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Clk post-transcriptional control denoises circadian transcription both temporally and spatially

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The transcription factor CLOCK (CLK) is essential for the development and maintenance of circadian rhythms in *Drosophila*. However, little is known about how CLK levels are controlled. Here we show that *Clk* mRNA is strongly regulated post-transcriptionally through its 3′ UTR. Flies expressing *Clk* transgenes without normal 3′ UTR exhibit variable CLK-driven transcription and circadian behaviour as well as ectopic expression of CLK-target genes in the brain. In these flies, the number of the key circadian neurons differs stochastically between individuals and within the two hemispheres of the same brain. Moreover, flies carrying *Clk* transgenes with deletions in the binding sites for the miRNA *bantam* have stochastic number of pacemaker neurons, suggesting that this miRNA mediates the deterministic expression of CLK. Overall our results demonstrate a key role of *Clk* post-transcriptional control in stabilizing circadian transcription, which is essential for proper development and maintenance of circadian rhythms in *Drosophila*.

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Most organisms use circadian clocks to keep temporal order and anticipate daily environmental changes. Circadian clocks appear to function on a cell-autonomous basis and are generated by interconnected complex transcriptional and post-translational feedback loops[1]. In *Drosophila*, the master genes *Clock* (*Clk*) and *cycle* (*cyc*) activate the circadian system by promoting rhythmic transcription of a number of key genes[2–4]. Three of these target gene products, PERIOD (PER)[5], TIMELESS (TIM)[6] and CLOCKWORK ORANGE (CWO)[7–9], repress CLK-CYC-mediated transcription on a daily basis. CLK activates transcription only while associated with CYC. The latter is constitutively expressed, whereas CLK is present in limiting amounts[4,10]. The CLK-CYC heterodimer also activates the expression of VRILLE (VRI) and PAR-DOMAIN-PROTEIN 1 (PDP1), which have been postulated to be responsible for the oscillation of *Clk* mRNA[11,12]. Although the role of VRI in repressing *Clk* transcription is well established, recent reports suggest that PDP1 might not directly regulate *Clk*[13]. In addition to transcriptional control, post-transcriptional and post-translational regulations play also a central role in circadian timekeeping (for reviews see refs 14–16).

Circadian clocks are widespread through the fly body[17]. However, a group of ~150 neurons in the brain drives circadian rhythms in locomotor activity[18]. These neurons have been divided into several subgroups based on their anatomical location and expression of the core clock genes[19,20]. These groups are small and large ventral lateral (sLNvs and lLNvs), dorsal lateral (LNds), and dorsal (DN1s, DN2s and DN3s) neurons. The neuropeptide pigment dispersing factor (PDF), which is expressed exclusively in the LNvs, is essential for normal circadian patterns of activity in light and dark and for persistent circadian rhythms in constant darkness (DD)[18].

Despite the central role of *Clk* in the circadian system, it is still not well understood how CLK levels and activity are controlled. The current model states that most (if not all) of this control is through post-translational regulation such as phosphorylation, ubiquitination and other types of modifications[21–25]. Indeed, although *Clk* mRNA levels display strong transcriptional oscillations[11,12], CLK protein levels are nearly constant though the day[26]. Moreover, expression of CLK under the *tim* or *per* promoter in ClkARK fly strains (in which transcription has the opposite daily phase relative to that under control of the *Clk* promoter[21]) does not disrupt circadian behaviour, indicating that flies can adapt or compensate for high levels of CLK[21]. As these experiments were performed in a wild-type background, whether constant or shifted *Clk* transcription alone can drive circadian behavioural rhythms was not determined.

In addition, it is well established that the levels or activity of CLK markedly alter the circadian period and amplitude[27–29]. Moreover, uncontrolled expression of CLK leads to death. For example, expression of CLK using the circadian driver *tim*-gal4 leads to embryonic lethality[30], and induction of CLK-driven transcription using the CLKGR system results in adult death (Ron Weiss, personal communication). Some of the toxic effects of CLK overexpression are likely due to a central role of *Clk* in cell determination. Indeed, CLK expression in non-circadian cells is enough to generate ectopic circadian clocks in the fly brain[30]. In addition, CLK-driven transcription may have a role in the development of the circadian system, as *Clk* and *cyc* fly mutants display abnormalities in the morphology of circadian neurons[29,31]. These data demonstrate that post-translational control alone may not be enough to buffer big changes in CLK levels. Indeed, post-transcriptional regulation might also be important, as we demonstrated in the past that *Clk* is strongly regulated by miRNAs[32]. In brief, *Clk* is strongly bound to AGO1 and the miRNA *bantam* can regulate *Clk* levels in *Drosophila* S2 cells[32]. Moreover, we showed that the putative *bantam* binding sites on *Clk* 3′ UTR are essential for normal circadian rhythms[32]. However, how this regulation influences CLK levels and activity in time and space is still unknown.

Circadian clocks are extraordinarily robust systems; they are able to keep time accurately without any timing cues. This robustness is likely the result of multiple layers of regulation that assure accurate timekeeping by buffering stochastic changes of clock-relevant activities. For example, it has been proposed that the main function of the redundancy and interlocked transcriptional feedback loops of the circadian system is to provide robustness to circadian transcription[33–36]. These layers of regulation extend even beyond the single-cell level. Circadian neurons in the brain are organized in a network that synchronizes and likely amplifies the individual neuronal oscillators thereby contributing to a coherent and robust behavioural output[2,37–39]. Circadian clocks must also control or buffer transcriptional noise and its consequences, especially for genes that are expressed at limiting levels (that is, *Clk*), as it is well known that transcriptional noise is inversely correlated with transcriptional rate[40].

Here we demonstrate that the *Clk* 3′ UTR sets a threshold for meaningful circadian gene expression. Flies in which *Clk* is expressed without post-transcriptional control (*ClkSV40* flies) have ectopic circadian cells in the brain. Surprisingly the levels of CLK per cell are normal in these flies, indicating that *Clk* post-transcriptional regulation does not control the overall CLK levels. Interestingly, *ClkSV40* flies have aberrant circadian transcription and behaviour, as well as widespread expression of CLK-target gene products in the brain. These behavioural deficits are accompanied by the vary stochastic development of *pdf*-expressing LNvs cells that vary in number between individuals and even between the two lobes of the same brain. Similarly, flies carrying *Clk* transgenes with deletions in the binding sites for the miRNA *bantam* display stochastic number of pacemaker neurons, strongly suggesting that this miRNA is the key factor mediating the deterministic expression of CLK. We backed up this role for post-transcriptional control by a mathematical model that predicts the central role of *Clk* mRNA turnover in minimizing noise of the circadian feedback loops. All together, our results demonstrate that post-transcriptional control of a master transcriptional regulator is an efficient way of assuring deterministic transcriptional responses.

Results

***Clk* mRNA is under strong post-transcriptional control**. We recently showed that *Clk* mRNA levels are regulated by microRNAs (miRNAs)[32]. To address the importance of *Clk* post-transcriptional regulation, we compared *Clk* mRNA levels with those of other circadian mRNAs using previously published microarray and RNA-seq data from fly heads and brains[7,41,42]. In these data sets *Clk* mRNA levels were the lowest of the core circadian components (Fig. 1a, Supplementary Fig. 1a,b).

To determine whether the low mRNA levels are due to low transcription and/or high post-transcriptional control, we estimated the extent of *Clk* post-transcriptional regulation by comparing the ratio of pre-mRNA with steady-state mRNA using quantitative scaled RT-PCR. This comparison revealed that *Clk* as well as *vri* are under very strong post-transcriptional regulation (Fig. 1b). This finding is in agreement with calculations made using previously published data comparing the signal obtained from nascent mRNA with steady-state mRNA[41] (Supplementary Fig. 1c). Additional lines of evidence also support strong *Clk* post-transcriptional regulation. For example, we observed no difference between the temporal profiles of mature and

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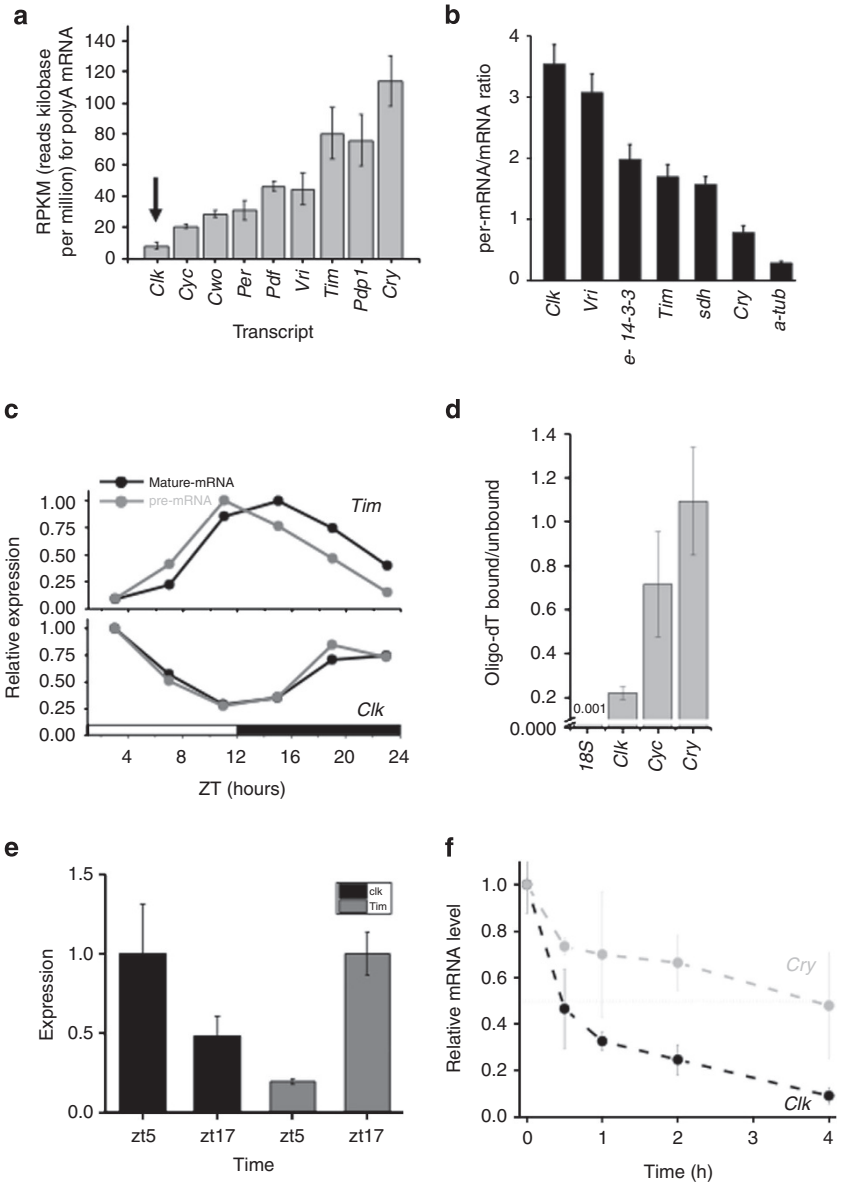

Figure 1 | *Clk* is under strong post-transcriptional regulation (**a**) Comparison of clock transcript levels. RNA-seq data[41] were used to compare expression levels of core circadian components in fly heads; an average of six timepoints ± s.e. in LD conditions (ZT 2, 6, 10, 14, 18, 22) were analysed. (**b**) The levels of pre-mRNA and mRNA of core clock components were measured by real-time PCR (RT-PCR) from a mixture of six circadian time points and scaled using an equimolar mixture of the PCR products to obtain absolute measurements between the different mRNAs or pre-mRNAs in each sample (mean ± s.e.; three biological replicas). As the values in each sample (pre-mRNA and mRNA) are normalized, the pre-mRNA/mRNA ratio is relative, not absolute (see methods for details). (**c**) RT-PCR measurements of transcriptional (grey) and steady state (black) levels of *Clk* and *tim*. Transcriptional/pre mRNA transcripts were detected using primers for intronic sequences of the genes, whereas steady-state/mature mRNAs were detected using primers flanking two exons. Expression was normalized to RP49 and rpS18. Representative experiment of three repeats is shown. (**d**) RT-PCR results presenting the ratio of mRNA bound to oligo-dT beads versus unbound mRNA. Data pooled from a mixture of six time points (mean ± s.e.; three biological replicates). (**e**) A *Drosophila* fly wing system was developed to monitor circadian gene mRNA expression levels (normalized to RP49 at ZT5, 17). *Clk* and *tim* levels cycle with the expected phase. The measurements were performed in triplicates for each time point using the Canton-S strain. (**f**) RT-PCR mRNA half-life measurements for *Clk* and *cry* mRNAs from fly wings treated with actinomycin D in five different time points (0–4 h after actinomycin D exposure, 12 h light/12 h dark conditions,mean ± s.e.; three biological replicates). See also Supplementary Fig. 1.

pre-mRNAs encoding CLK, suggesting that *Clk* is a very short-lived mRNA (Fig. 1c). A delay, such as that observed in the profiles for *tim* mature and pre-mRNAs (Fig. 1c), indicates a longer mRNA half-life. *Clk* mRNA binds weakly to oligo-dT beads (Fig. 1d), likely reflecting a short polyA-tail[43] and strongly binds to the miRNA-effector protein AGO1 demonstrating miRNA-mediated regulation of this mRNA[32].

To determine whether *Clk* post-transcriptional regulation is at least partially due to rapid mRNA turnover, we estimated the *Clk* mRNA half-life in an oscillating system. As *Drosophila* cells in culture do not exhibit circadian oscillations and mRNA half-lives cannot be easily measured in living flies, we first examined whether fly-wing cultures could be used for mRNA measurements and estimation of mRNA half-lives. These cultures are

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widely utilized to monitor circadian rhythms with luciferase reporters[17,44] but have never been validated for mRNA measurements. We measured the amounts of *Clk* and *tim* mRNAs at two different time points in cultured wings. As observed in fly heads and brains, *Clk* and *tim* mRNAs displayed oscillations with opposite phase (high levels in the morning and night respectively; Fig. 1e). We then determined the half-life of *Clk* and *cry* mRNAs by treating wings with the transcriptional inhibitor actinomycin D. The *Clk* mRNA half-life was very short (~0.5 h), considerably shorter than the half-life of *cry* mRNA (half-life ~4 h, Fig. 1f), another circadian mRNA that oscillates with a similar phase.

***Clk* 3′ UTR sets a threshold for CLK-driven transcription**. To study the functional significance of the short *Clk* mRNA half-life, we utilized *Drosophila* S2 cells, which do not express endogenous *Clk* mRNA or protein[45]. We co-transfected these cells with two different fluorescent reporters, both under the control of the copper-inducible metallothionein (MT) promoter, harbouring either a control or a *Clk* 3′ UTR (Fig. 2a). These reporters do not respond to transcriptional feedback (by VRI) and hence only evaluate the effect of the 3′ UTR. Expression from the reporter with the *Clk* 3′ UTR was significantly lower than that from control reporters, demonstrating that this 3′ UTR is strongly regulated also in S2 cells (Fig. 2b). Importantly, the *Clk* 3′ UTR rendered the reporter insensitive to increasing levels of transcription at low concentrations of inducer (Fig. 2b, inset), suggesting a suppression/threshold mechanism to prevent leaky *Clk* expression from stochastic transcription events.

To study the functional significance of the rapid *Clk* mRNA turnover in the context of the normal gene regulation, we generated a 14.5-kilobase (kb) *Clk* construct that includes all known *Clk* coding and regulatory sequences but also carries a *SV40* 3′ UTR inserted immediately downstream the open reading frame (*ClkSV40*; Fig. 2c). We transfected *ClkSV40* or *ClkWT* (same transgene but carrying the wild-type *Clk* 3′ UTR[32]) into *Drosophila* S2 cells along with a newly generated *tim*-YFP reporter (*tim* promoter driving the expression of YFP; *tim* is a direct CLK-CYC target). The *ClkWT* construct did not detectably activate expression of the *tim*-YPF reporter, indicating very low CLK expression (most cells show only background fluorescence; Fig. 2d, blue line). In contrast, the presence of the *ClkSV40* construct significantly increased *tim*-YFP levels compared with YFP levels in the presence of the *ClkWT* construct (Fig. 2d; compare $\text{mean}_{ClkWT} = 15.9$ with $\text{mean}_{ClkSV40} = 127$). Importantly, *tim*-YFP reporter levels were highly variable among *ClkSV40*-transfected cells, suggesting that the *Clk* 3′ UTR not only decreases *Clk* mRNA levels but makes them more uniform.

***Clk* 3′ UTR prevents leaking CLK activity in the brain**. To extend these observations, we generated transgenic flies carrying the *ClkSV40* transgenes. These flies express a V5 tag fused to the CLK C-terminus to allow the identification of the encoded mRNAs and proteins (Fig. 2c). We examined flies with the *ClkWT* or *ClkSV40* transgene in a wild-type *Clk* genetic background (*ClkWT* or *ClkSV40* flies, respectively). Like the endogenous *Clk* gene, the *ClkWT* transgene was under tight post-transcriptional control: the mRNAs expressed from the transgene were strongly associated with AGO1 and bound poorly to oligo-dT beads (Fig. 3a, b). In contrast, *ClkSV40* mRNAs did not associate with AGO1 and bound strongly to oligo-dT beads (Fig. 3a, b). This indicates that *Clk* post-transcriptional regulation is directed predominantly if not exclusively by the 3′ UTR.

To address the ectopic and variable expression issue suggested by the S2 cells experiments, we assayed the expression of CLKV5 protein by immunohistochemistry. In *ClkWT* flies, expression

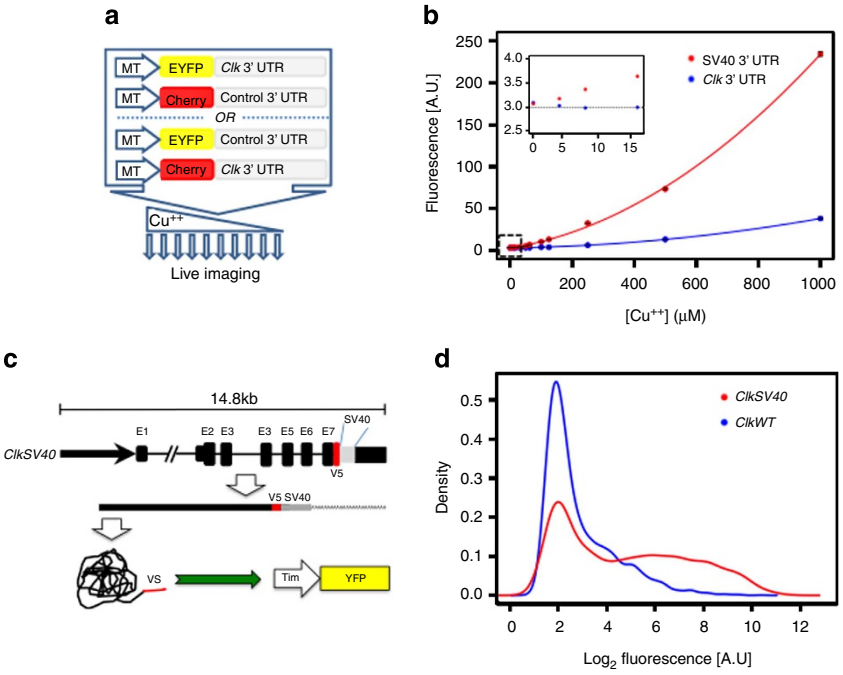

Figure 2 | *Clk* 3′ UTR establishes a threshold for CLK-driven transcription (**a**) Schematics of the system for monitoring post-transcriptional regulation in single *Drosophila* S2 cells. (**b**) Mean fluorescent levels of S2 cell population expressing the *Clk* and *SV40 3′ UTR* fluorescent reporters at different copper levels. The inset in the left represents a closer view of the signal at low copper concentrations (0–15 μM). Cyan fluorescent protein (CFP) expressed from a pAc-CFP plasmid was utilized as transfection control. (**c**) Schematics of the *ClkSV40* and *tim*-YFP constructs. (**d**) Histogram representing the intensity of signal due to the *tim*-YFP reporter on transfection with *ClkSV40* or *ClkWT* plasmids. See also Supplementary Fig. 2. Red fluorescent protein (RFP) expressed from a pAc-RFP plasmid was utilized as a transfection control.

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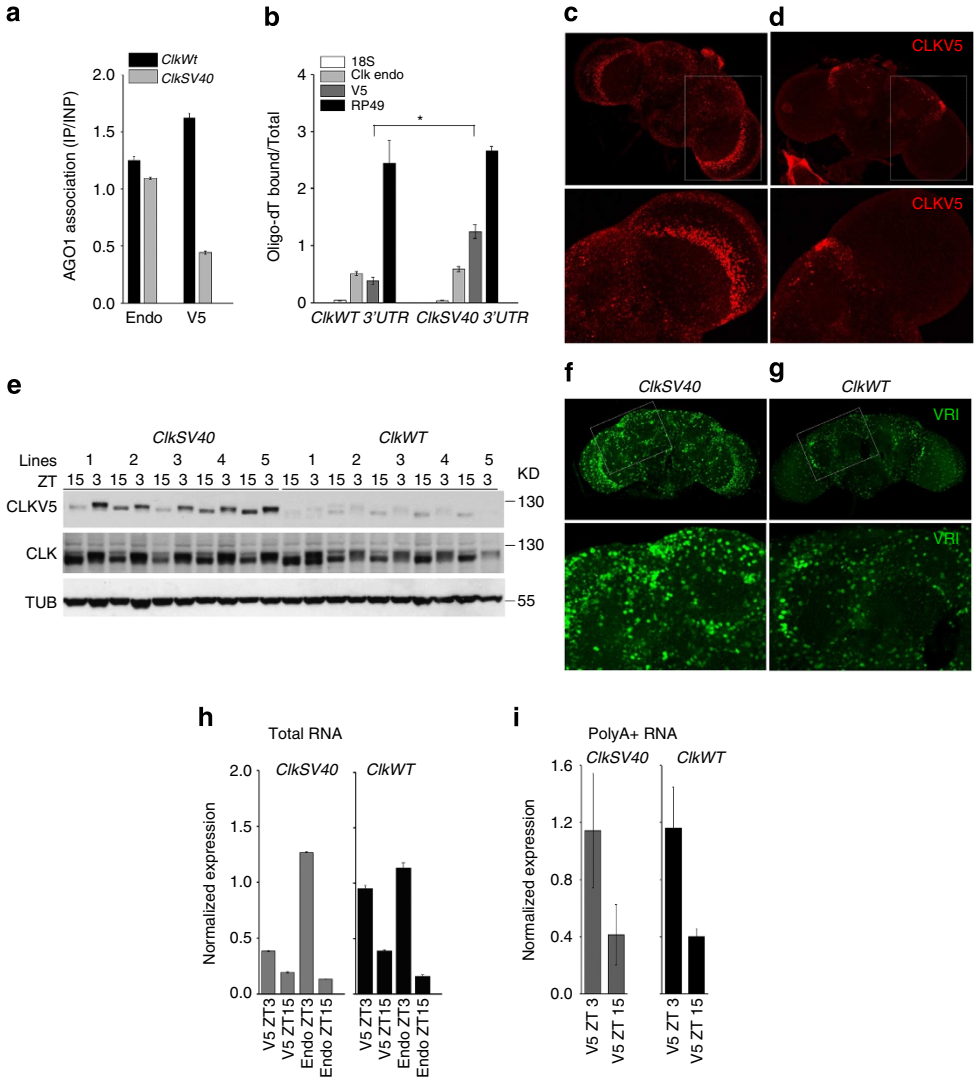

Figure 3 | *ClkSV40* flies express a stable mRNA that generates ectopic clock cells (**a**) AGO1 binding to endogenous genomic *Clk* mRNA or *ClkV5* from *ClkWT* or *ClkSV40* transgenic fly heads (measured by RT-PCR, ratio of IP vs input is shown, one representative experiment out of three is presented). (**b**) RT-PCR results showing the ratio of *Clk* mRNA bound versus unbound- to oligo-dT beads in *ClkWT* and *ClkSV40* fly heads (*$P<0.036$, student *t*-test; mean ± s.e. $n=6$ for each genotype, average of five independent insertions of these transgenes were plotted). (**c**,**d**) Immunofluorescence (IF) analysis of (**c**) *ClkSV40* or (**d**) *ClkWT Drosophila* brains using an anti-V5 antibody (lower panel represents magnification of rectangle area). (**e**) Expression levels of CLKV5, total CLK and tubulin proteins determined by western blot analysis of head lysates from *ClkWT* or *ClkSV40* flies (five independent insertions for each transgene). The shown experiment is one out of four representative experiments. (**f**,**g**) Immunofluorescence analysis of (**f**) *ClkSV40* or (**g**) *ClkWT Drosophila* brains using an anti-VRI antibody. (**h**,**i**) *ClkV5* and endogenous *Clk* mRNAs levels from *ClkSV40* and *ClkWT* fly heads determined by RT-PCR from (**h**) total RNA and (**i**) polyA$^+$-selected RNA (for each time point, the average of five independent insertions for each transgene is plotted). See also Supplementary Movies 1 and 2.

of CLKV5 was restricted to the normal pattern (approx. 150 circadian neurons[2]). In *ClkSV40* flies, however, there was widespread expression of CLKV5 throughout the brain (Fig. 3c, d). Importantly, this ectopic expression was observed in five independent insertions of the *ClkSV40* transgene and in none of the four *ClkWT* insertions that we tested (Supplementary Fig. 2a). Moreover, *ClkSV40* fly heads contained significantly higher levels of V5-tagged CLK than *ClkWT* heads as determined by western blot (Fig. 3e). We assume that this increase is due to the presence of the ectopic circadian cells in the brain.

To determine whether this ectopic expression also generated ectopic CLK-driven transcription, we analysed levels of two CLK-transcriptional targets: VRI and TIM. Immunocytochemistry using anti-VRI and anti-TIM antibodies revealed a massive increase in the number of VRI- and TIM-positive cells in *ClkSV40* compared with *ClkWT* fly brains (Fig. 3f,g and Supplementary Fig. 2a,b; also see Supplementary Movies 1 and 2 for 3D visualization of VRI-positive cells in brains of *ClkWT* and *ClkSV40* flies).

To rule out the possibility that CLK ectopic expression is due to enhanced transcription mediated by the introduction of the SV40 sequence, we compared *ClkV5* mRNA levels between *ClkWT* and *ClkSV40* fly heads at two different times of the day. Surprisingly mRNA levels from the *ClkSV40* transgene were even lower than those from the *ClkWT* transgene, demonstrating that the higher levels of CLK in *ClkSV40* flies are not due to an increase in *Clk* transcription (Fig. 3h, compare levels of V5-tagged *Clk* mRNAs). The substantial differences in the levels of CLKV5 protein (Fig. 3e) indicate that the *Clk* 3′ UTR also contributes to *Clk* translational regulation. This lack of a transcriptional effect was

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confirmed by examining the levels of V5-tagged *Clk* polyA$^+$ mRNAs, which were similar in flies that expressed the *ClkWT* and the *ClkSV40* transgenes (Fig. 3i).

***ClkSV40* flies have abnormal and variable circadian behaviour.** To test the importance of the *Clk* 3′ UTR on circadian behaviour, we assessed locomotor activity rhythms in *ClkWT* and *ClkSV40* flies. In flies with one copy of *ClkWT*, we observed a slightly shortened circadian period and little effect on the overall rhythm strength (Fig. 4a and Supplementary Fig. 3a) as previously described[28]. However, in flies with one copy of the *ClkSV40* transgene, we observed a slightly more pronounced shortening of the circadian period, which was accompanied by a higher proportion of arrhythmic flies (Fig. 4a and Supplementary Fig. 3a). Interestingly, there was also high variability among the nine tested *ClkSV40* fly insertion lines (Fig. 4a and Supplementary Fig. 3a). Moreover, a subpopulation of *ClkSV40* rhythmic flies displayed atypical locomotor activity rhythms after a few days in constant darkness (for example, variable and in some cases split behaviour; Fig. 4b, c and Supplementary Fig. 3a).

The variable behaviour observed in *ClkSV40* flies could be due to higher CLK levels, higher CLK-driven transcription in circadian cells or ectopic circadian gene expression. Importantly, we found no correlation between the number of ectopic VRI-expressing cells and the behavioural defects (Supplementary Fig. 3b). We then carefully quantified CLKV5 levels in flies carrying *ClkWT* and *ClkSV40* transgenes using immunostaining. Surprisingly, and despite the overall difference in total protein levels and the larger number of CLKV5-positive cells (Supplementary Fig. 4a), there were no significant differences in the CLKV5 protein level per cell between *ClkWT* and *ClkSV40* flies (Supplementary Fig. 4b). In contrast, we observed a strong effect on the levels of VRI, which was expressed in more cells and was also significantly upregulated on a per cell basis in *ClkSV40* compared with *ClkWT* flies (Supplementary Fig. 4c, d). VRI and CLK form a well-characterized transcriptional feedback loop: CLK is a strong transcriptional activator of *vri*, and VRI is the only known repressor of *Clk* transcription. Therefore, we surmised that the similar CLK protein levels per cell arise from very different levels of *Clk* transcription (high in *ClkWT* and low in *ClkSV40*) due to feedback transcriptional regulation by VRI. We assume that in steady state, in *ClkSV40* flies CLK activity per cell is slightly higher than that in *ClkWT* flies, which could explain the increased levels of VRI as well as the slightly shorter period observed in these flies.

A mathematical model of the CLK-VRI feedback loop. It has been previously suggested that post-transcriptional control (for example, by miRNAs) could be used to diminish variability in biological systems[46–51]. In this context, we speculate that the high-transcriptional/high-turnover *Clk* profile stabilizes the circadian system by minimizing the consequences of transcriptional noise and/or by allowing rapid transcriptional feedback control. To evaluate this hypothesis, we formulated a

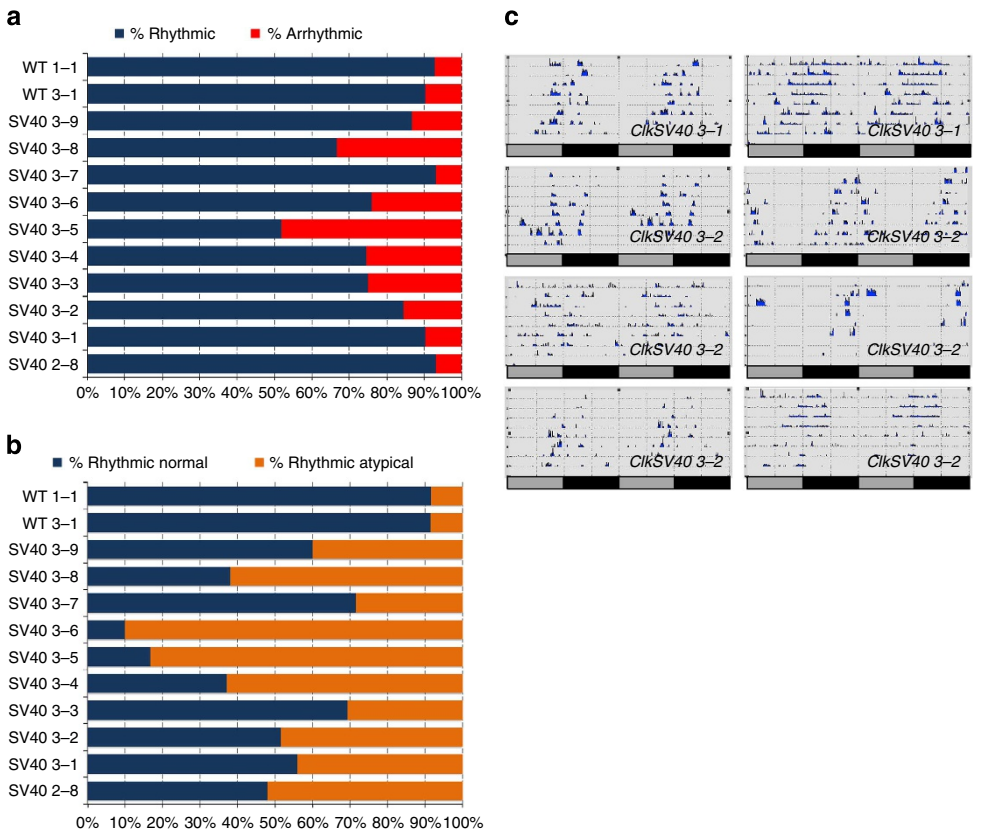

Figure 4 | *ClkSV40* flies display variable circadian behaviour (**a**) Bar graph summarizing the behavioural analyses of *ClkWT* and *ClkSV40* flies. Each row and set of numbers (for example, 1–1) represents a strain with an independent insertion of the wild-type (*ClkWT*) or SV40 (*ClkSV40*) transgene. In all cases flies carry one extra copy of the *Clk* gene (either *ClkWT* or *ClkSV40*) in a wild-type background. Flies were classified as rhythmic or arrhythmic in the first 5 days in constant darkness using the Behavioural toolbox[44]. (**b**) Classification of rhythmic flies as normal and atypical. The classification was done manually after 10 days in constant darkness. (**c**) Examples of individual *ClkSV40* flies displaying atypical circadian behaviour. See also Supplementary Figs 3 and 4.

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mathematical model for uncovering factors that could modulate noise in the levels of *Clk* (see Supplementary Methods). Interestingly, our model predicts that for a given target level of CLK protein, increasing the rate of *Clk* transcription while increasing the rate of mRNA degradation is an effective way of decreasing noise in the system (Supplementary Fig. 5). As stated above, the levels of CLK per cell are similar in *ClkWT* and *ClkSV40* flies; however, due to the strong post-transcriptional regulation provided by the *Clk* 3′ UTR, the same levels of protein are achieved by much lower transcriptional activity of the *Clk* promoter in *ClkSV40* flies. In agreement with the established inverse relationship between transcriptional levels and noise[52–55], our model predicted that in *ClkSV40* flies CLK-driven activity would be more sensitive to transcriptional noise and hence more variable. *Clk* is involved in the development of the circadian neurons as well as in the control of accurate circadian transcription. Therefore, our model predicts that *ClkSV40* flies might display stochastic development of the circadian neurons, which should be accompanied by random/variable circadian transcription.

Decanalized development of pacemaker cells in *ClkSV40* flies. To test these possibilities, we first determined whether *ClkSV40* flies show developmental abnormalities in the main pacemaker cells, the *pdf*-expressing neurons or LNvs. PDF is expressed in eight neurons in each brain lobe[18]. Most (69 out of 70) examined *ClkWT* brain hemispheres had eight canonical LNvs per brain lobe (Fig. 5a). However, flies from three independent insertions of the *ClkSV40* transgene had brains with more than eight *pdf*-expressing cells (Fig. 5a–d). Interestingly, the number of *pdf*-expressing cells varied between brains in these three *ClkSV40* insertions. In particular, one of the lines had between eight and fourteen *pdf*-expressing cells per brain hemisphere (Fig. 5a–d; see Supplementary Movies 3 and 4 for 3D visualization of *pdf*-positive cells in brains of *ClkWT* and *ClkSV40* flies). Although these cells resemble sLNvs due to their smaller size and lower intensity (Supplementary Fig. 6a,b), we did not detect them in larvae brains, suggesting that they develop later than canonical sLNvs. In contrast, we found that most brains of *ClkSV40* pupae had many additional *pdf*-expressing cells (Fig. 5e and Supplementary Fig. 7a,b). Interestingly, we found that most

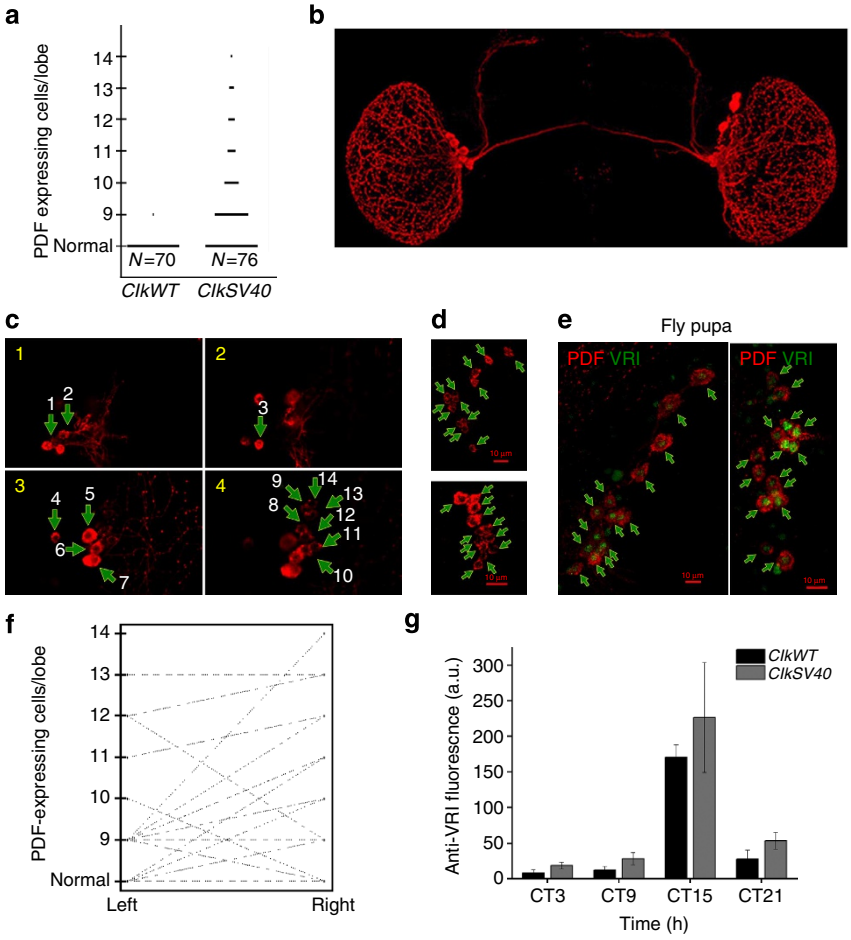

Figure 5 | Stochastic development of the circadian system in *ClkSV40* flies (**a**) Number of PDF-positive cell bodies in *ClkWT* and *ClkSV40* brains. Data are from the *ClkSV40* (2–8) and *ClkWT* (1–1) fly strains. (**b**) Representative example of a *ClkSV40* fly brain in which the position of extra *pdf*-expressing cells can be visualized. (**c**) Representative example of a *ClkSV40* fly brain in which 14 *pdf*-expressing cells were observed in one of the brain hemispheres. *Z*-stack of three images (each taken every 1 μm). Numbers in the upper left corner of each picture indicate the relative position of the *Z*-stack. Arrows indicate the position of the *pdf*-expressing cells. (**d**) *Z*-stack (maximal projection) of two brain hemispheres of *ClkSV40* flies immunostained for PDF showing the number of *pdf*-expressing cells (13 and 12 for the top and bottom brain hemispheres respectively). Arrows indicate the position of the *pdf*-expressing cells. Red scale bar indicates 10 μm. (**e**) *Z*-stack (maximal projection) of two brains of *ClkSV40 pupae* immunostained for PDF (in red) and VRI (in green) showing the number of *pdf*-expressing cells (14 and 15 for the left and right images respectively). Arrows indicate the position of the *pdf*-expressing cells. Red scale bar indicates 10 μm. (**f**) Comparison of left and right hemispheres in *ClkWT* and *ClkSV40* flies based on PDF immunofluorescent staining. (**g**) Brain-to-brain variation in the levels of VRI (measured by IF) protein in the sLNvs at 4 time points (day 10 in DD), in *ClkSV40* (line 2–8, red) and *ClkWT* (line 1–1, black) flies. See also Supplementary Figs 5–8 Supplementary Movies 3 and 4.

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ClkSV40 pupae brain hemispheres have larger number of *pdf*-expressing cells than adults from the same strain (see Supplementary Fig. 7b), suggesting that some of the additional *pdf*-expressing cells might die or lose *pdf* expression later in development (or after eclosion).

Interestingly, we observed that the two lobes of individual flies carrying *ClkSV40* transgenes flies did not necessarily have the same number of *pdf*-expressing neurons, demonstrating that the number of these neurons is variable in the absence of *Clk* post-transcriptional regulation (Fig. 5b,f). We observed the same phenomenon in pupae of *ClkSV40* flies (Supplementary Fig. 7c) demonstrating that this asymmetry is established during development.

To determine whether *Clk* 3′ UTR also diminishes variability in daily CLK-driven transcription within adult flies, we determined VRI levels throughout the day in flies carrying *ClkWT* or *ClkSV40* transgenes. We focused on one *ClkWT* (1-1) and one *ClkSV40* (2–8) insertions of the transgenes. To minimize the effects of an endogenous WT *Clk* gene, we performed the experiment in the background of the *Clk* hypomorphic mutant Clk^{AR} (ref. 29). In these transgenic lines, the *ClkWT* transgene rescued the arrhytmicity of the Clk^{AR} mutation much more effectively (>75%) than the *ClkSV40* transgene (<40%). Therefore, we stained *ClkWT*; Clk^{AR} and *ClkSV40*; CLK^{AR} flies for VRI and PDF at four different circadian time points after 8 days in constant darkness (DD8). We focused on the sLNvs, which display strong protein oscillations in constant darkness[37]. Whereas *ClkWT*; Clk^{AR} flies displayed robust VRI protein oscillations and little brain-to-brain variability, *ClkSV40*; Clk^{AR} flies expressed higher and more variable VRI levels with a significant number of flies showing inappropriate VRI staining at the early circadian time points (CT3 and CT7; Fig. 5g and Supplementary Fig. 8).

A role of *bantam* in the development of circadian neurons. We recently showed that the miRNA *bantam* regulates the *Clk* 3′ UTR, at least in *Drosophila* S2 cells[32]. Hence, we decided to define whether this regulation is key to the deterministic development and maintenance of the circadian neuronal network. For doing so, we examined by immunocytochemistry the number and intensity of circadian neurons (VRI- and PDF-positive cells) in flies carrying a *Clk* transgene with deletion of the putative *bantam* binding sites (*ClkΔban*[32]). Indeed, and similarly to *ClkSV40* flies, *ClkΔban* displayed ectopic expression of VRI across the fly brain (Fig. 6a). In addition, *ClkΔban* showed variable and increased number of *pdf*-expressing cells (Fig. 6b,c). Importantly, and as in *ClkSV40* flies, we did not observe a correlation between the numbers of *pdf*-expressing cells in each hemisphere of individual fly brains (Fig. 6d). These results demonstrate the importance of the *bantam* binding sites in the *Clk* 3′ UTR for normal development of the *pdf*-expressing cells. Interestingly, we found that some of these brains also have heterogeneous VRI expression in the PDF-positive cells. For example, we found brains in which VRI expression differs or is even absent in some of the PDF-positive cells (that is, see Fig. 6b). We observed a similar phenomenon in *ClkSV40* pupae (see Fig. 5e and Supplementary Fig. 6a). This strongly suggests that CLK activity is variable across circadian neurons within the same brain.

Discussion

Here we found that the circadian master regulator *Clk* is under very strong post-transcriptional control. One consequence of this tight control is the creation of a threshold-like regulation that inhibits ectopic expression of *Clk* and CLK-transcriptional targets. Indeed, flies devoided of this regulation have more circadian cells in the brain and aberrant and variable circadian rhythms. Interestingly, in these flies, the levels of CLK on a per cell basis are not altered compared with flies with wild-type CLK, suggesting that the main role of *Clk* post-transcriptional regulation is to limit the variability of CLK activity rather than CLK protein levels. Moreover, we observed an increase in the number of *pdf*-expressing cells in *ClkSV40* flies and pupae. To understand these results, we formulated a mathematical model of the circadian CLK-VRI feedback loop. Our model predicts that *Clk* post-transcriptional control diminishes the noise in this transcriptional feedback loop. Indeed, we found that in *ClkSV40* flies there were not only more *pdf*-expressing cells, but also that their numbers were variable among individuals or between the two lobes of the same brain, demonstrating that in the absence of *Clk* post-transcriptional control LNvs development and maintenance is stochastic. Last, flies carrying *Clk* transgenes with mutation in the putative binding sites for the miRNA *bantam* also displayed stochastic number of *pdf*-expressing cells between and within individuals, suggesting that the phenotypes observed in *ClkSV40* flies are mediated through this miRNA.

Clk is strongly regulated at the post-transcriptional level. Indicative of regulation by miRNAs, *Clk* mRNA is strongly bound to AGO1 and does not bind efficiently to oligo-dT beads. In addition, we observed significant differences between the CLK protein and *Clk* mRNA ratios in *ClkWT* and *ClkSV40* flies (compare Fig. 3h to Fig. 3e), which suggests that an important part of this regulation is at the translational level. Interestingly, mRNA turnover accounts at least partially for the post-translational control, as *Clk* mRNA is short lived when compared with other circadian mRNAs like *cry* (Fig. 1f). Therefore, *Clk* post-transcriptional regulation appears to be complex and redundant. Our results suggest that the *Clk* 3′ UTR is responsible for the decrease in CLK levels in most (if not all) cell types, likely by miRNAs. *Bantam* seems to have a predominant role but it might not be unique. The presence of this type of regulation is not surprising given the large amount of reports demonstrating the importance of RNA metabolism for circadian timekeeping[28,41,56–60].

The general assumption in the field is that *Clk* transcriptional oscillations are not essential for behavioural rhythms. This is based on the fact that the circadian behaviour of clk^{AR} mutants can be rescued using the cry-GAL4 driver and a UAS-*Clk* transgene[29] and that there are negligible effects on expression of CLK under the *per* or *tim* promoters (ARK flies[21]). However, the same UAS-*Clk* transgene expressed under control of other gal4 drivers like *tim-gal4* leads to developmental lethality[29] and the behavioural deficits of Clk^{AR} mutants are not rescued by *Clk* ARK transgenes (our own unpublished observation). Moreover, addition of extra *Clk* gene copies leads to a copy-number-dependent shortening of the period[28], demonstrating that CLK levels cannot be regulated solely at the post-translational level. Interestingly, the ARK transgenes carry a *per* 3′ UTR, which is strongly regulated post-transcriptionally (our unpublished data). This could explain why flies carrying this transgene are rhythmic in a wild-type background.

Our S2 cells and fly brains experiments demonstrate a key role of *Clk* 3′ UTR in avoiding ectopic CLK-driven transcriptional activity. Moreover, these results suggest that the *Clk* 3′ UTR is necessary for establishment of a threshold for meaningful *Clk* mRNA expression. This type of threshold might be important not only in circadian cell determination but also to prevent stochastic or random pulses of CLK-driven activity that could alter or shift the circadian clock. miRNA-mediated thresholding of master developmental regulators has been previously demonstrated for the miR-9a/*senseless*[61] and for the miR-263a/*hid* pairs[62]. We

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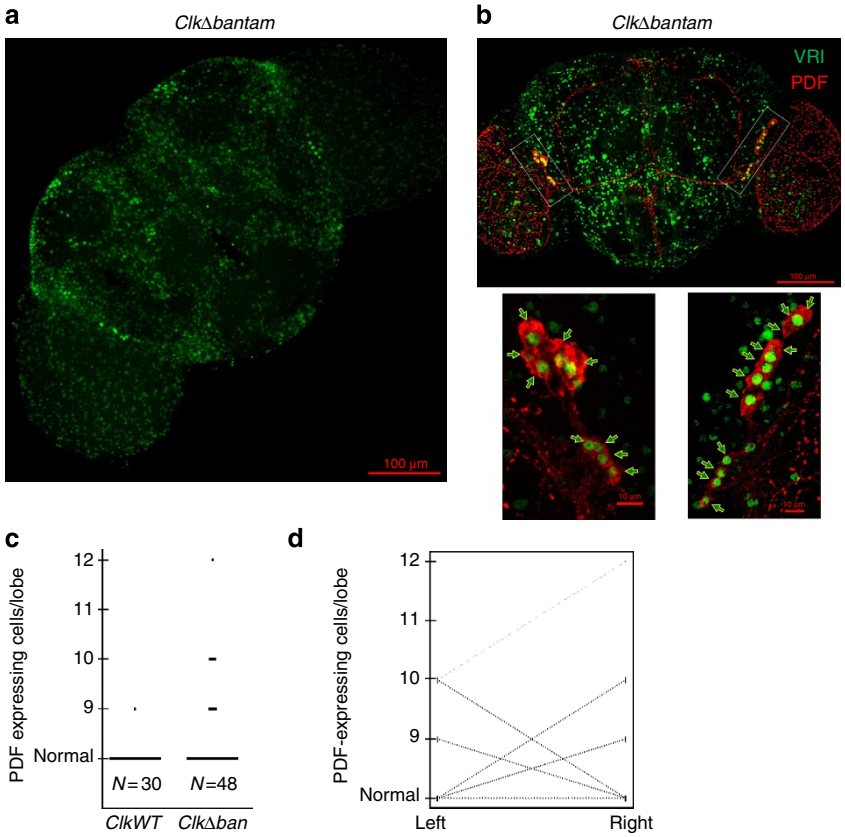

Figure 6 | Deletion of the *bantam* binding sites on the *Clk* 3′ UTR leads to stochastic development of the *pdf*-expressing cells (**a**) Immunofluorescence (IF) analysis of a representative *ClkΔban* (3–7) *Drosophila* brain using an anti-VRI antibody. Flies were collected and dissected at ZT15. (**b**) Representative example of a *ClkΔban* fly brain in which 21 LNvs cells were observed, 9 in one hemisphere and 12 in the other (top panel). Lower panel represents magnification of rectangle area. Of the 12 PDF-positive cell bodies in the right hemisphere: 9 cells are VRI positive, one cell display low intensity of VRI immunostaining and 2 cells are VRI negative. In the left hemisphere of the brain, 9 PDF-positive cells are observed: 8 cells are VRI positive and one cell displays low intensity of VRI immunostaining. Flies were collected and dissected at ZT15. Arrows indicate the position of each PDF-positive cell. (**c**) Number of PDF-positive cell bodies in *ClkWT* and *ClkΔban* brains. Data are taken from the *ClkΔban* (3–7) and *ClkWT* (1–1) fly strains. (**d**) Comparison of left and right hemispheres in *ClkWT* and *ClkΔban* flies.

believe that our failure to detect signal from the *Clk* 3′ UTR reporters is not related to a sensitivity issue. This is based on our observation of the abnormally high number of circadian cells in the brains of *ClkSV40* flies, which demonstrates that the threshold has a biological meaning.

Our work demonstrates a central role for *Clk* in the development of the *pdf*-expressing LNvs cells, as three of the fly lines with *ClkSV40* insertions as well as *ClkΔban* flies display larger number of LNvs than observed in wild-type brains. Although this work constitutes the first report of flies with more than 16 LNvs per brain, a previous report suggests that *Clk* affects the development of these cells: *Clk* and *cyc* mutants have reduced numbers of LNvs, and *Clk* can activate (albeit weakly) a pdf-luciferase reporter[31]. From our results it is not clear whether these cells are part of the neuronal circadian network or whether their development and maintenance is independent. Indeed, the transgenic line with the largest number of extra LNvs (*SV40* 2–8) was fairly rhythmic at the behavioural level, suggesting that these extra cells do not markedly influence the timekeeping process, at least early in constant darkness. The abnormal behaviour cannot be explained neither by the presence of additional VRI-expressing cells, as we see no correlation between the number of them and any of the behavioural anomalies (Supplementary Fig. 3b). We favour the possibility that the behavioural defects are due to stochastic changes in CLK-driven transcription. Stochastic variation in CLK-driven transcription could also explain the brain-to-brain variation in VRI levels in *ClkSV40* flies and the observed variations in VRI levels between *pdf*-expressing cells within the same brain in the *ClkSV40* pupae and *ClkΔban* fly brains (that is, see Figs 5e and 6b bottom, respectively). Moreover, we observed that *ClkSV40* pupae have generally larger numbers of extra LNvs, suggesting that many of these cells might lose *pdf* expression during adulthood (as we did not observed high levels of lethality in *ClkSV40* flies). We believe that in the future and given the advances in CRISPR technology, it would be interesting to repeat some of the experiments in flies in which the deletion the 3′-UTR is in the endogenous *Clk* locus.

Our results suggest that the main role of *Clk* post-transcriptional control is to limit the variability in CLK activity rather than to control the total levels of CLK. This is based on the fact that the levels of CLK per cell were not altered in *ClkSV40* flies despite the large increase in the total number of CLK-positive cells. We argue that the normal CLK levels in individual cells are due to an increase in VRI levels that reduce *Clk* transcription and hence total CLK levels (see model in Fig. 7). Indeed, heads of *ClkSV40* flies have lower levels of *ClkV5* mRNA than do *ClkWT* flies (Fig. 3h) despite much higher protein levels (Fig. 3e). Therefore, our data suggest that the main difference between *ClkWT* and *ClkSV40* flies is that the latter produce CLK by a low transcription/low degradation rather than the high transcription/high degradation profile observed in wild-type flies (Fig. 7). We

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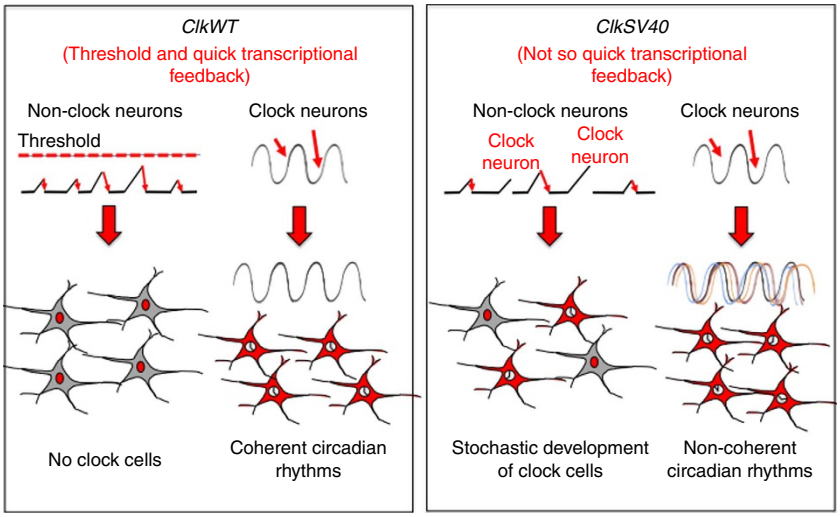

Figure 7 | Role of *Clk* post-transcriptional regulation in clock and non-clock neurons Small red arrows represent random or environmentally driven fluctuations in *Clk* gene expression.

speculate that the increase in VRI levels keeps the overall levels of CLK per cell constant. In contrast, the low transcription/low turnover *Clk* mRNA profiles observed in *ClkSV40* flies lead to more random, less fixed levels of *Clk* mRNA and protein (Fig. 7). We can then imagine a model in which under wild-type conditions (either no *Clk* transgene or one copy of the *ClkWT* transgene) stochastic pulses of *Clk* transcription in non-circadian neurons are filtered by the transcriptional threshold and do not lead to the development of circadian cells (Fig. 7). This is essential during development as well as in adult animals, as ectopic clocks in the fly brain are dependent on continuous expression of *Clk*[63]. In theory, *Clk* post-transcriptional control could help stabilize CLK-driven transcription in the circadian cells both by thresholding and by diminishing cell-to-cell variability in CLK-driven oscillations (Fig. 7).

In sum, here we found that post-transcriptional control of a master transcriptional regulator provides an efficient mechanism that assures canalized development and that stabilizes a transcriptional network during development and in adult animals. The role of post-transcriptional control in stabilizing the activities and levels of key activators of systems based on feedback loops has been previously proposed[46,47,51], but to the best of our knowledge, our work constitutes the first theoretical and experimental example of this control principle.

Methods

Fly strains. The CantonS (CS) strain used as wild type is CSIso3H (Bloomington Stock Centre, Indiana). Clk^{AR} mutants were described[29]. *ClkSV40* flies were constructed by injecting *ClkSV40* transgene into *yw* embryos (service provided by Best Gene). *ClkΔban* flies were described[32].

Analysis of gene expression by real-time PCR. Total RNA was prepared from adult fly heads using TRI Reagent (SIGMA) according to the manufacturer's protocol. RNA was DNase treated (DNaseI, NEB), and cDNA derived from this RNA (using iScript, Bio-Rad) was utilized as a template for quantitative real-time PCR performed with the C1000 Thermal Cycler Bio-Rad. The utilized primers are summarized in Supplementary Methods. mRNA values from heads were normalized to levels of mRNAs encoding ribosomal proteins RP49 and rpS18.

Assessment of post-transcriptional regulation. We utilized a mixture of RNA extracted at six different time points from fly heads. The three biological replicas were generated by three independent RNA extractions from each of the six time points. After extraction, the RNA was DNase treated, ethanol precipitated and retrotranscribed using iScript (Bio-Rad) with random primers. We performed real-time PCR using the diluted cDNA template and an equimolar mixture of the DNA products. This DNA mixture was utilized in experiments to determine the absolute levels of the pre-mRNA and mature mRNA. We generated this mixture by pooling equimolar concentrations of the purified PCR products. We designed two sets of primers for each gene, one intronic and one exonic. For intronic PCRs, real-time PCR was performed using the cDNA as a template, and amounts were plotted on a standard curve constructed using different concentrations of the genomic DNA as templates to normalize the different efficiencies of the primers. For each gene a pre-mRNA/mRNA ratio was calculated. The primer sequences are detailed in Supplementary Information. Three independent biological replicates were performed.

Isolation of different fractions of RNAs. Oligo-dT-bound fractions were isolated from total RNA using Invitrogen $(dT)_{25}$ Dynabeads following the manufacturer's protocol. The RNA not bound was precipitated with ethanol and dissolved in RNase-free water. For quantification, the unbound fraction was normalized to histone H1 and the bound fraction to RP49. Data were obtained from a pooled mixture of six time points, and three biological replicates were performed.

RNA measurements from cultured fly wings. Thirty adult male Canton-S fly wings were dissected and cultured as described[64] under 12 h light/12 h dark (LD) conditions for 3 days. In mRNA half-life measurements actinomycin D was added in ZT3 ($10\,\mu g\,ml^{-1}$), wings were transferred into TRI Reagent (SIGMA) at relevant time points and mRNA was extracted. For each time point, the experiments were performed in triplicates.

Plasmids. To generate the *tim*-YFP reporter, we amplified by PCR the YFP coding sequence and the *tim* promoter region (760 bp from the timwt760-luciferase construct[65]). The fragments were ligated sequentially into pBluescript. pAc-Cherry and pAc-CFP were constructed by amplifying the Cherry or CFP coding sequences by PCR and ligating these products into pAcB V5/His6 (Invitrogen). The *ClkSV40* plasmid was generated through steps similar to those used to construct the *ClkWT* (described in[28]) with the following modification: a fragment containing the *SV40* 3′ UTR sequence was amplified by PCR from pAc V5/His6 (Invitrogen) and cloned by overlap PCR, exactly downstream the stop codon, into a plasmid containing a Sph-NotI fragment derived from a plasmid containing the last portion of the *Clk* transgene (from position 7741779 to 7736982, *pdClk*4). The Sph-NotI fragment containing the *SV40* 3′ UTR was re-cloned into the *pdClk*4, which was then used to generate the whole *ClkV5* transgene as described previously[28]. The resulting plasmid was fully sequenced.

S2 cells maintenance and transfection. S2 cells were maintained in 10% fetal bovine serum (Invitrogen) insect tissue culture medium (HyClone). Cells were seeded in a six-well plate. Transfection was performed using 6 µl of MIRUS transfection reagent TransIT 2,020 and 2 µg of total DNA as per the manufacturer's instruction. For the 3′ reporter experiments, 250 ng each of pMT-Cherry and pMT-YFP reporters (one carrying the *SV40* 3′ UTR and one carrying the *Clk* 3' UTR) were co-transfected into *Drosophila* S2 cells along a pAc-CFP transcriptional control. To avoid bias due to the different fluorophores, we performed the analysis comparing the levels of the same fluorescent protein under the different 3′ UTRs (*SV40* versus *Clk* 3′ UTR). Shown are data obtained with the Cherry reporter, but we obtained similar results when YFP reporters were compared. For the experiments to follow CLK-driven activity, we utilized 150 ng of

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pAcCherry, 150 ng of the *tim*-YFP reporter and 500 ng of the *ClkWT* or *ClkSV40* plasmids. pBS-KS (Stratagene) was used to bring the total amount of DNA to 2 µg.

High-throughput reporter expression analysis in S2 cells. One day after transfection, cells were transferred to optic-suitable 96-well plates and visualized over 24 h using Scan^R high-throughput fluorescent microscope (Olympus). The intensity from the transfection control reporter (CFP for 3′ UTR and Cherry for the CLK-activity experiments), cell size and roundness were evaluated. Cherry or YFP levels were assessed only for cell populations that met intensity and cell morphology criteria.

AGO-1 immunoprecipitation. AGO-1 immunoprecipitations were performed as previously described[32]. The transcripts were quantified by RT-PCR using primers that exclusively identify the endogenous (endo) or the V5-tagged *Clk* mRNA (V5). Data are shown as ratio of immunoprecipitate to input.

Immunofluorescence. Male flies were entrained for at least 3 days in LD and then transferred to DD for the indicated time. Third stage larvae and last stage pupae were collected after 3 days entrainment in LD. Whole flies/larvae/pupae were collected into fixative solution, PBS 4% paraformaldehyde 0.1% Triton X-100, and incubated for 30 min at 4 °C followed by 2 h rotation at room temperature. After fixation, flies/larvae/pupae were transferred to PBS, and the brains were dissected. The brains were washed three times (PBS 0.1% Triton X-100) and transferred to blocking solution (PBS, 0.1% Triton X-100, 2% horse serum). After three washes, primary antibody solution was added and samples were incubated at 4 °C overnight. The primary antibody solution contained PBS, 0.1% Triton X-100, 2% horse serum, and 1:3,000 guinea pig anti-VRI (gift from Paul Hardin), 1:1,000 mouse anti-PDF (gift from the Justin Blau lab), 1:1,000 mouse anti-V5 (Sigma), 1:1,000 rat anti-TIM (a gift from Michael Rosbash) or 1:1,000 guinea pig anti-CLK (a gift from Paul Hardin). Brains were washed three times and then exposed to secondary antibody solution: PBS, 0.1% Triton X-100, 2% horse serum and 1:500 Alexa Fluor 488 goat anti-guinea pig (Invitrogen), 1:500 Dylight 550 donkey anti-mouse (Abcam) or 1:500 Alexa Fluor donkey anti-rat (Jackson) for 1 h in room temperature. Brains were washed three times and mounted in VECTASHIELD mounting medium (VECTOR) on microscope slides. Images were captured using a Nikon A1R confocal microscope and analysed with NIS-Elements (Nikon). Quantification of brain cell expression of V5 or VRI proteins was performed as follows: stained brains were placed between spacers to avoid pressure on the tissue. Confocal images were taken every 3 µm (on the Z axis) over the entire brain depth.

Western blotting. Fly heads (20 heads per sample) were collected on dry ice. Heads were homogenized in RIPA lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, **1** mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1 mM DTT, supplemented by protease inhibitor cocktail and phosphatase inhibitors) using a motorized pestle. Head lysates were then centrifuged at top speed for 10 min. The supernatant was boiled with protein sample buffer (Bio-Rad). Samples were resolved on Criterion XT Bis-Tris gels (Bio-Rad). Antibodies used for western blotting were those described above and anti-tubulin (DM1A, SIGMA).The complete blots of Fig. 3e are provided as Supplementary Fig. 9.

Locomotor activity. Male flies were monitored using trikinetics *Drosophila* activity monitors. Analyses were performed with a signal processing toolbox[44]. Flies were considered rhythmic if the rhythm index was >0.15 for the first 5 days in DD. Rhythmic flies after the 5-day period were followed for 5 more days and, if alive after this period, were classified as normal or atypical rhythmic by visual inspection after examination of the behavioural pattern and the MESA analysis.

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Author contributions

I.L., O.B., V.W. and U.W. performed the experiments. S.A. analysed the single cell microscopy data. C.G. generated the *ClkSV40* flies and performed the initial characterization. D.H. and N.F. formulated the mathematical model; M.R. supported J.M., discussed the experiments and help writing the manuscript. S.K. designed the experiments and wrote the manuscript.

Additional information

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