The circadian output gene takeout is regulated by Pdp1e

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The circadian clock controls many circadian outputs. Although a large number of transcripts are affected by the circadian oscillator, very little is known about their regulation and function. We show here that the Drosophila takeout gene, one of the output genes of the circadian oscillator, is regulated similarly to the circadian clock genes Clock (Clk) and cry. takeout RNA levels are at constant high levels in ClkJRK mutants. The circadian transcription factor PAR domain protein 1 (Pdp1e) is a transcription factor that had previously been postulated to control clock output genes, particularly genes regulated similarly to Clk. In agreement with this, we show here that Pdp1e is a regulator of takeout. Takeout RNA levels are low in flies with reduced Pdp1e and high in flies with increased amounts of Pdp1e. Furthermore, flies with reduced or elevated Pdp1e levels in the fat body display courtship defects, identifying Pdp1e as an important transcriptional regulator in that tissue.

Genetic and molecular analyses have yielded significant insight into the genes that constitute the core components of the Drosophila circadian clock (reviewed in refs. 1–3). It is regulated by two interlocked transcription/translation–based feedback loops, the period/timeless (per/tim) and Clock (Clk) loops (4). In addition, the regulation of the nuclear entry of proteins, their degradation rate, and phosphorylation state are crucial regulatory steps that are controlled by and contribute to the circadian clock. The per/tim cycle starts with the binding of CLK/CYC heterodimers to E-box promoter elements of the per and tim genes and their subsequent transcriptional activation. Eventually, the newly formed PER and TIM proteins will enter the nucleus and inhibit CLK/CYC action, thereby inhibiting the transcription of their own genes (5–7). Transcription of per and tim will resume once their protein levels have decreased sufficiently to release the inhibition of CLK/CYC. Rhythmic expression of Clock mRNA is regulated in the second loop, the Clock loop. CLK/CYC activate vri/e (vri) and Pdp1e, a transcriptional repressor and activator respectively, that have been shown to bind the Clock promoter competitively (8, 9). Although it was thought that this competition accounts for the oscillatory regulation of Clock mRNA, the fact that Clock mRNA levels are still high in Clock\textsuperscript{RKR} mutants (4) and that the core oscillator is only minimally impacted in flies with increased or decreased PAR domain protein 1 (Pdp1e) levels (depending on the allele/transgene and tissue examined) (10–12), indicate that an as yet unknown activator is required for Clock transcription. It has recently been demonstrated that Clock protein levels are constant in cells and that it is the phosphorylation state of the protein that determines its binding to DNA and its transcriptional activator function (7).

Although locomotor activity is the best-characterized circadian output, the circadian clock regulates numerous other outputs such as sleep (13–16), neuronal activity in olfactory neurons (17), and metabolism (18), and a number of tissues have been found to harbor autonomous clocks (19). This poses the question of the nature of the output genes that mediate these processes. Not surprisingly, because many of the main regulators are transcription factors, molecular screens for transcripts that are under circadian control, and change in mutants that affect the core clock, have revealed a large number of such transcripts (20–27). However, very little is known about the function of these potential output genes and the processes they regulate. The takeout (to) gene has been consistently identified in these screens. Its RNA and protein have been shown to cycle with a circadian rhythm, with a peak at late night/early morning and a trough in the late morning, closely resembling the cycling pattern described for Clock mRNA (24, 25). In contrast to what has been described for Clock mRNA, however, takeout levels were found to be down-regulated in Clock\textsuperscript{RKR}, cycl\textsuperscript{11}, and tim\textsuperscript{25} circadian mutants (25). The simplest way to explain takeout down-regulation in Clock and cycl mutants was to assume that its transcription is regulated by Clock. This would make takeout a Clock target with an unusual circadian rhythm because the CLK targets per and tim cycle in almost perfect antiphase to takeout. This suggested a more complicated and unusual way of takeout transcriptional regulation.

In this paper we show that in wild-type strains, there are two types of takeout expressers: High level takeout expressers and low level takeout expressers. When transcript levels were examined in outcrossed strains that contain only the high-expressing variant, we found circadian regulation of takeout transcription similar to that of Clock. Consistent with this, we show that Pdp1e, a circadian activator that had previously been postulated to control clock output genes, controls takeout levels. Flies with disrupted Pdp1e levels in the fat body display courtship defects, identifying Pdp1e as an important transcriptional regulator in that tissue.

Results

Circadian takeout RNA Expression Is Regulated Similarly to Clock RNA.

In the process of studying the transcriptional regulation of the takeout gene, we noticed that some laboratory strains expressed takeout at the high levels reported earlier (28), whereas others showed much lower levels of expression. None of the low expressing lines carried the previously described takeout\textsuperscript{1} (to\textsuperscript{1}) mutation (in which no takeout RNA can be detected (24, 28). Two wild-type strains, Canton-S (CS) and Crimea showed high levels of expression, whereas the other lines showed much lower levels of takeout RNA. The effect is due to a cis-effect (Fig. S1). The finding that there are two distinct kinds of takeout expressing alleles has implications for the analysis of takeout expression in mutant flies.


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If, for example, the mutant strain carries a low expressing copy, whereas the strain it is compared to is a high expresser, it is impossible to determine whether *takeout* levels in the mutant are low because of the mutation or because there are inherent differences in the *takeout* alleles between the two strains. It is therefore necessary to have equally expressing *takeout* alleles present in all strains to be compared.

It has previously been demonstrated that *takeout* RNA levels cycle in a circadian manner with a peak between circadian time (CT) 21 and 1 and a trough around CT9, a rhythm similar to that of *Clk* and *cry* RNAs. But, whereas *Clk* and *cry* RNA levels are constant high in *ClkJRK* mutants, *takeout* RNA levels were reported to be nondetectable in *ClkJRK* and *cry*01 mutants (24, 25), a regulation more like that found for *per* and *tim*, which both cycle with a different circadian phase than *takeout*. Thus, the regulation of *takeout* was unusual given the circadian profile of *takeout* RNA and protein. Given our findings of high- and low-expressing *takeout* alleles, we wondered whether the presence of differing *takeout* alleles in the strains compared might have influenced the results. To facilitate further examination of the circadian control of *takeout* in a *Clk* mutant background, we recombined the “high expressing” *takeout* allele from the Canton-S (CS) wild-type strain onto the *ClkJRK* mutant chromosome. We refer to this line as *ClkJRK (CS)*. We verified the presence of the *ClkJRK* mutation by sequencing and monitoring circadian behavior and the presence of *takeout* (CS) by expression (see below). We then analyzed *takeout* RNA levels in these lines by quantitative real-time PCR (qPCR) and compared them to the levels in the original *ClkJRK* mutants (Fig. 1 A and B). We found that *takeout* RNA levels are constant at near-peak levels under light-dark (LD) and dark-dark (DD) conditions in *ClkJRK (CS)* flies in comparison with wild-type flies. Therefore, transcriptional regulation of *takeout* may be similar to that of *Clk*, consistent with their similar circadian profiles. The *ClkJRK* line was found to be a *takeout* low-expressor line.

**Complex Posttranscriptional Regulation of Takeout Protein Levels.**

We next examined protein levels using a Takeout antibody we had previously generated (29). Flies were entrained and collected at different time points under 12-h light:12-h dark (LD) conditions, or under constant dark (DD) conditions following 3 days of LD entrainment. We found that under LD conditions, *takeout* protein levels in wild-type closely follow RNA cycling with a peak around Zeitgeber Time (ZT) 1 (Fig. 1C). In the absence of light (DD conditions), the *takeout* protein peak in wild-type is broader, suggesting a role for light in the degradation of the protein (Fig. 1D). Unexpectedly, in the *ClkJRK* (CS) mutants where RNA levels are at near-peak constant levels, *takeout* protein amounts are very low, indicating posttranscriptional control of the protein. Under LD conditions, a small amount of protein is induced in the mutant in the beginning of the day, suggesting that protein levels can be directly controlled by light (Fig. 1C). In DD, *takeout* protein levels are constant below trough levels in *ClkJRK* (CS) mutants (Fig. 1D).

**Takeout is a Target of PDP1e Regulation.** The results presented above indicate that the circadian regulation of *takeout* might be similar to that of *Clk*: both transcripts peak in the beginning of the day and are constant high in *ClkJRK* mutant flies. It has previously been suggested that in *ClkJRK* mutants an activator of *Clk* is constantly high, thus creating the high levels of *Clk* RNA. Because *ClkJRK* mutant RNA cannot give rise to a functional protein due to a nonsense mutation, the feedback loop that would normally reduce the levels of the activator is interrupted. It was previously shown that the CLOCK-CYCLE heterodimer activates the two transcription factors VRI and Pdp1e. Because both VRI and PDP1 bind the same regulatory element in *Clk*, the VRI/PDP1 ratio was thought to control the level of *Clk* transcription. This would predict that in flies with constitutively high levels of PDP1, *Clk* RNA levels should be very high. Flies with low levels of PDP1 should have low levels of *Clk* RNA. However, it was shown recently that *Clk* RNA and protein are not absent in flies expressing high or low levels of PDP1e. Depending on which Pdp1 allele/transgene was used and which tissue was tested, *Clk* levels and the core circadian oscillator are either not affected (10, 11) or moderately impacted (12). However, interestingly, flies expressing high or low levels of PDP1e were consistently arrhythmic in a locomotor activity assay, indicating that PDP1e levels regulate oscillator output and thus control circadian output genes (10–12). *takeout* has been identified in several screens as an output gene of the circadian oscillator. Given its similarity in circadian regulation to *Clk*, we hypothesized that it might be one of the genes controlled by Pdp1e. We therefore examined *takeout* RNA and protein levels in flies with altered Pdp1e levels. We made use of previously described transgenes that allow either overexpression of Pdp1e (UAS-Pdp1e), or its reduction by RNAi (UAS-Pdp1ei) using the Gal4/UAS system (10). We used the tim-Gal4 driver to express the transgenes. *tim-Gal4* is expressed in all clock cells, including the fat body (30). This is significant because *takeout* is preferentially expressed in the fat body of adult males (28). *takeout* RNA levels in the mutants were measured by qPCR and protein levels were assessed by Western blots using our Takeout antibody. Flies were entrained in a 12-h LD cycle for 3 days and collected at different time points during the first day of DD. RNA and protein was prepared from the heads of males and analyzed. Figs. 2 and 3 show expression of *takeout* RNA and protein in flies with either reduced PDP1 levels (timGal4/UAS-Pdp1ei) or in flies that overexpress PDP1 (timGal4/UAS-Pdp1e).
UAS-Pdp1i flies, we observed very low levels of *takeout* RNA in comparison with the levels in the respective control strains, suggesting that PDP1 is required for *takeout* expression at wild-type levels (Fig. 2A). In agreement with the observed RNA levels, we also observed reduced protein levels in the *timGal4/UAS-Pdp1i* mutants (Fig. 2B). In contrast, when PDP1 was overexpressed, *takeout* RNA levels were significantly higher than in the control flies and so were protein levels (Fig. 3A and B). Taken together these data indicate that *takeout* is a target of Pdp1 regulation. Because *takeout* is preferentially expressed in head associated fat body, and *tim-Gal4* is expressed in these cells, we conclude that PDP1 is likely to be an important transcriptional regulator in the fat body.

**PDP1 Mutants Show Reduced Male Courtship Behavior.** We have previously shown that the disruption of *takeout* leads to reduced male courtship (28). Given the low levels of *takeout* in the *timGal4/UAS-Pdp1i* mutants, we were wondering whether these males might have courtship defects when paired with target control females. As shown in Fig. 4A, males with low Pdp1 levels (*timGal4/UAS-Pdp1i*) show a significantly reduced courtship index. The courtship index is a measure of the fraction a male spends courting a female during the observation period. Courtship is also reduced in *a* *Pdp1* (29), indicating that Pdp1 levels affect additional proteins that are important for courtship. The observed reduction in courtship is more pronounced than that found in *takeout* mutant males alone (28), indicating that Pdp1 levels affect additional “courtship” genes. We have previously shown a similar courtship reduction to the one seen in the *Pdp1* experiments in males with feminized fat body (29), because *timGal4* is expressed in fat body, we wondered whether the observed effect was due to Pdp1 regulation of additional fat body genes besides *takeout*. To test this hypothesis, we used a fat body specific Gal4 driver that we had previously generated, *Lsp2-Gal4* (29), to express UAS-Pdp1 and UAS-Pdp1i specifically in fat body (Figs. S2 and S3). We found similarly reduced courtship defects in these males (Fig. 4B), indicating that Pdp1 plays a role in regulating genes in the fat body that control courtship. The reductions are somewhat less pronounced than with the *timGal4* driver. This may reflect differences in the temporal expression of the two drivers or the fact that *Lsp2-Gal4* is a slightly weaker fat body driver than *tim-Gal4* (Fig. S2). Alternatively, it may be due to the contribution of cells outside of the fat body.

To examine whether the effect on courtship we observed in flies with altered *Pdp1* levels was due to the disruption of the circadian clock in the fat body of these flies, we examined PER protein expression. We observed circadian oscillation of PER in all PER expressing tissues, including the fat body, indicating that the circadian clock was not abolished (Fig. S4). To test whether disruption of *Pdp1* levels by a mutation in the circadian clock would be able to disrupt courtship, we next tested courtship behavior of flies with a disrupted circadian clock in the fat body. We made use of a previously described transgene that allows expression of a dominant negative form of Clock (UAS-dnClk) to disrupt the circadian clock (31). We first used the fat body specific *Lsp2-Gal4* driver to express UAS-dnClk. However, fat body expression of dnClk using this driver proved lethal, possibly because of dominant effects of the transgene when expressed during development. Even when the Gal80<sup>ts</sup> system (32) was used to selectively induce dnClk only in adults, few adults were recovered, probably due to some inherent leakiness of the Gal80<sup>ts</sup> transgene during development. In the Gal80<sup>ts</sup>/UAS-dnClk; *Lsp2-Gal4* males that could be recovered, we induced expression of dnClk in adult males by overnight exposure to 32 °C, and tested the flies the next day. Males treated in this way showed reduced courtship, as shown in Fig. 4C, although the reduction was slightly less than that observed in *Pdp1* mutant flies. This might be due to limited disruption of the circadian clock in the surviving flies. It has recently been demonstrated that flies that express dnCLK in the fat body driven by a *takeout-Gal4* (to- *Gal4*) driver (28), which is adult-specific, survive and show a disrupted circadian clock in the fat body (18). We therefore repeated the courtship experiments using *to-Gal4* to express UAS-dnClk. The results are shown in Fig. 4D. We observed a strong reduction in courtship, similar to that found in *Pdp1* mutant flies. Taken together these results indicate that the observed phenotype in flies with altered *Pdp1* levels is at least in part due to the circadian function of Pdp1. Although it has previously been shown that these flies have defects in their metabolism, they were normal in a short term activity assay (Fig. 4E), indicating that the courtship defect is not caused by general sickness of the flies but rather due to the disruption of Pdp1 and/or circadian regulation.

**Discussion**

**Regulation of *takeout* RNA.** We have found that there are *takeout* high expressers and *takeout* low expressers. Interestingly, the observation of high- and low-expressing alleles may not be limited to

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**Fig. 2.** *takeout* RNA and protein levels are low in flies with reduced *Pdp1* levels. *takeout* RNA (A) and protein (B) levels are strongly reduced under constant low levels of *Pdp1*. *Pdp1* levels were reduced by expression of UAS-Pdp1i using the *timGal4* driver. RNA and protein from heads of *timGal4/UAS-Pdp1i* males was compared to that of the corresponding control genotypes (+UAS-Pdp1i and +*timGal4*). Flies were entrained for 3 days and collected at the indicated times on the first day of DD. Data are from three independent repeats.
the takeout gene but may extend to other fat body genes. Fuji et al. (33) have described strain differences in expression levels of other sex specific fat body genes. It is unknown what the biological significance of this dimorphism is. Strains collected from the wild from across Africa that differ in their hydrocarbon pheromone profiles (34) were found to be takeout low expressers (Fig. S1), indicating selection against high levels of Takeout. The different expression levels clearly have implications for the analysis of expression of these genes in mutants. We have found that the original ClkJRK strain contains a low expressing copy of takeout. When a high expressing copy was recombined onto the ClkJRK chromosome, it became apparent that takeout RNA in ClkJRK flies is constant at near-peak levels under LD conditions. Because takeout is mainly expressed in fat body, our results further confirm

![Fig. 3](image_url)

**Fig. 3.** takeout RNA and protein levels are elevated in Pdp1ε over-expressing flies. takeout RNA (A) and protein (B) levels are increased in flies that over-express PDP1ε. PDP1 was over-expressed by expression of UAS-Pdp1 by the timGal4 driver. RNA and protein from heads of timGal4/UAS-Pdp1 males was compared to that of the corresponding control genotypes (+/UAS-Pdp1 and +/timGal4). Flies were entrained for 3 days and collected at the indicated times on the first day of DD. Data are from three independent repeats.

![Fig. 4](image_url)

**Fig. 4.** Males with decreased or increased Pdp1ε levels or disrupted fat body clock show reduced courtship. Courtship indices (± SEM) of males toward wild-type virgin females. (A) timGal4 driven Pdp1 overexpression (timGal4/UAS-Pdp1) or reduction (timGal4/UAS-Pdp1) results in reduced male courtship indices. Mutant males are compared to the corresponding control males. (B) Males with decreased or increased levels of PDP in the fat body show reduced courtship. The fat body specific Lsp2-Gal4 driver was used to express UAS-Pdp1 or UAS-Pdp1. n = 10 for all genotypes. (C and D) The courtship indices of males expressing a dominant negative form of Clk (dnClk) in the fat body is shown. (C) The fat body specific Lsp2-Gal4 driver was used to express dnClk. The presence of Gal80ts allows induction of dnClk only in adult males. Expression was induced by exposing mature males to 32 °C overnight. Experimental and control males were treated equally. Testing occurred 2–4 h later. (D) The to-Gal4 driver was used to express dnClk predominantly in fat body. (E) Activity assay of the genotypes in D. The number of line crossings was counted.
the presence of a functional circadian clock in that tissue, as has recently also been shown by Xu et al. (18). We have found that in wild-type, Takeout protein cycling closely follows RNA cycling under LD conditions, as has previously been described (25). In the absence of light, the Takeout protein peak in wild-type is broader, suggesting a role for light in the degradation of the protein. In ClkJRK(CS) mutants, despite near-wild-type constant levels of RNA, Takeout protein levels are constant below trough levels in DD, indicating additional posttranscriptional regulation. Under LD conditions, a small amount of protein is induced in the mutant at the beginning of the day, again suggesting that protein levels can be directly controlled by light. Control by light in addition to the circadian clock has also been observed for fat body regulated feeding rhythms (18).

Pdp1ε: Regulates takeout RNA Levels. In addition to regulation by Cirk our data show that takeout RNA expression is also regulated by Pdp1 levels. takeout RNA levels are elevated approximately fivefold in flies that overexpress Pdp1. That this is unlikely to reflect unspeciﬁc activation is demonstrated by the fact that in the opposite situation, when Pdp1 levels are low due to reduction by RNAi, takeout levels are affected in the opposite way. In fact, this mode of regulation by Pdp1 ε had previously been suggested for the Cirk gene. However, recent experiments indicate that Pdp1 ε is not a major activator of Cirk transcription (10), but both increasing and decreasing the levels of Pdp1 ε in clock cells disturbed circadian locomotor activity, indicating that Pdp1 ε is required to regulate clock output genes. The molecular nature of these targets remains unknown. Genes with circadian rhythms that resemble those of Cirk and takeout (peaks near dawn or early in the morning) are candidates for being Pdp1 ε regulated output genes. Some of these are likely to be genes that are involved in locomotor activity, but there are probably others, like takeout, that have different roles and may be involved in other rhythmic outputs. A circadian function for very few of these genes has been identiﬁed to date. takeout was initially found in these screens and is among the best characterized clock output genes so far. We show here that takeout is regulated by Pdp1 ε and that Pdp1 ε is a transcriptional regulator in the fat body, a metabolic tissue. Disturbance of the circadian clock in fat body does not affect activity rhythms (18). Indicating that fat body speciﬁc outputs are not involved in locomotor control. A role for the fat body clock in the control of feeding rhythms and circadian starvation resistance has recently been demonstrated, and at least one cyclically expressed metabolic gene identiﬁed (18). Interestingly, takeout has previously been implicated in the control of larval feeding behavior (24). It remains to be seen how the two observations are linked.

Although our data indicate similarities in the regulation of takeout and Cirk or cry transcripts, there are important differences in the role of Pdp1 ε as a regulator of to versus Cirk that may be related to the more prominent role of Pdp1 in the control of output genes. It is unclear whether previous observations regarding the effect of peri ε and tim ε mutations on transcript levels need to be revised based on the status of the genetic backgrounds in these lines as high or low to expressors. Future experiments addressing this issue will shed further light on the extent of coregulation of to and Cirk or cry.

We have shown here that the RNA levels of takeout are regulated by Pdp1 ε; they are high when Pdp1 ε is overexpressed and low when Pdp1 ε levels are reduced. It has previously been shown that Pdp1 ε levels are low in ClkJRK(CS) mutants when measured in whole heads (8, 35). This would predict that takeout levels in ClkJRK(CS) mutants should be low due to lowered PDP1 levels. However, we have observed fairly high constant takeout levels in ClkJRK(CS) mutants. This suggests that the low levels of Pdp1 ε in the mutants are sufﬁcient to activate takeout, or that there is a separate activator present. That there is an appreciable amount of PDP1 in ClkJRK(CS) ﬁles is evident because these ﬁles live, whereas null mutants for Pdp1 ε die during development (8, 35). In contrast to what we have observed in ClkJRK(CS) mutants, takeout RNA and protein levels are directly correlated in Pdp1 ε over- and underexpressing ﬂies, suggesting a disruption of the circadian translational control of takeout in the mutants. The role of Pdp1 ε in the regulation of takeout transcription is likely to be indirect because we have not been able to ﬁnd PDP1 binding sequences or PDP1 binding to the takeout promoter. Furthermore, the regulatory elements in takeout that mediate circadian expression have not been identiﬁed yet.

Pdp1 ε: Regulated Fat Body Genes Are Involved in Male Courtship. Takeout is predominantly expressed in male fat body and takeout mutant males have reduced courtship (28). That male speciﬁc factors from the fat body play an important role in male courtship has been demonstrated by the fact that speciﬁc feminization of just this tissue signiﬁcantly reduces courtship to a degree that is beyond the reduction observed in takeout mutants (29). This indicates that male factors other than takeout also play a role. We speculate that some of these factors are also regulated by Pdp1 ε because ﬂies with disturbed PDP1 levels in the fat body show courtship defects similar to those observed in ﬂies with feminized fat body.

Circadian control of mating (but not courtship) has been described. However, in both of these studies it was noted that male courtship did not show a rhythm but that the rhythm was set by the female. Tauber et al. (36) have found preferential mating around dusk and overall higher levels during the subjective night than in the subjective morning. These rhythms were dependent on the clock gene per. Sakai and Ishida (37) observed mating rhythms in wild-type females, which were abolished in tim ε and per mutants. The only description of a male circadian courtship activity rhythm to date is by Fuji et al. (38). These authors observed a distinct shift in activity pattern when a male and a female ﬂy were housed together. Male–female couples show high levels of “close-proximity” (courtship) activity throughout the night and early morning. The rhythm is dependent on the clock genes in the brain and antennae and is dependent on the male’s circadian rhythm. It remains to be seen whether this circadian output behavior is regulated by Pdp1 ε-regulated genes in the brain and/or the fat body.

Materials and Methods
RNA Northern blots and hybridizations were performed as described in (28).

RNA Quantification. takeout mRNA levels were assayed by qPCR. Flies were entrained in a 12-h LD cycle for at least 3 days, collected every 4 h, and immediately stored at −80 °C. For DD collections, flies were entrained for 3 days in a 12-h LD cycle and collected every 4 h on the ﬁrst day of DD. Total RNA was isolated from male head using TRIZOL (Invitrogen). To eliminate genomic DNA contamination, each sample was treated with DNaseI (Promega). First-strand cDNA was synthesized from 1 µg of RNA using oligo(dT) primers and SuperScript II (Invitrogen). For qPCR, TaqMan assays were performed using the following primers and probes: forward primer, 5′-GCTCGTGTGGCTCGTGGTAT-3′; reverse primer, 5′-GCCATACCATACCTCAAAAGGTTT-3′; probe 6FAM-TCCCCGAAGATC-MGBNFQ. Ribosomal protein rp49 mRNA (rp49) was used as the internal loading control. The primers and TaqMan probe for rp49 were as follows: forward primer, 5′-CTGCCCACCG-GATTCAAG-3′; reverse primer, 5′-CGATTCGCCGAGTAAC-3′; probe VICCTCAGCTGCAGCGGATGT-MGBNFQ. Reactions were run on an Applied Biosystems Prism 7000. The relative levels of to and rp49 RNAs were calculated based on standard curves for to and rp49 that were run in each assay. to levels were normalized to rp49 at each time point.

Takeout Western blots were performed as described in ref. 29. Flies were entrained in a 12-h LD cycle for at least 3 days, collected every 4 h, and immediately stored at −80 °C. For DD collections, flies were entrained for 3 days in a 12-h LD cycle and collected every 4 h on the ﬁrst day of DD. Protein was extracted from male heads. Quantitation of Western blots: The relative levels of TAKEOUT (TO) were quantified as the ratio of the TO band intensity to that of a nonspeciﬁc background band using Quantity One-D Analysis software (Bio-Rad). These relative TO levels were normalized to TO levels in the wild-type control at ZT1 or its highest level.
Fly Stocks. The UAS-PDPI and UAS-PDPI transgenic strains were as described in ref. 10. The timGal4 driver (39), the fat body driver 2.1 Kb Lsp2-Gal4 (29) and the to-Gal4 driver (28, 29) have been described before. CLK<input>sup</input> were crossed to CS flies. Individual recombinant progeny of CLK<input>sup</input> were screened for the absence of input and the presence of CLK<input>sup</input> arrhythmia in a locomotor assay (10). (Location of genes: CLK 66A, ry 87D, to 96C). The DNA region around the C to T amino acid replacement that changes Q776 into a stop codon in CLK<input>sup</input> was amplified by PCR and sequenced to verify the presence of the mutation. At the same time, the number of glutamines in the longest polyglutamine repeat were confirmed to be 25, as had previously been described for CLK<input>sup</input>. (40). UAS-dnCLK transgenic lines were described as (31). The Gal80ts system was used to conditionally express dnCLK (32).

Fly Stocks. The wild-type strains Canton-S (CS) and Oregon-R (OreR) were lab strains, Crimea and Pi2 flies were obtained from the Bloomington Stock Center. Recently caught wild-type strains were as described in (1). Northern blots using the flies collected from the wild were performed in 2000, shortly after their collection.

Hot-Stop PCR. This method allows for linear quantitation of alleles and was performed as described in (2). A high- and a low-expressing takeout strain were crossed and RNA extracted from progeny males. cDNA was synthesized as described below. Twenty rounds of PCR were performed with takeout specific, nonlabeled primers (3). One more round of PCR was performed with 32P-labeled primers. PCR products were purified over a column (Qiagen) and digested with the restriction enzyme Fnu4H1. Only fragments originating from RNA produced by the low-expressing OreR strain contain the enzyme site and will show a smaller fragment size after digestion.

Immunohistochemistry was carried out as described (4) using the Vector ABC Elite staining kit. Unspecific fat body staining was blocked using the Vector Avidin/Biotin Blocking kit following the supplier's "fast protocol". Antibodies: Guinea pig anti-Pdp1 (GP40) (5) was used at 1:300 dilution, anti-guinea-pig (Vector) at 1:200. Rabbit anti-PER antibody (a gift of Michael Rosbash, Brandeis University, Waltham, MA) was preabsorbed against dissected heads of per01 flies and used at 1:4,000. Anti-Rabbit was from Vector and used as suggested by the ABC Elite staining protocol.

X-Gal staining was carried out as described (3).

Fig. S1. Varying takeout RNA levels in wild-type strains are due to a cis-effect. Strains freshly collected from the wild show low levels of takeout expression. Some laboratory strains expressed takeout at the high levels reported earlier, whereas others showed much lower levels of expression. Two wild-type strains, Canton-S (CS) and Crimea showed high levels of expression, whereas the other lines showed much lower levels of takeout RNA. To examine whether this effect was due to the differential levels of a transcriptional regulator (a trans effect) or due to inherent properties of the takeout promoter (a cis effect) in these lines, we crossed high-expressers with low-expressers and examined takeout RNA expression from the corresponding alleles in the progeny. To distinguish between the two alleles we made use of single nucleotide polymorphisms that we had identified in the coding regions of the two takeout alleles. If the difference is due to a trans effect, we expect both alleles to be equally expressed in the heteroallelic flies. In contrast, in the case of cis regulation we expect each allele to be expressed like it is in the parental line. This is indeed what we observed (B). The takeout copy from the CS and Crimea strain were still expressed at the high levels observed in the respective strains, whereas copies from the other lines maintained their low expression levels. We conclude that the difference in expression levels is due to a cis regulatory effect. (A) takeout expression in males (m) and females (f) of different wild-type strains was examined by Northern blot. Ribosomal protein 49 (rp49) hybridization to the same blot is shown as a control for the amount of RNA loaded. (B) RT-PCR analysis of takeout transcripts of RNA from parents and progeny of crosses between different wild-type strains. A fragment of the takeout coding region was amplified using the “HotStop” method and digested with Fnu4H1. This site is only present in the takeout variant from the low-expressing OreR strain. (C) takeout expression in male and female whole flies collected from across Africa (1) was examined by Northern blot. RNA from the CS and OreR laboratory strains was included on the blot for comparison. Origin of strains: 1, Ivory cost (Tai strain); 2, Malawi; 3, Seychelles; 4, Madagascar; 5, Cotonou (Benin); 6, Guinea-Bisseau; (a) In a short exposure RNA is visible only in CS males. (b) long exposure shows low levels of takeout RNA in OreR and all of the other strains. (c) Ribosomal protein 49 (rp49) hybridization to the same blot is shown as a control for the amount of RNA loaded.

Fig. S2. Comparison of the timGal4 and the Lsp2-Gal4 drivers. Cryosections of (A) Lsp2-Gal4/UAS-lacZ and (B) tim-Gal4/UAS-lacZ flies were stained side by side to compare the strength of the respective drivers. Lsp2-Gal4 shows slightly lower staining in fat body cells (marked by arrow).
**Fig. S3.** Pdp1 protein expression in the fat body of *Lsp2-Gal4/UAS-Pdp1* flies and *Lsp2-Gal4/Pdp1i* flies. Cryosections of mutant/transgenic flies and CS control flies collected at ZT21 and mounted and processed side by side were stained with a Pdp1 antibody (5). Slightly elevated and decreased protein levels can be observed in the respective mutants. (A and B) Pdp1 immunoreactivity in fat body cells of CS (A) and *Lsp2-Gal4/UAS-Pdp1* (B) flies. (C and D) Pdp1 immunoreactivity in fat body cells of CS (C) and *Lsp2-Gal4-Pdp1i* (D) flies.

**Fig. S4.** PER protein amounts in fat body cycle in *Lsp2-Gal4/UAS-Pdp1i* and *Lsp2-Gal4/UAS-Pdp1* flies. Flies were entrained for 3 days and either collected at ZT9 and ZT21, or placed at DD for one day and collected at CT9 and CT21. Head cryosections were incubated with anti-PER antibody. Heads of flies collected at different time points were mounted and processed next to each other on the same slide. Fat body cells are indicated by arrowhead, photoreceptor cells are marked by asterix.