clockwork orange Encodes a Transcriptional Repressor Important for Circadian-Clock Amplitude in *Drosophila*

Chunghun Lim,1,2,3 Brian Y. Chung,1,3 Jena L. Pitman,1 Jermaine J. McGill,1 Suraj Pradhan,1 Jongbin Lee,2 Kevin P. Keegan,3 Joonho Choe,2,3 and Ravi Allada1,4
1 Department of Neurobiology and Physiology Northwestern University Evanston, Illinois 60208
2 Department of Biological Sciences Korea Advanced Institute of Science and Technology Daejeon 305-701 Republic of Korea

Summary

Gene transcription is a central timekeeping process in animal clocks. In *Drosophila*, the basic helix-loop helix (bHLH)-PAS transcription-factor heterodimer, CLOCK/CYCLE (CLK/CYC), transcriptionally activates the clock components *period* (per), *timeless* (tim), *Par domain protein 1* (Pdp1), and *vrille* (vr), which feed back and regulate distinct features of CLK/CYC function [1]. Microarray studies have identified numerous rhythmically expressed transcripts [2–7], some of which are potential direct CLK targets [7]. Here we demonstrate a circadian function for one such target, a bHLH-Orange repressor, CG17100/CLOCKWORK ORANGE (CWO). CWO is rhythmically expressed, and levels are reduced in *Clk* mutants, suggesting that CWO is CLK activated in vivo. *cwo* mutants display reduced-amplitude molecular and behavioral rhythms with lengthened periods. Molecular analysis suggests that CWO acts, in part, by repressing CLK target genes. We propose that CWO acts as a transcriptional and behavioral rhythm amplifier.

Results and Discussion

Only two (out of five) microarray studies had initially identified CG17100 as a rhythmically expressed gene [4, 5]. To test whether CG17100 exhibits robust rhythms, we used real-time quantitative RT-PCR and found significant rhythms in both 12 hr light/12 hr dark (LD) and constant dark (DD) conditions (Figures 1A and 1B). We also assayed CG17100 in *Clk* mutants and found that *cwo* levels are at trough levels, suggesting that CG17100 is a CLK-activated gene (Figure 1C). We identified a remarkable 20 CLK target CACGTG E box sequences in the 5′ region and in the large first intron, suggesting direct CLK activation (Figure 1D). Given its potential clock function and the presence of an Orange domain, commonly found in basic helix-loop helix (bHLH) repressors [8], we dubbed it *clockwork orange* [9].

We then examined mutants containing transposon insertions—*cwoe004207* (*cwo*0) and *cwoe006957*—in the first *cwo* intron (Figure 1D). To determine whether these insertions disrupt *cwo*, we performed qRT-PCR by using primers spanning this 7 kb intron. Amplification in homozygous mutants was reduced to ~10% of wild-type levels (p < 0.001; Figure 1E). The only amplicon detected in the mutants was of wild-type size, although we probably failed to detect intron-containing transcripts because of the large size of the potential amplified product (>7 kb). Assaying of levels of downstream exon 3 suggests that *cwo*−containing transcript destabilizes the unspliced and/or misspliced transcript, resulting in reduced levels (Figure 1F). In *cwo−*, reduced apparent transcript amplification across intron 1 (Figure 1E) probably reflects inefficient amplification of the large unspliced and/or misspliced product. Nonetheless, because exon 1 contains the putative initiating ATG (Figure 1D), disruption of splicing between exon 1 and 2 in both *cwo* mutants would have a dramatic consequence on protein function.

We then tested trans-heterozygous mutants (*cwoe/cwo−*) as well as homozygous *cwo* mutants (*cwoe/cwoe*, *cwoe/cwo−*) and observed dramatic reductions in the strength of behavioral circadian rhythms in DD (Figures S1A–S1D and Table S1 in the Supplemental Data available with this article online; p < 0.001). Those flies that demonstrated detectable rhythms often had lengthened circadian periods with reduced strength (Table S1). Although rhythmicity is evident immediately upon transfer of flies to DD, rhythms dampen in DD (Figure 2C, Figure S1F). This phenotype is recessive, and excision of either insertion can substantially improve mutant rhythmicity and period (~24 hr; Table S1, Figure 2A, Figure S1E; p < 0.05). We also performed complementation testing with flies heterozygous for a deletion that removes *cwo*, *Df(3R)ED5495*. Flies trans-heterozygous for *cwoe* and *cwo−* with *Df(3R)ED5495* have very poor rhythms, indicating a failure to complement (Table S1, Figures 2A–2C, Figure S1F; p < 0.001). These data reveal a critical role for *cwo* in rhythm amplitude and additionally in setting period length.

Under light-dark conditions, wild-type flies display a morning peak around the time of lights-on and an evening peak around the time of lights-off. Flies increase their activity in anticipation of these transitions, reflecting circadian-clock function. Quantitative analysis of morning anticipation indicates a reduction in the degree of anticipation—i.e., the magnitude of the activity increase preceding lights-on, in *cwoe/Df(3R)ED5495* relative to heterozygous *cwoe/cwo−* or *Df(3R)ED5495*/+ control flies (p < 0.05)—consistent with a long-period clock or other defect in LD clock function (Figures 2D–2F).

To independently confirm the *cwo* phenotype, we also expressed a dsRNA targeting *cwo* in transgenic flies (Figure 1D). Expression using the circadian *tim*GAL4 driver, which is expressed in all ~100 pacemaker neurons controlling circadian behavior [10], also resulted

*Correspondence: jchoe@kaist.ac.kr (J.C.), r-allada@northwestern.edu (R.A.)

3 These authors contributed equally to this work.
Figure 1. CG17100/clockwork orange Transcript Rhythms in Drosophila Heads with Real-Time PCR

(A and B) Quantitative real-time RT-PCR (A and B) experiments performed in light-dark (LD; [A]) and dark-dark (DD; [B]) conditions. The x axis indicates either zeitgeber time (ZT; [A]), where ZT0 is lights-on, or circadian time (CT; [B]). For real-time experiments (A and B), relative transcript levels have been normalized with the peak level (ZT or CT13) set to 100.

(C) Quantitative real-time RT-PCR analysis of cwo transcript levels in wild-type (+/+ ) and ClkJrk (ClkJrk) mutants at ZT1 and ZT13. Relative transcript levels have been normalized with the wild-type peak (ZT13) set to 100.

(D) Full-length transcript profile and domain organization of wild-type cwo. White and gray boxes indicate untranslated and protein-coding regions, respectively. Positions of the various cwo transposon insertions used are shown as black triangles above the diagram. The genomic region used for the UAS-cwoRNAi construct is shown above the transcript profile (labeled as RNAi). Arrows over the diagram denote the location of the primer sets used in real-time PCR experiments. Asterisks indicate the physical location of canonical E box elements (CACGTG) within the promoter and first intron, as identified by Fly Enhancer (http://genomeenhancer.org/fly). The figure has been drawn to scale, and all units are provided in kilobases.

(E and F) cwo transcript levels in wild-type (+/+ ; =100) and homozygous cwo mutants cwoe (e/e) and cwof (f/f). Primer sets spanning either exons 1 and 2 (E) or exon 3 (F), as shown in (D), were used to measure relative transcript levels. n experiments ≥ 3. Error bars represent standard error of the mean (SEM).
RNAi phenotypes are due to specific knockdown of vrille. In different eye as opposed to the cvof/cvof mutants tested, we observed elevated vrille transcript levels at trough times CT1 (cwo+/ cwo−/ cwo+/ only) and CT5 (all cwo mutants tested, p < 0.05). In all cwo mutants, we also found reduced transcript levels at the peak CT13 (p < 0.05). We observed similar results when examining another CLK target, Pdp1+. Pdp1+ levels exhibited increased trough levels at CT1 and CT5 in cwo mutants (Figure 3B), consistent with a CWO role as a repressor of CLK-activated transcripts.

per mRNA (Figure 3C) and pre-mRNA levels (Figure S5A) were also altered but with reduced peak levels at CT13 in cvof/cvof mutants (p < 0.05). Clk expression in cvof/cvof mutants is comparable to wild-type levels, indicating that reduced peak per levels cannot be explained by reduced Clk expression (Figure S5B). cwo mutant effects were not evident by DD day 4, although oscillations were also not detectable because of damping of eye clocks (data not shown). The finding of transcript phenotypes on DD day 1 when behavioral phenotypes are subtle suggest that eye clocks may be more sensitive to cwo loss than behaviorally relevant pacemaker neurons. Importantly, two CLK target genes, vrille and Pdp1+, show increased transcript levels at trough times in cwo mutants.

cwo encodes for bHLH-Orange (bHLH-O) proteins that are often DNA-binding transcriptional repressors [8]. Interestingly, two proteins implicated in control of mammalian circadian rhythms, Dec1 and Dec2, are also bHLH-O proteins that can repress mouse Clock action [15]. However, genetic inactivation of Dec1 (also known as Stra13) does not have a core clock phenotype [16]. Deinfective tests of the in vivo function of Dec1 and Dec2 will require the analysis of double-knockout animals.

To determine whether CWO can repress CLK function, we cotransfected cwo and Clk into Drosophila S2 cells.
activate transcription in an E box-dependent manner (p < 0.001). Gel-shift analyses with recombinant CWO indicated specific binding to a CACGTG E box but not mutated E box probes. This binding is partially competed by an unlabeled E box, but not a mutated E box fragment, and is super shifted by GST antibodies (Figure 4D). We found similar results with extracts from FLAG-tagged CWO bHLH-domain-transfected 293T cells (Figure S7). These results suggest that CWO specifically binds to E boxes and represses CLK-activated promoters. Importantly, these in vitro results are consistent with our in vivo data indicating elevated vri and Pdp1ε transcript levels in cwo mutants at times of maximum repression (Figures 3A and 3B).

Here we have demonstrated an in vivo role for CWO in the Drosophila circadian clock. Our data demonstrate reduced morning anticipation, lengthened periods, and damping rhythms in DD. Given that these alleles may not be nulls (Figures 1E and 1F), we cannot determine definitively whether CWO is essential for clock function. Nonetheless, our data argue strongly for a CWO role in driving high-amplitude transcriptional oscillations. Indeed, the strength of the observed phenotypes is comparable to or greater than those of loss-of-function alleles in the PDP1/VRI feedback loop [17–19]. Mechanistic analysis suggests this may be accomplished, in part, by binding to CLK target E boxes and repressing E box-driven transcription. Two other groups have also found similar in vivo results for CWO (M. Rosbash and H. Ueda, personal communications).

It is interesting to compare the CWO repressor with the well-studied transcriptional repressor PER. Both are rhythmically expressed [20], are CLK activated in vivo [21], and in turn repress CLK activation in S2 cells [22], and genetic disruption leads to circadian molecular and behavioral phenotypes in both. Interestingly, both display differential effects on CLK target genes. In pep01, vri, and Pdp1ε, transcripts are at wild-type peak levels consistent with PER’s proposed repressor function [18], whereas the per transcript or transcription is intermediate between peak and trough [20, 23]. Reduced per transcription has been explained by low Clk levels in pep01, but then why do vri and Pdp1ε levels remain at peak levels? In cwo mutants, vri and Pdp1ε transcripts are elevated at trough times, whereas per transcript is reduced only at peak times (Figure 3). One possible explanation for the complexity of per regulation is that full repression by PER and/or CWO may be required to get subsequent full per activation. Alternatively, CWO and/or PER may activate per transcription under some conditions.

The identification of clockwork orange further emphasizes the pivotal role of the Clk gene in the circadian clock. Clk appears to directly activate five clock components, all of which feed back and control Clk gene activity at distinct steps (Figure S3). PER/TIM regulate Clk/CYC DNA binding [24, 25], PDP1/VRI control Clk transcription [18, 26], whereas CWO is activated by CLK, and feeds back by binding and repressing through CLK/CYC target sites. Taken together, the multiplicity of feedback controls highlights the central role of Clk, one consistent with a master-regulator function [27].

We propose that CWO and CLK are principally involved in regulating pacemaker amplitude [28], whereas

cwo represses CLK activation of several clock-gene promoters (Figure 4A; p < 0.005). CWO does not directly interact with CLK, nor does it significantly affect CLK levels (data not shown). Interestingly, cwo transfection alone also reduced baseline activity of CLK target promoters (Figure 4B; p < 0.05). Importantly, CWO does not repress (or only weakly represses) promoters that are not CLK activated: Clk itself and the heterologous thymidine kinase (TK) promoter (Figure 4B). CWO also selectively represses a CACGTG E box-containing promoter but not other artificial promoters (Figure S6; p < 0.005). CWO repression depends on intact CLK target CACGTG sequences (Figure 4C; p < 0.005). To independently test whether CWO acts through E boxes, we expressed a form of CWO fused to the VP16 transcriptional-activation domain. This CWO-VP16 fusion

Figure 3. Altered Rhythmic Expression of vri, Pdp1ε, and per in cwo Mutants
Quantitative real-time RT-PCR analysis of vri (A), Pdp1ε (B), and per (C) expression during the first day of DD. The x axis indicates circadian time. Wild-type (+/+) levels at CT 13 (peak) are set to 100 and indicated as a closed line. cwo01/cwo01 (e/f), cwo01/Df(3R)ED5495 (f/Df), and cwo01/cwo01 (f/f) are indicated as dashed lines. Data for f/f are not shown for vri but are similar to e/f. Statistical significance (p < 0.05) is indicated as a closed line. The x axis indicates circadian time. Wild-type (+/+) levels at CT 13 (peak) are set to 100 and indicated as a closed line. cwo01/cwo01 (e/f), cwo01/Df(3R)ED5495 (f/Df), and cwo01/cwo01 (f/f) are indicated as dashed lines. Data for f/f are not shown for vri but are similar to e/f. Statistical significance (p < 0.05) is indicated as a closed line. The x axis indicates circadian time. Wild-type (+/+) levels at CT 13 (peak) are set to 100 and indicated as a closed line. cwo01/cwo01 (e/f), cwo01/Df(3R)ED5495 (f/Df), and cwo01/cwo01 (f/f) are indicated as dashed lines. Data for f/f are not shown for vri but are similar to e/f. Statistical significance (p < 0.05) is indicated as a closed line.
the PER/TIM loop plays a pre-eminent role in dictating period or phase of the rhythms. Interestingly, our CWO results are similar to those of Clk mutants in both flies and mice in which reduced-amplitude circadian rhythms are observed[21, 29, 30]. Mutants in per and tim (time-less) and their phosphorylation regulators [31] can lead to large (>2 hr) period changes while largely sparing rhythmicity. Given their evolutionary conservation, we predict that genetic inactivation of both Dec1 and Dec2 will reveal similar roles in mammals.

Experimental Procedures

Plasmids

Total RNA from adult fly heads was isolated with the TRIzol reagent (Invitrogen) and reverse-transcribed with the M-MuLV reverse transcriptase according to the manufacturer’s instructions (Roche). The cwo cDNA was amplified by PCR with the appropriate primer set, inserted into pAcnF for N-terminally FLAG-tagged expression in S2 cells, and confirmed by sequencing. It was also inserted into pAcVP16 for VP16-activation-domain fusion-protein expression in S2 cells. The cDNA corresponding to the CWO bHLH domain (aa 1–126) was cloned into pFLAG-CMV2 (Sigma) for N-terminally FLAG-tagged expression in mammalian cells and pGEX-4T-1 (Amersham Biosciences) for glutathione-S-transferase (GST)-fusion-protein expression in bacteria. The dClk cDNA [24] were similarly inserted into pAc5/V5-His (Invitrogen) for V5- and His-tagged expression in S2 cells. The per-luc, tim-luc, dClk-luc, and tk-luc constructs were described previously [22]. Promoter regions of vri gene (from 22.8 kb to 48 bp relative to the transcriptional start site) [17] and Pdp1 gene (from –3.5 kb to 196 bp relative to the transcriptional start site) were amplified from fly genomic DNA, inserted into pGL3-basic (Promega)—which was modified to remove a putative binding site of E4BP-4/VRI/PDP1 [18, 32]—and designated as vri-luc and Pdp1-luc, respectively. The promoter region from 21.0 kb to 196 bp of Pdp1 gene was similarly subcloned and designated as Pdp1-Ebox-luc. This region contains one canonical E box element (CACGTG) at –758 bp upstream from the transcriptional start site; this E box element was mutated (CCCGGG) in the Pdp1-mutEbox-luc construct.
Drosophila Stocks
All flies were reared with standard commenl-yeast-agar medium at 25°C under 12 h light/12 h dark (LD) cycles. The GAL4 line, 55-\(\text{GAL4-64,}\) and \(\text{pdf-GAL80}\) were described previously [10, 12, 33]. UAS-GFP\(\text{RNA\_}\) [34] was obtained from Bloomington Drosophila Stock Center. For CWO overexpression in a transgenic fly via the GAL4/ UAS system [35], cwo cDNA was inserted into pUAST that was modified to express N-terminally FLAG-tagged CWO. The RNAi construct for cwo gene was designed according to the genomic cDNA hybrid method [36]. cDNA corresponding to the second and third exons of cwo gene (nt 60–460) and genomic DNA including the cDNA with the internal and adjacent 3' introns were ligated together into the pUAST. Transgenic constructs were injected with pUChsp\(\text{p2\_3}\) into \(\text{w}\) embryos, from which several germline transmitters were established.

Behavioral Analysis
Locomotor activity of individual male flies was measured with Drosophila Activity Monitors (Trikinetics). Monitoring conditions included LD cycles for 2–5 days, followed by constant dark (DD) cycles for a week. Data were analyzed with ClockLab analysis software (Actimetrics) with the significance level of the \(\chi^2\) periodogram set to \(x = 0.05\). Flies with a \(\chi^2\) statistic \(> 10\) over the significance line were scored as arrhythmic. Results from at least two independent experiments were averaged. Normalized activity plots for LD and DD were generated by normalizing the average activity of each individual fly to 1. Flies with little or no activity over the final day of the analysis, or throughout the entire analysis, were considered potentially sick and removed. To calculate values of the morning-anticipatory phase, we determined the largest 2 hr increase in normalized average activity of each genotype over the last 6 hr of the dark phase. For all genotypes, the largest 2 hr increase in activity occurred between zeitgeber time 22 (ZT22) and ZT24. Anticipation-index values were compared between genotypes and their appropriate controls with one-way ANOVA and Tukey post-hoc tests at a significance level of \(p = 0.05\).

Cell Culture and Transient Transfection
Drosophila Schneider 2 (S2) cells were maintained in Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). 293T cells were maintained in Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal bovine serum. Cells were transiently transfected with the standard calcium-precipitation method. The quantity of total DNA used in transfection was kept constant by including an appropriate blank vector. For reporter assay, 293T cells in 6-well plates were transfected with blank vector or expression vector for FLAG-tagged CWO by Lipofectamine 2000 (Invitrogen) or a mutated E box (Invitrogen) and were imaged with laser-scanning confocal microscopy (Nikon).

Electrophoretic-Mobility-Shift Assay
293T cells in 6-well plates were transfected with blank vector or expression vector for FLAG-tagged CWO bHLH domain (aa 1–128) of bacterially purified GST-fusion proteins in binding buffer (4 mM HEPES [pH 7.9], 100 mM KCl, 2% glycerol, 0.3 mM MgCl\(\text{2}\)). Cells were harvested 24 hr after transfection and lysed in 0.5 M Tris-Cl (pH 7.5), 10 mM EDTA, 0.2 mM dithiothreitol, 0.1% Nonidet P-40, and 1 mM phenylmethylsulfonl fluoride at 4°C for 30 min and stored at −70°C before use. Synthetic oligonucleotides containing an E box (5'-\text{AAAGCCGCGCTCACTGGAAGTACGTCGGG-3')} or a mutated E box (mE) (5'-\text{AAAGGCGCGCTACGTCGGCGAATGCGT-3'}) were labeled with \(\gamma\)-\text{P}ATP by use of T4 polynucleotide kinase and annealed to complementary strands. Labeled probe (approximately 200 fmol per reaction) was incubated with 5 ng of nuclear extract or 50 ng of bacterially purified GST-fusion proteins in binding buffer (4 mM HEPES [pH 7.9], 100 mM KCl, 2% glycerol, 0.3 mM MgCl\(\text{2}\), 0.06 mM EDTA, 0.04 mM dithiothreitol, 0.02% Nonidet P-40, 0.2 mM phenylmethylsulfonl fluoride, and 1 μg poly(dI-dC) at room temperature for 30 min. For a competition assay, a 10- or 50-fold molar excess of unlabelled probes containing a wild-type or mutated E box was preincubated with the proteins prior to the addition of labeled probe. For a supershift assay, 2 μg of FLAG antibody (Sigma) or GST antibody (Upstate) was preincubated with the proteins prior to the addition of labeled probe. The reactions were terminated by electrophoresis on a 6% native polyacrylamide gel in Tris-borate-EDTA running buffer. The gel was dried and subjected to autoradiography.

Statistical Analysis
Time-point and genotype experiments were performed with one-way ANOVA with Tukey post-hoc tests at a significance level \(p = 0.05\).

Supplemental Data
Seven figures and two tables are available at http://www.current-biology.com/cgi/content/full/17/12/688/DC1.

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References


Supplemental Data

clockwork orange Encodes a Transcriptional Repressor Important for Circadian-Clock Amplitude in Drosophila

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Figure S1. Circadian and Diurnal Behavior of cwo Mutants

Normalized activity profiles during diurnal (left) and constant-darkness (right) conditions of +/+ (A), cwo°/cwo° (B), cwo°/cwo° (C), cwo°/cwo° (D), cwo°/+ (E), and cwo°/Df(3R)5495 (F). Light and dark bars indicate activity during the light and dark phase. White and black boxes indicate light and dark periods. Gray boxes indicate subjective day in constant darkness. Values on diurnal activity profiles indicate largest 2 hr increase in normalized average activity of each genotype over the last 6 hr of the dark phase (morning-anticipation index—see Experimental Procedures for details). n = 24–93 (diurnal), and n = 13–86 (constant darkness). n experiments = 2–5 (diurnal, constant darkness). Error bars (diurnal) represent SEM.

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Figure S2. Expression of UAS-cwoRNAi Transgenes by timGAL4 Driver Reduce Expression of Endogenous cwo Gene Transcript

RT-PCR shown from total RNA of adult heads at ZT 3 with primer sets specific for cwo, cyc, and pdf gene.

Figure S3. The cwo Enhancer-Trap GAL4 Line, c632a, Labels Circadian Pacemaker Cells

(A) One hemisphere showing c632aGAL4-driven UASeGFP expression throughout the brain. Large and small ventral-lateral neurons (in box) are expanded in (B), (C), and (D). Note additional expression in pars intercerebralis (left arrow) and circadian dorsal-lateral neurons (right arrow).

(B) eGFP expression in ventral-lateral neurons.

(C) PDF expression in ventral-lateral neurons.

(D) Merged image showing eGFP and pigment-dispersing factor (PDF) expression in large and small ventral-lateral neurons.
The CLK/CYC heterodimer binds the E box (CACGTG) and activates transcription of per, tim, Pdp1, and vri. PER and TIM repress CLK/CYC E box binding; PDP1 and VRI activate and repress, respectively, Clk transcription. CLK also activates clockwork orange (cwo), which competes for the E box to repress transcription of CLK target genes.

Figure S5. Rhythmic Expression of per pre-mRNA and Clk in cwo Mutants
Quantitative real-time RT-PCR analysis of per pre-mRNA (A) and Clk (B) expression during the first day of DD. The x axis indicates circadian time. Wild-type (+/+) levels at CT 13 (peak) are set to 100 and indicated with a blue line. cwo mutants cwo+/cwo− (e/f) and cwo+/cwo− (f/f) are indicated by red and green lines, respectively. Statistical significance (p < 0.05) is indicated with a 1 for comparing +/- with e/f and 2 for comparing +/- with f/f. n experiments ≥ 3. Error bars represent SEM.
Figure S7. CLOCKWORK ORANGE Specifically Binds E Boxes
Nuclear extracts from 293T cells transfected with blank vector (FLAG-CMV2) or expression vector for FLAG-tagged CWO bHLH domain (aa 1–126) were incubated with radio-labeled oligonucleotides containing a wild-type or mutant E box at room temperature for 30 min. Where indicated, a 50-fold molar excess of unlabeled probes containing wild-type or mutant E box or 2 μg of FLAG antibody was preincubated with the nuclear extracts prior to the addition of labeled probe. The reactions were terminated by electrophoresis on a 6% native polyacrylamide gel in Tris-borate-EDTA running buffer. The gel was dried and subjected to autoradiography.

C1 indicates shift by FLAG-CWO bHLH:DNA complex; C2 indicates supershift by FLAG-CWO bHLH:DNA:anti-FLAG antibody complex; one asterisk indicates E box-dependent shift by a nuclear protein in 293T cells; and two asterisks indicate E box-independent shift by a nuclear protein in 293T cells.

Figure S6. Selective CLOCKWORK ORANGE Repression of a Clock Target Promoter
293T cells in 6-well plates were cotransfected with reporter plasmids (1 μg) and with increasing amounts of CWO expression vector (0.25 μg; + and 1 μg; ++). Repression fold was calculated by inversely normalizing values to luciferase activity in the presence of reporter plasmid, which was set to 1. Results represent the averages of three independent experiments, and standard deviations are depicted by error bars. Artificial promoters contain synthetic tandem repeats of transcription-factor binding sites. These include ATF/CREB (ATFx3-luc), Sp1 (5xSP1-luc), E2F (2xE2F-luc), p53 (G13-luc), TCF (OT), STAT (2xSIE-luc), and mouse CLOCK/BMAL1 (54bp-luc).
Table S1. Circadian Behavior in clockwork orange Mutants

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<td>cwo excision #7</td>
<td>24.0 ± 0.1</td>
<td>61.1 ± 6.3</td>
<td>55</td>
<td>78</td>
</tr>
<tr>
<td>cwo excision #8</td>
<td>24.1 ± 0.1</td>
<td>74.7 ± 9.9</td>
<td>26</td>
<td>84</td>
</tr>
</tbody>
</table>

*a Power is a measure of rhythmic strength.
* n indicates number of flies analyzed.
* %R indicates percent flies with detectable rhythmicity.

Table S2. Circadian Behavior in clockwork orange RNAi Flies

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Period ± SEM</th>
<th>Power ± SEM</th>
<th>n</th>
<th>%R</th>
</tr>
</thead>
<tbody>
<tr>
<td>pdf-GAL4/+</td>
<td>23.7 ± 0.0</td>
<td>116.1 ± 5.0</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>tim-GAL4-62/+</td>
<td>24.2 ± 0.1</td>
<td>47.2 ± 5.6</td>
<td>32</td>
<td>84</td>
</tr>
<tr>
<td>UAS-cwo #2-4/+</td>
<td>23.6 ± 0.1</td>
<td>101.1 ± 8.4</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>tim-GAL4-62/UAS-cwo #2-4</td>
<td>23.6 ± 0.1</td>
<td>42.8 ± 7.6</td>
<td>14</td>
<td>93</td>
</tr>
<tr>
<td>UAS-cwoRNAi #44</td>
<td>23.8 ± 0.1</td>
<td>36.4 ± 4.8</td>
<td>31</td>
<td>81</td>
</tr>
<tr>
<td>pdf-GAL4-62; UAS-cwoRNAi #44</td>
<td>25.2 ± 0.1</td>
<td>71.9 ± 7.2</td>
<td>26</td>
<td>96</td>
</tr>
<tr>
<td>tim-GAL4-62; UAS-cwoRNAi #44</td>
<td>26.9 ± 0.1</td>
<td>30.7 ± 3.1</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>tim-GAL4-62/pdf-GAL80; UAS-cwoRNAi #44</td>
<td>24.6 ± 0.1</td>
<td>26.8 ± 4.0</td>
<td>41</td>
<td>66</td>
</tr>
<tr>
<td>tim-GAL4-62/UAS-cwo #2-4; UAS-cwoRNAi #44</td>
<td>25.8 ± 0.1</td>
<td>39.4 ± 3.9</td>
<td>61</td>
<td>82</td>
</tr>
<tr>
<td>UAS-cwo #3-3/+</td>
<td>23.5 ± 0.1</td>
<td>72.7 ± 10.8</td>
<td>12</td>
<td>92</td>
</tr>
<tr>
<td>UAS-cwo #3-3</td>
<td>23.1 ± 0.1</td>
<td>41.0 ± 7.2</td>
<td>21</td>
<td>81</td>
</tr>
<tr>
<td>pdf-GAL4; UAS-cwo #3-3</td>
<td>24.2 ± 0.1</td>
<td>80.2 ± 12.0</td>
<td>22</td>
<td>86</td>
</tr>
<tr>
<td>tim-GAL4-62; UAS-cwo #3-3/+</td>
<td>23.6 ± 0.1</td>
<td>49.2 ± 7.5</td>
<td>29</td>
<td>86</td>
</tr>
<tr>
<td>tim-GAL4-62; UAS-cwo #3-3</td>
<td>23.9 ± 0.1</td>
<td>16.5 ± 4.4</td>
<td>21</td>
<td>43</td>
</tr>
<tr>
<td>tim-GAL4-62/pdf-GAL80; UAS-cwo #3-3</td>
<td>23.9 ± 0.1</td>
<td>37.7 ± 7.1</td>
<td>19</td>
<td>74</td>
</tr>
<tr>
<td>UAS-cwo #3-7</td>
<td>23.5 ± 0.1</td>
<td>103.4 ± 10.3</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>UAS-cwo #3-7</td>
<td>23.5 ± 0.1</td>
<td>81.8 ± 7.2</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>pdf-GAL4; UAS-cwo #3-7</td>
<td>24.1 ± 0.0</td>
<td>64.6 ± 5.8</td>
<td>56</td>
<td>91</td>
</tr>
<tr>
<td>tim-GAL4-62; UAS-cwo #3-7/+</td>
<td>23.7 ± 0.1</td>
<td>55.0 ± 6.2</td>
<td>29</td>
<td>93</td>
</tr>
<tr>
<td>tim-GAL4-62; UAS-cwo #3-7</td>
<td>24.3 ± 0.1</td>
<td>18.4 ± 3.8</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>tim-GAL4-62/pdf-GAL80; UAS-cwo #3-7</td>
<td>23.9 ± 0.1</td>
<td>30.1 ± 7.3</td>
<td>16</td>
<td>75</td>
</tr>
</tbody>
</table>

*a Power is a measure of rhythmic strength.
* n indicates number of flies analyzed.
* %R indicates percent flies with detectable rhythmicity.