An ecdysone-responsive nuclear receptor regulates circadian rhythms in Drosophila

Shailesh Kumar1, Dechun Chen1, Christopher Jang1, Alexandra Nall1, Xiangzhong Zheng1 & Amita Sehgal1,2

Little is known about molecular links between circadian clocks and steroid hormone signalling, although both are important for normal physiology. Here we report a circadian function for a nuclear receptor, ecdysone-induced protein 75 (Eip75/E75), which we identified through a gain-of-function screen for circadian genes in Drosophila melanogaster. Overexpression or knockdown of E75 in clock neurons disrupts rest:activity rhythms and dampens molecular oscillations. E75 represses expression of the gene encoding the transcriptional activator, CLOCK (CLK), and may also affect circadian output. PER inhibits the activity of E75 on the Clk promoter, thereby providing a mechanism for a previously proposed de-repressor effect of PER on Clk transcription. The ecdysone receptor is also expressed in central clock cells and manipulations of its expression produce effects similar to those of E75 on circadian rhythms. We find that E75 protects rhythms under stressful conditions, suggesting a function for steroid signalling in the maintenance of circadian rhythms in Drosophila.
Timekeeping in *Drosophila* relies upon transcription-translational feedback loops, in which rhythmically expressed clock genes negatively regulate their own expression. In the major loop, the CLOCK-CYCLES (CLK-CYC) heterodimer activates transcription of the *period* (*per*) and *timeless* (*tim*) genes during the day, and in the middle of the night, the *PER* and *TIM* proteins heterodimerize and enter the nucleus to repress the activity of CLK-CYC. In a second interlocked loop, CLK-CYC activates expression of the PAR Domain Protein 1 (*Pdp1*) and *vrille* (*vri*) genes, which encode an activator and a repressor, respectively, of the *Clk* gene. PDP1e activates *Clk* transcription during the late night to early morning. However, it is important to note that *Clk* mRNA levels are maintained at peak levels even in *Clk* heterozygous and *clk* null mutant flies that have very low PDP1, suggesting other transcriptional regulators of *Clk* expression. In addition, structure-function analyses of the *Clk* promoter suggested that *Clk* expression can be regulated by transcription factors other than PDP1 and VRI. Taken together, these studies implicate other transcription factors in *Clk* expression and possibly in the *Drosophila* molecular clock.

The mammalian circadian clock is generated through similar mechanisms, whereby the negative regulators, CRYPTOCHROME (CRY) and PER, regulate the transcriptional activity of CLOCK and BMAL1 (mammalian orthologue of CYC). As in the *Drosophila* clock, the second loop is generated through autoregulation of one of the transcriptional activators, but in this case it is *Bmal1* rather than *Clock*. Nuclear receptors, REV-ERB and *β* are transcriptional repressors of the *Bmal1* gene, whereas ROR*α* is an activator and both of these targets are regulated by CLOCK-BMAL1. The closest *Drosophila* homologue of REV-ERB is the nuclear receptor and ecdysone-induced protein, *Eip75* (also known as *E75*), and *E75* is an activator that is known to regulate ecdysone and nitric oxide (NO) signalling during development and is also implicated in haem metabolism and signalling of gases such as carbon monoxide (CO) and nitric oxide. However, although some components of the ecdysone signalling pathway are implicated in circadian rhythms, it is not known if *E75* has a role in the *Drosophila* circadian clock.

In the present study, we identified *E75* as a component of the *Drosophila* clock through an unbiased gain-of-function genetic screen for novel circadian genes. Overexpression as well as knockdown of *E75* in clock neurons leads to arrhythmic or weak circadian behaviour. These manipulations also attenuate the molecular cycling of PER, indicating that they directly impact the molecular clock. We found that *E75* acts as a repressor of *Clk*, and is itself subject to inhibition by PER. Thus, we have identified a mechanism for the previously proposed deressor function of PER on *Clk* expression. Given the role of *E75* in steroid signalling, which is involved in the response to stress, we also investigated its function under conditions of environmental stress. We found that expression of *E75* protects the central clock against environmental stressors.

**Results**

**E75 is a novel gene that regulates circadian rhythms.** As previously described, we conducted a genetic screen for new circadian clock genes by overexpressing genes downstream of a randomly inserted EP enhancer and promoter element and assaying rest:activity rhythms. Of the 3,662 lines screened, one line (NE-30-49-10) contained an insertion in the promoter region of the *E75* gene. This insertion lies upstream of all known isoforms of *E75*, and its expression by the *tim*-G4 driver increases expression of *E75* approximately threefold in adult heads (Supplementary Fig. 1a). This overexpression, which may be even greater in the targeted clock cells, rendered 96% of the flies arrhythmic under constant dark (DD) conditions (Table 1A; Supplementary Fig. 1b). *E75* is an ecdysone-induced protein and, as noted above, its closest homologue is REV-ERB*α* in mammals. As *TG27* is expressed very broadly, we next overexpressed *E75* under the control of the *cry3* and *Pdf-Gal4* drivers, which are expressed at lower levels and more specifically in clock cells. *Pdf-Gal4*, in particular, is expressed only in the ventral lateral neurons, which are the central clock cells critical for behaviour in constant darkness. Overexpression of *E75* by *cry2* and *Gal4* resulted in loss of rhythms in 50% of the flies and those that were rhythmic, displayed significantly longer periods as well as weaker rhythms (Table 1A; Supplementary Fig. 1b). *Pdf-Gal4*-mediated *E75* overexpression also produced a modest increase in period length and significantly reduced rhythm strength. In addition about 20% of the flies were arrhythmic under these conditions (Table 1A; Supplementary Fig. 1b).

To verify that phenotypes obtained with the NE-30-49-10 insertion were due to overexpression of the *E75* gene, we also overexpressed it using two independent UAS-*E75* transgenes on the 2nd and 3rd chromosomes. *E75* occurs as six different isoforms (see below), and we determined by sequencing that these transgenes overexpress the RC isofrom. Overexpression of *E75* by the *TG27* driver causes an approximately twofold increase in *E75* mRNA levels (Supplementary Fig. 1a). The use of the *TG27* driver with either UAS-*E75* transgene reduced the number of rhythmic flies. However, only *TG27* > UAS-*E75* (II) flies showed a significant lengthening of period and decreased rhythm strength (Table 1A). On the other hand, *Pdf-Gal4*-driven expression of UAS-*E75* (III) rendered ~25% of the flies arrhythmic, although it did not produce a significant effect on other rhythm parameters. The weaker effect of the *Pdf-Gal4* driver may be due to its restricted expression or to weaker strength. Regardless, these data support the idea that overexpression of *E75* affects circadian behaviour.

We next sought to determine whether loss of *E75* impacts circadian rhythms. As null mutations of *E75* are homozygous lethal, we used RNA interference (RNAi) to reduce the expression of *E75* in clock cells, and analysed effects on circadian behaviour. We employed four independent lines carrying transgenic RNAi constructs targeted to the common region of *E75*, two from the VDRC collection (GD and KK) and two from the Bloomington Stock Center (IF02257 and HMS01530). *TG27*-mediated knockdown of *E75* levels led to ~75%, ~25% and ~42% arrhythmicity with JF, KK and GD RNAi lines, respectively, whereas knockdown via HMS RNAi was lethal (Table 1B). The GD RNAi lines showed a stronger effect on *E75* transcript levels than KK lines (Supplementary Fig. 1c). Rhythmic flies for all three viable RNAi lines displayed significantly weaker rhythms and the JF line also yielded a significantly shorter period (Table 1). *Pdf-Gal4*-mediated *E75* knockdown with the GD and JF RNAi lines also significantly reduced rhythmicity, but not circadian period (Table 1B). To improve the efficacy of knockdown, we co-expressed *dicer2* with the RNAi constructs. Combining *dicer2* with *TG27* caused lethality, so we coupled a slightly weaker *tim* driver, *tim-UAS-Gal4* (TUG) and *Pdf-Gal4* with *dicer2*. TUG > *dicer2* or *Pdf-Gal4* > *dicer2*-mediated *E75* knockdown led to a significant reduction in the number of rhythmic flies, and in most cases also in the strength of rhythmicity, with either the GD or JF RNAi transgene (Table 1B).

To map the RNAi phenotype to *E75*, we coupled the RNAi knockdown with genetic mutations of *E75*. Although complete loss of *E75* causes lethality, flies carrying one copy of the null allele (heterozygotes) survive and have normal rhythms (Table 1B). For instance, the *E75* strain contains an ~30 kb deletion that removes exons shared by all *E75* isoforms, and the
heterozygotes display robust rhythms (Table 1). However, restricted knockdown of E75 using Pdf-Gal4 in flies heterozygous for E75<sup>AS1</sup> resulted in markedly fewer rhythmic flies than produced by Pdf-Gal4 in a wild-type background (Table 1B), indicating that the phenotype is attributable to loss of E75 function.

As E75 manipulations had robust effects on circadian behaviour, we sought to determine if its expression was regulated in a circadian manner. E75 expresses six different mRNA isoforms (Flybase), three of which encode the same protein (RB, RE, RF). We designed PCR primers for the four that vary in protein sequence (RA, RB/E/F, RC, RD) and assayed expression at different times of day in adult brains. We detected moderate to robust cycling of four E75 isoforms in wild-type flies, with no cycling in the arrhythmic mutant Clk<sup>KK</sup> (Supplementary Fig. 1d–g), suggesting that expression of E75 is regulated by the circadian clock. In fact, a recent chromatin immunoprecipitation study indicated that CLK binds directly to the E75 promoter<sup>19</sup>.

Alterations in levels of E75 affect the molecular clock. To determine whether E75 affects molecular clock components, we first examined transcript levels of per and Clk in whole head extracts of flies overexpressing E75 (UAS-E75 II) via the Tg<sup>27</sup> driver. Oscillations of per and Clk were dampened by E75 overexpression, in particular through a reduction in peak levels (Fig. 1a,b). We also measured PER and CLK protein levels through western blots of whole head lysates and found that these protein levels were significantly different at ZT08 (Fig. 2a,c,d). mRNA and protein levels were also significantly higher at specific times of day (Fig. 2b,c,e). Notably, under these conditions, mRNA and protein levels were significantly higher at specific times of day (Fig. 2b,c,e). Notably, under these conditions, mRNA and protein levels were significantly different at ZT08 (Fig. 2a,c,d).

| Genotype | % Rhythmic (n) | Period (h) ± s.e.m. | FFT ± s.e.m. |
|----------|----------------|---------------------|-------------|
| (A)      |                |                     |             |
| TG<sup>27</sup> | 93.75 (64) | 24.16 ± 0.07 | 0.04 ± 0.005 |
| Pdf<sub>cy</sub> | 95.06 (61) | 24.03 ± 0.08 | 0.062 ± 0.004 |
| crs<sup>24</sup> | 87.50 (24) | 24.12 ± 0.12 | 0.066 ± 0.007 |
| NE30-49-10 | 96.82 (63) | 23.65 ± 0.25 | 0.083 ± 0.003 |
| UAS-E75 (II) | 93.75 (32) | 23.32 ± 0.64 | 0.076 ± 0.009 |
| UAS-E75 (III) | 96.87 (32) | 23.67 ± 0.14 | 0.092 ± 0.003 |
| Tg<sup>27</sup>&gt; E75 (II) | 4.20 (72) | 25.64 ± 0.35<sup>*</sup> | 0.018 ± 0.003<sup>*</sup> |
| Tg<sup>27</sup>&gt; E75 (III) | 33.87 (62) | 25.54 ± 0.54<sup>*</sup> | 0.031 ± 0.004<sup>*</sup> |
| Tg<sup>27</sup>&gt; NE30-49-10 | 40.66 (31) | 24.38 ± 0.24 | 0.087 ± 0.007 |
| Pdf &gt; NE30-49-10 | 80.60 (62) | 24.53 ± 0.23<sup>*</sup> | 0.035 ± 0.007<sup>*</sup> |
| Pdf &gt; E75 (II) | 90.32 (31) | 24.47 ± 0.45 | 0.078 ± 0.005 |
| Pdf &gt; E75 (III) | 73.91 (23) | 24.35 ± 0.21 | 0.054 ± 0.009 |
| crs<sup>24</sup>&gt; NE30-49-10 | 52.17 (23) | 25.03 ± 0.15<sup>*</sup> | 0.022 ± 0.005<sup>*</sup> |

ar, arrhythmic; DD, constant dark; D2, UAS-dcr2; FFT, fast Fourier transform; n, number of flies assayed; ND, not determined.

- *Significant difference (P<0.05, unpaired t-test). Statistical analysis cannot be conducted for % rhythmic flies.

Table 1 | Circadian behaviour of flies with altered E75 expression levels under DD conditions.
CLK expression under free-running conditions. CLK levels were significantly higher at CT08 and CT14 on the first day of DD in E75 knockdown flies (Supplementary Fig. 2c and d).

Clock proteins in adult head extracts are derived largely from the eyes, which do not contribute to the behavioural rhythm. Therefore, we also assayed PER levels through

---

**Figure 1 | Effect of E75 overexpression on the expression of per and Clk in adult heads.** (a) per mRNA expression in TG27 controls and TG27 > UAS-E75 (II) flies during the indicated phases of an LD cycle. (b) Clk mRNA expression in TG27 controls and TG27 > UAS-E75 (II) flies under LD cycle. (c) PER and Clk levels in the genotypes indicated above. A representative western blot is shown. PER and Clk levels are significantly lower in the TG27 > UAS-E75 (II) flies than in TG27 control flies particularly at peak time points. HSP70 antibodies are used to control for loading. Quantification of four independent experiments shows significantly decreased (d) PER and (e) Clk levels in TG27 > UAS-E75 (II) flies relative to the TG27 control flies. Asterisks above the bars denote significant differences between genotypes. *P < 0.05 using unpaired Student’s t-test. Error bars depict s.e.m. A molecular marker (Precision Plus Protein Dual Color Standards) was run to detect the exact molecular size of different proteins.

---

**Figure 2 | Effects of E75 knockdown on the expression of per and Clk in adult heads.** (a) per mRNA expression in TG27 controls and TG27 > UAS-E75 RNAi (GD) flies at the indicated time points of an LD cycle. (b) Clk mRNA expression in TG27 controls and TG27 > UAS-E75 RNAi (GD) flies under LD cycle. (c) PER and Clk levels in the same genotypes as above. A representative western blot is shown. HSP70 antibodies are used to control for loading. Quantification of six independent experiments shows (d) PER and (e) Clk levels in TG27 > UAS-E75 RNAi (GD) flies and TG27 control flies. Asterisks above the bars denote significant differences between genotypes. *P < 0.05 using unpaired Student’s t-test. Error bars depict s.e.m. A molecular marker (Precision Plus Protein Dual Color Standards) was run to detect the exact molecular size of different proteins.

CLK expression under free-running conditions. CLK levels were significantly higher at CT08 and CT14 on the first day of DD in E75 knockdown flies (Supplementary Fig. 2c and d).
immunohistochemistry (IHC) in circadian behaviour‐relevant brain clock neurons of flies with reduced levels of E75. As the behavioural phenotype produced by E75 knockdown was somewhat variable (perhaps due to inefficient knockdown), we first selected arrhythmic flies by assaying their behaviour and then collected eight to ten flies from each experimental and control group at four different times of day. TG27‐mediated knockdown of E75 resulted in dampened cycling of PER in constant darkness in different subsets of clock neurons (Fig. 3a,b). The dampening appeared to arise from significantly higher expression at trough times (CT8 and CT14; Fig. 3a,b). As the PDF cells are the ones most relevant for free‐running behaviour, we quantified PER cycling was also dampened under LD cycles; in fact, under these conditions, PER levels appeared to be relatively higher at all times in LNv s and LNds of the TG27, RNAi (GD) flies (n = 10) relative to TG27 controls (n = 9). Asterisks above the bars denote significant differences between genotypes (P < 0.05 using unpaired Student’s t‐test. Error bars depict s.e.m. Time is indicated as CT (circadian time), where CT0 is 12 h after lights‐off of the last LD cycle. Scale bar, 10 μm.

**Figure 3 | Knockdown of E75 increases PER expression in brain clock cells.** Flies from TG27 and TG27 > E75 RNAi genotypes were tested for their circadian behaviour under DD conditions and on the 6th day 8–10 rhythmic TG27 controls and 8–10 arrhythmic E75 knockdown flies were used for IHC at each of the indicated time points. PER expression at different times of day in TG27 > UAS–E75 RNAi (GD) flies and TG27 controls in the (a) small and large LNv s and (b) dorsal LNs (LNds). Quantification of PER staining from (c) sLNv s and (d) ILNv s subset of neurons in the TG27 > UAS–E75 RNAi (GD) flies (n = 10) relative to TG27 control flies (n = 9). Asterisks above the bars denote significant differences between genotypes. *P < 0.05 using unpaired Student’s t‐test. Error bars depict s.e.m. Time is indicated as CT (circadian time), where CT0 is 12 h after lights‐off of the last LD cycle. Scale bar, 10 μm.
E75 affects transcriptional activity and levels of VRI. Clk transcription is also known to be repressed by a well-known bZIP transcriptional factor, VRI, which directly competes with PDP1 to bind at the V/P box. Interestingly, a genome-wide study aimed at transcriptional factor, VRI, which directly competes with PDP1 to transcription is also known to be repressed by a well-known bZIP significantly de-repressed the E75-mediated repression of transcription by inhibiting CLK-mediated per transcription (Supplementary Fig. 4d). These experiments strongly indicate specific repression by E75 at the Clk promoter.

As vri expression is increased by ecdysone signalling, we also examined whether E75 affects VRI levels. Knocking down E75 levels in clock cells significantly reduced VRI levels and overexpression slightly elevated trough levels of VRI, indicating that E75 interacts with VRI on multiple levels (Supplementary Fig. 5c,d).

PER interacts with E75 and acts as a de-repressor for Clk transcription. Low levels of Clk mRNA in mutants lacking PER (per01) are thought to reflect a de-repressor function of PER, in other words suggesting that PER suppresses activity of some repressor. However, PER is not known to affect VRI-dependent repression of Clk, as the effect of VRI on Clk expression is similar in wild-type and per null backgrounds. Because E75 had robust effects on Clk transcription in cell culture assays, we asked if PER affects repression of Clk by E75.

To address this question, we used the same luciferase-based transcription assays in cell culture. As above, the native Clk-luc promoter was activated by PDP1 and repressed by E75, and subsequently PER (driven by the CMV promoter) was added in a dose-dependent manner. Interestingly, PER strongly inhibited repression of Clk by E75, demonstrating that the de-repression did not result merely from the presence of another transfected protein (Fig. 4a). A construct expressing GFP did not affect repression by E75. To further address whether the two proteins act together to regulate transcription, we used a VRI-VP16 construct to directly activate the artificial Clk promoter, and found that the activation was potentiated by E75 (Supplementary Fig. 5a).

As the experiments above utilized a Clk promoter that only contained sites for PDP1/VRI, we asked if direct binding of E75 to its own target sites on the Clk promoter could also modulate effects of VRI. Thus, we used a native Clk promoter, and found that E75 significantly reduced VRI-VP16-mediated activation of Clk (Supplementary Figure 5b). These data indicate that E75 can repress Clk directly, but probably also affects VRI repression of the Clk promoter.
levels in wild-type flies relative to TG27 controls at ZT14. This effect is more striking in mRNA levels in wild-type and (II) in wild-type and (with GABA-T, PER also binds strongly with E75-RC. The RA isoform was poorly expressed, hence weaker interaction with PER (lane 5 in IP: (GABA-T) V5 and empty CMV vector. Anti-V5 antibody was used to pull down protein complexes. PER specifically binds with CRY but not with E75. 100 ng of CMV- E75-PA expression in the presence of the de-repressor PER. 

We also assayed Clk mRNA levels in wild-type and per0 backgrounds under conditions where E75 was overexpressed with the TG27 driver. As noted in Fig. 2, overexpression of E75 reduced Clk mRNA in wild-type flies. In per0 flies also, overexpression of E75 reduced the expression of Clk mRNA, although the difference was small, perhaps because Clk levels were already low (Fig. 5c). The effect of knockdown and overexpression of E75 on Clk mRNA levels in a per0 background indicate that endogenous PER affects E75 action at the Clk promoter.

Ecdysone signalling regulates circadian behaviour. Previous studies have reported that steroid hormone signalling induces E75 expression to regulate critical developmental processes. However, ecdysone signalling is also present in adult stages, and mutations that alter hormone or receptor levels affect diverse processes such as behaviour, stress resistance, reproduction and lifespan. In fact, disruption of ecdysone signalling has been associated with alterations in circadian behaviour and in sleep. Therefore, we assayed effects of manipulating
expressed EcR-B1 and thus indirectly affecting the Clk expression. Under stressed (nutritional and temperature) conditions, E75 is required to maintain robust rhythms. In addition, E75 also regulates VRI expression in such a way that overexpression or knockdown of E75 increases or reduces the VRI levels, respectively, thus indirectly affecting the Clk expression. Under stressed (nutritional and temperature) conditions, E75 is required to maintain robust rhythms.

The EcR-A antibody is specific for EcR-A isoform, and the EcR-C antibody is known to detect all three isoforms of EcR. We obtained antibodies to EcR isoforms and verified that they recognize these specific proteins, based on their reduced levels in EcR RNAi lines and increased levels in flies that overexpress EcR (data not shown). The EcR-A antibody is specific for EcR-A isoform, and the EcR-C antibody is known to detect all three isoforms of EcR. Through IHC experiments, we identified distinct expression of the EcR-A-specific isoform and perhaps other isoforms (as detected by EcR-C antibody) in adult LNvs as well as in the 3rd instar larval stage (Fig. 6a,b). To alter EcR activity in clock cells, we utilized RNAi, dominant negative and overexpression approaches, as null mutations of EcR are lethal. The dominant negative form of EcR (EcR\textsuperscript{A}) cannot be activated by ecdysone and interferes with the activity of endogenous EcR, leading to deficiencies in EcR function\textsuperscript{26}. Expression of EcR-B1\textsuperscript{A} by Pdf-Gal4 resulted in a significant increase in period and decreased rhythm strength (Table 2A). Using TUG, UAS-EcR-B1\textsuperscript{A} was expressed in broader sets of clock cells, and resulted in a much longer period (~26 h) and ~30% arrhythmicity, but surprisingly less of an effect on the strength of rhythms in rhythmic flies (Table 2A). We also expressed EcR-B1\textsuperscript{A} using the even stronger clock cell Gal4 (TG\textsuperscript{27}), which resulted in 100% lethality. As reported by Itoh et al.\textsuperscript{15}, knockdown of EcR using RNAi also yielded circadian phenotypes. EcR-A RNAi in PDF-positive cells did not alter period but significantly reduced rhythm strength (Table 2A). On the other hand, EcR-A RNAi using the TUG driver led to a significantly longer period (Table 2A), although again, with less of an effect on rhythm strength. We also overexpressed different isoforms of the EcR gene using Pdf-Gal4 and TG\textsuperscript{27} drivers. Pdf-Gal4-mediated overexpression of the different isoforms produced phenotypes of varying strength, with two isoforms (A and B2) reducing rhythm strength and one (A) also reducing the number of rhythmic flies (Table 2B). TG\textsuperscript{27}-mediated overexpression of EcR-A, B2 and C, which expresses a common region from all three isoforms of EcR, that is, A, B1 and B2 (ref. 26), resulted in phenotypes that included significantly longer periods, reduced rhythm strength and increased arrhythmia (Table 2B). However, the effects varied somewhat from one isoform to the other (Table 2B).

EcR is a well-known transcription factor regulating E75 levels, and so it likely contributes to E75 expression in clock cells\textsuperscript{28}. We assayed the levels of different isoforms of E75 in the brains of flies where EcR-B1 levels were knocked down. Consistent with previous findings, we found that E75-RA, RB and RC isoforms were reduced by knockdown of EcR (Supplementary Fig. 7a). EcR knockdown also increased Clk expression, although the effects were milder than seen with E75 reduction (Supplementary Fig. 7b and c), perhaps because EcR can affect Clk in multiple ways through different signals\textsuperscript{15}. These data indicate though that effects of EcR, like those of E75, are not going solely through the molecular clock (see Discussion).

**E75 protects the central clock under conditions of stress.** In adults, ecdysone signalling is increased upon exposure to stressful environments\textsuperscript{25,29}. As E75 is a direct target of EcR, we asked if signalling through E75 is important in the presence of environmental stressors. To test this idea, we subjected adult flies to two different stressful conditions that are known to increase ecdysone signalling: low nutrition and high temperature\textsuperscript{30,31}. For nutritional stress, we varied the amount of sucrose (1, 2 and 5%) in the 2% agar medium (see Methods for details), whereas for temperature stress, we tested temperatures of 25 (regular), 28 and 32 °C. Control flies (iso31 or UAS/Gal4 alone) did not show changes in circadian period upon temperature or nutritional stress (Table 3 and Supplementary Table 1) and for the most part they remained rhythmic, although the lines carrying the drivers alone...
Table 2 | Circadian behaviour of flies with altered EcR expression levels under DD conditions.

| Genotype       | Condition | % Rhythmic (n) | Period (h) ± s.e.m. | FFT ± s.e.m. |
|----------------|-----------|----------------|---------------------|--------------|
|                |           |                |                     |              |
| (A)            |           |                |                     |              |
| Pdf; D2        |           | 100 (16)       | 23.69 ± 0.047       | 0.065 ± 0.008 |
| TUG; D2        |           | 100 (16)       | 23.78 ± 0.045       | 0.054 ± 0.008 |
| UAS-EcR-B1A    |           | 100 (14)       | 24.11 ± 0.075       | 0.135 ± 0.013 |
| UAS-EcR-A RNAi |           | 100 (16)       | 24.53 ± 0.047       | 0.080 ± 0.007 |
| Pdf > EcR-B1A  |           | 93.75 (16)     | 25.32 ± 0.147       | 0.031 ± 0.004* |
| TUG > EcR-B1A  | 68.75 (16) | 26.01 ± 0.089* | 0.056 ± 0.024       |
| Pdf;D2>EcR-A RNAi |   | 100 (15)     | 24.24 ± 0.204       | 0.038 ± 0.004* |
| TUG;D2>EcR-A RNAi | 93.75 (16) | 25.65 ± 0.149* | 0.057 ± 0.009       |
| (B)            |           |                |                     |              |
| UAS-EcR-A      | 100 (16)  | 23.81 ± 0.029  | 0.059 ± 0.006       |
| UAS-EcR-B2     | 100 (13)  | 23.69 ± 0.076  | 0.035 ± 0.005       |
| UAS-EcR-C      | 100 (11)  | 24.16 ± 0.084  | 0.063 ± 0.013       |
| Pdf > EcR-A    | 62.5 (16) | 24.06 ± 0.205  | 0.029 ± 0.003*      |
| TUG27 > EcR-A  | 93.75 (16) | 27.32 ± 0.092* | 0.026 ± 0.003*      |
| Pdf > EcR-B2   | 100 (13)  | 23.65 ± 0.113  | 0.021 ± 0.003*      |
| TUG27 > EcR-B2 | TUG27 > EcR-C | 50 (14) | 25.14 ± 0.048*     | 0.040 ± 0.006 |
| TUG27 > EcR-C  | 96.77 (31) | 24.59 ± 0.057  | 0.066 ± 0.006       |
| TUG27 > EcR-C  | 40.62 (32) | 25.88 ± 0.397* | 0.029 ± 0.005*      |

Table 3 | Circadian behaviour of flies with low E75 under nutritional stress conditions.

| Genotype       | Condition | % Rhythmic (n) | Period (h) ± s.e.m. | FFT ± s.e.m. |
|----------------|-----------|----------------|---------------------|--------------|
|                |           |                |                     |              |
| (A)            |           |                |                     |              |
| Iso31          | 5% Sucrose| 97.83 (46)     | 23.92 ± 0.060       | 0.062 ± 0.006 |
| Iso31          | 2% Sucrose| 97.87 (47)     | 23.89 ± 0.055       | 0.066 ± 0.005 |
| Iso31          | 1% Sucrose| 88.89 (45)     | 23.97 ± 0.114       | 0.048 ± 0.004 |
| (B)            |           |                |                     |              |
| E75 RNAi (GD)  | 5% Sucrose| 100 (34)       | 23.54 ± 0.092       | 0.041 ± 0.004 |
| E75 RNAi (GD)  | 2% Sucrose| 96.88 (32)     | 24.01 ± 0.135       | 0.052 ± 0.006 |
| E75 RNAi (GD)  | 1% Sucrose| 84.62 (26)     | 24.07 ± 0.188       | 0.042 ± 0.004 |
| TUG            | 5% Sucrose| 93.75 (16)     | 23.83 ± 0.161       | 0.039 ± 0.017 |
| TUG            | 2% Sucrose| 100 (16)       | 23.57 ± 0.125       | 0.066 ± 0.006 |
| TUG            | 1% Sucrose| 93.75 (16)     | 23.01 ± 0.165       | 0.048 ± 0.007 |
| TUG > E75 RNAi (GD) | 5% Sucrose| 62.5 (16) | 24.49 ± 0.42       | 0.040 ± 0.011 |
| TUG > E75 RNAi (GD) | 2% Sucrose| 33.34 (12) | 23.63 ± 0.71       | 0.034 ± 0.006 |
| TUG > E75 RNAi (GD) | 1% Sucrose| 6.25 (16) | 23.92            | 0.042       |
| Pdf;D2        | 5% Sucrose| 100 (19)       | 23.59 ± 0.08        | 0.043 ± 0.007 |
| Pdf;D2        | 2% Sucrose| 81.25 (16)     | 24.07 ± 0.376       | 0.054 ± 0.006 |
| Pdf;D2        | 1% Sucrose| 75 (16)        | 23.48 ± 0.334       | 0.042 ± 0.016 |
| Pdf;D2 > E75 RNAi (GD) | 5% Sucrose| 58.82 (34) | 23.59 ± 0.142        | 0.025 ± 0.003* |
| Pdf;D2 > E75 RNAi (GD) | 2% Sucrose| 40.74 (27) | 23.53 ± 0.164        | 0.031 ± 0.008* |
| Pdf;D2 > E75 RNAi (GD) | 1% Sucrose| 30.43 (23) | 23.52 ± 0.150        | 0.025 ± 0.008 |

D2, Dicer2; FFT, fast Fourier transform; n, number of flies assayed.
Values in bold indicate noticeable differences with respect to UAS and Gal4 controls.
*Significant difference (P<0.05, unpaired t-test). Statistical analysis cannot be conducted for % rhythmic flies.

To exclude the possibility that any manipulation of the clock renders it more sensitive to environmental stressors, we subjected a Clk allele, Clkhypo, which has dampened molecular oscillations and a long period (~26.5 h; unpublished observations; see Methods for details regarding the lesion), to the same stress conditions. Approximately 60–70% of the Clkhypo flies remained rhythmic under low-nutrient or high-temperature conditions (Supplementary Table 2). Thus, the effect of the stressors was specific for flies that had low levels of E75. To test whether EcR also impacts the circadian clock under stressful conditions, we assayed locomotor activity rhythms of flies in which EcR had

showed some loss of rhythm. Environmental stressors (temperature and nutritional) produced a much stronger effect when E75 was knocked down in all clock neurons (TUG > E75 RNAi), with flies displaying increased arrhythmia (Table 3B and Supplementary Table 1). Notably, the period of the rhythmic flies did not change significantly even with E75 knocked down (Table 3B and Supplementary Table 1). We also used Pdf-Gal4; dicer2 to knock down E75 levels under these two conditions, and observed a significant decrease in fast Fourier transform (FFT) values (that is, rhythm strength) and number of rhythmic flies (Table 3B and Supplementary Table 1).
been knocked down under high-temperature conditions. As with E75 knockdown, a reduction in EcR levels (A and B1 isoforms) in clock cells led to weakened rhythm strength as well as increased arrhythmicity under conditions of stress (Supplementary Table 3). Importantly, control flies and flies with reduced E75 had similar responses to stress in terms of locomotor activity levels, lifespan and food intake (Supplementary Fig. 8). Taken together, these data suggest that ecdysone signalling promotes maintenance of rhythms under stressful conditions without significantly affecting daily activity levels, food consumption and longevity.

Discussion
We report here that E75, a nuclear hormone receptor induced by ecdysone signalling in Drosophila, regulates circadian behaviour. This finding was based upon an unbiased, forward genetic screen, in which E75 stood out as a robust modulator of behavioural rhythms. Although the focus of this study is on rest:activity rhythms, which are controlled by the central clock in the brain, the western blot data suggest that E75 is also a component of peripheral clocks in the head. It is likely that not just E75, but ecdysone signalling in general impacts peripheral circadian function. We have also identified a molecular mechanism by which E75 affects the clock. Finally, we show that, in addition to its role in the clock under normal conditions, E75 protects the clock in times of stress. The latter may be related to its function in a steroid signalling pathway.

In vertebrates, the closest homologues of E75 are members of the REV-ERB family. Although REV-ERB is a part of the mammalian clock mechanism, the Drosophila orthologue, that is, E75, was previously known to have a circadian function. Here we show that E75 is an inhibitor of Clk transcription, by itself and also in conjunction with VRI. Before this work, it was thought that the role of nuclear hormone receptors in mammalian clocks was served by PAR domain containing proteins, PDP1 and VRI, in Drosophila\(^2\). Thus, although REV-ERB and ROR regulate expression of Bmali in mammals, PDP and VRI regulate expression of the other transcriptional activator, Clk, in flies. Our data indicate that E75 does indeed function in the Drosophila clock, much as its mammalian counterpart does (Fig. 6c). One may ask why E75 is required if the second feedback loop is maintained by PDP1 and VRI. We suggest that E75 couples the clock to extracellular cues. Induction of E75 by the steroid hormone, ecdysone, likely allows the clock to respond to endocrine signals and perhaps other ligands (further discussed below). As reported here, E75 signalling may be particularly relevant under conditions of stress.

Tissue culture experiments do not indicate a direct effect of E75 on per expression, although we cannot exclude the possibility that it does so in flies, as suggested by the robust effect of E75 overexpression on per mRNA and protein levels (Fig. 1). However, E75 interacts with PER to regulate transcription of Clk. Indeed, this work reveals a new role for Drosophila PER as a de-repressor. As noted above, earlier studies showed that PER promotes expression of Clk, but the underlying mechanisms were not identified. We find that it does so by reducing the inhibitory effect of E75 on Clk. It may do so by affecting DNA binding of E75 or perhaps even by destabilizing it. Regardless, these data are reminiscent of mammalian PER, which acts as a de-repressor with some nuclear receptors and a co-activator with others\(^3^4\). Indeed, mammalian PER2 and REV-ERB-\(\alpha\) physically interact\(^32\), as we show here for PER and E75. We suggest that crosstalk between components of the two loops is a conserved mechanism that serves to maintain a robust cycle. On the one hand, Drosophila PER inhibits activity of the CLK/CRYC complex to generate a negative feedback loop; meanwhile, it interacts with nuclear hormone receptors like E75 to promote Clk gene expression in the positive feedback loop. Although this study only examined effects of PER on E75-mediated repression of Clk, it is likely that there are other circadian targets of E75 that are modulated by PER. Future studies should help to clarify the extent to which E75 impacts transcription within the circadian network. Importantly, the data on E75 presented here provide insight into some of the unresolved questions in the clock field—for instance, why Clk mRNA levels are low in per\(^{\text{−}}\) flies.

Although E75 appears to act as a component of the molecular clock, its effects on behaviour are probably not going entirely through the clock. Knockdown of E75 increases Clk, which typically shortens period\(^1^5\), but does not cause arrhythmia. Along the same lines, per rhythms dampen, but are not eliminated upon E75 knockdown, and this dampening is not expected to render flies arrhythmic. We suggest that effects of E75 on circadian period are mediated by Clk, but in addition E75 affects circadian output, which contributes to the arrhythmia caused by knockdown. As mentioned above, E75 may affect the transcription of other genes, perhaps even in a circadian manner. We suggest that E75 is regulated by the clock, which is supported by experiments showing direct binding of CLK to the E75 promoter\(^1^6\).

Although this is the first report of an ecdysone-induced nuclear receptor in the Drosophila central clock mechanism, ecdysone signalling has been previously linked to circadian function. Early gene at 23 (E23), which suppresses the response to ecdysone, is required for normal circadian rhythms in Drosophila\(^1^5\). E23 encodes a membrane-bound ATP-binding cassette (ABC) transporter that is induced by ecdysone in central clock neurons, and its knockdown in these neurons (LN\(_{\text{s}}\)) increases expression of the clock gene, vrille, and lengthens circadian period\(^1^5\). The relationship between E75 and E23 is not known; it is possible that ecdysone signalling activates both molecules, which then have independent effects on the clock. Alternatively, effects of E75 (overexpression and knockdown) on VRI expression as well as cell culture data indicate that E75 may also regulate VRI to modulate CLK expression and circadian behaviour. Importantly, disruption of EcR signalling also affects rhythms. Loss of EcR can have developmental effects on clock cells\(^3^5\), but we note that many of our manipulations of EcR change circadian period without causing arrhythmia, indicating a more specific effect on clock function. Both EcR and E75 are required to maintain rhythmicity under conditions of stress, perhaps through modulation of clock molecules and circadian output.

Of the \(~\)18 known nuclear receptors in Drosophila\(^3^6\), only one other, unfulfilled, has been implicated in clock function\(^1^7\). However, the mechanism by which it affects the clock is unknown, as is the ligand that activates it. In case of E75, a few natural ligands have been identified such as haem, CO and NO\(^1^2\). REV-ERB also binds to haem and may reset the clock in response to it\(^3^8\). It is possible that NO and CO also affect the clock. Future studies of E75 in Drosophila could elucidate mechanisms by which nuclear receptors mediate effects of signalling molecules on circadian clock function.

Methods
Fly stocks. tim-Gal4\(^2^7\), (TG\(^2^7\)), trp\(^{-}\)-Gal4, Paf-Gal4 UAS-E75 RNAi (F022357 and HMS0130), UAS-EcR B13G653, UAS-EcR A RNAI UAS-EcR-C, UAS-EcR-C and E75\(^{51}\) lines were provided by the Bloomington Stock Center. UAS-E75 II and III were provided by Henry Krause (University of Toronto). UAS-E75 RNAi GD and KK were obtained from the Vienna Drosophila Resource Center. TUG; UAS-Dicer2 and Paf-Gal4; UAS-Dicer2 lines were from our laboratory. NSE1-49-19 EP was an overexpression line generated in our laboratory\(^3^9\). The insertion was determined by Inverse PCR in accordance with protocols from the Berkeley Drosophila Genome Project. Clk\(^{\text{Drosophila}}\) is a Piggybac [WH]06808
analyses suggest that it is a hypomorphic allele of the insertion line and was obtained from the Bloomington Stock Center. This element of Drosophila melanogaster (strain TRi2, containing two copies of the genomic region with the transposable element LipoZon060b (Life Technologies) was obtained via transfection using the ClockLab software (ActiMetrics). The periodicity and the strength of the activity rhythm of each fly was determined by visual examination of activity records and FFT analysis. Flies displaying a single well-defined peak in the periodogram and an FFT value of greater than 0.01 were classified as rhythmic and were included for determining the average period and rhythm strength. Individuals with multiple or broad peaks in the periodogram analysis were not counted for period determination, whereas those that showed random activity patterns and no clear peak by periodogram were categorized as arrhythmic. Note that rhythm strength (FFT values) was calculated only for rhythmic individuals.

**Western blot analysis.** Western blot assays were performed as previously described. Briefly, 4 to 5-day-old flies were entrained in LD 12:12 h cycles for 3 days. Exactly ten heads were collected on dry ice at indicated time points. For constant dark (DD) experiments, the flies were sampled on the second day. The fly heads were lysed in a homogenization buffer containing 10 mM HEPES (pH 7.5), 100 mM KCl, 10 mM NaCl, 50% glycerol, 1 mM dithiothreitol, and 5 mM phenylmethylsulphonyl fluoride along with the phosphatase inhibitors okadaic acid and 1 mM sodium vanadate. A protease inhibitor cocktail (Boehringer) was also added to the buffer according to the manufacturer’s instructions. Homogenates were spun twice for 10 min at 12,000 r.p.m., and the supernatant was transferred to microcentrifuge tubes kept on ice. 15 μl of each sample was run on a 4–12% SDS-polyacrylamide mini-gels, and transferred onto nitrocellulose membranes overnight. PER protein was detected with a 1:2000 dilution of GP anti-PER antibody. This antibody is highly specific to PER with the most intense band detected at approximately 150 kDa, the predicted size of Drosophila PER. The other antibodies used in different assays were guinea pig anti-CLK (1:3,000) and mouse anti-HSP70 (1:15,000; Sigma). The western blots were developed by a horseradish peroxidase enzymatic activity-based assay followed by enhanced chemiluminescence (ECL reagent; Thermo Scientific). Blots were stripped using a western blot stripping buffer (Thermo Scientific) and re-probed with a guinea pig anti-CLK antibody (1:3,000). Images were obtained using a Kodak image station or through exposure to X-ray film. These images were enhanced chemiluminescence (ECL reagent; Thermo Scientific). Blots were scanned and quantified by ImageJ software (NIH) for quantification of individual bands. Analysis of the expression constructs constructed the expression vectors pMT-paraformaldehyde (made in PBS) and fixed for the indicated time points on the last day of the LD cycle. Total RNA was isolated using TRIzol (Life Technologies). A total of 500 ng of DNA was used in each case. Following transfection, the cells were collected after 48 h, lysed and assayed for luciferase activity (Promega). The luciferase activities were normalized to renilla luciferase activity counts and presented as a ratio.

**Immunohistochemistry.** Three- to five-day-old adults were entrained to a 12:12 h LD cycle for 3 days at 25°C and then collected at indicated time points. Adult fly heads were washed in 70% alcohol and brains were dissected in 4% paraformaldehyde (made in PBS) and fixed for 20 min, washed 1 h in PBS buffer and followed by overnight incubation with primary antibody (in PBS buffer with 3% normal donkey serum and 0.3% Triton X-100) at 4°C. Brain samples were washed four to five times with PBS-T (PBS with 0.3% Triton-X) buffer and incubated with Cy3 donkey anti-rabbit (or guinea pig) and fluorescein isothiocyanate or Cy3 donkey anti-rabbit (mouse) secondary antibodies (Jackson ImmunoResearch Laboratories) for 2 h at room temperature, followed by an additional four to five washes in PBS-T. A Leica TCS SP5 confocal microscope (Leica) was used to obtain the fluorescent images. The primary antibody dilutions used in these assay were as follows: PER, 1:1,000; CLK (GP50), 1:1,000; PDF, 1:1,000; mouse Ecr common (AgO10.2) 1:150; mouse Ecr-A (15G1a), 1:50. Secondary antibody dilutions were 1:500. The fluorescent intensity of individual cells was measured from confocal images with NIH ImageJ software. The background intensity from the adjacent area was subtracted from fluorescence values, followed by averaging of the normalized values.

**Expression constructs.** For co-transfection studies, the CR-luc- and 4.6 kb per-luc plasmids were generated by inserting genomic DNA upstream of the basal promoter in the luciferase reporter vector pGL3 (Promega). DNA templates for E75, kindly provided by Carl Thummel (University of Utah), were used for constructing the expression vector pMT-E75A and pMT-E75B, which contain full-length E75-R and E75-RC, respectively, in the NotI and XbaI sites of pMTV5HSa (Invitrogen). All constructs were verified by sequencing.

**Cell culture luciferase assay.** For the luciferase reporter-based transcriptional assays, either human embryonic kidney (HEK293T) or Drosophila S2 cells were used. For HEK 293T cell transfection assays, a total of ~150,000 cells were transfected into 24-well plates with the following expression plasmids—CMV-E75, CMV-pdpl1a, CMV-per-HA, CMV-GFP—together with the reporter CR-luc and the renilla-luciferase as internal control. Empty pcDNA3.1 vectors were added to control the uniform DNA amounts across transfections using Lipofectamine (Life Technologies). A total of 500 ng of DNA was used in each case. Following transfection, the cells were collected after 48 h, lysed and assayed for luciferase activity (Promega). The luciferase measurements were normalized to renilla luciferase activity counts and presented as a ratio.

**Quantitative real-time PCR.** Protocols for total RNA isolation and cDNA synthesis have been described previously. Briefly, 3- to 5-day-old adults were entrained to a 12:12 h LD cycle for 3 days at 25°C and then collected on dry ice at indicated time points on the last day of the LD cycle. Total RNA was isolated using the manufacturer’s protocol (TRizol; Life Technologies), and cDNAs were synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems; Life Technologies). Real-time assays were performed using an ABI prism 7000 with a SYBR Green kit (Applied Biosystems). The oligo dT primer was used as the probe. Assays were performed with five (5'-AGTCAACCAGAATCCACC-3') and Act5C reverse (5'-GGGGTTCATTCTTCTACA-3') primers for 10 cycles (5'-TGTGCTGCGCCTCAATG-3'). RNA expression was normalized to Act5C controls and values were taken after the threshold was reached. The expression levels were calculated by the comparative ΔΔCT method.

**References**

1. Zheng, X. & Sehgal, A. Speed control: cogs and gears that drive the circadian clock. Trends Neurosci. 35, 574–585 (2012).
2. Cyran, S. A. et al. vPv1l, Pdpl1, and dClock form a second feedback loop in the Drosophila circadian clock. Cell 112, 329–341 (2003).
3. Zheng, X. et al. An isoform-specific mutant reveals a role of PDP1 epsilon in the circadian oscillator. J. Neurosci. 29, 10920–10927 (2009).
4. Glossop, N. R., Lyons, L. C. & Hardin, P. E. Interlocked feedback loops within the Drosophila circadian oscillator. Science 286, 766–768 (1999).
5. Gummadova, J. O., Coutts, G. A. & Glossop, N. R. Analysis of the Drosophila Clock promoter reveals heterogeneity in expression between subgroups of central oscillator cells and identifies a novel enhancer region. J. Biol. Rhythms 24, 353–367 (2009).
6. Mohawk, J. A., Green, C. B. & Takahashi, J. S. Central and peripheral circadian clocks in mammals. Annu. Rev. Neurosci. 35, 445–462 (2012).
7. Duez, H. & Staels, B. Rev-erb-alpha: an integrator of circadian rhythms and metabolism. J. Appl. Physiol. (1985) 107, 1972–1980 (2009).
8. Preattier, N. et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110, 231–242 (2002).
9. Yin, L., Wu, N. & Lazar, M. A. Nuclear receptor Rev-erbalpha: a heme receptor that coordinates circadian rhythm and metabolism. Nucl. Recept. Signal. 8, e001 (2010).
10. Segraves, W. A. & Hogness, D. S. The E75 eyecside-inducible gene responsible for the 75B early puff in Drosophila encodes two new members of the steroid receptor superfamily. Genes Dev. 4, 204–219 (1990).
11. Schwedes, C. C. & Carney, G. E. Ecdysone signaling in adult Drosophila melanogaster. J. Insect. Physiol. 58, 293–302 (2012).
12. Caceres, L. et al. Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. Genes Dev. 25, 1476–1485 (2011).
13. Reinking, J. et al. The Drosophila nuclear receptor e75 contains heme and is gas responsive. Cell 122, 195–207 (2005).
14. Thummel, C. S. Powered by gas-a ligand for a fruit fly nuclear receptor. Cell 122, 151–153 (2005).
15. Itoh, T. Q., Tanimura, T. & Matsumoto, A. Membrane-bound transporter controls the circadian transcription of clock genes in Drosophila. Genes Cells 16, 1159–1167 (2011).
16. Zheng, X., Yang, Z., Yue, Z., Alvarez, J. D. & Sehgal, A. FOXO and insulin signaling regulate sensitivity of the circadian clock to oxidative stress. Proc. Natl Acad. Sci. USA 104, 15899–15904 (2007).
17. Pardeé, K. L. et al. The structural basis of gas-responsive transcription by the liver nuclear hormone receptor Rev-ERBbeta. PLoS Biol. 7, e43 (2009).
18. Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W. A. & Thummel, C. S. Loss of the ecdysoid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in Drosophila. Dev. Cell 3, 209–220 (2002).
19. Abruzzi, K. C. et al. Drosophila CLOCK target gene characterization: implications for circadian tissue-specific gene expression. Genes Dev. 25, 2374–2386 (2011).

20. Harding, H. P. & Lazar, M. A. The orphan receptor Rev-ErbA alpha activates transcription via a novel response element. Mol. Cell. Biol. 13, 3113–3121 (1993).

21. Harlington, T. K., Lyons, L. C., Hardin, P. E. & Kay, S. A. The period E-box is sufficient to drive circadian oscillation of transcription in vivo. J. Biol. Rhythms 15, 462–471 (2000).

22. Beckstead, R. B., Lam, G. & Thummel, C. S. The genomic response to 20-hydroxyecdysone at the onset of Drosophila metamorphosis. Genome Biol. 6, R99 (2005).

23. Morris, I. X. & Spradling, A. C. Steroid signaling within Drosophila ovarian epithelial cells sex-specifically modulates early germ cell development and meiotic entry. PLoS ONE 7, e46109 (2012).

24. Busczak, M. et al. Ecdysone response genes govern egg chamber development during mid-oogenesis in Drosophila. Development 126, 4581–4589 (1999).

25. Ishimoto, H. & Kitamoto, T. The steroid molting hormone Ecdysone regulates sleep in adult Drosophila melanogaster. Genetics 185, 269–281 (2010).

26. Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E. & Cherbas, P. EcR isoforms in Drosophila: testing tissue-specific requirements by targeted blockade and rescue. Development 130, 271–284 (2003).

27. Talbot, W. S., Swyryd, E. A. & Hogness, D. S. Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73, 1323–1337 (1993).

28. Johnston, D. M. et al. Ecdysone- and NO-mediated gene regulation by competing EcR/Usp and E75A nuclear receptors during Drosophila development. Mol. Cell 44, 51–61 (2011).

29. Simon, A. F., Shi, C., Mack, A. & Benzer, S. Steroid control of longevity in Drosophila melanogaster. Science 299, 1407–1410 (2003).

30. Rauschenbach, I. Y., Sukhanova, M. Z., Hirashima, A., Sutsugu, E. & Kiano, E. Role of the ecdysteroid system in the regulation of Drosophila reproduction under environmental stress. Dokl. Biol. Sci. 375, 641–643 (2000).

31. Terashima, J., Takaki, K., Sakurai, S. & Bownes, M. Nutritional status affects 20-hydroxyecdysone concentration and progression of oogenesis in Drosophila melanogaster. J. Endocrinol. 187, 69–79 (2000).

32. Schmutz, I., Ripperger, J. A., Barriwiol-Abbecher, S. & Albrecht, U. The mammalian clock component PERIOD2 coordinates circadian output by interaction with nuclear receptors. Genes Dev. 24, 345–357 (2010).

33. Ripperger, J. A., Schmutz, I. & Albrecht, U. PERsauding nuclear receptors to dance the circadian rhythm. Cell Cycle 9, 2515–2521 (2010).

34. Allada, R., Kadener, S., Nandakumar, N. & Rosbash, M. A recessive mutant of Drosophila clock reveals a role in circadian rhythm amplitude. EMBO J. 22, 3367–3375 (2003).

35. Gorostiza, E. A. & Ceriani, M. F. Retrograde bone morphogenetic protein signaling shapes a key circadian pacemaker circuit. J. Neurosci. 33, 687–696 (2013).

36. King-Jones, K. & Thummel, C. S. Nuclear receptors—a perspective from Drosophila. Nat. Rev. Genet. 6, 311–323 (2005).

37. Beuchle, D., Jaumouille, E. & Nagoshi, E. The nuclear receptor unfulfilled is required for free-running clocks in Drosophila pacemaker neurons. Curr. Biol. 22, 1212–1227 (2012).

38. Raghuram, S. et al. Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBBeta. Nat. Struct. Mol. Biol. 14, 1207–1213 (2007).

39. Kumar, S., Chen, D. & Sehgal, A. Dopamine acts through Cryptochrome to promote acute arousal in Drosophila. Genes Dev. 26, 1224–1234 (2012).

40. Luo, W. & Sehgal, A. Regulation of circadian behavioral output via a MicroRNA-1AK/STAT circuit. Cell 148, 765–779 (2012).

41. Houl, J. H., Ng, F., Taylor, P. & Hardin, P. E. CLOCK expression identifies developing circadian oscillator neurons in the brains of Drosophila embryos. BMC Neurosci. 9, 119 (2008).

Acknowledgements

We are grateful to Zhaohai Yang and Zhiheng Yee for the initial EP insertion screen, Henry Krause for the UAS-E75 (II) and (III) transgenic flies, Carl Thummel for the E75 cDNA constructs. Paul Hardin for GP-50 (anti-CLK), Justin Blau for Cik-luc, CSTK-luc, C3mTk-luc, CMV-Pdp1 and CMV-VRI VP16 constructs. The laboratory is supported by an R01NS048471 grant from the NIH. X.Z. is supported by a 2010 NARSAD Young Investigator Award. A.S. is an Investigator of the Howard Hughes Medical Institute.

Author contributions

S.K. and A.S. conceived the project. S.K. planned and carried out experiments, analysed data. S.K. and A.S. wrote the manuscript. D.C. carried out qPCR, WB and co-IP experiments. A.N. helped in behavioural assays. C.J. helped in cell culture assays. X.Z. helped plan experiments. All authors edited and approved the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Kumar, S. et al. An ecdysone-responsive nuclear receptor regulates circadian rhythms in Drosophila. Nat. Commun. 5:5697 doi: 10.1038/ncomms6697 (2014).