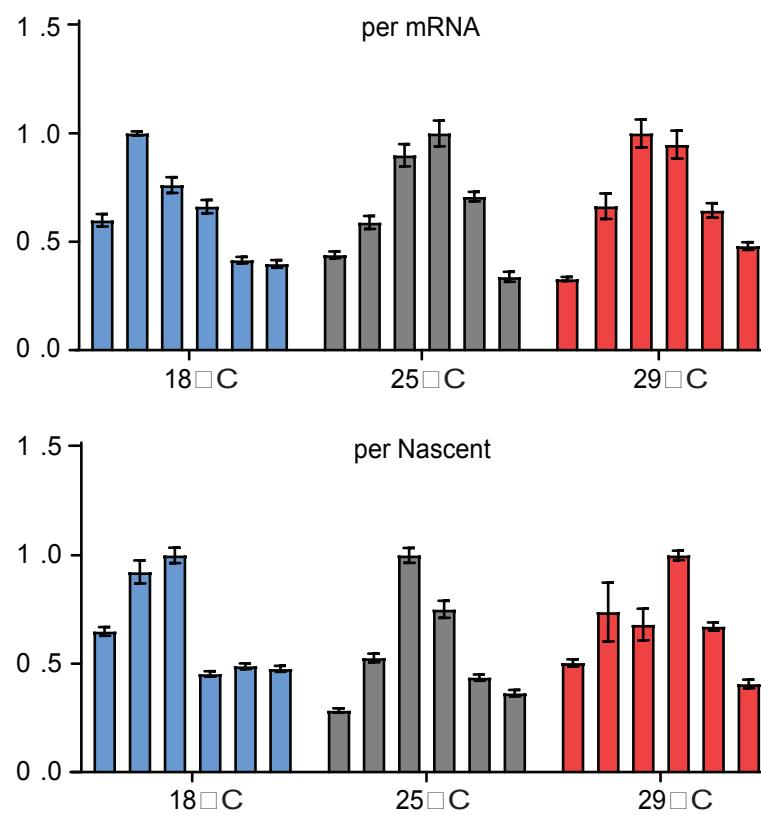
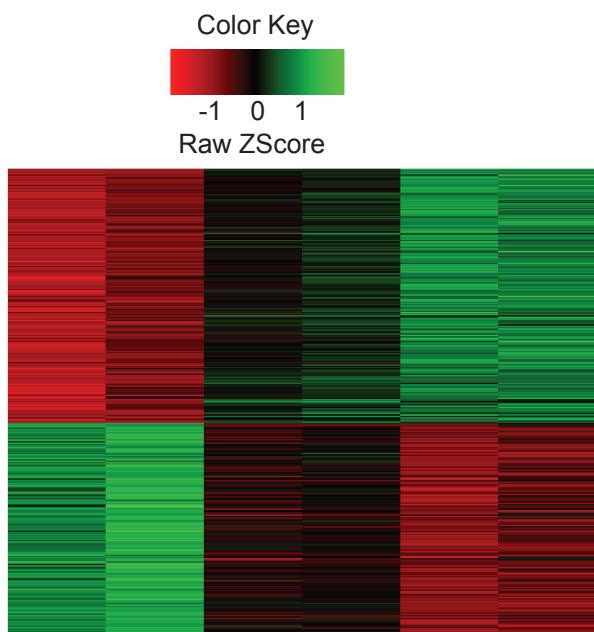


Figure S1

A.**B.****Figure S2**

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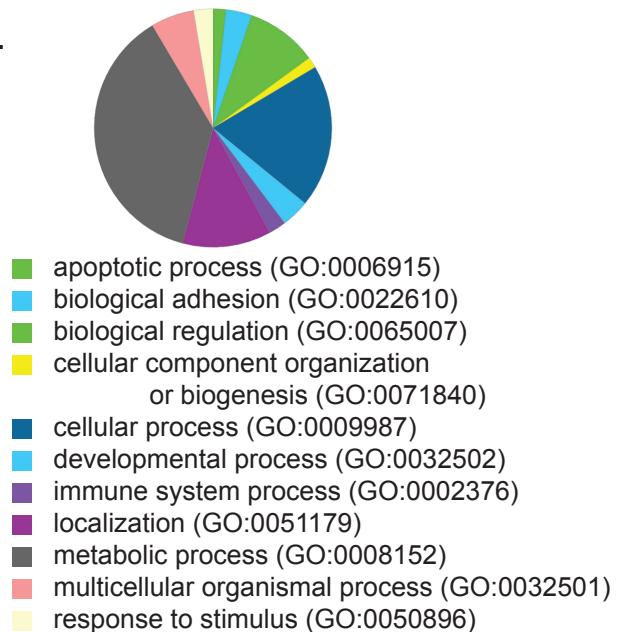


Figure S3

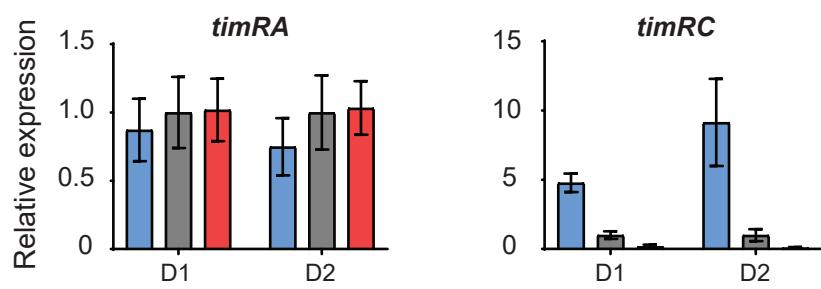


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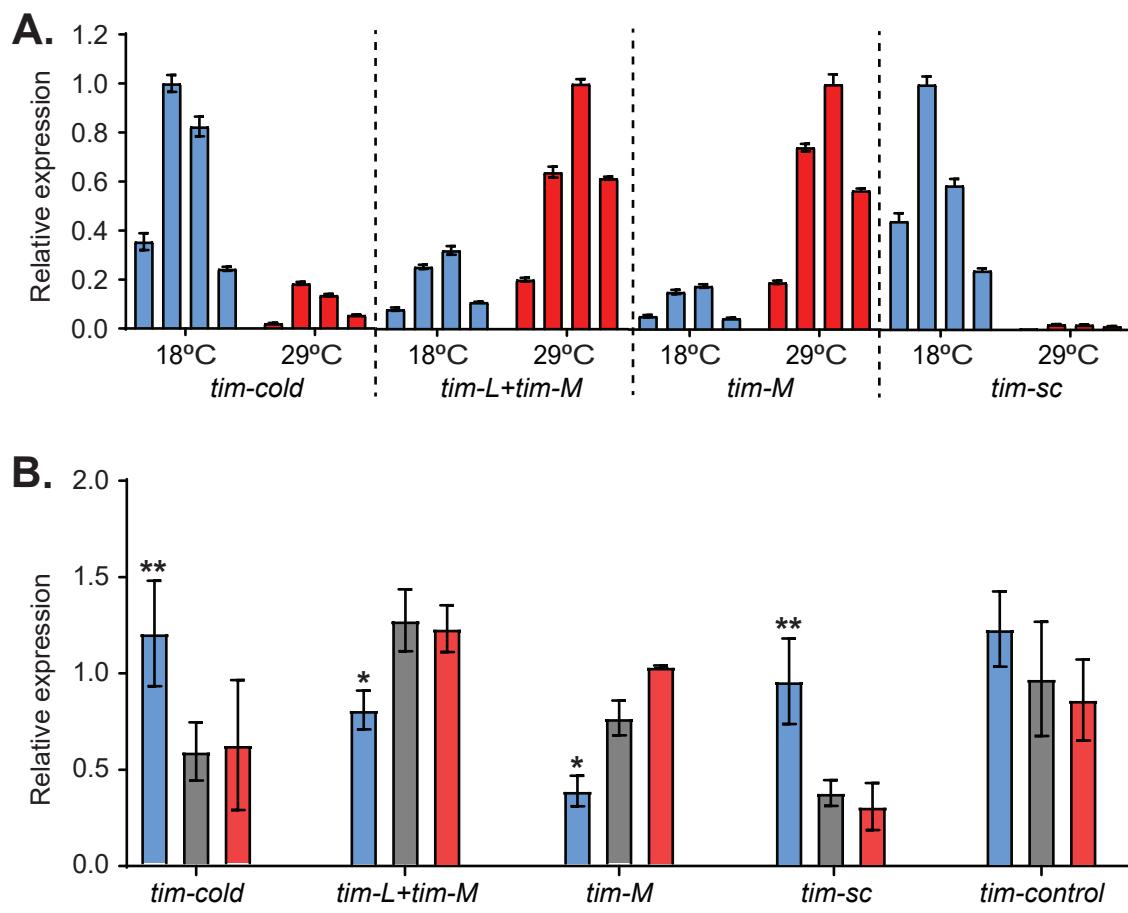


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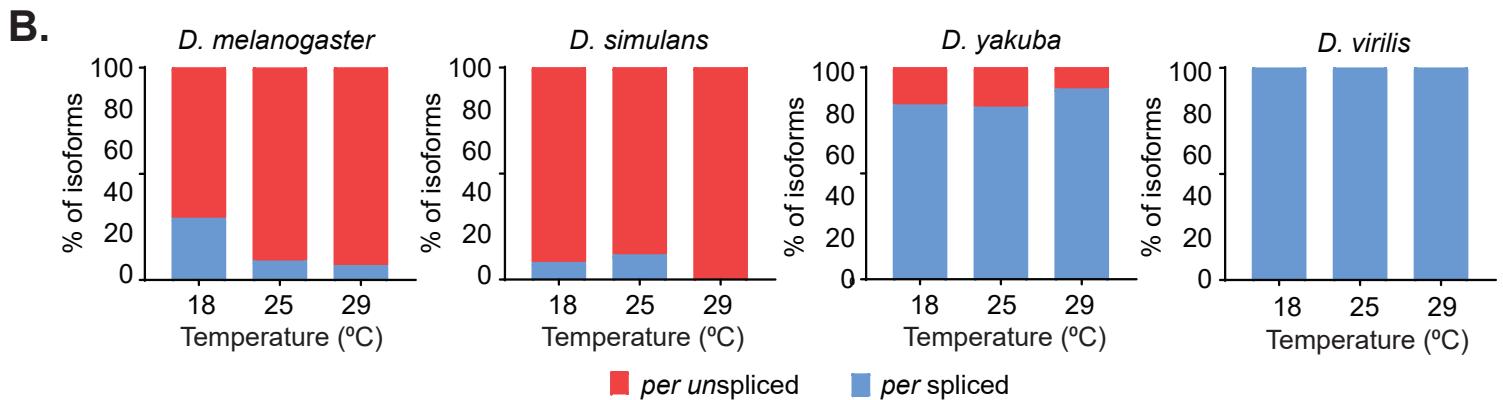
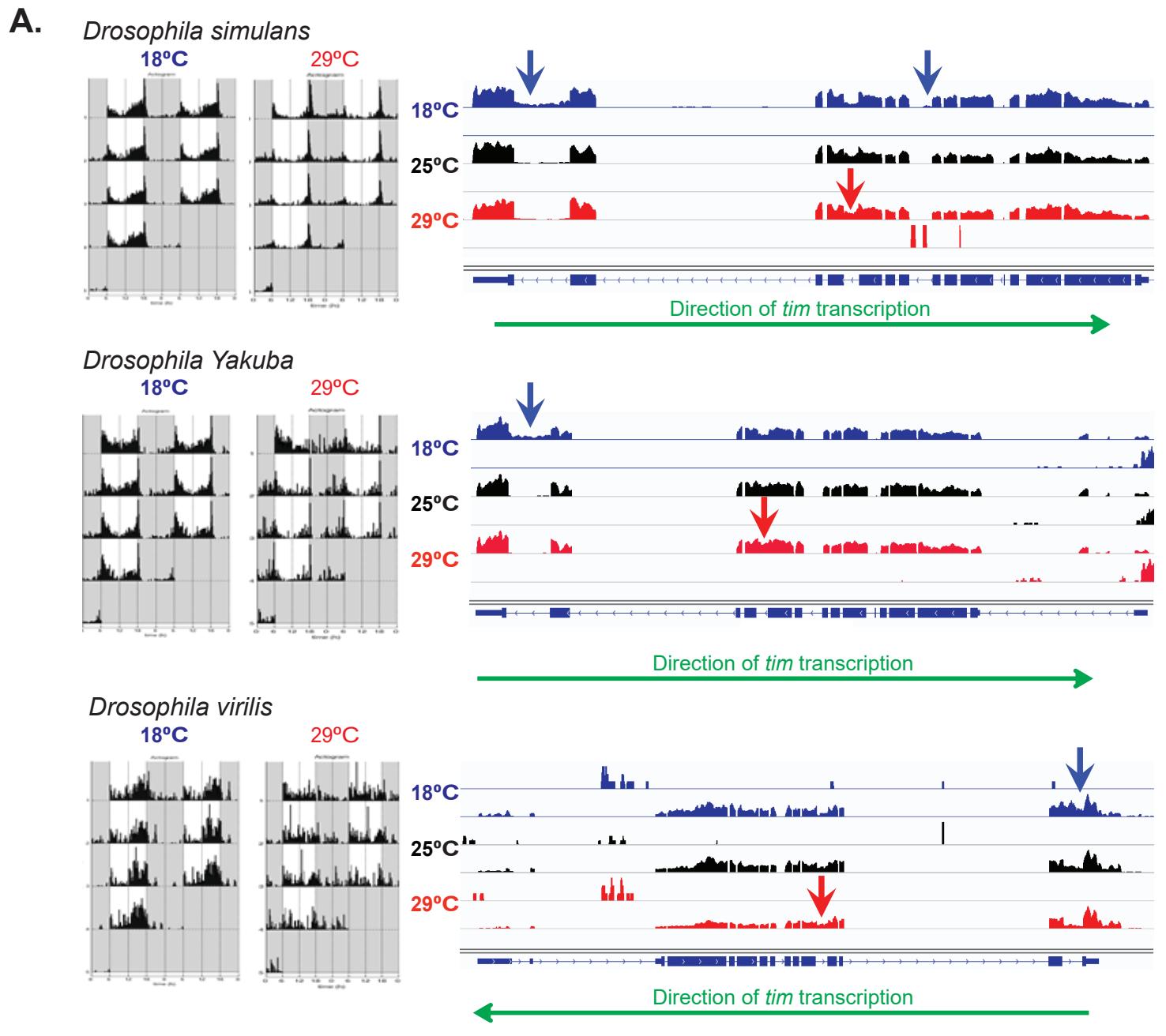


Figure S6

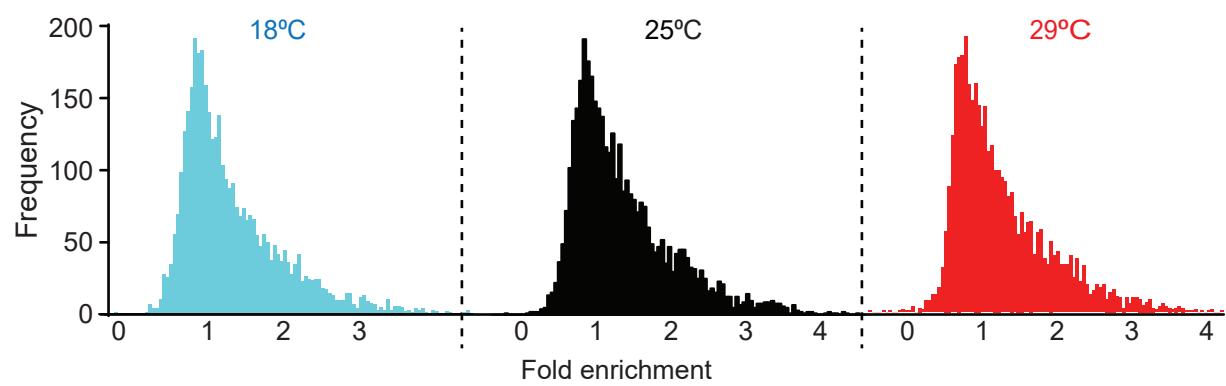


Figure S7

A.



B.

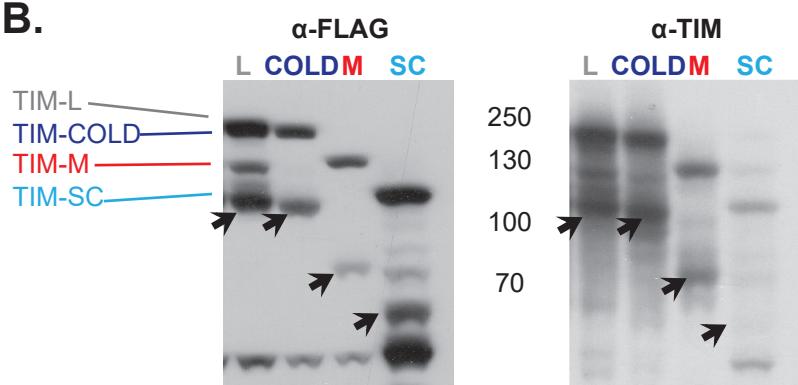


Figure S8

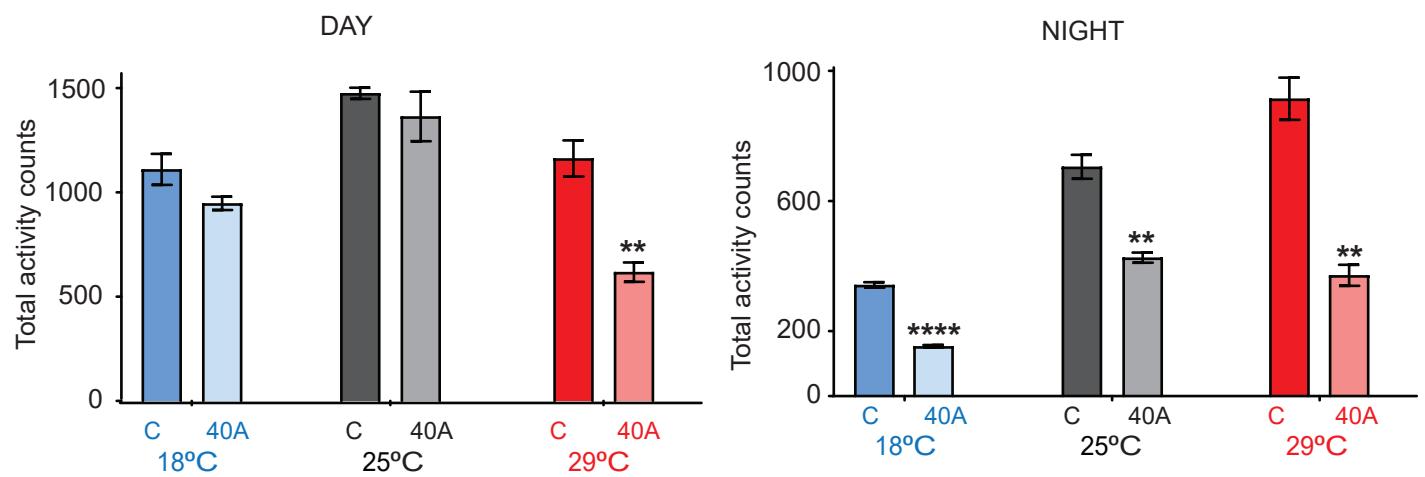


Figure S9

D. melanogaster			D. simulans			D. yakuba		
Intron #	5' SS	3' SS	Intron #	5' SS	3' SS	Intron #	5' SS	3' SS
2	0.92	0.97	2	0.96	0.92	2	0.85	0.20
3	0.93	0.84	3	0.98	0.98	3	0.93	0.98
4	0.98	0.97	4	0.89	0.62	4	0.88	0.89
5	0.90	0.82	5	0.82	0.59	5	0.91	0.91
6	0.82	0.69	6	0.03	---	6	0.22	0.97
7	0.29	0.98	7	0.22	0.93	7	0.88	0.79
8	0.92	0.94	8	0.92	0.88	8	0.88	0.94
9	0.88	0.96	9	0.88	0.96	9	0.90	0.34
10	0.69	0.58	10	0.76	0.92	10	0.98	0.60
11	0.94	0.97	11	0.96	0.94	11	0.95	0.84
12	0.90	0.32	12	0.90	0.18	12	0.82	0.84
13	0.95	0.88	13	0.95	0.75	13	0.79	0.13
14	0.94	0.93	14	0.94	0.95			
15	0.76	0.22	15	0.76	0.22			

	5' SS	3' SS
Short 1st	0.62	0.74
Long 1st	0.54	

Figure S10

Table S1. Circadian oscillation analysis. The circadian analysis was performed using the MetaCycle algorithm (see main text for details). In the table the p-value, phase and amplitude are reported for each gene along with its normalized expression at each circadian timepoint.

Table S2. Gene ontology analysis for the genes with circadian oscillation at each temperature. Genes are ordered by ranking in Fisher exact test. Three different ontologies were analyzed: Biological process. Molecular function. Cellular component. Top 100 genes are reported.

Table S3. Predicted miRNA binding sites (using TargetScan) for each *tim* RNA isoform 3' UTR. Putative miRNA binding sites are reported given the chromosome coordinates. Conservation in different insect species is also reported.

Table S4. Differential miRNA expression at each temperature. Log2(fold change) and p-adjusted value from DeSeq2 are reported for each miRNA.