

The circadian output gene *takeout* is regulated by *Pdp1ε*

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Edited by Jeffrey C. Hall, University of Maine, Orono, ME, and approved December 23, 2009 (received for review June 17, 2009)

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 *Clk^{JRK}* mutants. The circadian transcription factor *PAR domain protein 1 (Pdp1ε)* 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 *Pdp1ε* is a regulator of *takeout*. *Takeout* levels are low in flies with reduced *Pdp1ε* and high in flies with increased amounts of *Pdp1ε*. Furthermore, flies with reduced or elevated *Pdp1ε* levels in the fat body display courtship defects, identifying *Pdp1ε* as an important transcriptional regulator in that tissue.

fat body | courtship | clock | *Drosophila*

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* (*vri*) and *Pdp1ε*, 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 *Clk* mRNA, the fact that *Clk* mRNA levels are still high in *Clk^{JRK}* mutants (4) and that the core oscillator is only minimally impacted in flies with increased or decreased *PAR domain protein 1 (Pdp1ε)* levels (depending on the allele/transgene and tissue examined) (10–12), indicate that an as yet unknown activator is required for *Clk* transcription. It has recently been demonstrated that CLK 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 *Clk* mRNA (24, 25). In contrast to what has been described for *Clk* mRNA, however, *takeout* levels were found to be down-regulated in *Clk^{JRK}*, *cyc⁰¹*, and *tim⁰¹* circadian mutants (25). The simplest way to explain *takeout* down-regulation in *Clk* and *cyc* mutants was to assume that its transcription is regulated by CLK. This would make *takeout* a CLK 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 *Clk*. Consistent with this, we show that *Pdp1ε*, a circadian activator that had previously been postulated to control clock output genes, controls *takeout* levels. Flies with disrupted *Pdp1ε* levels in the fat body display courtship defects, identifying *Pdp1ε* as an important transcriptional regulator in that tissue.

Results

Circadian *takeout* RNA Expression Is Regulated Similarly to *Clk* 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 *takeout¹ (to¹)* 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.

Author contributions: J.B. and B.D. designed research; J.B., V.H., C.L., A.A.L., and B.D. performed research; J.B., V.H., C.L., A.A.L., and B.D. analyzed data; V.H., J.-F.F., P.E.H., and B.D. contributed new reagents/analytic tools; and B.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0906422107/DCSupplemental.

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 *Clk^{JRK}* mutants, *takeout* RNA levels were reported to be nondetectable in *Clk^{JRK}* and *cyc⁰¹* 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 *Clk^{JRK}* mutant chromosome. We refer to this line as *Clk^{JRK}(CS)*. We verified the presence of the *Clk^{JRK}* 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 *Clk^{JRK}* 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 *Clk^{JRK}(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 *Clk^{JRK}* line was found to be a *takeout* low-expresser 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 *Clk^{JRK}(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 *Clk^{JRK}(CS)* mutants (Fig. 1D).

Takeout Is a Target of PDP1 ϵ 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 *Clk^{JRK}* mutant flies. It has previously been suggested that in *Clk^{JRK}* mutants an activator of *Clk* is constantly high, thus creating the high levels of *Clk* RNA. Because *Clk^{JRK}* 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 *Pdp1 ϵ* . 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 PDP1 ϵ . 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 PDP1 ϵ were consistently arrhythmic in a locomotor activity assay, indicating that PDP1 ϵ 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 *Pdp1 ϵ* . We therefore examined *takeout* RNA and protein levels in flies with altered *Pdp1 ϵ* levels. We made use of previously described transgenes that allow either overexpression of *Pdp1 ϵ* (*UAS-Pdp1*), or its reduction by RNAi (*UAS-Pdp1i*) 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-Pdp1i*) or in flies that overexpress PDP1 (*timGal4/UAS-Pdp1*). In *timGal4/*

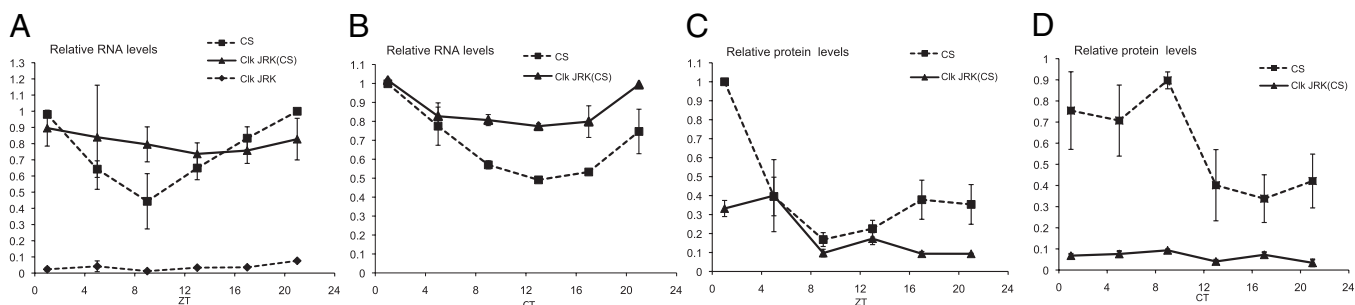


Fig. 1. (*A* and *B*) Circadian *takeout* RNA expression in *Clk^{JRK}(CS)* males is constant at high levels. *takeout* qPCR analysis of male head RNA from flies collected at the indicated times under (*A*) LD (ZT) and (*B*) DD conditions (CT). Relative to mRNA levels were quantified as described in *Materials and Methods*. RNA levels were normalized to the amount in control CS flies at ZT1. Data are from three independent repeats. Original *Clk^{JRK}* mutants, mutants carrying a high-expressing *takeout* allele *Clk^{JRK}(CS)*, and CS control flies were examined. (*C* and *D*) Takeout (TO) protein levels are under posttranscriptional control. Quantification of TO levels from Western blots. TO levels were quantified as described in *Materials and Methods* and normalized to wild-type levels. Data are from three independent repeats. Proteins from heads of wild-type CS and *Clk^{JRK}(CS)* males were examined. (*C*) Flies were collected at the indicated times under LD conditions (ZT). (*D*) Flies were entrained for 3 days and collected at the indicated times on the first day of DD.

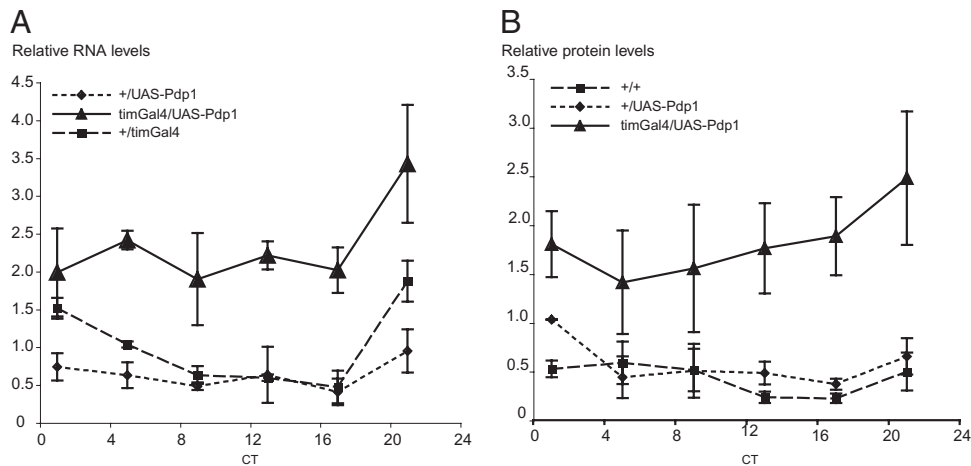


Fig. 3. *takeout* RNA and protein levels are elevated in *Pdp1 ϵ* over-expressing flies. *takeout* RNA (A) and protein (B) levels are increased in flies that over-express *PDP1 ϵ* . *PDP1* was over-expressed by expression of *UAS-Pdp1* by the *timGal4* driver. RNA and protein from heads of *timGal4/UAS-Pdp1* 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.

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 *Clk^{JRK}* strain contains a low expressing copy of *takeout*. When a high expressing copy was recombined onto the *Clk^{JRK}* chromosome, it became apparent that *takeout* RNA in *Clk^{Jrk}(CS)* flies is constant at near-peak levels under LD conditions. Because *takeout* is mainly expressed in fat body, our results further confirm

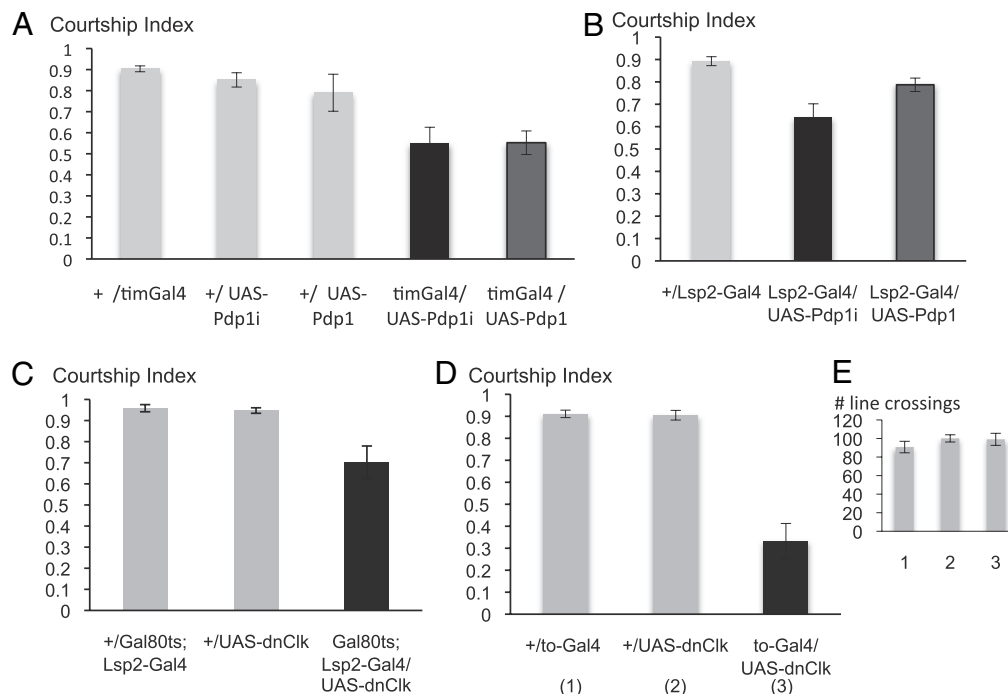


Fig. 4. Males with decreased or increased *Pdp1 ϵ* levels or disrupted fat body clock show reduced courtship. Courtship indices (\pm SEM) of males toward wild-type virgin females. (A) *timGal4* driven *Pdp1* overexpression (*timGal4/UAS-Pdp1*) or reduction (*timGal4/UAS-Pdp1i*) results in reduced male 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-Pdp1i* 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 *Gal80^{ts}* 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 *Clk^{JRK}(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).

Pdp1 ϵ Regulates *takeout* RNA Levels. In addition to regulation by *Clk*, 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 *Clk* gene. However, recent experiments indicate that PDP1 is not a major activator of *Clk* 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 *Clk* 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 *Clk* or *cry* transcripts, there are important differences in the role of PDP1 as a regulator of *to* versus *Clk* 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 *per⁰¹* and *tim⁰¹* mutations on *to* 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 *Clk* 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 *Clk* mutants when measured in whole heads (8, 35). This would predict that *takeout* levels in *Clk* mutants should be low due to lowered PDP1 levels. However, we have observed fairly high constant *takeout* levels in *Clk^{JRK}(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 *Clk^{JRK}* flies is evident because these flies live, whereas null mutants for *Pdp1 ϵ* die during development (8,

35). In contrast to what we have observed in *Clk^{JRK}(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 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* 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 fly heads 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 oligodT primers and SuperScript II (Invitrogen). For qPCR, TaqMan assays were performed using the following to primers and probes: forward primer, 5'-GCCTTTGGTCTCGGTGGAT-3'; reverse primer, 5'-GCCATCACCATACTTCAAGTTTT-3'; probe 6FAM-TCCCGAAGATC-MGBNFQ. Ribosomal protein 49 mRNA (*rp49*) was used as the internal loading control. The primers and TaqMan probe for *rp49* were as follows: forward primer, 5'-CTGCCACCGGATTCAAG-3'; reverse primer, 5'-CGATCTCGCCGAGTAAAC-3'; probe VICCTCCAGCTCGGCACGTTG-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 One1-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-PDP1i* and *UAS-PDP1* transgenic strains were as described in ref. 10. The *timGal4* driver (39), the fat body driver *3.1 kb Lsp2-Gal4* (29) and the *to-Gal4* driver (28, 29) have been described before. *Clk^{JRK}* flies were as described (40). Generation of *Clk^{JRK}(CS);Clk^{JRK}, ry* flies were crossed to *CS* flies. Individual recombinant progeny of *Clk^{JRK}(CS), ry/CS* females were then screened for the absence of *ry* and the presence of *Clk^{JRK}* by arrhythmicity 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^{JRK}* 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^{JRK}* (40). *UAS-dnClk* transgenic lines were as described (31). The Gal80^{ts} system was used to conditionally express dnCLK (32). *P{tubP-GAL80tsj20/UAS-dnClk; Lsp2-Gal4}* flies were grown and kept after eclosion at 18 °C. To inactivate Gal80^{ts}, 7- to 8-day-old males were transferred to the

restrictive temperature of 32 °C overnight for at least 18 h and tested after an additional 2–3 h at room temperature. Control flies were treated in the same way. All fly strains were reared on medium containing corn meal, yeast, agar, and Tegosept at 25 °C, except for the Gal80^{ts} experiments, for which flies were reared at 18 °C.

Courtship assays were performed as described in ref. 28.

ACKNOWLEDGMENTS. We thank William Mattox (University of Texas M.D. Anderson Cancer Center) for help with the identification of the polymorphisms in the wild-type strains and for discussions. We thank Gregg Roman (University of Houston) for providing bench space during parts of this project, and Fanny Ng and Pete Taylor for helpful discussions. We further thank Michael Rosbash (Brandeis University) for anti-PER antibodies. This work was supported by National Science Foundation Grants IOS-0416476 and IOS-0919697 to B.D.

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