

1 **CRYPTOCHROMES confer robustness, not rhythmicity, to circadian timekeeping**

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26 **In brief**

27 Circadian turnover of mammalian clock protein PERIOD2 persists in the absence of canonical
28 transcriptional feedback repression and rhythmic clock gene activity, demanding a re-
29 evaluation of cellular clock function and evolution.

30 **Summary**

31 Circadian (approximately daily) rhythms are a pervasive property of mammalian cells, tissues,
32 and behaviour, ensuring physiological and metabolic adaptation to solar time. Models of daily
33 cellular timekeeping revolve around transcriptional feedback repression, whereby CLOCK and
34 BMAL1 activate the expression of 'clock proteins' PERIOD (PER) and CRYPTOCHROME
35 (CRY), which in turn repress CLOCK/BMAL1 activity. CRY proteins are thus considered
36 essential negative regulators of the oscillation; a function supported by behavioural
37 arrhythmicity of CRY-deficient mice when kept under constant conditions. Challenging this
38 interpretation, however, we find evidence for persistent circadian rhythms in mouse behaviour
39 and cellular PER2 levels when CRY is absent. CRY-less oscillations are variable in their
40 expression and have a shorter period than wild type controls. Importantly, we find classic
41 circadian hallmarks such as temperature compensation and determination of period by casein
42 kinase 1 δ/ϵ activity to be maintained. In the absence of CRY-mediated transcriptional feedback
43 repression and rhythmic *Per2* transcription, PER2 protein rhythms are sustained for several
44 cycles, accompanied by circadian variation in protein stability. We suggest that, whereas
45 circadian transcriptional feedback imparts robustness and functionality onto biological clocks,
46 the core timekeeping mechanism is post-translational. Our findings suggest that PER proteins
47 normally act as signalling hubs that transduce timing information to the nucleus, imparting daily
48 rhythms upon the activity of transcriptional effectors.

49

50 **Highlights**

- 51 ➤ PER/CRY-mediated negative feedback is dispensable for mammalian circadian
52 timekeeping
- 53 ➤ Circadian variation in PER2 levels persists in the absence of rhythmic *Per2*
54 transcription
- 55 ➤ CK1 and GSK3 are plausible mechanistic components of a 'cytosillator' mechanism
- 56 ➤ CRY-mediated feedback repression imparts robustness to biological timekeeping

57 **Introduction**

58 The adaptive advantage conferred on organisms by anticipation of the 24-hour cycle of day
59 and night has selected for the evolution of circadian clocks that, albeit in different molecular
60 forms, are present throughout all kingdoms of life (Edgar et al., 2012; Rosbash, 2009).
61 Circadian rhythms are robust, in that they are “capable of performing without failure under a
62 wide range of conditions” (Merriam-Webster Dictionary, 2020). The mechanism proposed to
63 generate daily timekeeping in mammalian cells is a delayed transcriptional-translational
64 feedback loop (TTFL) that consists of activating transcription factor complexes containing
65 CLOCK and BMAL1 and repressive complexes, containing the BMAL1:CLOCK targets
66 PERIOD and CRYPTOCHROME (Dunlap, 1999; Reppert and Weaver, 2002; Takahashi,
67 2016). Various coupled, but non-essential, auxiliary transcriptional feedback mechanisms are
68 thought to fine-tune the core TTFL and coordinate cell-type specific temporal organisation of
69 gene expression programs; the best characterised being effected by the E-box mediated
70 rhythmic expression of REV-ERB α/β , encoded by the Nr1d1/2 genes ((Liu et al., 2008; Preitner
71 et al., 2002; Takahashi, 2016; Ueda, 2007). These auxiliary loops are not considered sufficient
72 to generate circadian rhythms in the absence of the core TTFL (Liu et al., 2008; Preitner et al.,
73 2002).

74 CRY1 and CRY2 operate semi-redundantly as the essential repressors of CLOCK/BMAL1
75 activity (Chiou et al., 2016; Ye et al., 2014), required for the nuclear import of PER proteins,
76 and together are considered indispensable for circadian regulation of gene expression *in vivo*
77 as well as in cells and tissues cultured *ex vivo* (Chiou et al., 2016; Kume et al., 1999; Ode et
78 al., 2017; Sato et al., 2006). Certainly, mice homozygous null for *Cry1* and *Cry2* do not express
79 circadian behavioural rest/activity cycles under standard experimental conditions (Horst and
80 Muijtjens, 1999; Thresher et al., 1998; Vitaterna et al., 1999).

81 The hypothalamic suprachiasmatic nucleus (SCN) is a central locus for circadian coordination
82 of behaviour and physiology, and research over the last two decades has stressed the strong
83 correlation between SCN timekeeping *in vivo* and its activity when cultured *ex vivo* (Anand et

84 al., 2013; Welsh et al., 2010). We were therefore intrigued by the observation that roughly half
85 of organotypic SCN slices prepared from homozygous *Cry1^{-/-},Cry2^{-/-}* (CRY knockout; CKO)
86 mouse neonates continue to exhibit approximately short period ~20h-hour rhythms, observed
87 using the genetically encoded PER2::LUC clock protein::luciferase fusion reporter (Maywood
88 et al., 2011; Ono et al., 2013), despite having previously been described as arrhythmic (Liu et
89 al., 2007). Moreover, short period circadian rhythms of locomotor activity have previously been
90 reported for CKO mice raised from birth under constant light (Ono et al., 2013). As CKO SCN
91 oscillations were only observed in cultured neonatal organotypic slices *ex vivo*, they were
92 suggested to be a network-level SCN-specific rescue by the activity of neuronal circuits, that
93 desynchronise during post-natal development (Ono et al., 2013; Welsh et al., 2010). In our
94 view, however, these observations are difficult to reconcile with an essential requirement for
95 CRY in the generation of circadian rhythms. Rather, they are more consistent with CRY making
96 an important contribution to circadian rhythm stability and functional outputs, as recently shown
97 for the genes *Bmal1* and *Clock* (Landgraf et al., 2016; Ray et al., 2020), which had both
98 previously been thought indispensable for circadian timekeeping in individual cells (Bunger et
99 al., 2000; DeBruyne et al., 2007).

100 Recent observations have further questioned the need for transcriptional feedback repression
101 to enable cellular circadian timekeeping. For example, circadian protein translation is regulated
102 by cytosolic BMAL1 through a transcription-independent mechanism (Lipton et al., 2015), and
103 isolated erythrocytes exhibit circadian rhythms despite lacking any DNA (Cho et al., 2014;
104 O'Neill and Reddy, 2011). Moreover, circadian timekeeping in some species of eukaryotic alga
105 and prokaryotic cyanobacteria can occur entirely post-translationally (Nakajima et al., 2005;
106 O'Neill et al., 2011; Sweeney and Haxo, 1961; Tomita et al., 2005). Whether non-
107 transcriptional clock mechanisms operate in other (nucleated) mammalian cells is unknown
108 however, and hence their mechanism and relationship with TFL-mediated rhythms is an open
109 question.

110 Here, we used cells and tissues from CRY-deficient mice, widely accepted not to exhibit
111 circadian transcriptional regulation (Edwards et al., 2016; Kume et al., 1999; Ukai-Tadenuma
112 et al., 2011) to test whether any timekeeping function remained from which we might begin to
113 dissect the mechanism of the postulated transcription-independent cytosolic oscillator, or
114 'cytoscillator' (Hastings et al., 2008).

115 **Results**

116 **Cell-autonomous circadian PER2::LUC rhythms in the absence of CRY proteins**

117 Consistent with previous observations, we observed no significant circadian organisation of
118 locomotor activity in CRY-deficient (CKO) mice following entrainment to 12h:12h light:dark
119 (LD) cycles or in constant light (LL). Upon transition from constant light to constant darkness
120 (DD) (described to be a stronger zeitgeber (Chen et al., 2008)) however, CKO mice expressed
121 rhythmic bouts of consolidated locomotor activity with a period of ~16.5h (Figure 1A, B, S1A).
122 CKO rhythms were shorter in period and more variable than wild type (WT) controls, but
123 persisted for >2 weeks, consistent with these mice possessing residual timing function that is
124 not engaged during standard environmental entrainment protocols. In support of this
125 interpretation, and in accordance with previous reports (Maywood et al., 2011; Ono et al.,
126 2013), longitudinal bioluminescence recordings of organotypic PER2::LUC SCN slices cultured
127 *ex vivo* from WT or CKO neonates revealed rhythmic PER2 expression in approximately 40%
128 of CKO slices (Figure 1C). In line with behavioural data and previous reports, these CKO SCN
129 rhythms exhibited significantly shorter periods compared with WT controls (Figure 1D).

130

131 Two explanations might account for the variable CKO SCN phenotype: (1) the previously
132 proposed explanation: genetic loss-of-function is compensated at a network-level by SCN-
133 specific neuronal circuits whose function is sensitive to developmental phase and small
134 variations in slice preparation (Evans et al., 2012; Liu et al., 2007; Ono et al., 2013; Tokuda et
135 al., 2015); or (2) CKO (SCN) cells have cell-intrinsic circadian rhythms that are expressed (or

136 observed) more stochastically and with less robustness than their WT counterparts, and can
137 be amplified by SCN interneuronal signalling (O'Neill and Reddy, 2012; Welsh et al., 2010).

138
139 To distinguish between these two possibilities, we asked whether PER2::LUC rhythms are
140 observed in populations of immortalised PER2::LUC CKO adult fibroblasts, which lack the
141 specialised interneuronal neuropeptidergic signalling that is so essential to SCN amplitude and
142 robustness *in* and *ex vivo* (O'Neill and Reddy, 2012; Welsh et al., 2010). We observed this to
143 be the case (Figure 1E and S1B-C). Across >100 recordings, using independently-generated
144 cell lines cultured from multiple CRY-deficient mice (male and female), we observed
145 PER2::LUC rhythms that persisted for several days under constant conditions. Again, the
146 mean period of rhythms in CRY-deficient cells was significantly shorter than WT controls, and
147 with increased variance within and between experiments (F-test p-value <0.0001, Figure 1F
148 and S1D, E). Consistent with SCN results, rhythmic PER2::LUC expression in CKO cells
149 occurred stochastically between experiments, being observed in ~30% of independently
150 performed assays. Importantly, there was very little variation in the occurrence of rhythmicity
151 within experiments meaning that in any given recording all CKO replicate cultures were
152 rhythmic or none, whereas WT cultures were always rhythmic. CKO PER2::LUC rhythms
153 damped more rapidly than wild type controls (Figure S1F), and were more sensitive to acute
154 changes in temperature than WT controls (Figure 2A, C), consistent with their oscillation being
155 less robust. Crucially though, the PER2::LUC rhythms in CKO cells were temperature
156 compensated (Figure 2A, B) and entrained to 12h:12h 32°C:37°C temperature cycles in the
157 same phase as WT controls (Figures 2C), and are thus circadian by definition (Pittendrigh,
158 1960).

159
160 These observations suggest that CRY-dependent transcriptional feedback repression primarily
161 confers robustness to rhythmic clock output, rather than generating circadian rhythms *per se*.
162 To test this in another model system we turned to *Drosophila melanogaster*, where TIMELESS
163 fulfils the functionally analogous role to mammalian CRY proteins as the obligate partner of

164 PER, required for repression of circadian transcription at E-box promotor elements. In assays
165 of PER:LUC (XLG-LUC) bioluminescence in freely behaving flies, we observed robust
166 circadian rhythms in 7 out of 36 TIMELESS knockout animals, compared with 11 out of 21 wild
167 type controls (Figure S2). As observed for CRY-deficient cells, rhythms in TIMELESS-deficient
168 flies persisted over several days, but were noisier and exhibited lower relative amplitude than
169 WT.

170
171 Considering recent reports that transcriptional feedback repression is not absolutely required
172 for circadian rhythms in the activity of FRQ, the functional orthologue of PER in the fungus
173 *Neurospora crassa* (Larrondo et al., 2015), that nascent transcription is not required for
174 circadian rhythms in the green lineage (O'Neill et al., 2011), or in isolated human red blood
175 cells (O'Neill and Reddy, 2011), we next asked whether residual rhythms of PER2:LUC in
176 CRY-deficient cells result from post-translational regulation.

177

178 **CRY-independent PER2::LUC rhythms are driven by a non-transcriptional process**

179 CRY has previously been described as the driving factor for feedback repression of
180 BMAL1/CLOCK-dependent transcriptional activation, and is therefore considered essential to
181 the rhythmic regulation of clock-controlled genes (CCGs). In fact, overexpression studies have
182 suggested PER requires CRY to exert its function as a BMAL1-CLOCK repressor (Chiou et
183 al., 2016; Ye et al., 2014). This importance of CRY for BMAL1-CLOCK repression (and auto-
184 repression of *Cry* and *Per*) was also suggested by the increased PER2::LUC levels observed
185 in CKO cells (Figure 1E, S1B). Indeed, at the peak of PER2::LUC expression, CKO cells
186 contain approximately twice as many PER2 molecules compared with their WT counterparts
187 (Figure 3A and S3A).

188 Although not sufficient to completely rescue rhythms in CKO cells, it seemed plausible that
189 increased PER protein expression might partially compensate for the loss of CRY function and
190 continue to exert auto-regulation through rhythmic BMAL1-CLOCK binding, thereby

191 accounting for the residual PER2::LUC rhythms in CKO cells. To test this possibility, we
192 compared BMAL1-PER2 binding at the expected peak of BMAL1-PER2 complex formation
193 (i.e. at the peak of PER2::LUC expression) in WT and CKO cells. To this end, we
194 immunoprecipitated BMAL1 and measured the associated PER2::LUC activity. In accordance
195 with CRY being required for PER2-BMAL1 binding, we did not find a PER2::LUC-BMAL1
196 complex in CKO cells, while the complex was readily detected in WT cells (Figure 3B and
197 S3B), strongly suggesting that residual oscillations in PER2::LUC cannot result from a residual
198 negative feedback upon the BMAL1-CLOCK complex.

199 In the absence of PER:CRY-mediated feedback repression, it seemed unlikely that CRY-
200 independent oscillations in PER2::LUC expression are driven directly by rhythms in *Per2*
201 transcription. Indeed, whereas PER2::LUC in co-recorded cells showed a clear variation over
202 24h, *Per2* mRNA in parallel replicate CKO cultures instead exhibited a gradual accumulation
203 (Figure 3C). In contrast and as expected, *Per2* mRNA in WT cells varied in phase with co-
204 recorded PER2::LUC oscillations. The gradual increase of *Per2* mRNA in CKO cells is
205 concordant with *Per2* transcriptional derepression predicted by the canonical TTFL model,
206 accounting for the generally increased levels of PER2::LUC we observed (Figure 3A), but not
207 their oscillation. In agreement with these findings and in contrast with WT cells, *Bmal1* mRNA
208 also showed no significant variation in CKO cells (Figure S3C), suggesting that E-box-
209 dependent circadian regulation of REV-ERB activity may not occur in the absence of CRY-
210 mediated feedback repression. In an independent validation we assessed the activity of the
211 circadian E-box-driven *Cry1*-promoter (Maywood et al., 2013) in mouse adult WT and CKO
212 lung fibroblasts (MAFs) (Figure S3D), as well as the *Per2*- and *Rev-erba* (*Nr1d1*-) promoters
213 in mouse embryonic fibroblasts (MEFs) (Figure 3D, S3E-G). No rhythmic *Cry1*- or *Per2*-
214 promoter activity was observed in either set of CKO cells under any condition, whereas
215 isogenic control cells showed clear circadian regulation of these promoters.

216 In recordings from *Nr1d1*:LUC MEFs however, we were most surprised to observe
217 temperature-compensated circadian rhythms in the activity of the *Nr1d1* promoter in CKO cells,

218 at just ~3% amplitude of WT cells, that persisted for several days (Figure 3D and S3E, red
219 traces). In the same experiments, similar but still noisier and lower amplitude rhythms were
220 also detected in quadruple knockout MEFs that were also deficient for PER1/2, as well as
221 CRY1/2 (CPKO, Figure 3D and S3E, blue traces), confirming these oscillations cannot be
222 attributable to any vestigial activity of PER proteins. We acknowledge it is conceivable that
223 some unknown TTFL-type mechanism might generate these residual oscillations in *Nr1d1*
224 promoter activity. However, we find it more plausible that residual oscillations of *Nr1d1*:LUC in
225 CKO cells are the output of a post-translational timekeeping mechanism, from which the
226 amplification and robustness conferred by CRY-dependent transcriptional feedback repression
227 has been subtracted. Indeed, we note that besides CRY, *Nr1d1* expression is regulated by
228 many other transcription factors, e.g., AP-1, NRF2, NF-KB and BMAL1/CLOCK (Preitner et al.,
229 2002; Wible et al., 2018; Yang et al., 2014), whose activity is regulated post-translationally by
230 the same rather promiscuous kinases that rhythmically regulate PER and BMAL1 in other
231 contexts (Eide et al., 2002; Iitaka et al., 2005; Narasimamurthy et al., 2018; Sahar et al., 2010)
232 e.g. casein kinase 1, glycogen synthase kinase (Jiang et al., 2018; Liang and Chuang, 2006;
233 Medunjanin et al., 2016; Preitner et al., 2002; Rada et al., 2011; Tullai et al., 2011).

234 **Circadian control of PER2 stability persists in absence of CRY**

235 The concentrations of luciferase substrates (Mg.ATP, luciferin, O₂) under our assay conditions
236 are >10x higher than their respective K_m (Feeney et al., 2016a) and so it is implausible that
237 PER2::LUC rhythms in CKO cells result from anything other than circadian regulation in the
238 abundance of the PER2::LUC fusion protein. Indeed, PER2::LUC levels measured in cell
239 lysates perfectly mirrored longitudinal PER2::LUC recordings from both WT and CKO cells
240 (Figure 4A). We observed that the addition of the proteasomal inhibitor MG132 to
241 asynchronous cells led to acute increases in PER2::LUC levels which were significantly greater
242 in CKO cells than in WT controls, indicating that CKO cells support higher basal rates of PER2
243 turnover (Figure 4B and 4C). In consequence therefore, relatively small changes in the rate of
244 PER2::LUC translation or degradation should be sufficient to affect the steady state

245 PER2::LUC concentration. CKO cells exhibit no rhythm in *Per2* mRNA (Figure 3C, D), nor do
246 they show a rhythm in global translational rate (Figure S4A, B), nor did we observe any
247 interaction between BMAL1 and S6K/eIF4 as occurs in WT cells (Lipton et al, 2015) (Figure
248 S4C). We therefore investigated whether changes in PER2::LUC stability might be responsible
249 for the persistent bioluminescence rhythms in CKO cells, by analysing the decay kinetics of
250 luciferase activity during saturating translational inhibition.

251
252 In the presence of 10 μ M cycloheximide (CHX) PER2::LUC bioluminescence decayed
253 exponentially (Figure 4D and S4D, $R_2 > 0.9$), with a half-life that was consistently <2 hours
254 (Figure 4D and S4E); much less than the half-life of luciferase expressed in fibroblasts under
255 a constitutive promoter (≥ 5 h, Figure S4D and E). Moreover, we observed a significant variation
256 ($\pm 50\%$) in the half-life of PER2::LUC between the rising and falling phases of its expression
257 (1.5 vs 1 h, respectively, Figure 4D and S4F) without any commensurate change in global
258 protein turnover (Figure S4G). Strikingly, we also observed a similar phase-dependent
259 variation of PER2::LUC stability in CKO cells, with a smaller ($\pm 20\%$) but significant difference
260 between opposite phases of the oscillation (Figure 4D). To test if a 20% variation in protein
261 half-life, in the absence of any underlying mRNA abundance rhythm, was sufficient to account
262 for our experimental observations given the intrinsically high turnover of PER2, we made a
263 simple mathematical model using experimentally derived values for mRNA level, protein half-
264 life and translation (Figure 3C and S4). We found that the model produced PER2::LUC levels
265 that closely approximate our experimental observations (Figure 4E). Thus whilst we cannot
266 absolutely discount the possibility that rhythmic translation contributes to the PER2::LUC
267 rhythms in CKO cells, we found no evidence to support this, whereas experimental
268 observations and theoretical modelling do suggest rhythmic PER2 degradation alone is
269 sufficient to explain the residual bioluminescence rhythms we observe in CKO PER2::LUC
270 fibroblasts.

271

272 **CK1 δ/ϵ and GSK3 contribute to CRY-independent PER2 oscillations**

273 PER2 stability is primarily regulated through phosphorylation by casein kinases (CK) 1 δ and
274 1 ϵ , which phosphorylate PER2 at phosphodegron sites to target it for proteasomal degradation
275 (Lee et al., 2009; Philpott et al., 2020). In this context CK1 δ/ϵ frequently operate in tandem with
276 glycogen synthase kinase (GSK) 3 α/β , as occurs in the regulation of β -catenin stability (O'Neill
277 et al., 2013; Robertson et al., 2018). Interestingly, both CK1 δ/ϵ and GSK3 α/β have a conserved
278 role in determining the speed at which the eukaryotic cellular circadian clock runs (Causton et
279 al., 2015; Hastings et al., 2008), both in the presence and absence of transcription (Beale et
280 al., 2019; Hirota et al., 2008; Meng et al., 2008; O'Neill et al., 2011). This is despite the fact
281 that the clock proteins phosphorylated by these kinases are highly dissimilar between animals,
282 plants, and fungi (Causton et al., 2015; Wong and O'Neill, 2018).

283
284 We hypothesised that the PER2::LUC rhythm in CKO cells reflects the continued activity of a
285 post-translational timekeeping mechanism that involves CK1 δ/ϵ and GSK3 α/β , which results
286 in the differential phosphorylation and turnover of clock protein substrate effectors such as
287 PER2 during each circadian cycle (O'Neill et al., 2013). To test this we incubated WT and CKO
288 cells with selective pharmacological inhibitors of CK1 δ/ϵ (PF670462; PF) and GSK3 α/β
289 (CHIR99021; CHIR), which have previously been shown to slow down, and accelerate,
290 respectively, the speed at which the cellular clock runs in a wide range of model organisms
291 (Badura et al., 2007; Causton et al., 2015; Hirota et al., 2008; O'Neill et al., 2011). As a control
292 we used KL001, a small molecule inhibitor of CRY degradation (Hirota et al., 2012), which has
293 previously been shown to affect cellular rhythms in WT cells via increased CRY stability.

294
295 We found that inhibition of CK1 δ/ϵ and GSK3- α/β had the same effect on circadian period in
296 CKO cells as WT controls (Figure 5A, B, S5A, B). In contrast, KL001 increased period length
297 and reduced amplitude of PER2::LUC expression in WT cells but had no significant effect on
298 post-translationally regulated PER2::LUC rhythms in CKO cells (Figure 5C and S5C). Besides
299 confirming the specific mode of action for KL001 in targeting CRY stability, these observations

300 implicate CK1 δ/ϵ and GSK3 α/β in regulating the post-translational rhythm reported by
301 PER2::LUC in CKO cells.

302

303 **Discussion**

304 We found that transcriptional feedback in the canonical TTFL clock model is dispensable for
305 cell-autonomous circadian timekeeping in animal and cellular models. In mice and flies,
306 deficient for CRY/PER or TIMELESS/PER-mediated feedback repression, the capacity for
307 circadian gene expression remained intact, though clearly impaired with respect to WT.
308 Circadian rhythms of PER2 abundance were observed in CKO SCN slices and fibroblasts,
309 indicating that the post-translational mechanisms that confer circadian rhythmicity onto PER
310 proteins in WT cells remain ostensibly intact in the absence of CRY-mediated transcriptional
311 feedback repression. Importantly however, CKO PER2 rhythms were only observed in a
312 minority of recordings (~30%), and when observed they showed increased variance of period
313 and sensitivity to perturbation. This reduced capacity to perform without failure under a wide
314 range of conditions means that CRY-deficient PER2 oscillations are less robust than those in
315 WT cells (Merriam-Webster Dictionary, 2020). We were unable to identify all of the variables
316 that contribute to the apparent stochasticity of CKO PER2::LUC oscillations, and so cannot
317 distinguish whether this variability arises from reduced fidelity of PER2::LUC as a circadian
318 reporter or impaired timing function in CKO cells. In consequence, we restricted our study to
319 those recordings in which clear bioluminescence rhythms were observed, enabling the
320 interrogation of TTFL-independent cellular timekeeping.

321 In the field of chronobiology, CKO cells and mice are often used as clock-deficient models.
322 Indeed, canonical circadian transcriptional output is essentially absent from these models
323 (Hoyle et al., 2017; Ode et al., 2017), and thus for studying TTFL-mediated control of overt
324 physiology they are appropriate negative controls. However, as the underlying timekeeping
325 mechanism seems at least partially intact, we therefore consider it inappropriate to describe
326 CKO cellular models as arrhythmic. Indeed, rest/activity behaviour of CKO mice does entrain

327 to daily cycles of restricted feeding (Iijima et al., 2005), which is SCN-independent (Storch and
328 Weitz, 2009), as well as a sufficiently strong synchronising zeitgeber (Figure 1A, (Chen et al.,
329 2008)). Thus, non-TTFL mediated timekeeping seems sufficient to serve as an (about) daily
330 interval timer *in vivo* (Crosby et al., 2019).

331 Previous studies have reported isolated CKO cells to be entirely arrhythmic (Ode et al., 2017;
332 Sato et al., 2006; Ukai-Tadenuma et al., 2011), in stark contradiction with our findings.
333 However, most such studies measured changes in transcription either by quantitative RT-PCR,
334 or with luciferase fusions to fragments of the *Bmal1*, *Per* and *Cry* promoters which we also
335 found to be arrhythmic in CKO cells. We did observe low amplitude oscillations in *Nr1d1*
336 promoter activity however. It may be pertinent to report that these MEF recordings only
337 revealed oscillations of *Nr1d1*-promoter activity, and only in bicarbonate-buffered medium
338 supplemented with 1mM luciferin and 10% serum (Figure 3D), but not in low serum or HEPES-
339 buffered media, as employed in other studies that used different circadian reporters and may
340 have employed sub-saturating concentrations of luciferin (Feeney et al., 2016a). It is also
341 plausible that the high sensitivity of the electron-multiplying CCD camera we used for these
342 bioluminescence assays allows the quantification of biological rhythms that were not
343 detectable using other approaches (Crosby et al., 2017).

344 Although several mechanisms for circadian regulation of translation have been described
345 (Jouffe et al., 2013; Lipton et al., 2015), we did not find any contribution of rhythmic translation
346 to CRY-independent rhythms. In fact, the BMAL1-S6K1 interaction that mediates BMAL1's
347 interaction with the translational apparatus is absent from CKO cells (Figure S4C), implying a
348 possible role for CRY proteins in this complex. Instead, we found an overt circadian regulation
349 of PER2::LUC stability that persists in the absence of CRY proteins and which was sufficient
350 to account for the observed PER2::LUC rhythms in a simple mathematical model. Persistent
351 post-translational regulation of PER stability/activity may also account for the results of earlier
352 over-expression studies, in mammalian cells and flies, where constitutive *Per* mRNA
353 expression resulted in rhythmic PER protein abundance (Fujimoto et al., 2006; Yamamoto et

354 al., 2005; Yang and Sehgal, 2001); whereas *Per* over-expression should really abolish rhythms
355 if *Per* mRNA levels are the fundamental state variable of the oscillation. This interpretation has
356 marked similarities with recent reports in the fungal clock model, *Neurospora Crassa*, where
357 experiments have suggested that post-translationally regulated cycles in the activity of the
358 FRQ clock protein, not its abundance, are the critical determinant of downstream circadian
359 gene regulation (Larrondo et al., 2015).

360 Indeed, our observations may not be particularly surprising when one considers that post-
361 translational regulation of circadian timekeeping is ubiquitous in eukaryotes, with the period-
362 determining function of CK1 δ/ϵ and GSK3 α/β being conserved between the animal, plant and
363 fungal clocks (Causton et al., 2015; Hirota et al., 2010; Lee et al., 2009; O'Neill et al., 2011;
364 Wong and O'Neill, 2018; Yao and Shafer, 2014), despite their clock protein targets being highly
365 dissimilar between phylogenetic kingdoms. Importantly we observed that pharmacological
366 inhibition of these kinases elicited the same period-lengthening and -shortening effects on
367 CRY-independent rhythms as on WT rhythms. This has implications for our understanding of
368 the role that these kinases play in the cellular clock mechanism, since in the absence of TTFL-
369 mediated timekeeping their effects cannot be executed through regulation of any known
370 transcriptional clock component.

371 Given similar findings across a range of model systems, including isolated red blood cells
372 (Wong and O'Neill, 2018), the simplest interpretation of our findings entails an underlying,
373 evolutionarily-conserved post-translational timekeeping mechanism: a “cytoscillator” (Hastings
374 et al., 2008) that involves CK1 δ/ϵ and GSK3 α/β , and can function independently of canonical
375 clock proteins, but normally reciprocally regulates with cycles of clock protein activity through
376 changes in gene expression (Qin et al., 2015). This cytoscillator confers 24-hour periodicity
377 upon the activity and stability of PER2, and most likely to other clock protein transcription
378 factors as well (Figure 3D). However, a purely post-translational timing mechanism should be
379 rather sensitive to environmental perturbations and biological noise (Ladbury and Arold, 2012),
380 as seen for CKO cells. Due to the geometric nature of their underlying oscillatory mechanism,

381 relaxation oscillators are known to be particularly insensitive to external perturbations and
382 prevalent in noisy biological systems (Muratov and Vanden-Eijnden, 2008). We therefore
383 suggest that in wild type cells, low amplitude, cytosillator-driven circadian cycles of clock
384 protein activity are coupled with, reinforced and amplified by a damped TTFL-based relaxation
385 oscillation of stochastic frequency (Chickarmane et al., 2007), resulting in high-amplitude,
386 sustained circadian rhythms in both clock and clock-controlled gene expression. Indeed,
387 mathematical modelling shows that such coupling can both drive the emergence of sustained
388 oscillations in overdamped systems (In et al., 2003) and play an important role in maintaining
389 robust oscillations in a random environment (Medvedev, 2010). This model is consistent with
390 recent observations in the clocks of the prokaryotic cyanobacterium *Synechococcus elongatus*
391 (Qin et al., 2010; Teng et al., 2013) as well as the fungus *Neurospora crassa* (Larrondo et al.,
392 2015), and the alga *Ostreococcus tauri* (Feeney et al., 2016b) (see supplementary information
393 for an extended discussion).

394 Interestingly, the concept of the eukaryotic post-translational clock mechanism we propose is
395 not new (Jolley et al., 2012; Meroow et al., 2006; Qin et al., 2010; Roenneberg and Meroow,
396 1998) and resembles the KaiA/B/C mechanism elucidated in cyanobacteria (Nakajima et al.,
397 2005; Teng et al., 2013). The challenge will now be to identify additional factors that, in concert
398 with CK1 and GSK3, and protein phosphatase 1 (Lee et al., 2011), serve as the functional
399 equivalents of KaiA/B/C; allowing reconstitution of the mammalian circadian clock *in vitro*.
400 (Millius et al., 2019; Nakajima et al., 2005).

401 Here we have uncovered PER2 as a node of interaction between a putative cytosillator
402 mechanism and the canonical circadian TTFL (Figure 5D). It is unlikely however that PER2 is
403 the only interaction between the two, as *Per2*^{-/-} knockout cells and mice exhibit competent
404 circadian timekeeping (Xu et al., 2007), suggesting redundancy in this respect. Indeed, the
405 residual noisy but rhythmic activity of the *Nr1d1*-promoter in the absence of both PER1/2 and
406 CRY1/2 (Figure 3D), suggests another point-of-connection between the cytosillator and
407 TTFL. Moreover, both CK1 and GSK have been implicated in the phosphorylation and

408 regulation of many other clock proteins (See table S2 in (Causton et al., 2015), also reviewed
409 in (O'Neill et al., 2013)). Some or all of these targets may play a role in coupling the cytos oscillator
410 with TTFL-mediated clock output. We believe that it is now imperative to delineate the specific
411 means by which the TTFL couples with the cytos oscillator to effect changes in circadian phase
412 in order that the two resonate with a common frequency.

413 **Conclusion**

414 Whilst the contribution of clock protein transcription factors to the temporal coordination of
415 gene expression, physiology and behaviour is unambiguous, the primacy of transcriptional
416 feedback repression as the ultimate arbiter of circadian periodicity within eukaryotic cells is
417 not. Similar to the conserved kinase-dependent regulation of the cell division cycle, we suggest
418 the circadian cycle in diverse eukaryotes is conserved from a common ancestor, with diverse
419 TTFL components having been recruited throughout speciation to impart robustness, signal
420 amplification and functional specificity to the oscillation.

421

422 **Experimental procedures**

423 Reagents were obtained from sigma unless stated otherwise. More detailed experimental
424 procedures can be found in the supplementary information (SI).

425 **Mouse work**

426 All animal work was licensed under the UK Animals (Scientific Procedures) Act 1986, with
427 Local Ethical Review by the Medical Research Council. *Cry1/2*-null mice were kindly provided
428 by G. T. van der Horst (Erasmus MC, Rotterdam, The Netherlands) (Horst and Muijtjens,
429 1999), *PER2::LUC* mice by J. S. Takahashi (UT Southwestern, USA) (Yoo et al., 2004) and
430 *Cry1::LUC* mice by M. Hastings (MRC LMB, Cambridge, UK) (Maywood et al., 2013). All lines
431 were maintained on a C57BL/6J background. For mouse behavioral studies, CKO *PER2::LUC*
432 female mice aged 2-5 months, and age-matched *PER2::LUC* controls, were singly housed in
433 running wheel cages with circadian cabinets (Actimetrics). They were then subject to 7 days
434 12h:12h LD cycles or 7 days constant light (400 lux), and then maintained in constant darkness
435 with weekly water and food changes. Locomotor activity was recorded using running wheel
436 activity and passive infrared detection, which was analysed using the periodogram function of
437 ClockLab (Actimetrics). SCN organotypic slices from 7-10 day old pups were prepared as
438 previously described (Hastings et al., 2005), and bioluminescence recorded using
439 photomultiplier tubes (Hamamatsu).

440

441 **Mammalian cell culture**

442 Primary fibroblasts were isolated from lung tissue (Seluanov et al., 2010) of adult wild type
443 (WT) and *Cry1^{-/-},Cry2^{-/-}* (CKO) *PER2::LUC* male and female mice, and WT and CKO *Cry1::LUC*
444 mice. Stable WT, CKO and *Cry1^{-/-},Cry2^{-/-}, Per1^{-/-}, Per2^{-/-}* (CPKO) mouse embryonic fibroblasts
445 (MEFs) expressing transcriptional luciferase reporters for clock gene activity were generated
446 by puromycin selection and cultured as described previously (Valekunja et al., 2013). MEFs
447 were seeded into 96-well white plates at 10⁴ cells/well and grown to confluency for 5 days
448 under temperature cycles (12h:12h, 32°:37°) to synchronise circadian rhythms. Primary

449 fibroblasts were cultured as described previously (O'Neill and Hastings, 2008) and
450 immortalised by serial passage (Xu, 2005). CRY deficiency was confirmed by PCR (see SI
451 and Western blotting (guinea pig-anti-CRY1 and CRY2 antibodies (Lamia et al., 2011)).
452 NIH3T3 fibroblasts expressing SV40::LUC have been described before (Feeney et al., 2016a).

453

454 **Luciferase recordings**

455 Fibroblast recordings were performed in air medium (either HEPES or MOPS buffered (20mM),
456 either in airtight sealed dishes (in non-humidified conditions) or open in humidified conditions
457 (0% CO₂). Air medium stock was prepared as described previously (O'Neill and Hastings,
458 2008) and supplemented with 2% B-27 (Life Technologies, 50X), 1 mM luciferin (Biosynth AG),
459 1X glutamax (Life Technologies), 100 units/ml penicillin/100 µg/ml streptomycin, and 1%
460 FetalClone™ III serum (HyClone™). Final osmolarity was adjusted to 350 mOsm with NaCl.
461 Recordings were preceded by appropriate synchronisation (see SI for details) in presence of
462 0.3 mM luciferin to prevent artificially high bioluminescence activity at the start of the recording,
463 and started immediately after a medium change from culture medium into air medium. The
464 presented MEF recordings were performed in an ALLIGATOR (Crosby et al., 2017), and
465 employed bicarbonate-buffered Dulbecco's Modified Eagle Medium (10569010) with
466 penicillin/streptomycin and 1 mM luciferin in a humidified incubator at 5% CO₂, also
467 supplemented with 2% B-27 and 10% FetalClone™ III serum. A range of other media
468 conditions were explored but did not produce detectable bioluminescence rhythms in CKO or
469 CPKO cells (not shown). For pharmacological perturbation experiments (unless stated
470 otherwise in the text) cells were changed into drug-containing air medium from the start of the
471 recording. Mock-treatments were carried out with DMSO or ethanol as appropriate.

472 Bioluminescence recordings were performed in a lumicycle (Actimetrics), a LB962 plate reader
473 (Berthold technologies) or an ALLIGATOR (Cairn Research). Acute luciferase assays were
474 performed using a Spark 10M microplate reader (Tecan).

475

476 **Biochemistry**

477 The number of PER2 molecules was determined by harvesting a known number of
478 synchronised WT and CKO cells at the peak of PER2 expression and comparing the Luciferase
479 activity to a standard curve of recombinant Luciferase (see SI for details). Three technical
480 replicates were measured in every experiment and the experiment was carried out three times.
481 A representative experiment is shown.

482 For determining *Per2::Luc* and *Bmal1* mRNA levels, synchronised cells were harvested from
483 constant conditions in triplicate every four hours from 24 hours up to 48 hours after media
484 change. RNA extraction and qPCR were performed as detailed in the SI. Analysis involved
485 three technical and three biological replicates. Relative amounts of mRNA were determined by
486 comparing the samples to a standard curve, and expressed relatively to ribosomal RNA
487 Rns18s.

488 For comparing longitudinal PER2::LUC recordings to the actual PER2::LUC protein levels
489 (longitudinal versus acute luciferase assays), synchronised WT and CKO cells (cultured in
490 absence of luciferin) were harvested every hour (in triplicate) from 16 hours up to 64 hours
491 after media change, while co-cultures were recorded for bioluminescence in presence of
492 luciferin. Luciferase activity in acute assays was determined as detailed in SI.

493 For assaying the interaction between BMAL1 and PER2::LUC, synchronised cells were
494 harvested directly from temperature cycles at the expected peak of PER2::LUC expression (4
495 hours after change to 32°C) and BMAL1 was precipitated as described in SI. PER2::LUC co-
496 immunoprecipitation was measured in a luciferase assay by mixing the BMAL1-loaded beads
497 in luciferase assay buffer (15 mM MgSO₄, 30 mM HEPES, 300 μM luciferin, 1 mM ATP, 10
498 mM 2-mercaptoethanol) and measuring luciferase activity in a Berthold platereader. The
499 results were corrected for input and plotted relatively to the WT IgG pulldown.

500 To study the interaction of BMAL1 with S6K and eIF4, cells were synchronised by a 2-hour
501 dexamethasone pulse, after which they were changed into normal growth medium. 12 and 24
502 hours after the medium change, BMAL1 immunoprecipitation was executed as described in
503 SI. Samples were analysed by Western blot for presence of BMAL1, S6K and eIF4 (cell
504 signalling, resp. #2708 and #2013)

505

506 ***Drosophila* experiments**

507 All fly strains were kept in standard cornmeal food under 12 h:12 h LD cycles at constant
508 25°C (LD cycles). The following control strains were included in the experiments: *per⁰¹*,
509 *Canton S*, and *w¹¹¹⁸*. The generation of *Tim^{Out}* flies, crossings with XLG-luc flies (Veleri et al.,
510 2003), and details of recordings are described in SI. In short, three to seven days-old flies
511 were entrained for three day LD cycles before being loaded individually into the wells of a
512 microtiter plate containing the food-luciferin substrate (15mM luciferin). Recordings were
513 performed under constant darkness at 26°C over seven days. Bioluminescence from each fly
514 was background subtracted, summed into 2-hour bins, then detrended using a 24-hour
515 moving average. Rhythmicity of averaged traces was tested using the RAIN algorithm
516 (Thaben and Westermark, 2014) using 4-hour binned traces from 48 till 96 hours.
517 Normalised and detrended Single fly traces were manually divided over three categories:
518 “Robustly rhythmic”, “Poorly rhythmic”, and “Arrhythmic” according to examples in Figure
519 S2B: traces with clear ~24hr rhythms over 4 cycles were termed “robustly rhythmic”, traces
520 with lower amplitude but overt rhythms over 3 cycles “poorly rhythmic” and traces with <3
521 overt peaks were termed “arrhythmic”.

522

523 **Analysis**

524 All analyses were performed in Graphpad Prism versions 7 and 8. Where indicated, data was
525 detrended using moving average subtraction, where temporal window of the moving average
526 was refined iteratively until it matched with the period of oscillation derived as follows. Period
527 analysis was performed either manually, or by least-square fitting to a circadian damped sine
528 wave with a linear baseline:

$$529 \quad y = (mx + c) + a \exp^{-kx} \sin\left(\frac{2\pi x - r}{p}\right)$$

530 Where m is the gradient of the baseline, c is the y offset, k describes the rate of dampening, a
531 the amplitude, r the phase and p the period. Reported p -values for the curve fit are those

532 produced by the comparison of fits functions in Prism 8, where the null hypothesis was a
533 straight line ($y = mx + c$), i.e., change over time but with no oscillatory component. The simpler
534 model was preferred unless the sine wave fit produced a better fit with $p < 0.05$.

535
536 For the mathematical model in 4E we used assumed that PER2::LUC translation at time (t) is
537 a function of *Per2::Luc* mRNA abundance, corrected for the changes we observed for global
538 translation rate over time; and that PER2::LUC degradation rate follows one-phase exponential
539 decay kinetics where the decay constant is defined by a sine wave with 24-hour periodicity,
540 with the amplitude, phase and other parameters being derived entirely from experimental
541 measurements. See SI for details.

542

543 **Acknowledgements**

544 We thank biomedical technical staff at Medical Research Council (MRC) Ares facility and LMB
545 facilities for assistance, G.T. van der Horst and J.S. Takahashi for sharing rodent models, M.H.
546 Hastings and E.S. Maywood for providing reagents and input, K. Lamia for providing reagents,
547 and P. Crosby, D.S. Tourigny, J.E.C. Jepson, C.P. Kyriacou, H.R. Pelham for valuable
548 discussion. MP was supported by the Dutch Cancer Foundation (KWF, BUIT-2014-6637) and
549 EMBO (ALTF-654-2014). JON was supported by the Medical Research Council
550 (MC_UP_1201/4) and the Wellcome Trust (093734/Z/10/Z). NP and RF were supported by the
551 Deutsche Forschungsgemeinschaft FKZ (Pe1798/2-1). AS and CPS were supported by the
552 National Institutes of Health (GM118102).

553

554 **Author contributions**

555 MP and JON designed the study, analysed the data and wrote the manuscript; AZ and NR
556 performed mouse behavioural studies; MP, DW, ES, NH, KF and JON performed cell
557 experiments; CS and AS generated MEF cell lines; ME performed SCN experiments; KC, RF,

558 NP and JON performed fly experiments; JC performed tissue collection and husbandry; All
559 authors commented on the manuscript.

560

561 **Conflict of interest**

562 The authors declare that they have no conflict of interest.

563

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833

834

835 **List of abbreviations**

836

837	CCGs	Clock controlled genes
838	CHX	Cycloheximide
839	CK1	Casein kinase 1
840	CKO	CRY Knock out
841	CPKO	CRY PER Knock out
842	CPS	Counts per second
843	CP 20s	Counts per 20 seconds
844	CRY	Cryptochrome
845	GSK3	Glycogen synthase kinase 3
846	LL>DD	12h:12h Light:light to dark:dark transition
847	MAF	Mouse adult fibroblast
848	MEF	Mouse embryonic fibroblast
849	PER	Period
850	RLU	Relative light units
851	SCN	Suprachiasmatic nucleus
852	SD	Standard deviation
853	SEM	Standard error of the mean
854	<i>Tim_{out}</i>	Timeless knockout
855	TTFL	Transcriptional translational feedback loop
856	WCL	Whole cell lysates
857	WT	Wild type

858

859

860 **FIGURE 1. CRY-independent circadian timekeeping occurs cell-autonomously**

861 (A) Representative double-plotted actograms showing wheel-running activity of wild type

862 (WT) and CRY-deficient (CRY Knockout; CKO) mice during constant light (yellow shading)

863 and thereafter in constant darkness. Note the 48 hour X-axis for WT versus 32 hour for CKO.

864 (B) Mean period and amplitude (\pm SEM) of mouse behavioural data (n=4).

865 (C) Longitudinal bioluminescence recordings of organotypic SCN slices from WT (black) and

866 CKO (red) PER2::LUC mice (RLU; relative light units).

867 (D) Mean period and amplitude (\pm SEM) of rhythmic SCN bioluminescence traces.

868 (E) Circadian PER2::LUC expression in immortalised WT and CKO adult lung fibroblasts.

869 Left panel shows two raw traces of a representative longitudinal bioluminescence recording,

870 right panel shows same data detrended with a 24-hour moving average to remove

871 differences in baseline expression.

872 (F) Period of rhythmic fibroblast bioluminescence traces from at least 31 experiments (n \geq 3

873 per experiment). P-values were calculated using an unpaired t test with Welch correction.

874 Standard deviations differ significantly between WT and CKO (F test: p <0.0001).

875

876 **FIGURE 2. CRY-less oscillations are temperature compensated and entrained**

877 (A) Detrended traces of bioluminescence recordings of WT and CKO fibroblast at different
878 constant temperature conditions within the physiological range (n=4, mean \pm SEM).

879 Temperature was changed from 37°C to 32°C halfway through the experiment, as depicted
880 by red/blue shading. Arrows represent medium changes. Note the lack of rhythmicity in the
881 first three days in CKO and the appearance of rhythmicity after the first medium change.

882 (B) Quantification of period from recordings presented in (A). Both WT and CKO oscillations
883 are temperature compensated with respective Q_{10} s of 1.05 and 0.95.

884 (C) Bioluminescence of WT and CKO PER2::LUC cells during temperature entrainment (12h
885 32°C (blue) – 12h 37°C (red)). Two representative traces of two independent cell lines are
886 shown per genotype.

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893 **FIGURE 3. CRY-independent rhythms are regulated post-transcriptionally**

894 (A) Mean number of PER2::LUC molecules per cell at the estimated peak of PER2
895 expression for each cell line (mean of three experiments, n=3 each). P-values were
896 calculated in a paired t test.

897 (B) PER2::LUC binding to BMAL1 in WT and CKO cells. Cells were harvested at the peak of
898 PER2 expression, BMAL1 was immunoprecipitated, and PER2::LUC binding was measured
899 by bioluminescence measurements (n=3, mean \pm SD). P-values were calculated in an
900 unpaired t test.

901 (C) *Per2* mRNA levels in WT (left) and CKO (right) cells were determined by qPCR over one
902 circadian cycle (bottom), while PER2::LUC bioluminescence (min-max normalised) was
903 recorded from parallel cultures (top) (mean *Per2* mRNA relative to *Rns18s* (bottom) and
904 PER2::LUC signal (top), n=3, \pm SEM). The WT mRNA trace could be fitted with a circadian
905 damped sine wave ($p=0.0412$) whereas data of CKO cells could not (ns).

906 (D) Detrended *Per2* and *Nr1d1* promoter activity in WT, CKO and quadruple *Cry1/2-Per1/2*
907 knockout (CPKO) mouse embryonic fibroblasts (MEFs) recorded at 37°C. *Nr1d1* data were fit
908 with a circadian damped sine wave over straight line ($p<0.0001$) (right graphs). Similar
909 recordings performed at 32°C and an expanded view of *Per2* data are in figures S3E and F.

910

911 **FIGURE 4. PER2::LUC stability oscillates in CRY-deficient cells**

912 (A) Actual PER2::LUC levels (dark symbols (3-hour moving average, $n=3 \pm \text{SEM}$, 4 outliers
913 removed)) as assayed in acute luciferase assays on cell lysates from cells harvested every
914 hour over 48 hours, compared with parallel longitudinal co-recordings from cells in the
915 presence of 0.1mM luciferin (light lines ($n=6$, mean $\pm \text{SEM}$)).

916 (B) PER2::LUC recording of asynchronous WT and CKO cells pulsed with proteasome
917 inhibitor MG132 (10 μM , applied at the arrow) ($n=3$, mean $\pm \text{SEM}$).

918 (C) Quantification of relative PER2::LUC induction upon proteasome inhibition. P-value was
919 calculated by unpaired t test.

920 (D) Phase-dependent PER2::LUC half-life was determined by inhibiting translation at different
921 circadian phases and fitting the resulting data with a one-phase exponential decay curve ($n=3$,
922 mean $\pm \text{SEM}$). Left image depicts the timing of cycloheximide (CHX, 10 μM) pulses (labelled I
923 (PER2 levels going up) and II (PER2 levels going down)), plotted on PER2::LUC
924 bioluminescence traces of control cells (dark colours). A representative trace of CHX-treated
925 cells at time point I is shown in light colours. See FIG S4D-E for more raw data and time points.
926 Right image shows quantifications, p-values were calculated by unpaired t test.

927 (E) A simple model incorporating mRNA, protein translation and PER2::LUC stability we
928 measured experimentally (inputs) shows that the observed oscillating stability of PER2 is
929 sufficient to generate rhythmic PER2::LUC expression (output).

930

931 **FIGURE 5 A role for CK1 and GSK3 in the cytoplasmic oscillator**

932 (A) Period (right; n=3, mean \pm SEM) analyses of WT and CKO PER2::LUC cells in the
933 presence or absence of CK1 δ/ϵ inhibitor PF670462 (0.3 μ M; PF). P-values were calculated
934 by unpaired t test.

935 (B) As in (A), GSK3 inhibitor CHIR99021 (5 μ M; CHIR).

936 (C) As in (A), in presence of CRY inhibitor KL001 (1 μ M).

937 (D) Schematic model integrating CRY-independent timekeeping into the existing canonical
938 model of the circadian clock. The CRY-dependent gene expression feedback loop (TTFL) is
939 required for most circadian regulation of transcriptional clock controlled genes (CCGs) and
940 therefore for robustness and behavioural and physiological rhythmicity. However, it is
941 dispensable for circadian timekeeping *per se*, as reported by residual oscillations in PER2
942 protein levels, suggestive of the existence of a coupled underlying (cytosolic) timekeeping
943 mechanism involving CK1 and GSK3 (cytosillator).

944 See FIG S5 for raw data.

FIGURE 1

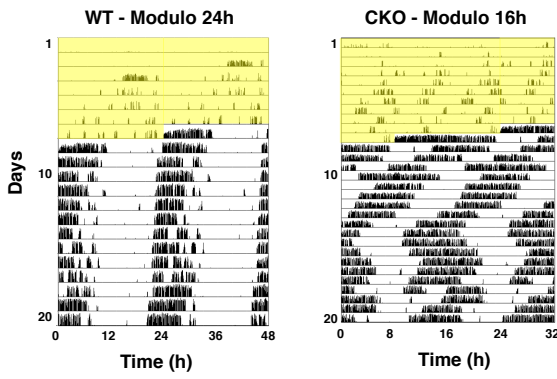
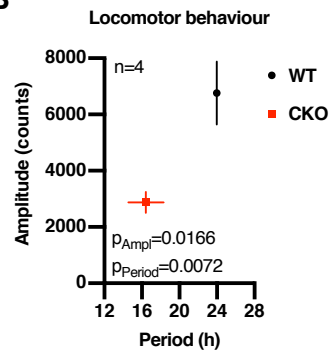
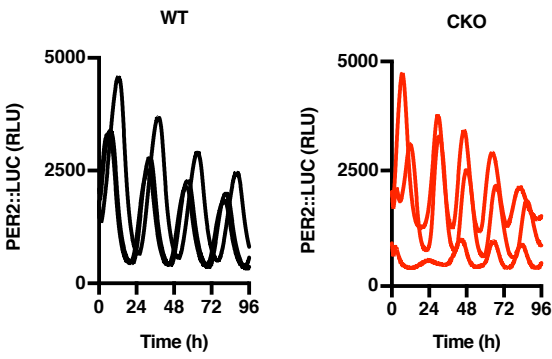
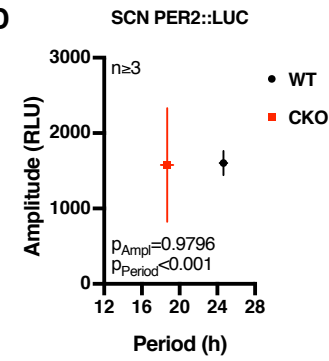
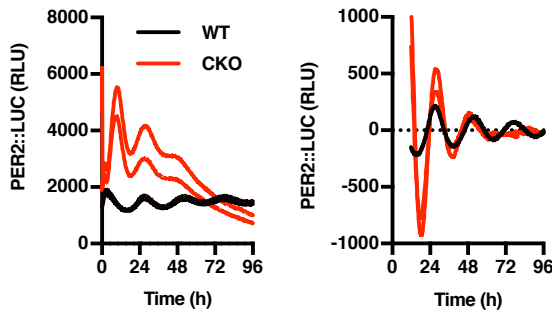
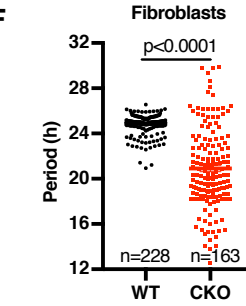
A**B****C****D****E****F**

FIGURE 2

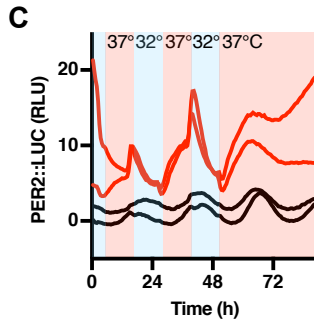
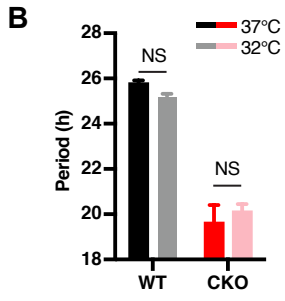
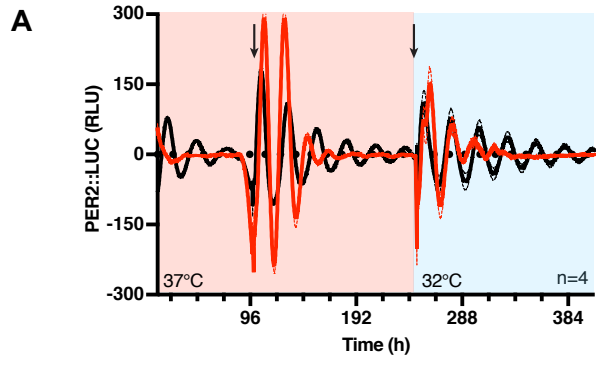


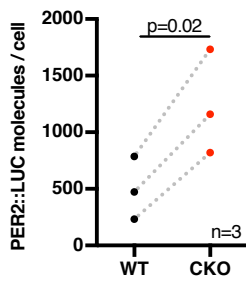
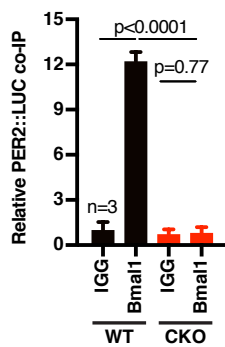
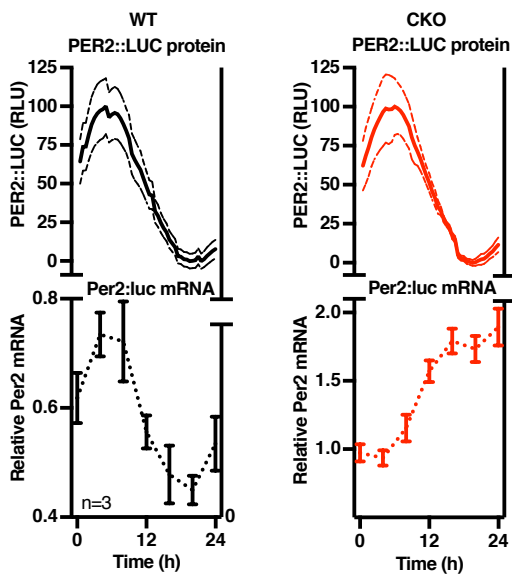
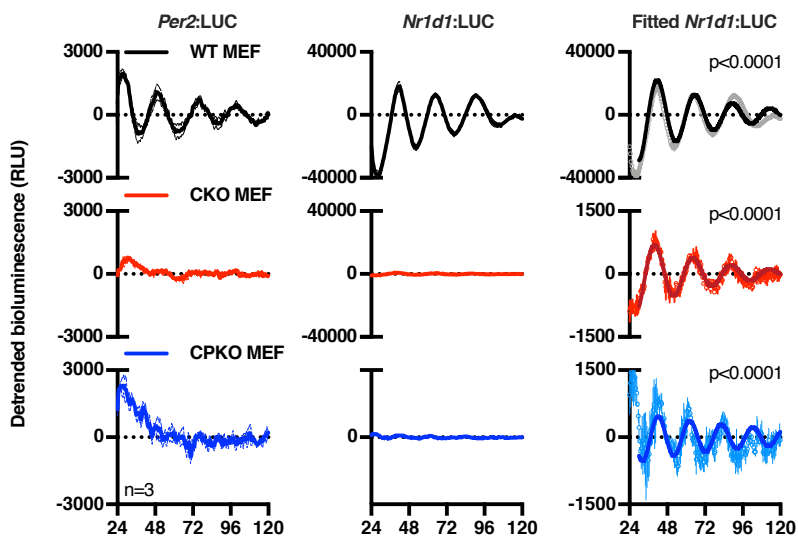
FIGURE 3**A****B****C****D**

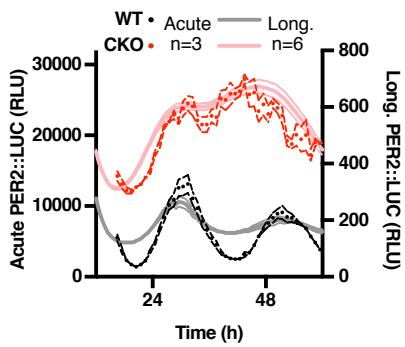
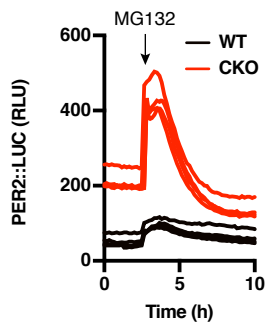
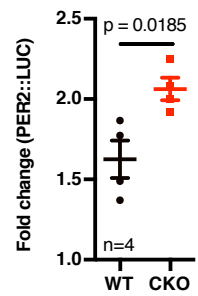
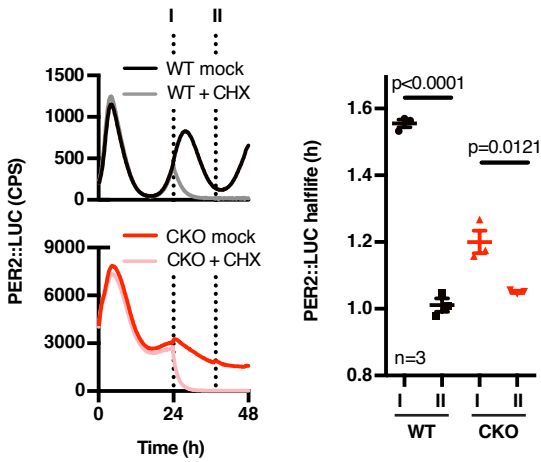
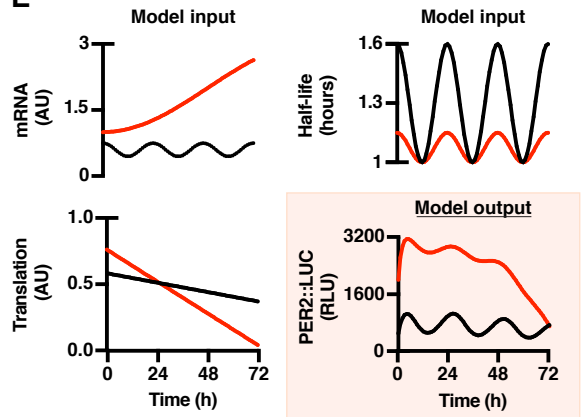
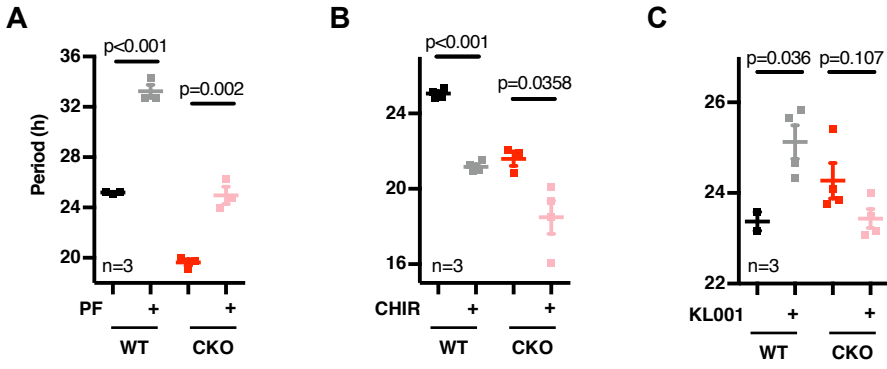
FIGURE 4**A****B****C****D****E**

FIGURE 5**D**