Crosstalk between \textit{vrille} transcripts, proteins, and regulatory elements controlling circadian rhythms and development in \textit{Drosophila}

**HIGHLIGHTS**

- \textit{vri}-E mRNA is sufficient for \textit{Drosophila} development and circadian behavior
- E-boxes upstream of the \textit{vri}-ADF promoter are required for \textit{Drosophila} development
- \textit{vri}-ADF mRNAs primarily produce short VRI protein rather than long VRI protein
- Short VRI protein primarily controls \textit{Drosophila} development and circadian behavior

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Crosstalk between *vrille* transcripts, proteins, and regulatory elements controlling circadian rhythms and development in *Drosophila*

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**SUMMARY**

The *vrille* (*vri*) gene encodes a transcriptional repressor required for *Drosophila* development as well as circadian behavior in adults. Alternate first exons produce *vri* transcripts predicted to produce a short VRI isoform during development and long VRI in adults. A *vri* mutant (*vri*D679) lacking long VRI transcripts is viable, confirming that short VRI is sufficient for developmental functions, yet behavioral rhythms in *vri*D679 flies persist, showing that short VRI is sufficient for clock output. E-box regulatory elements that drive rhythmic long VRI transcript expression are required for developmental expression of short VRI transcripts. Surprisingly, long VRI transcripts primarily produce short VRI in adults, apparently due to a poor Kozak sequence context, demonstrating that short VRI drives circadian behavior. Thus, E-box-driven long VRI transcripts primarily control circadian rhythms via short VRI, whereas the same E-boxes drive short VRI transcripts that control developmental functions using short VRI.

**INTRODUCTION**

The *vrille* (*vri*) gene from *Drosophila* encodes a bZIP transcriptional repressor that was initially identified as an enhancer of decapentaplegic (dpp) dorsoventral patterning defects (George and Terracol, 1997). *vri* null mutants show multiple embryo patterning defects that cause lethality before hatching as L1 larvae (George and Terracol, 1997), and patches of *vri* mutant tissue in adults show patterning and proliferation defects (George and Terracol, 1997; Szuplewski et al., 2003). Subsequently, *vri* was recovered in a screen for circadian clock-controlled transcripts present in adult heads (Blau and Young, 1999). As *vri* mutants are embryonic lethal, *vri* function in the clock was first characterized via overexpression, which lengthened or abolished behavioral rhythms (Blau and Young, 1999). Indeed, *vri* rhythmically represses transcription of the key clock activator *Clock* (*Clk*) as well as other genes whose mRNAs cycle with a peak around dawn (Cyran et al., 2003; Glossop et al., 2003; Hardin, 2011). Transgenic flies that permit conditional *vri* inactivation revealed that *vri* is not necessary for molecular clock function, but disrupts clock output in key brain pacemaker neurons to abolish behavioral rhythms (Gunawardhana and Hardin, 2017).

The *vri* gene produces two protein isoforms from five transcripts that are initiated at three different first exons (Gramates et al., 2017; Szuplewski et al., 2003). The 610-amino acid short VRI isoform (short VRI) is produced by the *vri*-E and *vri*-C transcripts that initiate at first exons 1a and 1b, respectively, whereas the 729-amino acid long isoform (long VRI) is produced by three transcripts that only differ in their 3′ UTR length, *vri*-A, *vri*-D, and *vri*-F (henceforth referred to as *vri*-ADF) initiated at first exon 1c. Each first exon splices onto common second and third exons to produce full-length mRNAs, but translation of *vri*-E and *vri*-C initiates within exon 2 to produce short VRI, whereas translation from *vri*-ADF initiates in exon 1c to produce long VRI. In adults, prominent ~85-kDa and minor >90-kDa VRI bands are rhythmically expressed, but migrate more slowly than the predicted ~65-kDa short and ~78-kDa long VRI isoforms (Glossop et al., 2003). The ~85- and >90-kDa VRI bands seen in wild-type adults likely correspond to short VRI and long VRI, respectively, but this predicted relationship has not been tested.

Mutants that eliminate transcription or translation of exons common to all *vri* transcripts are lethal (George and Terracol, 1997; Szuplewski et al., 2003). A 17-kb *vri* transgene that lacks only exon 1a fails to rescue developmental lethality, suggesting that the *vri*-E transcript is necessary for development (George and Terracol, 1997). Although RNA sequencing (RNA-seq) detects little if any *vri*-C mRNA, *vri*-E mRNA is detected...
in embryos, pupae, and adults, and vri-ADF mRNA is present at all developmental stages (Thurmond et al., 2019). Rhythmic vri transcription is thought to be driven by CLK-CYCLE (CLK-CYC) binding to E-box regulatory elements upstream of exon 1c (Abruzzi et al., 2011; Blau and Young, 1999; Cyran et al., 2003; Lim et al., 2007). Based on these observations, we hypothesize that short VRI produced by vri-E is responsible for vri developmental functions and long VRI produced by vri-ADF mediates circadian function. If true, eliminating transcription from exon 1c should eliminate vri circadian function but leave vri developmental functions intact.

Here we generate a vri mutant (vriΔ679) that eliminates vri-ADF mRNA production. This mutant is homozygous viable, demonstrating that vri-ADF mRNA is dispensable for fly development. Both vri-ADF and vri-E

Figure 1. vriΔ679 mutants lack vri-ADF transcript expression

(A) The vri genomic region (white box) showing vri exons (black boxes) and canonical E-box regulatory elements (purple lines) is depicted above the vri 70-kb BAC and vri 24-kb BAC transgenes (green boxes) that were used to rescue developmental and circadian function in vri mutants (see Figures 4 and 5). The five vri mRNA isoforms (RA, RC, RD, RE, RF) and the three alternate first exons (1a, 1b, 1c) are shown, where exons for different vri transcripts are shown as boxes denoting untranslated (yellow) or translated (blue) regions. The five vri transcripts produce short VRI or long VRI isoforms, depicted by orange boxes corresponding to their exon coding sequences. A magnified view of the vri genomic region bracketed by the red lines is shown below the short VRI and long VRI isoforms. Canonical E-box regulatory elements (purple boxes) are shown upstream of the vri d01873 P element (dashed triangle), which is located 4 bp upstream of exon 1c. Imprecise excision of vri d01873 produced a deletion removing most of exon 1 (stippled yellow and blue region) and a part of intron 1 (dashed line) and inserted an 8-bp sequence (red). The location and orientation (gray arrows) of the screen forward (screen F), screen reverse (screen R), D679 forward (Del F), and Deletion reverse (Del R) primers used to identify and characterize vri deletions are denoted.

(B) Sequence of the vri exon 1c region showing the vri d01873 P element insertion site, the vriΔ679 deletion (underlined nucleotides), the 5' untranslated region (orange line), the translated region (blue line), intron 1 (green line), and the 8-bp insertion (bold). (C) RNA-seq analysis of vri expression in heads from w1118 and vriΔ679 flies collected at ZT2 and ZT14 during LD cycles. The left graph shows composite mRNA expression levels in fragments per kilobase million (FPKM) for all vri mRNA isoforms (vri), the middle graph shows mRNA expression levels in read counts for exon 1c, and the right graph shows mRNA expression levels in read counts for exon 1a. Data from the two biological replicates are shown as black circles and white squares, and the average is shown by the bar height.
mRNAs are rhythmically expressed, but E-boxes situated near the vri-ADF promoter are required for viability, which suggests that they are important for vri-E transcription during development. Although vri-ADF mRNA and long VRI are absent in vriΔ679 flies, low levels of cycling short VRI protein derived from vri-E mRNA are sufficient for behavioral rhythms. Surprisingly, vri-ADF mRNA primarily produces short VRI, likely due to the poor Kozak sequence context of the long VRI initiation codon relative to that of short VRI. These results suggest that short VRI carries out both developmental and circadian functions in Drosophila, where vri-E transcripts primarily support developmental function and vri-ADF transcripts primarily support circadian function.

**RESULTS**

**A mutant that eliminates vri-ADF transcripts maintains clock function**

As vri-ADF transcripts are thought to mediate clock function based on the clustering of E-boxes in their promoter, we sought to determine the significance of vri-ADF transcripts for clock function by generating a mutant that eliminates these transcripts. The vriΔ679 P element is inserted 4 bp upstream of the exon 1c transcription start site (Figure 1A and 1B), is homozygous viable, and displays activity rhythms similar to those in w1118 wild-type controls (Table 1). The vriΔ679 P element was excised (see Transparent Methods), and progeny lacking the vriΔ679 insert were screened by PCR to identify flies carrying a deletion that disrupts exon 1c (Table S1 and Figure S1). The largest deletion mutant recovered, vriΔ679, removes a 679-bp region that includes the last 513 nucleotides of exon 1c and 166 bp from intron 1 (Figures 1A, 1B, and 1S). An 8-bp sequence was inserted within the deleted region (Figure 1B), possibly due to a P element.

| Genotype | N  | % Rhythmic | Period ± SEM | Strength ± SEM |
|----------|----|------------|--------------|----------------|
| w1118   | 32 | 93.8 ±     | 23.77 ± 0.05* | 310.08 ± 40.56 |
| vriΔ679b | 62 | 85.5 ±     | 23.30 ± 0.05  | 112.97 ± 11.59* |
| vriΔ679a | 63 | 93.5 ±     | 22.92 ± 0.05* | 202.14 ± 16.94 |
| vriΔ679, vri24 | 22 | 95.5 ±     | 23.29 ± 0.05  | 309.33 ± 38.59 |

Table 1. vriΔ679 mutants show activity rhythms with a shorter period

*The period of w1118 activity rhythms is significantly longer (\( p \leq 0.001 \)) than all other genotypes.

The power of w1118, vriΔ679, and S1). An 8-bp sequence was inserted within the deleted region (Figure 1B), possibly due to a P element.

The period of vriΔ679 activity rhythms is significantly shorter (\( p \leq 0.002 \)) than all other genotypes.

The period of vriΔ679 activity rhythms is significantly shorter (\( p \leq 0.002 \)) than all other genotypes.

*The power of vriΔ679 flies is significantly lower (\( p \leq 0.02 \)) than all other genotypes.

The power of vriΔ679 flies is significantly lower (\( p \leq 0.02 \)) than w1118 and vriΔ679. vri24. N, total number of flies tested.

Complete genotype is w1118, vriΔ679, vri24.

We expected vriΔ679 flies to be behaviorally arrhythmic because they lack the vri-ADF mRNAs thought to be required for clock output (Gunawardhana and Hardin, 2017). Surprisingly, >93% of vriΔ679 flies were rhythmic with an ~0.8 h shorter period than w1118 controls (Table 1). Given the quasi-normal vriΔ679 behavioral rhythms, we assessed VRI expression in vriΔ679 brains. VRI levels in all vriΔ679 brain pacemaker neurons were sharply reduced compared with w1118 control brains (Figure 2A). Likewise, VRI levels were much lower in heads from vriΔ679 flies than w1118 controls, but continued to cycle in abundance (Figure 2B). This result...
suggests that VRI expression in retinal photoreceptors, which comprise >80% of all clock cells in the head (Glossop et al., 2003), is also reduced in vriD679 flies. Indeed, as in brain pacemaker neurons VRI levels are greatly reduced in photoreceptor cells (Figure 2C). Likewise, VRI levels were low in Malpighian tubules (MTs) (Figure 2D), a clock-containing tissue that mediates renal function in Drosophila (Dow and Romero, 2010). Although greatly reduced, VRI levels in photoreceptor cells and MTs of vriD679 flies are rhythmically expressed (Figure 2E). Decreased levels of VRI in vriD679 flies likely shorten circadian period by increasing Clk expression because VRI acts to repress Clk transcription and increased Clk expression shortens circadian period (Allada et al., 2003; Cyran et al., 2003; Glossop et al., 2003). Consistent with this possibility, Clk mRNA is ~50% higher on average in vriD679 flies than w1118 controls (Figure S2). In addition, CLK-CYC targets per and tim are also upregulated in vriD679 flies, but cyc, which is not regulated by the circadian clock (Liu et al., 2017; Nagoshi et al., 2010), is expressed at about the same levels in vriD679 and w1118 flies (Figure S2). These results show that vri-ADF expression is not required for rhythmic behavior per se, although the lower levels of VRI in vriD679 flies likely shorten circadian period through their impact on Clk expression.

Figure 2. Low-level rhythms in VRI expression are observed in vriD679 mutants
(A) Brains from control w1118 and vriD679 flies were collected at ZT20 and were immunostained with VRI (red) and PER (green) antisera. Merged VRI + PER images are shown in yellow. The following brain pacemaker neuron groups were detected: dorsal neuron 1 (DN1), dorsal neuron 2 (DN2), dorsal neuron 3 (DN3), dorsal lateral neuron (LN), and small ventrolateral neuron (lLN). Scale bars, 50 μm.
(B) Proteins from the heads of w1118, vriD679, and Clkout flies collected during LD at zeitgeber time (ZT) 2, 8, 14, and 20 were used to prepare western blots. Westerns probed with VRI antisera showed rhythmically expressed high-and low-mobility VRI bands (arrowheads). β-Actin (ACT) was used as a loading control. Molecular weight marker positions to the right are 95 kDa (blue) and 72 kDa (orange) on the VRI blot and 37 kDa (light blue) on the ACT blot.
(C) Heads from w1118 and vriD679 mutant flies collected at ZT20 were cryosectioned and immunostained with VRI antisera. Images of compound eyes are shown, with VRI (red) staining detected in photoreceptor nuclei (white arrows). Scale bars, 20 μm.
(D) Malpighian tubules from w1118 and vriD679 mutant flies collected at ZT20 were immunostained with VRI (red) and PER (green) antisera. Merged VRI + PER images are shown as yellow. Scale bars, 50 μm.
(E) Proteins from Malpighian tubule tissue samples (left) and compound eyes (right) of w1118 and vriD679 flies collected during LD at ZT2 and ZT14 were used to prepare western blots. Westerns probed with VRI antisera showed rhythmically expressed high-and low-mobility VRI bands (arrowheads). β-Actin (ACT) was used as a loading control. Molecular weight marker positions to the right are 95 kDa (blue) and 72 kDa (orange) on the VRI blots and 37 kDa (light blue) on the ACT blots.
As vri-ADF expression is virtually eliminated in vriΔ679 adults, vri-E expression presumably accounts for the cycling VRI levels and rhythmic behavior in vriΔ679 flies. Indeed, RNA-seq analysis shows that vri-E expression, as reflected by exon 1a transcription, is even higher in vriΔ679 flies than w1118 controls, but continues to have higher expression levels at ZT14 than ZT2 like control w1118 flies (Figure 1C). These results suggest that vri-E mRNA is upregulated in vriΔ679 flies and cycles in abundance. To confirm vri-E mRNA upregulation and cycling in vriΔ679 flies, RT-qPCR was carried out on head RNA from control w1118 and in vriΔ679 flies collected every 6 h during LD. Total vri mRNA, vri-ADF mRNA, and vri-E mRNA in wild-type flies and vri-E mRNA in vriΔ679 flies all showed significant (p <= 0.006) rhythmicity by one-way ANOVA (see Transparent Methods). When the levels of vri-E in w1118 and vriΔ679 flies are plotted relative to total vri mRNA in w1118 flies, vri-E mRNA levels are >10-fold higher in vriΔ679 than w1118 flies at ZT15, when vri-E peaks in vriΔ679 flies, and are almost 3-fold higher at ZT9, when vri-E peaks in w1118 flies (Figure 3A). Thus, the loss of exon 1c results in higher levels of vri-E mRNA with a delayed ZT15 peak, which is the same peak phase as the major vri-ADF transcript in w1118 flies (Figures 3B and 1C). This shift in vri-E expression in vriΔ679 flies suggests that the level and phase of rhythmic vri expression is in part compensated in the absence of exon 1c. Importantly, given that vriΔ679 flies only express vri-E mRNA, vri-E is sufficient for both fly development and circadian behavioral rhythms.

E-boxes located upstream of vri exon 1c are essential for development

CLK-CYC binding to E-box regulatory elements having the canonical sequence CACGTG drives rhythmic transcription (Darlington et al., 1998; Yu and Hardin, 2006). Five canonical E-boxes are situated within 2.5 kb upstream of vri exon 1c (Figure 4). These E-boxes are centered on the strongest CLK-CYC binding site in the Drosophila genome (Abruzzi et al., 2011) and likely account for high levels of rhythmic vri transcription (Blau and Young, 1999). In addition, two canonical E-boxes <1.5 kb upstream of exon 1a are located within another strong CLK-CYC binding site (Abruzzi et al., 2011) and likely contribute to rhythmic expression of vri-E mRNA.

Previously we demonstrated that a ~70-kb BAC clone (CH321-28 × 1021), which covers the vri genomic region (Figure 1A), rescues molecular and behavioral rhythms in vriΔ null mutant flies (Gunawardhana and Hardin, 2017). To test whether E-boxes in the exon 1c promoter are necessary for high levels of rhythmic vri
transcription, four of the five E-boxes upstream of exon 1c were deleted in this BAC clone via site-directed mutagenesis (Figure 4). The resulting vri\(^{70kb}\)\(^{DE}/C0\)Box mutant was integrated into three independent docking sites (VK5, VK13, and VK20) on chromosome 3 using PhiC32-mediated recombination (Venken et al., 2006).

Surprisingly, none of the vri\(^{70kb}\)\(^{DE}/C0\)Box transgenic lines rescued the developmental lethality in the vri\(^5\) null mutant, which suggests that these E-boxes are essential for development. As we cannot distinguish between vri mRNA and protein produced by endogenous vri and the vri\(^{70kb}\)\(^{DE}/C0\)Box transgene, we could not determine whether the vri\(^{70kb}\)\(^{DE}/C0\)Box transgene was expressed in the presence of wild-type vri. Thus, even though vri\(-E\) transcripts are sufficient for development in vri\(^{D679}\) flies, the regulatory elements that remain in the vri\(^{70kb}\)\(^{DE}/C0\)Box transgene are incapable of driving sufficient vri expression during development to rescue lethality of the vri\(^5\) mutant.

**vri-ADF mRNA primarily produces the short VRI isoform**

To restore vri-ADF mRNA and long VRI protein expression in vri\(^{D679}\) flies, a ~24-kb BAC clone (CH322-102O15) was used to generate the vri\(^{24}\) transgene (Figure 5A). This transgene was unable to rescue developmental lethality of the vri\(^5\) null mutant, suggesting that vri-ADF mRNA is not sufficient for fly development. However, vri\(^{24}\) rescued wild-type levels of VRI expression in vri\(^{D679}\) brain pacemaker neurons and fly heads, where VRI in fly heads cycled with low levels at ZT2 and high levels at ZT14 (Figures 5B and 5C). Moreover, the vri\(^{24}\) transgene partially rescues the short period (~22.9 h) activity rhythms of vri\(^{D679}\) flies by lengthening period to ~23.3 h (Table 1). Thus, vri-ADF mRNA driven by vri\(^{24}\) supplements the low VRI levels derived from vri-E mRNA, thereby lengthening the short circadian period of vri\(^{D679}\) flies.

Although vri-E is the only vri transcript present in vri\(^{D679}\) flies (Figure 1C), VRI from vri\(^{D679}\) flies co-migrates with the major VRI band from control w\(^{1118}\) flies (Figure 2), which is surprising because vri-E mRNA encodes short VRI and the much more abundant vri-ADF mRNA encodes long VRI (Figures 1C and 3B). To unequivocally identify the VRI isoform produced by vri-ADF mRNA, a 5.3-kDa VS-3xHA epitope tag was added to the vri\(^{24}\) transgene at the N terminus of long VRI. Western blots containing proteins from the heads of vri\(^{24}\)HA flies were collected at ZT14 and probed with VRI and hemagglutinin (HA) antibodies. Surprisingly, no VRI band could be detected from vri\(^{24}\)HA flies with HA antibody (Figure S3). If the weak ~90-kDa VRI band seen in w\(^{1118}\) flies corresponds to long VRI (Figures 2B and 2E), then the VS-3xHA-tagged long VRI band may not be expressed at high enough levels to detect. To detect VRI from vri\(^{24}\)HA flies with greater sensitivity, we immunoprecipitated 10-fold more head extract from flies collected at ZT14 with VRI antibody. This immunoprecipitate, along with head extracts from vri\(^{24}\)HA and

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**Figure 4. Diagram of vri\(^{70kb}\)\(^{DE}/Box\) transgene**

A ~70-kb BAC clone (CH321-28 \(\times 10^3\)), which is ~42 kb upstream and ~3 kb downstream of the vri gene, was used to generate the vri\(^{70kb}\)\(^{DE}/Box\) transgene. Multiple E-box regulatory sequences are found nearby vri exons 1a and 1c (expanded diagram with red dashed lines) which are found within CLK binding peaks (aqua boxes) or outside CLK binding peaks (purple boxes). Four of the five E-boxes within 2.5 kb upstream of exon 1c were deleted (expanded diagram with brown dashed lines) to generate the vri\(^{70kb}\)\(^{DE}/Box\) mutant transgene containing one intact E-box.
Figure 5. vri24kb transgene rescues vriΔ679 mutant phenotypes
(A) Diagram depicting the ~24-kb BAC clone (CH322-102O15) that was used to generate the vri24 transgene (green rectangle), which produces a V5-3xHA epitope-tagged VRI protein. This BAC clone is ~10.5 kb upstream of the vri-ADF mRNA transcription start site and ~7.7 kb downstream of vri-ADF mRNAs.
(B) Brains from wild-type (w1118), vriΔ679 mutants, and vriΔ679; vri24 flies were collected at ZT19 and were immunostained with VRI antisera. The following brain pacemaker neuron groups were detected: dorsal neuron 1 (DN1), dorsal neuron 2 (DN2), dorsal neuron 3 (DN3), dorsal lateral neuron (LNl), large ventrolateral neuron (LNV), and small ventrolateral neuron (sLNv). Scale bar, 50 μm.
Positions (Figure 6C). These data suggest that the short VRI start codon possesses a strong Kozak sequence, while the long VRI start codon had uracil at three of four of these positions, whereas the short VRI start codon had no uracils in these positions.

Generally, translation initiation is most efficient when the start codon resides within a strong Kozak sequence, which is optimally GCCGCC[A|G]CCAGG in Drosophila (Feng et al., 1991). The short VRI start codon has an 8/13 identity with the optimal Kozak sequence for the long VRI and short VRI start codons and found that the short VRI start codon had a 5/13 identity (Figure 6C). Moreover, a uracil at the +2 position relative to the start codon weakens translation initiation (Feng et al., 1991). The long VRI start codon has an 8/13 identity with the optimal Kozak sequence versus the long VRI start codon, which had a 5/13 identity (Figure 6C). Moreover, a uracil at the −3, −2, −1, and +4 positions with respect to the translation initiation site weakens translation initiation (Feng et al., 1991). The long VRI start codon had uracil at three of four of these positions, whereas the short VRI start codon had no uracils in these positions (Figure 6C). These data suggest that the short VRI start codon possesses a strong Kozak sequence that more efficiently initiates translation.

**DISCUSSION**

Previous work demonstrated that vri regulates developmental and clock-controlled processes in Drosophila (Blau and Young, 1999; George and Terracol, 1997; Gunawardhana and Hardin, 2017). The vri gene produces multiple transcripts and two proteins during development and in adults (Blau and Young, 1999; George and Terracol, 1997; Glossop et al., 2003), but the extent to which each transcript and protein isoform contributes to developmental and clock functions is not known. The vri6679 mutant generated here specifically eliminates vri-ADF mRNA expression (Figure 1C), which shortens the period of activity rhythms (Table 1). As VRI represses Clk transcription (Cyran et al., 2003; Glossop et al., 2003), the low levels of vri expression in vri6679 flies likely shorten period by increasing Clk levels (Figure S2), consistent with previous results (Zhao et al., 2003). As vri6679 flies are homozygous viable, the only remaining vri mRNA expressed, vri-E, is sufficient to rescue vri developmental and clock function (Figures 1C, 2, and S1).

Although vri-E mRNA expression is much lower than vri-ADF in wild-type flies (Figures 1C and 3D), vri-E is rhythmically expressed (Figure 3). There is a strong CLK-CYC binding site containing two canonical E-boxes
~2.5 kb upstream of exon 1a (Abruzzi et al., 2011), which may account for vri-E mRNA cycling. The five canonical E-boxes present within 2.5 kb upstream of exon 1c coincide with the strongest CLK-CYC binding site in the Drosophila genome (Abruzzi et al., 2011) and presumably account for high-amplitude rhythms in vri-E mRNA levels. When we tested whether the materials were probed with VRI antisera, Long VRI, L-VRI; Long VRI-FLAG-HA, L-VRI*; Short VRI, S-VRI; Short VRI-FLAG-HA, S-VRI*. Molecular weight marker positions to the right are 130 kDa (green), 95 kDa (blue), and 72 kDa (orange) on the VRI blot and 37 kDa (light blue) on the ACT blot. Loading control and abbreviations are as described in (A). Molecular weight markers to the right are 130 kDa (green), 95 kDa (blue), and 72 kDa (orange) on the VRI blot and 37 kDa (light blue) on the ACT blot. (C) Sequence comparison of Kozak sequences associated with the long VRI translation initiation codon (first VRI start codon) and the short VRI translation initiation codon (second VRI start codon) to the optimal Kozak sequence for Drosophila melanogaster. An asterisk (*) indicates a match, a dash (−) indicates a difference, and the number of matches of the 13 residues is noted at end of the row. The numbering on top represents the location of each base with respect to the first base of the translation initiation codon (+1).

The long VRI and short VRI isoforms are encoded by the vri-ADF and vri-E transcripts, respectively (Gra-mates et al., 2017), but identifying short and long VRI bands on western blots has been a challenge because both VRI bands migrate much more slowly than the predicted short and long VRI molecular weights and the major VRI band in adults is the faster migrating band even though long VRI should be produced by abundant vri-ADF mRNA (Cyran et al., 2003; Glossop et al., 2003; Gunawardhana and Hardin, 2017). The vri24 transgene, which rescues vriD679 period shortening and restores high levels of rhythmic VRI (Figure 5), was modified to epitope tag long VRI with V5-3xHA at the N terminus. Based on gel migration, the HA-
tagged long VRI band corresponds to the slow-migrating VRI band that runs at >90 kDa, and we infer that the faster migrating band that runs at ~85 kDa is short VRI.

Both long and short VRI are produced by cDNAs or genomic DNA that encodes only long VRI (Figure 6). As short VRI is produced using an in-frame AUG codon 119 amino acids downstream of the first AUG that initiates long VRI, we reasoned that the ribosome may not interact with the long VRI AUG as efficiently as the short VRI AUG. Indeed, the consensus Kozak sequence at the long VRI AUG is much weaker than that at the short VRI AUG. Although this difference in Kozak sequence strength may account for the relative expression levels of long and short VRI, other mechanisms could account for this difference. For instance, IRES sequences may direct the ribosome primarily to the short VRI AUG. In fact, the mammalian VRI ortholog, NFIL3/E4BP4, contains an IRES in its 5’ UTR that directs cap-independent translation (Kim et al., 2017). Whether such a mechanism functions to promote short VRI translation awaits further analysis. In any case, our analysis shows that short VRI is the predominant VRI isoform during development and in adults, which implies that short VRI mediates vri developmental and circadian function.

Limitations of the study
Although we show that vri-E mRNA is sufficient for activity rhythms, vri-E mRNA and short VRI are both rhythmically expressed at lower levels, thus providing no new insight into whether VRI cycling is dependent on vri mRNA cycling or whether rhythmic behavior is dependent on VRI rhythms. E-boxes upstream of the vri-ADF transcription start site are necessary for viability, but these results do not reveal the developmental processes, tissue(s), or transcription factors involved. Our results imply that short VRI is expressed from long VRI transcripts due to a weak Kozak consensus, but additional experiments such as swapping Kozak consensus sequences for long and short VRI are needed to confirm this conclusion.

Resource availability
Lead contact
Further information and requests for resources and reagents not made available through national stock centers should be directed to and will be fulfilled by the lead contact, Paul Hardin (phardin@bio.tamu.edu).

Materials availability
All unique/stable reagents generated in this study will be made available on request by the Lead Contact.

Data and code availability
The RNA-seq datasets generated during this study are available at the Gene Expression Omnibus Repository and can be accessed under the accession number GSE154785 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154785).

METHODS
All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2020.101893.

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Conceptualization, P.E.H. and K.L.G.; Methodology, K.L.G. and G.B.S.R.; Investigation, K.L.G., C.C., and G.B.S.R.; Formal Analysis, K.L.G. and G.B.S.R.; Writing – Original Draft, K.L.G.; Writing – Review & Editing, P.E.H., K.L.G., C.C., and G.B.S.R.; Funding Acquisition and Supervision, P.E.H.
DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES

Abrouzzi, K.C., Rodriguez, J., Menet, J.S., Desrochers, J., Zadina, A., Luo, W., Tkachev, S., and Rosbash, M. (2011). Drosophila CLOCK target gene characterization: implications for circadian tissue-specific gene expression. Genes Dev. 25, 2374–2386.

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

Blau, J., and Young, M.W. (1999). Cycling vrille expression is required for a functional Drosophila clock. Cell 99, 661–671.

Cyan, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.C., Glossop, N.R., Hardin, P.E., Young, M.W., Storti, R.V., and Blau, J. (2003). vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. Cell 112, 329–341.

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steves, T.D., Weitz, C.J., Takahashi, J.S., and Kay, S.A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. Science 280, 1599–1603.

Dow, J.A., and Romero, M.F. (2010). Drosophila provides rapid modeling of renal development, function, and disease. Am. J. Physiol. Ren. Physiol 299, F1237–F1244.

Feng, Y., Gunter, L.E., Organ, E.L., and Cavener, D.R. (1991). Translation initiation in Drosophila melanogaster is reduced by mutations upstream of the AUG initiator codon. Mol. Cell Biol. 11, 2149–2153.

Gallant, P., Shiio, Y., Cheng, P.F., Parkhurst, S.M., and Eisenman, R.N. (1996). Mxc and max homologs in Drosophila. Science 274, 1523–1527.

George, H., and Terracol, R. (1997). The vrille gene of Drosophila is a maternal enhancer of decapentaplegic and encodes a new member of the bZIP family of transcription factors. Genetics 146, 1345–1363.

Glossop, N.R., Hou, J.H., Zheng, H., Ng, F.S., Dudek, S.M., and Hardin, P.E. (2003). VRILLE feeds back to control circadian transcription of Clock in the Drosophila circadian oscillator. Neuron 37, 249–261.

Gramates, L.S., Marygold, S.J., Santos, G.D., Urbano, J.M., Antonazzo, G., Matthews, B.B., Rey, A.J., Tabone, C.J., Crosby, M.A., Emmert, D.B., et al. (2017). FlyBase at 25: looking to the future. Nucleic Acids Res. 45, D663–D671.

Gunawardhana, K.L., and Hardin, P.E. (2017). VRILLE controls PDF neuropeptide accumulation in small ventrolateral neurons to drive rhythmic behavior in Drosophila. Curr. Biol. 27, 3442–3453.e4.

Hardin, P.E. (2011). Molecular genetic analysis of circadian timekeeping in Drosophila. Adv. Genet. 74, 141–173.

Kim, H.J., Lee, H.R., Seo, J.Y., Ryu, H.G., Lee, K.H., Kim, D.Y., and Kim, K.T. (2017). Heterogeneous nuclear ribonucleoprotein A1 regulates rhythmic synthesis of mouse Nfil3 protein via IRES-mediated translation. Sci. Rep. 7, 42982.

Lim, C., Chung, B.Y., Pitman, J.L., Mcgill, J.J., Pradhan, S., Lee, J., Keegan, K.P., Choe, J., and Allada, R. (2007). Clockwork orange encodes a transcriptional repressor important for circadian-clock amplitude in Drosophila. Curr. Biol. 17, 1082–1089.

Liu, T., Mahesh, G., Yu, W., and Hardin, P.E. (2017). CLOCK stabilizes CYCLE to initiate clock function in Drosophila. Proc. Natl. Acad. Sci. USA 114, 10972–10977.

Nagoshi, E., Sugino, K., Kula, E., Okazaki, E., Tachibana, T., Nelson, S., and Rosbash, M. (2010). Dissecting differential gene expression within the circadian neuronal circuit of Drosophila. Nat. Neurosci. 13, 60–68.

Szuplewski, S., Kottler, B., and Terracol, R. (2003). The Drosophila bZIP transcription factor Vrille is involved in hair and cell growth. Development 130, 3651–3662.

Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N.A., Singh, C.M., Buchholz, R., Demsky, M., Favcett, R., Francis-Lang, H.L., et al. (2004). A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat. Genet. 36, 283–287.

Thurmond, J., Goodman, J.L., Strelets, V.B., Attrill, H., Gramates, L.S., Marygold, S.J., Matthews, B.B., Millburn, G., Antonazzo, G., Trovisco, V., et al. (2019). FlyBase 2.0: the next generation. Nucleic Acids Res. 47, D759–D765.

Tower, J., Karpen, G.H., Craig, N., and Spradling, A.C. (1993). Preferential transposition of Drosophila P elements to nearby chromosomal sites. Genetics 133, 347–359.

Venken, K.J., He, Y., Hoskins, R.A., and Bellen, H.J. (2006). Flacman: a BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. Science 314, 1747–1751.

Yu, W., and Hardin, P.E. (2006). Circadian oscillators of Drosophila and mammals. J. Cell Sci. 119, 4795–4799.

Zhao, J., Kilman, V.L., Keegan, K.P., Peng, Y., Emery, P., Rosbash, M., and Allada, R. (2003). Drosophila clock can generate ectopic circadian clocks. Cell 113, 755–766.
Supplemental Information

Crosstalk between *vrille* transcripts, proteins, and regulatory elements controlling circadian rhythms and development in *Drosophila*

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Figure S1. PCR amplification products from $w^{1118}$ and $vri^{Δ679}$ genomic DNA. Related to Figure 1. Screen Forward and Screen Reverse primers produced an 1120bp band from $w^{1118}$ DNA and a 411bp band from $vri^{Δ679}$ DNA, whereas Deletion Forward and Deletion Reverse primers (Del primers) produced a 267bp band from $w^{1118}$ DNA, but no band from $vri^{Δ679}$ DNA. The $Δ679$ Forward primer contains the first 24bp of $vri^{Δ679}$ sequence from the $vri$ transcription start consisting of the 4 remaining bases of the 5’ UTR, an 8bp sequence insert (italics), and 12 bps from intron 1 (see panel A), and was used with Screen Reverse ($Δ679$ primers) to amplify a 275bp band from $vri^{Δ679}$ DNA, but no band from $w^{1118}$ DNA.
Figure S2. Clock gene expression in vriΔ679 flies. Related to Figure 2. RNA-seq analysis of Clk, cyc, per and tim expression in heads from w1118 (white bars) and vriΔ679 (gray bars) flies collected at ZT2 and ZT14 during LD cycles. Each graph shows mRNA expression levels in Fragments Per Kilobase Million (FPKM). Data from the two biological replicates are shown as black circles and white squares, and the average is shown by the bar height.
Figure S3. VRI-HA is not detected on western blots by anti-HA antibody. Related to Figure 5. \( w^{118} \), \( w; cwo-HA \); + (cwo-HA), and \( w; vri24HA \); + (vri24HA) flies were entrained for 3 days in LD, collected at ZT16 and protein extracts were prepared from heads. Protein extracts were used to prepare western blots probed with anti-HA and anti-VRI antibodies. Short (top) and long (bottom) exposures of the blot are shown. Arrows point to the CWO-HA, VRI-HA and a non-specific (ns) HA cross-reacting protein bands. Molecular weight markers (M) are 130kDa (blue, top), 95kDa (blue, second), 72kDa (red, third) and 56kDa (blue, bottom).
Table S1. Related to Figure 1. RNA-seq analysis of transcript reads in vri exons from w₁₁₁₈ control and vri^{D679} mutant flies.

| vri exon | w₁₁₁₈ ZT2_R1 | w₁₁₁₈ ZT2_R2 | w₁₁₁₈ ZT14_R1 | w₁₁₁₈ ZT14_R2 | vri^{D679} ZT2_R1 | vri^{D679} ZT2_R2 | vri^{D679} ZT14_R1 | vri^{D679} ZT14_R2 |
|----------|---------------|---------------|---------------|---------------|------------------|------------------|------------------|------------------|
| exon1a   | 11            | 9             | 38            | 26            | 53               | 72               | 74               | 416              |
| exon1c   | 75            | 78            | 829           | 477           | 1                | 0                | 0                | 0                |
| exon2    | 152           | 164           | 1724          | 1076          | 73               | 70               | 134              | 481              |
| exon3s   | 1815          | 1633          | 16210         | 11588         | 710              | 669              | 2811             | 7003             |

Biological replicates were collected as described above. Values for mRNA expression for vri exon1a, exon1c, exon2 and exon3s (the shortest vri exon3 variant) are presented as read counts.
Transparent Methods

Fly strains

The following fly strains were used in this study: \( w^{1118} \), st\([1]\) Blm[D2] Sb[1] \( P[ry+t7.2]=\Delta 2\text{-}3\) 99B \( (P[ry+\Delta 2\text{-}3]) \) (BDSC), \( tim\)-Gal4 (Emery et al., 1998)(Emery et al., 1998), \( tim(UAS)Gal4 \) (Blau and Young, 1999), \( gf^{60} \) (Moses et al., 1989), \( vri^{901873} \) (BDSC), cwo-HA (Zhou, 2017) and UAS-vri (Blau and Young, 1999). Flies were reared on standard cornmeal/agar medium supplemented with yeast and kept in 12 h light/12 h dark (LD) cycles at 25°C

\( vri^{901873} \) P-element mobilization and \( vri^{\Delta 679} \) mutant generation

The \( vri^{901873} \) insertional mutant line was initially backcrossed seven times to \( w^{1118} \) flies. Then \( vri^{901873} \) flies were crossed to \( P[ry+\Delta 2\text{-}3] \) flies containing the P-element transposase. Progeny of the cross that had white eyes were crossed to \( w^{1118} \), \( CyO/sco \) flies. Once the chromosome that contained the P-element was homozygous, the genomic DNA was extracted from 5-6 flies and screened for a deletion with Screen Forward (5' CGGCAATGTGATCGCTTGCAAC 3') and Screen Reverse (5' CATGCACGTACACTTAAGCGCTC 3') primers (Figure S1). The largest deletion strain, \( vri^{\Delta 679} \), was characterized via PCR using the Deletion Forward (5' GATCGGGTTCACAACCGC 3') and Deletion Reverse (5' GTTCACTTTCTGTTCCAGCGTG 3') primer pair and the \( \Delta 679 \) Forward (5' GCGTCATGATGATGCTCTTTTG 3') and Screen Reverse primer pair (Figure S1), sequenced, and backcrossed seven times to \( w^{1118} \) flies to generate an isogenized strain. The isogenized \( vri^{\Delta 679} \) mutant was used to carry out all subsequent experiments.

\( vri^{70kb\Delta E\text{-}Box} \) transgene construction and transgenic fly generation

BAC clone CH321-28E21 (BacPac Resources) was used to generate the \( vri^{70kb\Delta E\text{-}Box} \) transgene. Initially the E-box-containing region of this BAC clone was sub-cloned into the TA vector (Invitrogen) using the following primers: \( vri \) E-box region Forward, 5'

\[
\text{GTTCTTCTCTCGAAGGACCAC}3' \]

; \( vri \) E-box region Reverse, 5'

\[
\text{GCTGGGGAGTTGGGAAAACG}3'.
\]
The resulting plasmid, vriEbox-TA, was used to mutate the E-boxes using the QuickChange II XL site directed mutagenesis kit (Stratagene, La Jolla, CA). For each E-Box, the following complementary forward and reverse primers were used to generate the indicated deletion: *vri* E-box 1 mutation Forward, 5' GCTGGTGCCCTCCACGAACCCTCGTCCGC 3'; *vri* E-box 1 mutation Reverse, 5' GGCTAGTGCAACGTCCCCAACAGCC 3'; *vri* E-box 2&3 mutation Forward, 5' AATCGGGCGACTGATATCGCGCAG 3'; *vri* E-box 2&3 mutation Reverse, 5' TCCGGACCAATCGTAGTCGGATCCG 3'; *vri* E-box 4 mutation Forward, 5' CCCATCTGTCGATTGATTTCACTGCGTGCAG 3'; *vri* E-box 4 mutation Reverse, 5' CGCTTTGAAACTTTGAGTTTTAACAAGGGG 3'. E-box deletions in the resulting plasmid, vriΔEbox-TA, were confirmed by sequencing. The E-box-containing region of the BAC clone was replaced by a selectable galK gene using bacterial homologous recombination (Warming et al., 2005). The *galK* gene was then replaced with an amplicon containing the E-box mutant region from vriΔEbox-TA that was produced using the *vri* E-box GalK homology Forward (5' TTGGCTTGCAGCGACTCAAGTCGACAACATGTGAG 3') and *vri* E-box GalK homology Reverse (5' ATACGTACATGTATTTTGGGAAGCTTTCAACTCGTGAATT 3') primers (Venken et al., 2008). We used primers containing ~50 bp homology arms when carrying out the homologous recombination (see Table 2). After selection of recombinants and sequence verification, the resulting vri70kbΔE-Box transgene was inserted at VK5, VK13 and VK20 docking sites via PhiC32-mediated recombination (Venken et al., 2006).

**vri24 and vri24HA transgenes construction and transgenic fly generation**

BAC clone CH322-102O15 (BacPac Resources) was used to generate the vri24kb and vri24HA transgenes. The vri24kb transgene is a 24kb BAC clone containing partial *vri* gene sequence.
(chr2L5295134 - chr2L5319513) without any modifications. This transgene was inserted at the VK33 docking site via PhiC32-mediated recombination (Venken et al., 2006).

The vri24HA transgene has an N-terminal V5 and 3xHA epitope introduced to the vri24kb transgene using bacterial recombination (Venken et al., 2008). The V5-3xHA epitopes were inserted immediately downstream of the initiation codon of vri-ADF mRNA using a V5-3xHA recombination cassette comprised of the V5-3xHA epitope sequence, a Kanamycin (Kan) gene flanked by loxP sites, and two homology arms generated via PCR using the ‘V5-3xHA-vri Homology Forward’ and ‘V5-3xHA-vri Homology Reverse’ primers (Table 2). After selection for recombinants and sequence verification, the Kan cassette was removed using CRE recombinase as described (Venken et al., 2008). After sequence verification, the resulting vri24HA transgene was inserted at the VK20 docking site via PhiC32-mediated recombination (Venken et al., 2006).

**RNA-seq library preparation**

w1118 control and vriΔ679 mutant flies were entrained for three days in LD at 25°C. Flies were collected at ZT2 and ZT14, frozen on dry ice and heads were isolated as described (Oliver and Phillips, 1970). Total RNA from heads was extracted using TRIzol, treated with TURBO™ DNase (Thermo Fisher Scientific), precipitated and then purified with Lithium Chloride (Thermo Fisher Scientific) following manufacturer’s instructions. A total of 1ug of RNA was used to isolate mRNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). RNA library construction was conducted using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer’s instructions. Libraries were sent to Texas A&M AgriLife Genomics and Bioinformatics Facility where they were mixed and multiplexed at the same equimolar concentrations and sequenced on a Hi-seq 2500 (Illumina) using 75bp single-end reads. The sequenced reads were mapped to the *Drosophila melanogaster* genome (dm6 – UCSC) with STAR aligner version 2.6.1d (Dobin et al., 2013).
Procedures to quantify and estimate differential expression were done as previously described (Pertea et al., 2016). Only genes that have an adjusted p-value ≤ 0.05 and fold change ≥ 1.5 (high expression) or ≤ 0.5 (low expression) were considered as differentially expressed genes in \( vrr^{A679} \) flies.

**Drosophila activity monitoring and behavior analysis**

All fly strains used in behavior experiments were backcrossed seven times to \( w^{1118} \) flies to minimize effects due to differences in genetic background. Locomotor activity was monitored using the *Drosophila* Activity Monitor (DAM) system (Trikinetics). One to three-day old male flies were placed in monitors, and activity was recorded for 3 days in 12:12 light-dark (LD) cycles and for 7 days in constant darkness (DD) at 25°C. Analyses of period, power and rhythm strength during constant darkness (DD) was carried out using ClockLab (Actimetrics) software as previously described (Agrawal and Hardin, 2016).

**Immunostaining**

Fly tissues were processed for immunostaining as previously described (Houl et al., 2008), with minor modifications. Larval brains were dissected at the L3 stage and adult tissues were dissected 3-5 days after eclosion. Dissected tissues were fixed with 3.7% formaldehyde, washed and blocked in PAXD buffer (1X PBS, 5% BSA, 0.03% sodium deoxycholate, 0.03% tritonX100) with 5% donkey or goat serum for 1 hour, and incubated with primary and secondary antibodies diluted in PAXD buffer. The following primary antibodies were used: guinea pig anti-VRI GP3 1:25,000, rabbit anti-HA ab9110 (Abcam) 1:200, pre-absorbed rabbit anti-PER (a gift from Michael Rosbash Laboratory, Brandeis University). The following secondary antibodies were used at a 1:200 dilution: goat anti-rabbit Alexa 647 (Molecular Probes), goat anti-guinea pig Cy-3 (Jackson ImmunoResearch Laboratories, Inc.) as previously described (Liu et al., 2017).
**Cryosectioning**

Flies with a wild-type clock \((w^{118})\) were sectioned into 10\(\mu\)M thickness sections and collected on Superfrost plus microscopic slides (Fisher Scientific). All sections were fixed with 3.7% formaldehyde for 15 minutes. Subsequent immunostaining steps were identical to those described for whole mount immunostaining.

**Confocal microscopy and image analysis**

Confocal imaging was carried out as described (Agrawal et al., 2017), with minor modifications. Fly tissues were imaged using an Olympus FV1000 confocal microscope 20x /0.8 NA or 100x /1.4 NA oil immersion objective lenses. Serial optical scans were obtained at 3 \(\mu\)M intervals (with 20x objective lens) Original Olympus images were saved as 12-bit oib format. Preliminary image processing was carried out using either FV1000 confocal software or Adobe Photoshop.

**Western blotting**

Western blot analyses were conducted as described (Zhou et al., 2016), with minor modifications. MTs were collected for protein extraction intact along with some contaminating intestinal tissue. Compound eyes were dissected as described (Zeng et al., 1994). After protein extraction, equal amounts of RIPA S extract were run, transferred, and probed with guinea pig anti-VRI GP3, 1:5,000, rabbit anti-HA, 1:5,000 and mouse anti-beta-actin (Abcam), 1:20,000. Horseradish peroxidase-conjugated secondary antibodies (Sigma) against guinea pig, rabbit and mouse were diluted 1:5,000. Immunoblots were visualized using ECL plus (GE) reagent.

**RNA extraction, quantitative RT-PCR and data analysis**

RNA extraction and quantitative RT-PCR was performed as described (Zhou et al., 2016), with minor modifications. Flies were entrained in 12:12 light-dark (LD) for at least 3 days, collected at
the indicated time points, and frozen. For RNA extracted from embryos, larvae and pupae all sub-stages were pooled together to represent all sub-stages. Total RNA was isolated and cDNA was synthesized using Superscript II (Invitrogen). The reverse transcription (RT) product was amplified with SsoFast qPCR Supermix (Bio-Rad) in a Bio-Rad CFX96 Real-Time PCR System using the following gene or transcript-specific primers: vri-ADF qPCR Forward, 5’ ACGCTGGAACAGAAAGTGA 3’; vri-E qPCR Forward, 5’ CATAACGACCAACGGCCG 3’; vri-ACDEF qPCR Reverse, 5’ GCTAGTTTCTGCTGCAGTTG 3’; vri qPCR Forward, 5’ ATGAACAACGTCCGGCTATC 3’; vri qPCR Reverse, 5’ CTGCGGACTTATGGATCCTC 3’; rp49 qPCR Forward, 5’ TACAGGCCCAAGATGGATATGCTCTC 3’; rp49 qPCR Reverse, 5’ GCACTCTGTTGTCGATACCC 3’.

For each sample, mRNA quantity was determined as described (Zhou et al., 2016). rp49 mRNA levels were used as a normalization control when determining the relative levels of mRNA. Student two-tailed t-tests with unequal variances were performed to determine whether differences in levels were statistically significant. To evaluate the rhythmicity of vri isoform a One-Way ANOVA was conducted followed by a Tukey test for multiple comparison using the GraphPad Prism Software version 5.3 (Prism, La Jolla, CA) using as criteria to be considered rhythmic a p-value ≤ 0.05 and a significant difference in more than 2 timepoints (p ≤ 0.05 based on the Tukey test comparing all timepoints).

S2 cell culture experiments

S2 cell maintenance was carried out as described with minor modifications (Mahesh et al., 2014). Cells were maintained in Schneider’s Drosophila medium (Invitrogen) containing 10% fetal bovine serum with (100 units/ml) penicillin and streptomycin (100 μg/ml) (Invitrogen). S2 cells at 60–80% confluence were transiently transfected with C-terminally epitope tagged vriA-Flag-HA (FMO13008) or vriC-Flag-HA (FMO07976) using Effectene Transfection Reagent (Qiagen) (6.4 μL of Enhancer, 10 μL of Transfection Reagent, 0.8 μg of total DNA), and 24 hour after cells were collected for protein extractions as described for western blotting.
Supplemental References

AGRAWAL, P. & HARDIN, P. E. 2016. The Drosophila Receptor Protein Tyrosine Phosphatase LAR Is Required for Development of Circadian Pacemaker Neuron Processes That Support Rhythmic Activity in Constant Darkness But Not during Light/Dark Cycles. J Neurosci, 36, 3860-70.

AGRAWAL, P., HOUL, J. H., GUNAWARDHANA, K. L., LIU, T., ZHOU, J., ZORAN, M. J. & HARDIN, P. E. 2017. Drosophila CRY Entrain Clocks in Body Tissues to Light and Maintains Passive Membrane Properties in a Non-clock Body Tissue Independent of Light. Curr Biol, 27, 2431-2441 e3.

BLAU, J. & YOUNG, M. W. 1999. Cycling vrille expression is required for a functional Drosophila clock. Cell, 99, 661-71.

DOBIN, A., DAVIS, C. A., SCHLESINGER, F., DRENKOW, J., ZALESKI, C., JHA, S., BATUT, P., CHAISSON, M. & GINGERAS, T. R. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics, 29, 15-21.

EMERY, P., SO, W. V., KANEKO, M., HALL, J. C. & ROSBASH, M. 1998. CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell, 95, 669-79.

HOUL, J. H., NG, F., TAYLOR, P. & HARDIN, P. E. 2008. CLOCK expression identifies developing circadian oscillator neurons in the brains of Drosophila embryos. BMC Neurosci, 9, 119.

LIU, T., MAHESH, G., YU, W. & HARDIN, P. E. 2017. CLOCK stabilizes CYCLE to initiate clock function in Drosophila. Proc Natl Acad Sci U S A, 114, 10972-10977.

MAHESH, G., JEONG, E., NG, F. S., LIU, Y., GUNAWARDHANA, K., HOUL, J. H., YILDIRIM, E., AMUNUGAMA, R., JONES, R., ALLEN, D. L., EDERY, I., KIM, E. Y. & HARDIN, P. E. 2014. Phosphorylation of the transcription activator CLOCK regulates progression through a approximately 24-h feedback loop to influence the circadian period in Drosophila. J Biol Chem, 289, 1747-51.

MOSES, K., ELLIS, M. C. & RUBIN, G. M. 1989. The glass gene encodes a zinc-finger protein required by Drosophila photoreceptor cells. Nature, 340, 531-6.

OLIVER, D. & PHILLIPS, J. P. 1970. Technical note. Drosophila Information Service, 45, 58.

PERTEA, M., KIM, D., PERTEA, G. M., LEEK, J. T. & SALZBERG, S. L. 2016. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc, 11, 1650-67.

VENKEN, K. J., HE, Y., HOSKINS, R. A. & BELLEN, H. J. 2006. P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. Science, 314, 1747-51.

VENKEN, K. J., KASPROWICZ, J., KUENEN, S., YAN, J., HASSAN, B. A. & VERSTREKEN, P. 2008. Recombineering-mediated tagging of Drosophila genomic constructs for in vivo localization and acute protein inactivation. Nucleic Acids Res, 36, e114.

WARMING, S., COSTANTINO, N., COURT, D. L., JENKINS, N. A. & COPELAND, N. G. 2005. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res, 33, e36.

ZENG, H., HARDIN, P. E. & ROSBASH, M. 1994. Constitutive overexpression of the Drosophila period protein inhibits period mRNA cycling. Embo J, 13, 3590-8.

ZHOU, J. 2017. Characterizing the function of CLOCKWORK ORANGE in the circadian feedback loops in Drosophila melanogaster. Biology PhD, Texas A&M University.

ZHOU, J., YU, W. & HARDIN, P. E. 2016. CLOCKWORK ORANGE Enhances PERIOD Mediated Rhythms in Transcriptional Repression by Antagonizing E-box Binding by CLOCK-CYCLE. PLoS Genet, 12, e1006430.