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 *vrille* (*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}, cyc^{01}, and tim^{01} 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 $takeout^{1}$ (to^{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.

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 cyc^{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 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. 1*C*). 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. 1*D*). 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. 1*C*). In DD, Takeout protein levels are constant below trough levels in Clk^{JRK}(*CS*) mutants (Fig. 1*D*).

Takeout Is a Target of PDP1ε 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 *Pdp1ε*. 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 PDP1ε. 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 PDP1ε were consistently arrhythmic in a locomotor activity assay, indicating that PDP1ε 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 *Pdp1ε*. We therefore examined *takeout* RNA and protein levels in flies with altered *Pdp1ε* levels. We made use of previously described transgenes that allow either overexpression of Pdp1ε (*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*/

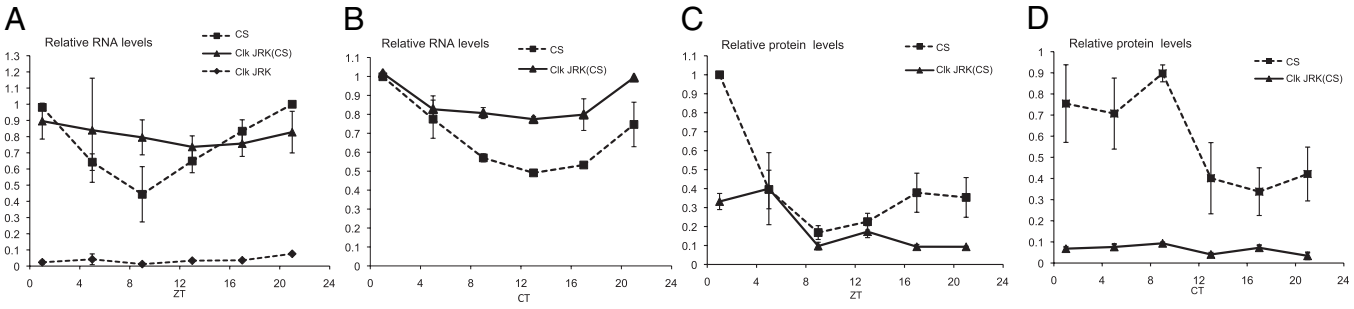

Fig. 1. (*A* and *B*) Circadian *takeout* RNA expression in Clk^{JRK}(*CS*) males is constant at high levels. *takeout* qPCR analysis of male head RNA from flies collected at the indicated times under (*A*) LD (ZT) and (*B*) DD conditions (CT). Relative *to* mRNA levels were quantified as described in *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.

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. 2*A*). In agreement with the observed RNA levels, we also observed reduced protein levels in the *timGal4*/*UAS-Pdp1i* mutants (Fig. 2*B*). In contrast, when PDP1 was overexpressed, *takeout* RNA levels were significantly higher than in the control flies and so were protein levels (Fig. 3 *A* 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. 4*A*, 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 *timGal4*/*UAS-PdP1* males that overexpress *Pdp1* (Fig. 4*A*), suggesting that PDP1 is involved in maintaining controlled levels of 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 *Pdp1ε* 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 scores in these males (Fig. 4*B*), indicating that *Pdp1ε* has 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^{ts} system (32) was used to selectively induce *dnClk* only in adults, few adults were recovered, probably due to some inherent leakiness of the Gal80^{ts} transgene during development. In the *Gal80^{ts}/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. 4*C*, although the reduction was slightly less than that observed in *Pdp1ε* 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. 4*D*. We observed a strong reduction in courtship, similar to that found in *Pdp1ε* mutants. Taken together these results indicate that the observed phenotype in flies with altered *Pdp1ε* levels is at least in part due to the circadian function of *Pdp1ε*. Although it has previously been shown that these flies have defects in their metabolism, they were normal in a short term activity assay (Fig. 4*E*), indicating that the courtship defect is not caused by general sickness of the flies but rather due to the disruption of Pdp1ε 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

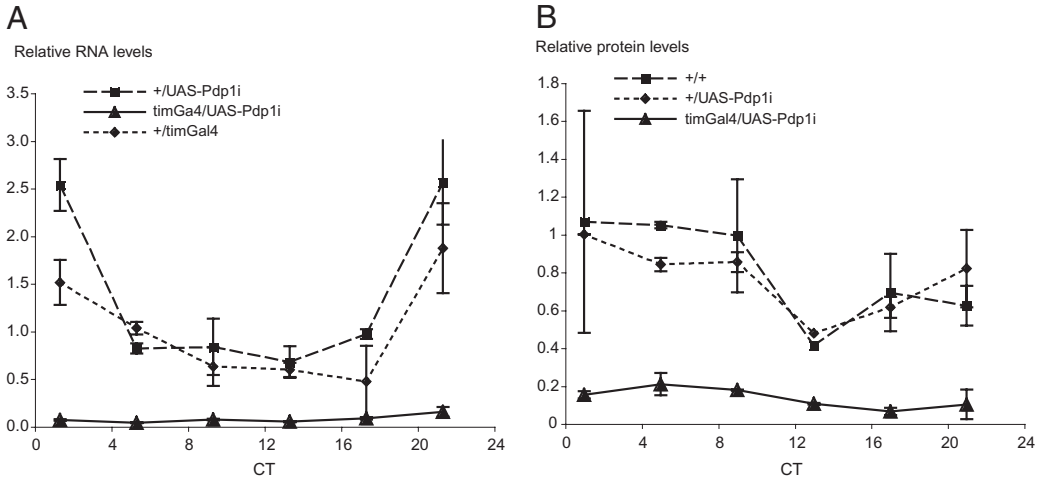

Fig. 2. *takeout* RNA and protein levels are low in flies with reduced Pdp1ε levels. *takeout* RNA (*A*) and protein (*B*) levels are strongly reduced under constant low levels of PDP1ε. PDP1 levels were reduced by expression of *UAS-Pdp1i* using the timGal4 driver. RNA and protein from heads of *timGal4*/*UAS-Pdp1i* 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.

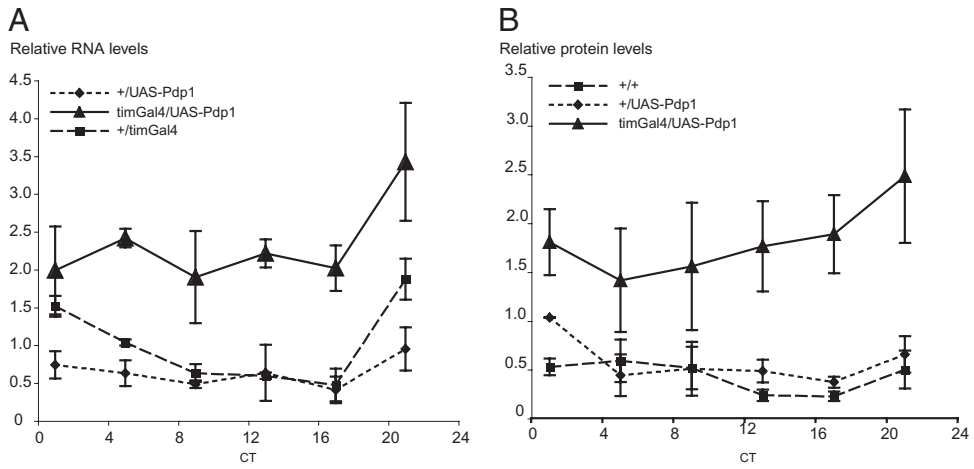

Fig. 3. *takeout* RNA and protein levels are elevated in Pdp1ε over-expressing flies. *takeout* RNA (*A*) and protein (*B*) levels are increased in flies that over-express PDP1ε. PDP1 was over-expressed by expression of *UAS-Pdp1* by the timGal4 driver. RNA and protein from heads of *timGal4/UAS-Pdp1* 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.

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

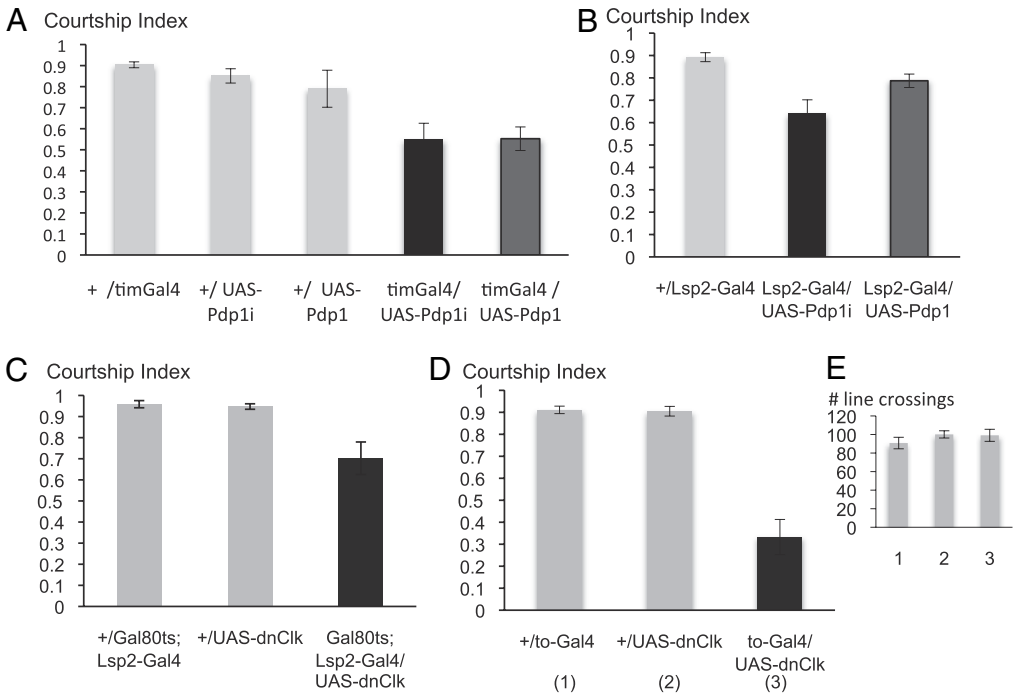

Fig. 4. Males with decreased or increased Pdp1ε levels or disrupted fat body clock show reduced courtship. Courtship indices (± SEM) of males toward wild-type virgin females. (*A*) timGal4 driven Pdp1 overexpression (*timGal4/UAS-Pdp1*) or reduction (*timGal4/UAS-Pdp1i*) results in reduced male courtship 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).

Pdp1ε 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 *Pdp1ε* 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 *Pdp1ε* in clock cells disturbed circadian locomotor activity, indicating that *Pdp1ε* 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 *Pdp1ε* 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 *Pdp1ε* and that *Pdp1ε* 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 per^{01} and tim^{01} 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 Pdp1ε; they are high when Pdp1ε is overexpressed and low when Pdp1ε levels are reduced. It has previously been shown that Pdp1ε 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 Pdp1ε 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 *Pdp1ε* 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 Pdp1ε over- and underexpressing flies, suggesting a disruption of the circadian translational control of *takeout* in the mutants. The role of *Pdp1ε* 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.

Pdp1ε 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 Pdp1ε-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′-GCCTTTTGGTCTCGGTGGAT-3′; reverse primer, 5′-GCCATCACCATACTTACAAGGTTTT-3′; probe 6FAM-TCCCCGAAGATC -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′-CTGCCCACCGGATTCAAG-3′; reverse primer, 5′-CGATCTCGCCGCAGTAAAC-3′; probe VICCCTCCAGCTCGCGCACGTTG-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-GAL80ts}20/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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