Figures and figure supplements

Dissection of central clock function in Drosophila through cell-specific CRISPR-mediated clock gene disruption

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Figure 1. Toolbox for cell-specific, CRISPR-mediated disruption of core circadian regulators. (A) Schematic of the transcriptional/translational negative feedback loop that drives rhythmic expression and activity of the four core circadian regulators. Figure 1 continued on next page.
circadian regulators: Period (Per), Timeless (Tim), Clock (Clik), and Cycle (Cyc). (B) Diagram of CRISPR-Cas9 mediated DNA damage and repair pathways. (C) Diagram of plasmid (pCFD6, adapted from Port and Bullock, 2016) used to generate UAS-sgRNA transgenic flies. D.m. = Drosophila melanogaster. O.s. = Oryza sativa, Asian rice. (D) Diagram showing sgRNA target sites for acp98AB (acp, gray), period (per, orange), and timeless (tim, blue), numbered in order of 5′–3′ position in the respective UAS-sgRNA construct. Arrows = exons; shaded rectangles = promoters and UTRs. *tim sgRNA one has a single base pair deletion in the Cas9-binding scaffold region (see Materials and methods). (E) Diagram of ~150 clock neurons organized into the following anatomical and functional clusters in the Drosophila brain: dorsal neurons (DN1, DN2, DN3), lateral posterior neurons (LPN), dorsal lateral neurons (LNd), and small and large ventral lateral neurons (s-LNv, 5th s-LNv, l-LNv).

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Figure 2. Cell-specific disruption of per or tim in circadian (Tim⁺) cells but not glial (Repo⁺) cells causes loss of behavioral rhythmicity. (A) Diagram of clock neurons targeted for CRISPR-mediated gene disruption using tim-Gal4. (B) Disruption of per or tim in all clock neurons caused complete loss of rhythmicity.

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behavioral rhythmicity. (C) Scatter plot showing the period of rhythmic flies with tim-Gal4-driven disruption of acp, per, or tim. (D) Actograms showing average activity in constant darkness of flies with tim-Gal4-driven disruption of acp, per, or tim. Activity data is double-plotted, with six days of activity displayed. Dark gray rectangles = subjective day, black rectangles = subjective night. (E) Relative mRNA levels, measured by qRT-PCR over a 24-hour period, of the core circadian genes timeless (left), period (middle), and clock (right) in heads of tim-Gal4 CRISPR flies. JTK analysis revealed that only acp-targeted flies display statistically significant rhythmic cycling indicative of circadian oscillation of all three genes. (F) repo-Gal4 targets most glia for CRISPR-mediated gene disruption. (G) repo-Gal4-driven, CRISPR-mediated gene disruption in glia had no effect on behavioral rhythmicity. (H) Scatter plot showing the period of rhythmic flies with repo-Gal4-driven disruption of acp, per, or tim. (I) Actograms show average activity of flies in constant darkness with repo-Gal4-driven disruption of acp, per, or tim in glia.

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Figure 2—figure supplement 1. Cell-specific disruption of per or tim in circadian (Tim⁺) cells but not glial (Repo⁻) cells causes loss of rhythm strength. χ² peak height values (rhythm strength) for (A) tim-Gal4, (B) repo-Gal4, targeting of acp (gray), per (orange), or tim (blue). Significance determined by one-way ANOVA followed by Tukey’s multiple comparisons test (for normally distributed samples; B) or Kruskal-Wallis nonparametric ANOVA (to account for non-normality of samples; A) followed by Dunn’s multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons. ****: p<0.0001; *: p<0.05; n.s.: not significant, p>0.05.

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**Figure 2—figure supplement 2.** Average actograms for tim-Gal4 and repo-Gal4 targeted flies under 12 hr:12 hr light:dark conditions. Average actograms for the first day in LD for (A) tim-Gal4 and (B) repo-Gal4 targeting of acp (gray), per (orange), or tim (blue). DOI: https://doi.org/10.7554/eLife.48308.005
Figure 2—figure supplement 3. Rhythmicity analysis of control flies. (A) Average actograms showing the activity in constant darkness of flies with single copies of the CRISPR tool transgenes used in this study. None of the genotypes should have CRISPR-mediated gene disruption. Activity data are double-plotted, with seven days of activity displayed. Dark gray rectangles = subjective day, black rectangles = subjective night. (B) Presence of tim-Gal4 or CRISPR transgenes does not affect behavioral rhythmicity. (C) Scatter plot showing the period of rhythmic control flies. (D) $\chi^2$ peak height values (rhythm strength).

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Figure 2—figure supplement 4. CRISPR-targeting of per or tim in Tim\(^{+}\) neurons leads to overall loss of Per and Tim protein. (A) Maximum intensity projections of tim-Gal4>acp\(^{CRISPR}\), tim-Gal4>per\(^{CRISPR}\), tim-Gal4>tim\(^{CRISPR}\), and per\(^{D1}\) null brains at ZT0 with immunohistochemistry for Period (green) and Timeless (magenta) with schematic of clock neuron clusters; scale bar = 50 \(\mu\)m. (B) Higher magnification inset from boxed region in A; scale bar = 10 \(\mu\)m (C) Schematic showing expected staining pattern based on Per and Tim stability and requirements for nuclear entry.

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Figure 2—figure supplement 5. CRISPR-targeting of per or tim in Repo⁺ glia leads to overall loss of Per protein in glia but not neurons. (A) 15 μm-thick (10 slices x 1.5 μm slice thickness) posterior maximum intensity projections of repo-Gal4>acpCRISPR, repo-Gal4>perCRISPR, and repo-Gal4>timCRISPR.

Figure 2—figure supplement 5 continued on next page
brains at ZT0 with immunohistochemistry for Repo (magenta) and Period (green); scale bar = 50 µm. Insets show high-magnification views of the same projections; scale bar = 5 µm. Arrowheads indicate Repo⁺ nuclei; asterisks indicate Repo⁻ (likely neuronal) nuclei. (B) Schematic of clock neuron clusters (left) with full-thickness maximum intensity projections of samples shown in (A) with immunohistochemistry for Period (grayscale); scale bar = 50 µm.
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Figure 3. Cell-specific disruption of per or tim in Mai179 neurons causes complete loss of behavioral rhythmicity and efficient loss of the targeted protein. (A) Diagram of the circadian neuron subset marked by Mai179-Gal4. (B) Disruption of per or tim in Mai179 neurons caused complete loss of...
behavioral rhythmicity. (C) Scatter plot showing the period of rhythmic flies with Mai179-Gal4-driven disruption of acp, per, or tim. (D) Average actograms showing the activity of flies in constant darkness with Mai179-Gal4-driven disruption of acp, per, or tim. Six days of activity are displayed, double-plotted. Dark gray rectangles = subjective day, black rectangles = subjective night. (E and F) Background-subtracted nuclear fluorescence intensity of Per (E) or Tim (F) at ZT0 in GFP<sup>+</sup> neurons, grouped by brain with mean ± SEM shown. Individual brains are shown in the same order in both E and F. (G and H) Mean nuclear fluorescence intensity of Per (G) or Tim (H) at ZT0 in GFP<sup>+</sup> neurons, averaged per brain (acp n = 18, per n = 16, tim n = 15). **: p<0.01; ****: p<0.0001; n.s.: not significant, p>0.05. Significance determined by Kruskal-Wallis nonparametric ANOVA (to account for non-normality of samples) followed by Dunn's multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons. DOI: https://doi.org/10.7554/eLife.48308.009
Figure 3—figure supplement 1. Cell-specific disruption of per or tim in Mai179° cells causes loss of rhythm strength. χ² peak height values (rhythm strength) for Mai179-Gal4 targeting of acp (gray), per (orange), or tim (blue). Significance determined by Kruskal-Wallis nonparametric ANOVA (to account for non-normality of samples) followed by Dunn’s multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons. ****: p<0.0001; n.s.: not significant, p>0.05.
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Figure 3—figure supplement 2. Period and Timeless immunohistochemistry in Mai179-Gal4-driven CRISPR flies. Maximum intensity projections of Mai179-Gal4-driven GFP+ dorsal lateral (LNd, top) and ventral lateral (LNv, bottom) neurons with immunohistochemistry for GFP (yellow), Period (green) and Timeless (magenta); scale bar = 10 μm. Arrowheads indicate GFP+ (CRISPR-targeted) cells. DOI: https://doi.org/10.7554/eLife.48308.012
Figure 3—figure supplement 3. Cell-specific disruption of per or tim in Mai179^+ neurons is most efficient in dorsal lateral (LNd) and small ventral lateral (s-LNv) neurons. Background-subtracted nuclear fluorescence intensity of Per (A, C, E) or Tim (B, D, F) at ZT0 in GFP^+ neurons, grouped by brain.
and separated by cell type (A, B: dorsal lateral neurons; C, D: small ventral lateral neurons; E, F: large ventral lateral neurons) with mean ± SEM shown. Individual brains are shown in the same order in all graphs (acp n = 18, per n = 16, tim n = 15 brains).

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**Figure 3—figure supplement 4.** Average actograms for Mai179-Gal4 targeted flies under 12 hr:12 hr light:dark conditions. Average actograms for the first day in LD for Mai179-Gal4 targeting of acp (gray), per (orange), or tim (blue).

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Figure 4. Cell-specific disruption of per or tim in Pdf+ neurons causes incomplete loss of behavioral rhythmicity and loss of morning anticipation in constant darkness. (A) Diagram showing Pdf+ circadian neurons. (B) CRISPR-mediated disruption of per or tim in Pdf+ neurons using the Pdf-Gal4 driver caused incomplete loss of behavioral rhythmicity. (C) Scatter plot showing the period of rhythmic flies with Pdf-Gal4-driven disruption of acp, per, or tim. (D) Actograms showing average activity of flies in constant darkness with Pdf-Gal4-driven disruption of acp, per, or tim. Nine days of activity are Figure 4 continued on next page.
Figure 4 continued
displayed, double-plotted. Dark gray rectangles = subjective day, black rectangles = subjective night. (E) Average hourly activity counts during the second day of complete darkness (DD Day 2; gray bars = CT 0–11, black bars = CT 12–23). Mean number of beam breaks per hour is shown ± SEM. (F) Morning Anticipation Index (MAI) was calculated by dividing the average hourly activity for CT 21–23 by the average hourly activity for CT 18–23. (G) Evening Anticipation Index (EAI) was calculated by dividing the average hourly activity for CT 9–11 by the average hourly activity for CT 6–11. For scatter plots, each point represents an individual fly and mean ± SEM is shown; ***: p<0.001; ****: p<0.0001; n.s.: not significant, p>0.05. Significance determined by Kruskal-Wallis nonparametric ANOVA (to account for non-normality of samples) followed by Dunn’s multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons.

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Figure 4—figure supplement 1. Representative actograms for each phenotypic class. Representative single-fly actograms of Pdf-Gal4>acp\textsuperscript{CRISPR} (gray), per\textsuperscript{CRISPR} (orange), or tim\textsuperscript{CRISPR} (blue) flies blindly scored as rhythmic (top row), weakly rhythmic (middle row), or arrhythmic (bottom row; no Pdf-Gal4>acp\textsuperscript{CRISPR} flies were scored as arrhythmic).

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**Figure 4—figure supplement 2.** Cell-specific disruption of per or tim in Pdf+ cells causes reduction in rhythm strength. χ² peak height values (rhythm strength) for Pdf-Gal4 targeting of acp (gray), per (orange), or tim (blue). Significance determined by one-way ANOVA followed by Tukey’s multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons. ***: p<0.001; **: p<0.01; n.s.: not significant, p>0.05.

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Figure 4—figure supplement 3. Average actograms for Pdf-Gal4 targeted flies under 12 hr:12 hr light:dark conditions. Average actograms for the first day in LD Pdf-Gal4 targeting of acp (gray), per (orange), or tim (blue).
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Figure 4—figure supplement 4. Cell-specific disruption of per or tim in Pdf+ neurons causes loss of the morning anticipatory peak under constant conditions. (A and D) Average hourly activity counts for one day under a 12 hr:12 hr light-dark schedule (A; white bars = ZT 0–11, black bars = ZT 12–23) or averaged over days 3–9 of complete darkness (D; gray bars = CT 0–11, black bars = CT 12–23); mean number of beam breaks per hour is shown ± SEM. (B and E) Morning Anticipation Index (MAI) calculated by dividing the average hourly activity for ZT (B) or CT (E) 21–23 by the average hourly activity for ZT or CT 18–23. (acp n = 42; per n = 31; tim n = 48). (C and F) Evening Anticipation Index (EAI) calculated by dividing the average hourly activity for ZT (C) or CT (F) 9–11 by the average hourly activity for ZT or CT 6–11. For scatter plots, each point represents an individual fly and mean ± SEM is shown. Significance determined by one-way ANOVA followed by Tukey’s multiple comparisons test (for normally distributed samples; (B and E) MAI = (***/***).
and F) or Kruskal-Wallis nonparametric ANOVA (to account for non-normality of samples; (C and E) followed by Dunn’s multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons. *: p<0.05; ****: p<0.0001; n.s.: not significant, p>0.05.

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Figure 5. CRISPR-mediated disruption of per or tim in Pdf+ neurons leads to efficient loss of the targeted protein. (A) Maximum intensity projections of Pdf-Gal4-driven GFP+ small and large ventral lateral neurons (s- and l-LNv) with immunohistochemistry for GFP (yellow), Period (green) and Timeless (magenta) at ZT0. Scale bar = 10 μm; arrows indicate CRISPR-targeted nuclei with residual protein signal. (B) Diagram showing Pdf-Gal4 expression in the 4 large and four small ventral lateral neurons (l- and s-LNv), bilaterally. (C) Quantification of the number of GFP+ neurons per brain (acp n = 14; per n = 15; tim n = 13 brains). (D and E) Background-subtracted nuclear fluorescence intensity of Per (D) or Tim (E) at ZTO in GFP+ neurons, grouped by brain with mean ± SEM shown. (F and G) Mean nuclear fluorescence intensity of Per (G) or Tim (H) at ZTO in GFP+ neurons, averaged per brain (acp n = 14; per n = 16; tim n = 14 brains). ****: p<0.0001; n.s.: not significant, p>0.05. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons.

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**Figure 5—figure supplement 1.** Cell-specific disruption of *per* or *tim* in Pdf+ neurons has similar efficiency in small ventral lateral (s-LNv) and large ventral lateral (l-LNv) neurons. Background-subtracted nuclear fluorescence intensity of Per (A, C) or Tim (B, D) at ZT0 in GFP+ neurons, grouped by brain and separated by cell type (A, B: small ventral lateral neurons; C, D: large ventral lateral neurons) with mean ± SEM shown. Individual brains are shown in the same order in all graphs (acp n = 14; per n = 16; tim n = 14 brains).

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Figure 6. Cell-specific disruption of per or tim in Mai179+Pdf− neurons does not affect behavioral rhythmicity. (A) Diagram showing circadian neurons targeted using Mai179-Gal4; Pdf-Gal80. (B) CRISPR-mediated disruption of per or tim in Mai179+Pdf− neurons did not affect overall rhythmicity. (C) Figure 6 continued on next page.
Figure 6 continued

Scatter plot showing the period of rhythmic flies with Mai179-Gal4; Pdf-Gal80-driven disruption of acp, per, or tim. (D) Average actograms showing the activity of flies in constant darkness with Mai179-Gal4; Pdf-Gal80-driven disruption of acp, per, or tim. Six days of activity are displayed, double-plotted. Dark gray rectangles = subjective day, black rectangles = subjective night. (E and F) Background-subtracted nuclear fluorescence intensity of Per at ZT0 in (E) RFP+ LNds (magenta) and the 5th s-LNv (light green) and (F) PDF+ s- (purple) and l-LNv neurons (dark green), grouped by brain with mean ± SEM shown (acp n = 10; per n = 9; tim n = 10). *: p<0.05; n.s.: not significant, p>0.05. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons.

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Figure 6—figure supplement 1. Cell-specific disruption of tim, but not per, in Mai179 Pdf cells causes a slight reduction in rhythm strength. $\chi^2$ peak height values (rhythm strength) for Mai179-Gal4; Pdf-Gal80 targeting of acp (gray), per (orange), or tim (blue). Significance determined by Kruskal-Wallis nonparametric ANOVA (to account for non-normality of samples) followed by Dunn's multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons. *: $p<0.05$; n.s.: not significant, $p>0.05$.

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Figure 6—figure supplement 2. Mai179-Gal4; Pdf-Gal80-driven CRISPR-targeting protects PDF⁺ cells from gene disruption. Maximum intensity projections of Mai179-Gal4; Pdf-Gal80-driven RFP⁺ dorsal lateral (LNd, bottom) and ventral lateral (s-LNv, 5th s-LNv, l-LNv; bottom) neurons with Figure 6—figure supplement 2 continued on next page.
immunohistochemistry for RFP (yellow), Period (green) and PDF (magenta); scale bar = 10 μm. Arrowheads indicate RFP⁺ (CRISPR-targeted) cells; arrow indicates rare PDF⁺ cell that has escaped Pdf-Gal80 protection and undergone CRISPR-mediated gene disruption.

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Figure 6—figure supplement 3. Cell-specific disruption of per in Mai179<sup>Pdf</sup><sup>−</sup> neurons causes loss of the evening anticipatory peak under constant conditions. (A) Average hourly activity counts during the second day of complete darkness (DD Day 2; gray bars = CT 0–11, black bars = CT 12–23). Mean number of beam breaks per hour is shown ± SEM. (ACP n = 58; per n = 63; tim n = 55) (B) Morning Anticipation Index (MAI) was calculated by dividing the average hourly activity for CT 21–23 by the average hourly activity for CT 18–23. (C) Evening Anticipation Index (EAI) calculated by dividing the average hourly activity for CT 9–11 by the average hourly activity for CT 6–11. For scatter plots, each point represents an individual fly and mean ± SEM is shown. Significance determined by Kruskal-Wallis nonparametric ANOVA (to account for non-normality of samples) followed by Dunn’s multiple comparisons test; reported p-values are multiplicity adjusted to account for multiple comparisons. ***: p<0.001; n.s.: not significant, p>0.05. DOI: https://doi.org/10.7554/eLife.48308.025
Figure 6—figure supplement 4. Average actograms for Mai179-Gal4; Pdf-Gal80 targeted flies under 12 hr:12 hr light:dark conditions. Average actograms for the first day in LD for Mai179-Gal4; Pdf-Gal80 targeting of acp (gray), per (orange), or tim (blue).
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