TOXICOLOGICAL SCIENCES **115(2)**, 513–520 (2010)
doi:10.1093/toxsci/kfq083
Advance Access publication March 26, 2010

Circadian Clock Regulates Response to Pesticides in *Drosophila* via Conserved *Pdp1* Pathway

Laura Michelle Beaver,[1] Louisa Ada Hooven, Shawn Michael Butcher,[2] Natraj Krishnan, Katherine Alice Sherman, Eileen Shin-Yeu Chow, and Jadwiga Maria Giebultowicz

Department of Zoology, Oregon State University, Corvallis, Oregon 97331

[1] To whom correspondence should be addressed at Department of Zoology, Oregon State University, 3029 Cordley Hall, Corvallis, OR 97331. Fax: (541) 737-0501. E-mail: beaverl@science.oregonstate.edu.
[2] Present address: Department of Public Health, Oregon State University, Corvallis, OR 97331.

Received January 7, 2010; accepted March 5, 2010

Daily rhythms generated by the circadian clock regulate many life functions, including responses to xenobiotic compounds. In *Drosophila melanogaster*, the circadian clock consists of positive elements encoded by *cycle* (*cyc*) and *Clock* (*Clk*) and negative elements encoded by *period* (*per*) and *timeless* (*tim*) genes. The ε-isoform of the *PAR-domain protein 1* (*Pdp1ε*) transcription factor is controlled by positive clock elements and regulates daily locomotor activity rhythms. *Pdp1* target genes have not been identified, and its involvement in other clock output pathways is not known. Mammalian orthologs of *Pdp1* have been implicated in the regulation of xenobiotic metabolism; therefore, we asked whether *Pdp1* has a similar role in the fly. Using pesticides as model toxicants, we determined that disruption of *Pdp1ε* increased pesticide-induced mortality in flies. Flies deficient for *cyc* also showed increased mortality, while disruption of *per* and *tim* had no effect. Day/night and *Pdp1*-dependent differences in the expression of xenobiotic-metabolizing enzymes *Cyp6a2*, *Cyp6g1*, and *α-Esterase-7* were observed and likely contribute to impaired detoxification. *DHR96*, a homolog of constitutive androstane receptor and pregnane X receptor, is involved in pesticide response, and *DHR96* expression decreased when *Pdp1* was suppressed. Taken together, our data uncover a pathway from the positive arm of the circadian clock through *Pdp1* to detoxification effector genes, demonstrating a conserved role of the circadian system in modulating xenobiotic toxicity.

Key Words: **circadian clock; *Pdp1*; permethrin; *Cyp6g1*; *α-Esterase-7*; *DHR96*.**

The circadian clock generates daily rhythmic patterns in sleep, activity, and other physiological variables, including daily fluctuations in metabolism, cellular function, and gene expression (Schibler, 2007). Circadian rhythms are entrained by exogenous zeitgebers (time givers), especially light/dark cycles. Remarkably, most clock genes, their network properties, and the basic multioscillatory organization of the circadian systems are highly conserved between flies and humans (Giebultowicz, 2001; Schibler, 2007; Stanewsky, 2003). The core circadian clock in *Drosophila melanogaster* consists of two interacting molecular feedback loops organized into 24-h feedback cycles. In the first loop, positive clock elements encoded by genes *Clock* (*Clk*) and *cycle* (*cyc*) activate the transcription of *period* (*per*) and *timeless* (*tim*) in the early night (Hardin, 2005). This leads to daily increases in the levels of *per*/*tim* messenger RNA (mRNA) and PER/TIM proteins. These negative clock elements accumulate in cell nuclei late at night and inhibit CLK-CYC–mediated transcription of *per* and *tim* (Glossop *et al*. 2003). The second feedback loop consists of the genes *vrille* (*vri*) and the *PAR-domain protein 1* (*Pdp1*) (Cyran *et al*., 2003; Glossop *et al*., 2003; Zheng, *et al*., 2009). *Pdp1* is a complicated gene because multiple promoters and alternative splicing events produce seven annotated isoforms of this transcription factor (Reddy *et al*., 2000). Two isoforms RD and RJ are regulated in a circadian manner and are collectively called *Pdp1ε* (Zheng *et al*., 2009). *Pdp1ε* is controlled by positive clock elements and regulates *Clk* expression through a feedback loop. In addition, *Pdp1ε* is necessary for daily rhythms in locomotor activity (Benito *et al*., 2007; Cyran *et al*., 2003; Lim *et al*., 2007; Zheng *et al*., 2009). It is not known whether this gene regulates other output rhythms from the circadian clock as specific target genes of *Pdp1* have not been identified.

Several studies have suggested that the circadian clock plays a role in regulating the response to xenobiotics, including therapeutics and toxins (Hooven *et al*., 2009; Levi and Schibler, 2007). Microarray studies have shown daily molecular oscillations in expression of xenobiotic-metabolizing genes in mammals (Yan *et al*., 2008) and flies (Wijnen and Young, 2006). Furthermore, studies have shown that the nuclear receptor and xenosensors constitutive of androstane receptor (CAR) and pregnane X receptor (PXR also known as steroid and xenobiotic receptor [SXR]) are expressed rhythmically (Yang *et al*., 2006; Zhang *et al*., 2009). While these studies

illustrate daily rhythms in expression of genes important for xenobiotic metabolism, they did not address functional significance of these rhythms. A recent study in *Drosophila* identified daily rhythms in activity of specific xenobiotic-metabolizing enzymes and daily variation in mortality after exposure to specific pesticides, suggesting that circadian clock genes may play an important functional role in responses to toxins (Hooven *et al*., 2009).

Another line of evidence for a role of circadian clock genes in xenobiotic metabolism comes from genetic studies in mammals. Disruption of the genes *Clk* and the *cyc* homolog (BMAL1) renders mice highly sensitive to the anticancer drug cyclophosphamide, while disruption of negative clock elements did not (Gorbacheva *et al*., 2005). Furthermore, mice lacking all three paralogs of *Pdp1*, the PAR bZip transcription factors dbp, tef, and hlf, are hypersensitive to pentobarbital and some anticancer drugs (Gachon *et al*., 2006). These triple knockout mice have altered expression of genes related to drug metabolism and detoxification processes like cytochrome P450 monooxygenases (P450s), carboxlyesterases, and the CAR (Gachon *et al*., 2006).

The present study sought to determine whether circadian clock genes regulate the response to toxicants. An additional goal of this study was to identify links between the central circadian mechanism and oscillations in output pathways related to detoxification. Using pesticides as a model for toxicant exposure, we identified a new output pathway from the circadian clock in *Drosophila* through *cyc* and *Pdp1* to genes implicated in pesticide metabolism. This novel finding is functionally significant as flies lacking *cyc* or *Pdp1* appear to have an impaired detoxification response and are thus more sensitive to pesticide-induced death. Taken together with mammalian studies, our study demonstrates a conserved pathway from positive clock elements through *Pdp1* to xenobiotic metabolism effector genes. Given this conservation, studies in the fly may provide a fundamental understanding of the circadian system in modulating xenobiotic toxicity in humans and suggest that the fly may be an effective model for chronopharmacological and chronotoxicological studies.

MATERIALS AND METHODS

Fly rearing, strains, and activity. *Drosophila melanogaster* were raised on yeast (35 g/l), cornmeal and molasses diet at 25 ± 1°C, at low density to attain uniform size, under a 12-h light/12-h dark regimen (where Zeitgeber time 0 [ZT 0] is time of lights on and ZT 12 is time of lights off). Flies were separated 1–2 days after emergence, and 5-day-old males were used for all experiments. cyc^{01} (Rutila *et al*., 1998), per^{01} (Konopka and Benzer, 1971), and tim^{01} (Sehgal *et al*., 1994) mutants were backcrossed to the Canton-S (CS) control line, allowing free recombination for at least six generations to isogenize the genetic background. The control flies for each mutant isogenized to the CS background were designated as CS^C, CS^P, CS^T, and CS^D for cyc^{01}, per^{01}, tim^{01}, and $DHR96^0$, respectively. Attenuated expression of *Pdp1* was accomplished through RNA interference (RNAi) by crossing flies carrying *tim*Gal4 and UAS-*Pdp1* RNAi constructs. $Pdp1i_a$ and $Pdp1i_b$ denote a UAS-*Pdp1* RNAi line from the laboratory of Dr Hardin (Benito *et al*., 2007) or Dr Choe (Lim *et al*., 2007), respectively. Control flies for RNAi experiments were generated by crossing the *tim*Gal4 driver with w^{1118} flies, the genotype used to generate the transgenic lines. The fly lines used in this study were generously shared and included: $Pdp1i_a$ from P. Hardin (Benito *et al*., 2007), $Pdp1i_b$ from J. Choe (Lim *et al*., 2007), $Pdp1^{3135}$ and their respective isogenized control iso^{131} flies from X. Zheng (Zheng *et al*., 2009), and $DHR96^0$ and their respective isogenized control CS^D from C. Thummel (King-Jones *et al*., 2006). Rhythmic activity was measured in a standard Trikinetics locomotor activity monitor (Waltham, MA). Data were collected in 15-min bins. A quantitative measure of the rhythmicity data was obtained using a fast Fourier Transform (FFT) (ClockLab, Actimetrics, Wilmette, IL), and individuals with FFT < 0.04 were deemed arrhythmic and those with FFT > 0.06 were rhythmic. Total daily activity (counts/24 h) was averaged for all individuals tested and served as an overall measure of daily activity.

Chemicals and insect treatment. Pesticides were obtained from Chem-Service (Westchester, PA), handled under a chemical hood, and were of the highest purity available. For pesticide exposure, 0.2 µl of permethrin (0–640 µg/ml) or malathion (0–32 µg/ml) dissolved in acetone was administered topically via gastight microsyringe to each fly (10 flies/vial replicated thrice). Mortality was recorded 24 h after exposure. Each set of experiments was repeated three or more times. The concentration of pesticide that was lethal to 50% of flies (LC_{50}) was calculated using PROBIT analysis (Finney, 1978) with a code written for and executed in SAS software (SAS 9.1.3; SAS Institute Inc., Cary, NC).

Quantitative real-time PCR. Twenty-five whole males were collected, frozen, and homogenized in TriReagent following manufacturer's protocol (Sigma) using a Kontes handheld motor. Samples were purified using the RNeasy Mini Kit (Qiagen) with on-column DNAse digestion (Qiagen) according to manufacturer's protocol. Synthesis of complementary DNA was achieved with Sprint RT Complete kit (Clontech) or iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's protocol. Real-time PCR was performed with iTaq SYBR Green Supermix with Rox (Bio-Rad) on an ABI Prism 7300, 7500, or Step-One Plus real-time machine in triplicate under default thermal cycling conditions. Primers were designed for target gene sequences using standard practices (specific primer sequences are available upon request) and obtained from Integrated DNA Technologies. Data were analyzed using the standard $2^{-\Delta\Delta CT}$ method normalized to the gene *rp49* and expressed relative to control samples at ZT 4.

Enzymatic activity assays. Twenty-five male flies were homogenized in 250 µl of buffer (100mM K-PO_4, pH 7.0, containing 2mM EDTA and 0.1% by volume Triton X-100), sonicated on ice, centrifuged at 3000 × g for 5 min at 4°C, and the supernatant recentrifuged at 10,000 × g for an additional 15 min. The protein concentration of the diluted supernatant (1:10 in assay buffer, 100mM K-PO_4, pH 7.0) was quantified using the bicinchoninic acid assay. All assays were performed using a BioTek Synergy 2 plate reader (Winooski, VT).

Esterase activity was assayed using Van Asperen's method, with α- or β-naphthyl acetate as substrates in microtitre plates with some modifications (Bracco *et al*., 1999). Fast Blue B was added following incubation at 30°C for 10 min, and absorbance was read at 595 nm for α-naphthyl acetate–cleaving esterase and 540 nm for β-naphthyl acetate–cleaving esterase. The concentration of products was determined at end point from standard curves of α- or β-naphthol. The activity was expressed as millimole of product formed per minute per milligram protein.

4-Nitrophenyl acetate (PNPA; Sigma) hydrolysis measurements were assayed according to Kim and Lee (2000). Briefly, generation of 4-nitrophenol was monitored as changes in absorbance at 405 nm adjusted against negative-control wells (without enzyme source). A standard curve of 4-nitrophenol ranging from 0 to 1mM was used to calculate activity as 1 µmol of 4-nitrophenol per minute per milligram protein.

Glutathione S-transferase (GST) activity assay was adapted from the procedure described (Habig *et al*., 1974) using 1-chloro-2,4-dinitrobenzene (CDNB) solution in acetonitrile as substrate. Absorbance at 340 nm was recorded at intervals for 5 min at 25°C. The conjugation of CDNB with reduced

glutathione is accompanied by an increase in absorbance at 340 nm, directly proportional to the GST activity. Total enzyme activity (cytosolic and microsomal) was expressed as nanomole per minute per milligram of protein.

RESULTS

Disrupting cyc *and Pdp1 Expression Increases Sensitivity of Drosophila to Pesticides*

To identify components of the circadian system that modulate xenobiotic response, we exposed flies with disrupted expression of *Pdp1*, or null mutations in core clock genes, to pesticides. Flies were exposed to varying concentrations of each pesticide, and the lethal concentration to 50% of subjects was calculated (LC_{50}). We used permethrin and malathion insecticides, which are used worldwide for the control of many insects, including the malarial vector *Anopheles gambiae*. These compounds represent two mechanistically distinct classes of insecticides (pyrethroids and organophosphates, respectively). No daily rhythm for permethrin-induced death was detected (Supplementary figs. 1A–C); thus, the pesticides were applied at ZT 4 according to a previous study by Hooven *et al.* (2009). We first examined the response to pesticides in flies where *Pdp1* expression was suppressed in clock cells by RNAi. A 50–60% decrease in *Pdp1*ε and total *Pdp1* mRNA expression levels was detected in both RNAi lines at ZT 4 and ZT 20 time points (Supplementary fig. 1D). The effectiveness of RNAi was confirmed by decreased daily rhythms in behavioral activity (Supplementary table 1). Suppression of *Pdp1* in two independent fly lines resulted in a significant twofold and threefold reduction in the average LC_{50} for permethrin (Fig. 1A). Disruption of *Pdp1* expression also resulted in a significant twofold reduction in the average LC_{50} for malathion (Fig. 1B). These data suggest that *Pdp1* regulates the ability of flies to survive a toxic exposure. Given the similar mortality response observed with both pesticides when *Pdp1* was disrupted, we focused the remainder of our survival experiments on permethrin exposure.

To explore the specific role of clock-controlled *Pdp1*ε, we used the $Pdp1^{3135}$ mutant fly line where only the ε-isoforms are disrupted by a deletion in their second exon (Zheng *et al.*, 2009). We confirmed that $Pdp1^{3135}$ flies were arrhythmic in locomotor activity (Supplementary table 1) as described (Zheng *et al.*, 2009). Additionally, we showed that cycling of *tim* mRNA levels was significantly attenuated, which provides additional evidence that *Pdp1*ε is involved in modulation of the clock (Supplementary fig. 1E). Importantly, disruption of *Pdp1*ε alone resulted in a significant 1.7-fold reduction in the average LC_{50} for permethrin (Fig. 1C). These data suggest that clock-dependent *Pdp1*ε can regulate the ability of flies to survive a toxic exposure.

To determine whether clock elements upstream from *Pdp1*ε are essential for normal pesticide response, we exposed flies with null mutations in core clock genes to permethrin. Loss of *cyc* expression resulted in a significant decrease in the average LC_{50} for permethrin (Fig. 1D, $p = 0.029$), demonstrating that response to pesticides is regulated in part by the positive limb of the circadian clock. In contrast, loss of either *per* or *tim* function did not affect ability of flies to survive permethrin treatment (Figs. 1E and 1F), suggesting that repressive clock elements are not essential for a normal response to this compound. These data are consistent with *Pdp1* being a key player in pesticide response because both total and *Pdp1*ε expression levels are suppressed and nonrhythmic in cyc^{01} flies but remain high and constitutively expressed in tim^{01} and per^{01} flies (Fig. 2; Benito *et al.*, 2007; Cyran *et al.*, 2003; Zheng *et al.*, 2009). These data demonstrate that the circadian clock regulates the response of *Drosophila* to pesticides via the *cyc-Pdp1*ε axis.

DHR96 *Affects Susceptibility to Permethrin and May Be Regulated in Part by Pdp1*

The *Drosophila* genome contains a single ortholog of both CAR and PXR, named *DHR96* (Fisk and Thummel, 1995; King-Jones and Thummel, 2005; King-Jones *et al.*, 2006). Given the regulation of CAR by PAR bZip transcription factors in mammals (Gachon *et al.*, 2006) and its involvement in metabolism of xenobiotics (Goodwin and Moore, 2004), we asked whether *DHR96* was regulated by *Pdp1* in flies. Preliminary results using real-time PCR suggested that *DHR96* mRNA levels did not change based on the circadian time of day (data not shown); therefore, we evaluated *DHR96* mRNA levels in *Pdp1* mutants at the time when flies were tested with pesticides. Disruption of *Pdp1* via RNAi or mutation of *Pdp1*ε reduced *DHR96* mRNA expression from 20 to 50% (Figs. 3A and 3B). These results suggest that *Pdp1* may contribute to the regulation of *DHR96* in the fly but also suggest that this multifunctional gene may be regulated by other mechanisms (Fisk and Thummel, 1995; King-Jones *et al.*, 2006; Sieber and Thummel, 2009).

PXR and CAR are known to bind a wide range of xenobiotic chemicals in mammals and activate many different target genes involved in detoxification (Goodwin and Moore, 2004; Maglich *et al.*, 2002); thus, we asked in parallel experiments if flies with a mutation in *DHR96* are more sensitive to an acute permethrin exposure. We show that loss of *DHR96* expression significantly decreased the average LC_{50} for permethrin by 1.6-fold (Fig. 3C), demonstrating that *DHR96* regulates the survival response of insects to a pyrethroid.

Disruption of Pdp1 *Results in Altered Transcription of Xenobiotic-Metabolizing Genes and Esterase Activity*

Recent work in our laboratory showed that both expression and activity of specific xenobiotic-metabolizing enzymes fluctuate during the day in *Drosophila* (Hooven *et al.*, 2009). To determine the consequences of disrupted *Pdp1* expression on xenobiotic-related genes, three effector-level genes, *Cyp6a2*,

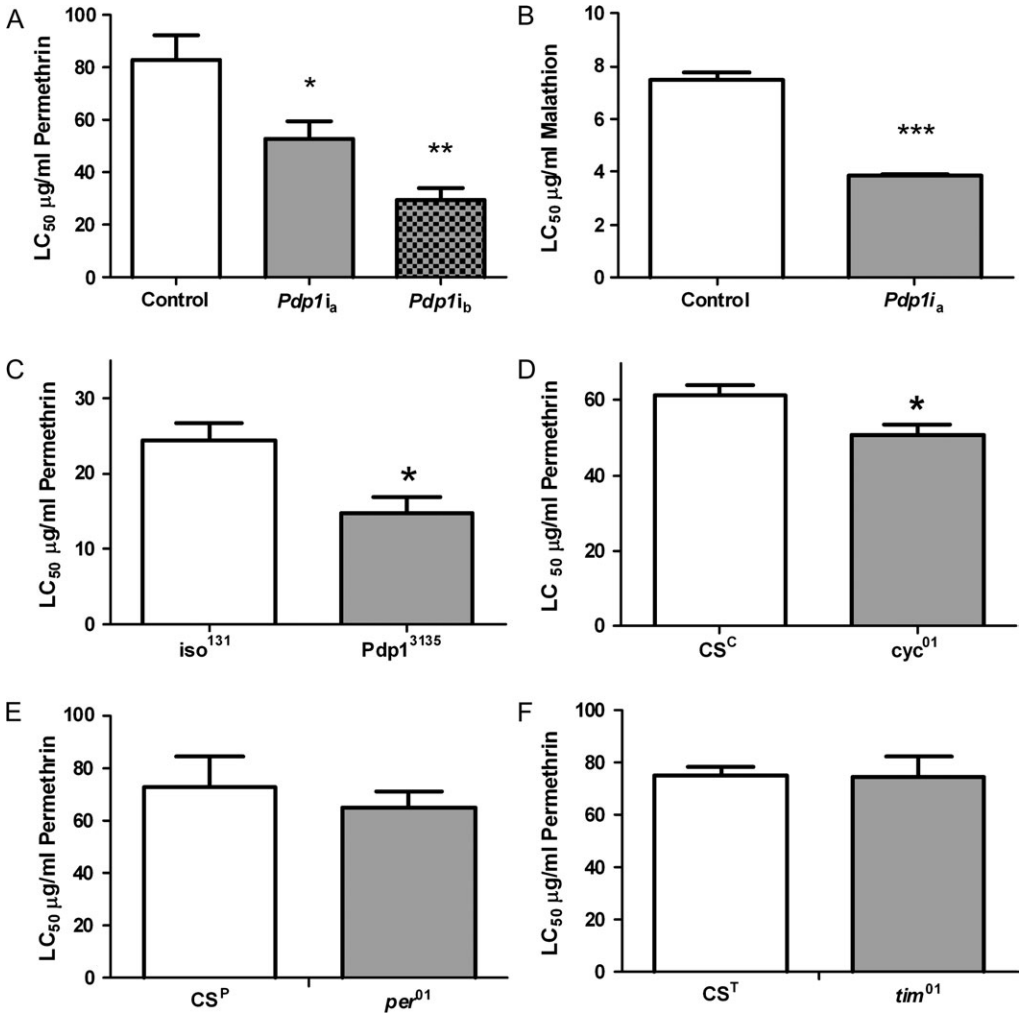

FIG. 1. Disruption of Pdp1 or *cyc* expression results in increased sensitivity to pesticides. Flies were exposed to permethrin or malathion at various concentrations and data represent the average LC_{50} (+ SEM) for each mutant and their matched control flies in three independent experiments unless otherwise noted. (A) Data represent five independent experiments for control flies, six for *Pdp*1i$_a$ flies, and three for *Pdp*1i$_b$. (D) Four independent experiments were completed for cyc^{01} and control flies. Statistical significance was determined by a one-way ANOVA and a Tukey's multiple comparison post-test for (A) and unpaired *t*-test for (B–F) where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. See also Supplementary figure 1 and Supplementary table 1.

Cyp6g1, and α-*Esterase-7*, were selected. These genes were chosen because microarray studies suggested that they were rhythmically expressed and they are associated with pesticide metabolism and resistance (Daborn *et al*., 2002; Dunkov *et al*., 1997; Heidari *et al*., 2005; Hooven *et al*., 2009; Wijnen and Young, 2006). Real-time PCR revealed that mRNA levels of *Cyp6g1* and *Cyp6a2* are significantly higher at ZT 4 than ZT 20 in control flies, suggesting regulation by the circadian clock (Fig. 4A, Supplementary fig. 2A). Disruption of *Pdp1* expression through RNAi resulted in significantly lower expression of both *Cyp6g1* and *Cyp6a2* (Figs. 4A and 4B). Disruption of *Pdp1*ε alone also resulted in a significant decline in the levels of *Cyp6g1* mRNA and a similar trend in *Cyp6a2* mRNA, suggesting regulation by the circadian clock (Supplementary fig. 2A).

Another group of phase I enzymes contributing to pesticide metabolism are esterases. Examination of α-*Esterase-7* mRNA levels revealed a significantly higher amount of mRNA at ZT 4 than ZT 20 in control flies (Fig. 4C, Supplementary fig. 2A). Furthermore, both *Pdp1*i$_a$ and $Pdp1^{3135}$ flies had significantly lowered α-*Esterase-7* mRNA at both time points (Fig. 4 and Supplementary fig. 2A). *Pdp1*i$_b$ flies also showed a trend of lowered α-*Esterase-7* at both ZT 4 and ZT 20 (Fig. 4C). These data suggest that *Pdp1* regulates α-*Esterase-7* transcription placing this gene as an effector in a clock-controlled pathway. To test functional significance of the transcriptional rhythm in α-*Esterase-7*, we examined esterase activity and show that disruption of *Pdp1* had substrate-specific effects. Activity toward α-naphthyl acetate, a substrate of α-*Esterase-7* (Heidari *et al*., 2005), was significantly reduced in *Pdp1* deficient flies (Fig. 4D). Thus, decreased esterase gene expression and enzymatic activity may contribute to the phenotype of increased sensitivity to pesticides. Additionally, esterase

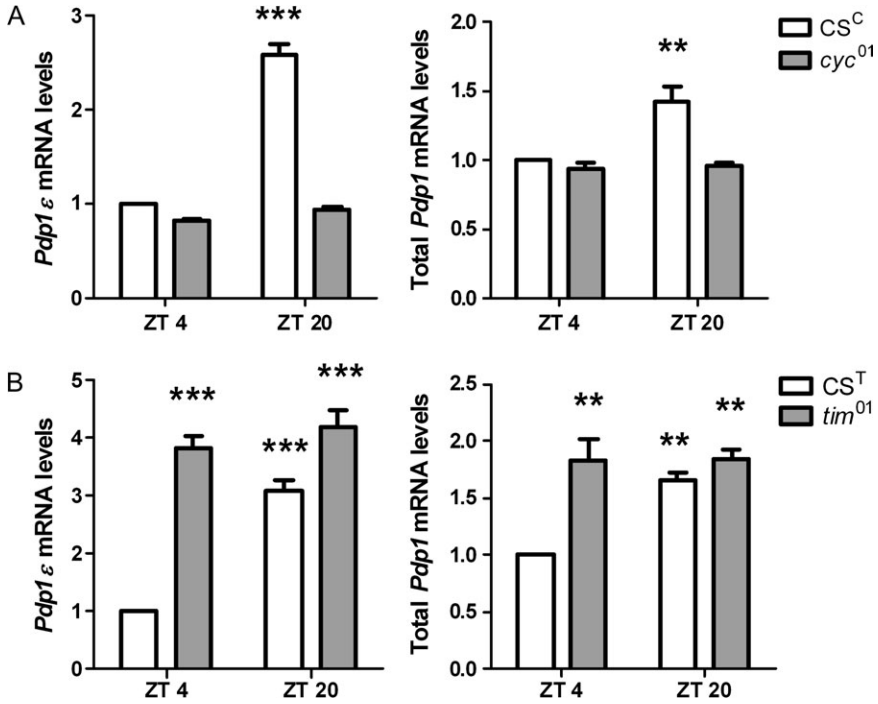

FIG. 2. *Pdp1* mRNA expression in *cyc* and *tim* mutants. *Pdp1*ε (left) and total *Pdp1* (right) mRNA levels are lower in (A) cyc^{01} flies with increased susceptibility to permethrin but not in (B) tim^{01} flies that do not show this sensitivity. Data represent mean mRNA levels + SEM for three independent experiments. Significant difference from control ZT 4 values, where values $^{**}p < 0.01$ and $^{***}p < 0.001$, as calculated by ANOVA and Tukey's multiple comparison post-test.

activity was significantly higher at ZT 20 than at ZT 4 for the substrates β-naphthyl acetate and PNPA, but this was not dependent on *Pdp1* (Supplementary figs. 2B and 2C). We also examined GST activity, which plays an important role in phase II detoxification but found no *Pdp1*- or time-dependent change in GST activity levels (Supplementary fig. 2D).

DISCUSSION

We identified a new circadian output pathway in *Drosophila* leading from the central clock gene *cyc*, to the transcription factor *Pdp1*, to xenobiotic-metabolizing genes *Cyp6a2*, *Cyp6g1*, and α-*Esterase-7* (Fig. 5). Functional importance of a clock regulated detoxification pathway was demonstrated by showing that deficiency in *cyc* and *Pdp1*ε genes increases the sensitivity of insects to pesticide exposure. To our knowledge, this is the first demonstration that the circadian clock regulates the response of *Drosophila* to toxicants via the *cyc-Pdp1*ε axis. We observed a statistically significant difference between the *cyc* and *Pdp1* mutants and their matched controls, e.g., a 1.7-fold reduction in the lethal dose for permethrin exposure was observed in *Pdp1*ε mutants. This signifies an important difference in the fly's ability to survive a toxic chemical; a 70%

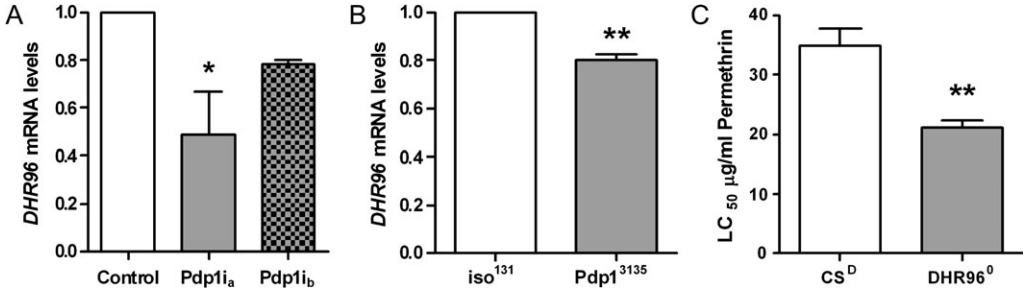

FIG. 3. Influence of *Pdp1* on *DHR96 expression*. (A–B) *DHR96* mRNA expression levels in *Pdp1* RNAi, $Pdp1^{3135}$, and their controls (control or iso^{131} flies, respectively) at ZT 4. Data represent mean relative expression (+ SEM) of three independent experiments. (C) Permethrin susceptibility is increased in the *DHR96* mutant as compared to its respective control (CS^D). Data are average LC_{50} (+ SEM) for four independent experiments. ANOVA and Tukey's multiple comparison post-test (A) or an unpaired *t*-test (B–C) were used to determine statistical significance from control samples where $^{*}p < 0.05$ and $^{**}p < 0.01$.

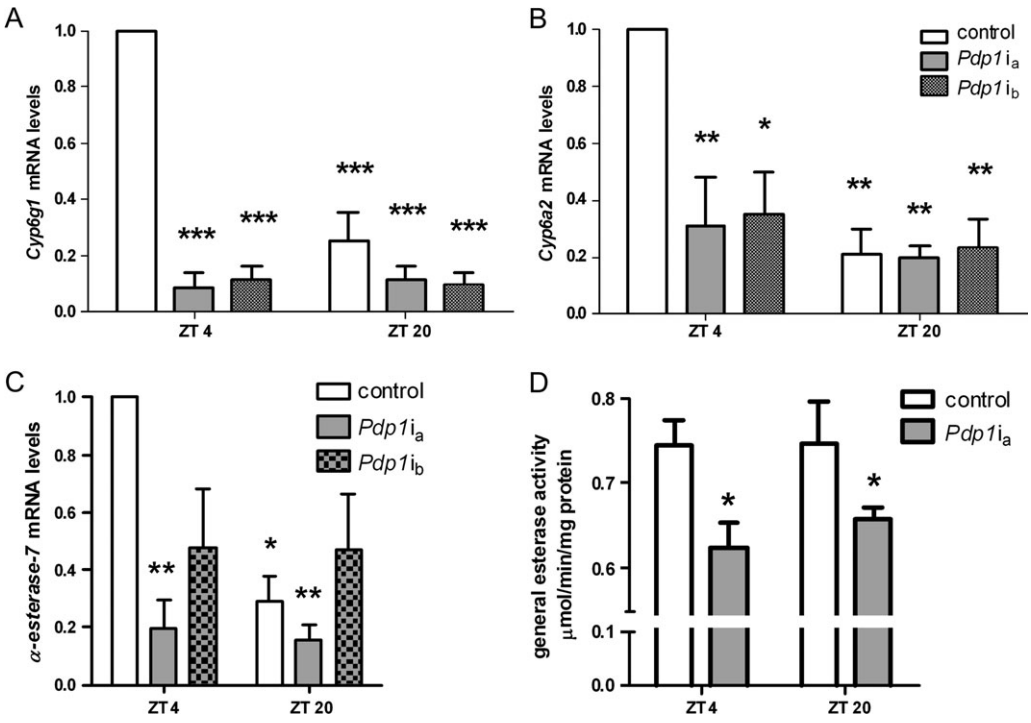

FIG. 4. *Pdp1* regulates components of xenobiotic metabolism. (A–C) Time of day and *Pdp1*-dependent *Cyp6g1*, *Cyp6a2*, and α-*Esterase-7* mRNA expression levels in control and *Pdp1* RNAi flies. Data represent mean relative expression (+ SEM) for three independent experiments. ANOVA and Tukey's multiple comparison post-test were used to determine statistical significance from control ZT 4 samples where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. (D) Flies with disrupted *Pdp1* expression have significantly lower general esterase activity for α-naphthyl acetate. Significant difference from control ZT 4 was determined by an ANOVA and Bonferonni post-test, $p < 0.05$. See also Supplementary figure 2.

greater concentration of the toxicant was necessary to induce death when *Pdp1*ε is not mutated. Furthermore, a 1.7-fold reduction in the LC_{50} means that a farmer could cut the dose of pesticide by nearly half, which has important financial and environmental implications.

Interestingly, abrogating expression of the *Pdp1*ε isoforms, or all *Pdp1* isoforms, renders flies more sensitive to pesticide exposure than flies lacking *cyc*. This may be due to the fact that in addition to influencing the expression of core clock genes, *Pdp1*ε also functions as an output gene of the circadian clock

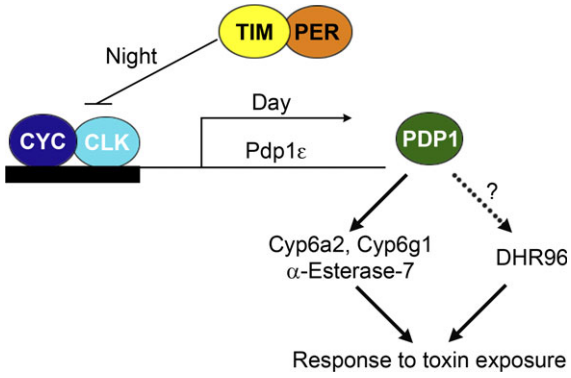

FIG. 5. The positive arm of the circadian clock regulates xenobiotic-metabolizing genes and the response of flies to a toxic exposure.

(Supplementary fig. 1E and Benito *et al.*, 2007; Cyran *et al.*, 2003; Lim *et al.*, 2007; Zheng *et al.*, 2009). It is also noteworthy that this susceptibility is observed following permethrin treatment since there was no apparent circadian rhythm in response to this pesticide throughout the day. Thus, the functional circadian clock appears to significantly contribute to the response to a toxicant even when the end point response does not show daily fluctuations.

We show a significant decline in mRNA levels of the nuclear receptor *DHR96* when *Pdp1* expression was disrupted. This finding in flies is similar to the role observed in mammals for PAR bZip transcription factors in regulating CAR transcription. Specifically, PAR bZip triple knockout mice express only a low and noncircadian basal level of CAR mRNA (Gachon *et al.*, 2006). The decline in *DHR96* mRNA was more apparent when all isoforms of *Pdp1* were suppressed by RNAi, suggesting that multiple isoforms of the gene may regulate *DHR96*. While our data support a role for *Pdp1* regulation of *DHR96*, this multifunctional gene is also regulated by the insect hormone 20-hydroxyecdysone and is likely to be regulated by other additional mechanisms (Fisk and Thummel, 1995; Horner *et al.*, 2009; Sieber and Thummel, 2009).

In murine liver, the xenosensors CAR, PXR (also known as SXR), aryl hydrocarbon receptor (AhR), and a number of other nuclear receptors are known to be expressed rhythmically

(Gachon *et al*., 2006; Teboul *et al*., 2008; Zhang *et al*., 2009). In contrast, we did not detect a rhythm in expression of the orthologous *DHR96* gene when whole flies were analyzed. While we cannot exclude that *DHR96* mRNA may cycle in specific tissues, this is not the first time a nuclear receptor was shown to be differentially regulated in flies as compared to mammals. The *Drosophila* equivalent of AhR, *spineless*, is not rhythmically expressed in the fly and plays a diminished role in response to xenobiotics compared to mammalian species (Brown *et al*., 2005). These examples illustrate that while much of the pathway from the clock to xenobiotic metabolism is conserved between flies and mammals, the details may vary between species.

In this paper, we additionally identified that flies with a mutation in *DHR96* are more susceptible to an acute exposure to permethrin, demonstrating that *DHR96* regulates the survival response of insects to a pyrethroid. This is consistent with the report by King-Jones *et al*. (2006) who showed that flies lacking *DHR96* are more sensitive to chronic exposure to the pesticide dichlorodiphenyltrichloroethane (DDT). At this time, it is not clear if *DHR96* directly regulates the response to toxins or if this phenotype is at least in part indirectly regulated by altered triacylglycerol and cholesterol homeostasis, which has been observed in these animals (Horner *et al*., 2009; Sieber *et al*., 2009).

Our study uncovered novel links between the central circadian mechanism and rhythmic output pathways related to detoxification and xenobiotic metabolism. P450s and esterases are phase I enzymes that modify foreign compounds to decrease their hydrophobicity and promote conjugation by phase II enzymes, in preparation for excretion from the organism. We identified day/night and *Pdp1*-dependent differences in the expression of *Cyp6a2*, *Cyp6g1*, and α-*Esterase-7*. These results confirm previous microarray studies that suggested that these genes may be regulated by the circadian clock (Hooven *et al*., 2009; Wijnen and Young, 2006;). Rhythmic expression of α-*Esterase-7* agrees well with the day/night and *Pdp1*-dependent differences in esterase activity. Differences in mRNA levels of *Cyp6a2* and *Cyp6g1* reported here are consistent with a previous report showing daily rhythm in P450 activity (Hooven *et al*., 2009). Upregulation of P450s may result in increased oxidative stress (Lewis, 2002). Coordinating the upregulation of P450s with the temporal window when the individual is active and ingesting food, and thus most likely to be exposed to toxins, would minimize oxidative stress. The timing of food intake is coordinated by the circadian clock; therefore, we speculate that clock genes also evolved to coordinate the expression of P450s when exposure to xenobiotics is most likely.

Taken together, the data suggest that circadian clock genes, and in particular *Pdp1*, play an important role in regulating xenobiotic metabolism across species and open a new avenue for understanding regulation of insect genes that have been associated with pesticide resistance. In particular, *Cyp6a2* and *Cyp6g1* are found to be overtranscribed in DDT-resistant isolates of *D. melanogaster* (Dunkov *et al*., 1997; Pedra *et al*., 2004), and cross-resistance to pyrethroids and organophosphates has been suggested to be mediated by insect orthologs of α-*Esterase-7* (Heidari *et al*., 2005). Managing pesticide resistance is critical for protecting the food supply, environment, and human health.

While it has been known that both the core circadian clock and xenobiotic metabolism are evolutionary conserved between insects and mammals (King-Jones and Thummel, 2005; Schibler, 2007; Stanewsky, 2003), our work connects these two pathways in flies. Circadian clock genes and circadian rhythms have been implicated in the response to therapeutic compounds (Levi and Schibler, 2007). Similar to our results with insecticides, disruption of *Clk* and the *cyc* homolog BMAL1 renders mice highly sensitive to the anticancer drug cyclophosphamide, but disruption of a negative element of clock does not (Gorbacheva *et al*., 2005). Furthermore, high sensitivity of PAR bZip deficient mice to pentobarbital and the anticancer drugs mitoxantrone and cyclophosphamide were demonstrated (Gachon *et al*., 2006). Thus, a protective pathway involving positive clock elements and PAR bZip transcription factors is evolutionarily conserved between flies and mammals. Studies in the fly may help to provide a fundamental understanding of the functional significance of the circadian system in modulating xenobiotic toxicity and serve as an effective model for chronopharmacological and chronotoxicological studies.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

National Institute of General Medical Sciences at the National Institute of Health (GM0737920 to J.M.G.); National Research Initiative at the United States Department of Agriculture (2007-04617 to J.M.G.); National Research Service Award at the National Institute of Health (5 F32 ES14994 to L.A.H.).

ACKNOWLEDGMENTS

We thank Drs A. Sehgal and X. Zheng for sharing $Pdp1^{3135}$ flies and data prior to publication. We thank David Chin for assistance with fly rearing; Dr B. Pittendrigh for helpful discussions, and Drs P. Hardin, J. Choe, X. Zheng, and C. Thummel for flies.

REFERENCES

Benito, J., Zheng, H., and Hardin, P. E. (2007). PDP1epsilon functions downstream of the circadian oscillator to mediate behavioral rhythms. *J. Neurosci.* **27,** 2539–2547.

Bracco, J. E., Barata, J. M., and Marinotti, O. (1999). Evaluation of insecticide resistance and biochemical mechanisms in a population of Culex quinquefasciatus (Diptera: Culicidae) from Sao Paulo. *Brazil. Mem. Inst. Oswaldo Cruz* **94,** 115–120.

Brown, R. P., McDonnell, C. M., Berenbaum, M. R., and Schuler, M. A. (2005). Regulation of an insect cytochrome P450 monooxygenase gene (CYP6B1) by aryl hydrocarbon and xanthotoxin response cascades. *Gene* **358,** 39–52.

Cyran, S. A., Buchsbaum, A. M., Reddy, K. L., Lin, M. C., Glossop, N. R., Hardin, P. E., Young, M. W., Storti, R. V., and Blau, J. (2003). *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. *Cell* **112,** 329–341.

Daborn, P. J., Yen, J. L., Bogwitz, M. R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., *et al.* (2002). A single p450 allele associated with insecticide resistance in Drosophila. *Science* **297,** 2253–2256.

Dunkov, B. C., Guzov, V. M., Mocelin, G., Shotkoski, F., Brun, A., Amichot, M., Ffrench-Constant, R. H., and Feyereisen, R. (1997). The Drosophila cytochrome P450 gene Cyp6a2: structure, localization, heterologous expression, and induction by phenobarbital. *DNA Cell Biol.* **16,** 1345–1356.

Finney, D. J. (1978). In *Statistical Method in Biological Assay*, 3rd ed.. Charles Griffin and Company Ltd, London.

Fisk, G. J., and Thummel, C. S. (1995). Isolation, regulation, and DNA-binding properties of three Drosophila nuclear hormone receptor superfamily members. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10604–10608.

Gachon, F., Olela, F. F., Schaad, O., Descombes, P., and Schibler, U. (2006). The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metab.* **4,** 25–36.

Giebultowicz, J. M. (2001). Peripheral clocks and their role in circadian timing: insights from insects. *Phil. Trans. R. Soc. B* **356,** 1791–1799.

Glossop, N. R., Houl, J. H., Zheng, H., Ng, F. S., Dudek, S. M., and Hardin, P. E. (2003). VRILLE feeds back to control circadian transcription of *Clock* in the *Drosophila* circadian oscillator. *Neuron* **37,** 249–261.

Goodwin, B., and Moore, J. T. (2004). CAR: detailing new models. *Trends Pharmacol. Sci.* **25,** 437–441.

Gorbacheva, V. Y., Kondratov, R. V., Zhang, R., Cherukuri, S., Gudkov, A. V., Takahashi, J. S., and Antoch, M. P. (2005). Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 3407–3412.

Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249,** 7130–7139.

Hardin, P. E. (2005). The circadian timekeeping system of Drosophila. *Curr. Biol.* **15,** R714–R722.

Heidari, R., Devonshire, A. L., Campbell, B. E., Dorrian, S. J., Oakeshott, J. G., and Russell, R. J. (2005). Hydrolysis of pyrethroids by carboxylesterases from Lucilia cuprina and Drosophila melanogaster with active sites modified by in vitro mutagenesis. *Insect Biochem. Mol. Biol.* **35,** 597–609.

Hooven, L. A., Sherman, K. A., Butcher, S., and Giebultowicz, J. M. (2009). Does the clock make the poison? Circadian variation in response to pesticides. *PLoS One* **4,** e6469.

Horner, M. A., Pardee, K., Liu, S., King-Jones, K., Lajoie, G., Edwards, A., Krause, H. M., and Thummel, C. S. (2009). The Drosophila DHR96 nuclear receptor binds cholesterol and regulates cholesterol homeostasis. *Genes Dev.* **23,** 2711–2716.

Kim, D. H. D. H., and Lee, S. S. S. S. (2000). Origin of rate-acceleration in ester hydrolysis with metalloprotease mimics. *Bioorg. Med. Chem.* **8,** 647–652.

King-Jones, K., Horner, M. A., Lam, G., and Thummel, C. S. (2006). The DHR96 nuclear receptor regulates xenobiotic responses in Drosophila. *Cell Metab.* **4,** 37–48.

King-Jones, K., and Thummel, C. S. (2005). Nuclear receptors—a perspective from Drosophila. *Nat. Rev. Genet.* **6,** 311–323.

Konopka, R. J., and Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* **68,** 2112–2116.

Levi, F., and Schibler, U. (2007). Circadian rhythms: mechanisms and therapeutic implications. *Annu. Rev. Pharmacol. Toxicol.* **47,** 593–628.

Lewis, D. F. V. (2002). Oxidative stress: the role of cytochromes P450 in oxygen activation. *J. Chem. Technol. Biotechnol.* **77,** 1095–1100.

Lim, C., Lee, J., Koo, E., and Choe, J. (2007). Targeted inhibition of Pdp1epsilon abolishes the circadian behavior of Drosophila melanogaster. *Biochem. Biophys. Res. Commun.* **364,** 294–300.

Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T., and Kliewer, S. A. (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol. Pharmacol.* **62,** 638–646.

Pedra, J. H., McIntyre, L. M., Scharf, M. E., and Pittendrigh, B. R. (2004). Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 7034–7039.

Reddy, K. L., Wohlwill, A., Dzitoeva, S., Lin, M. H., Holbrook, S., and Storti, R. V. (2000). The Drosophila PAR domain protein 1 (Pdp1) gene encodes multiple differentially expressed mRNAs and proteins through the use of multiple enhancers and promoters. *Dev. Biol.* **224,** 401–414.

Rutila, J. E., Suri, V., Le, M., So, V., Rosbash, M., and Hall, J. C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell* **93,** 805–814.

Schibler, U. (2007). The daily timing of gene expression and physiology in mammals. *Dialogues Clin. Neurosci.* **9,** 257–272.

Sehgal, A., Price, J., Man, B., and Youngs, M. (1994). Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science* **263,** 1603–1606.

Sieber, M. H., and Thummel, C. S. (2009). The DHR96 nuclear receptor controls triacylglycerol homeostasis in Drosophila. *Cell Metab.* **10,** 481–490.

Stanewsky, R. (2003). Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. *J. Neurobiol.* **54,** 111–147.

Teboul, M., Guillaumond, F., Grechez-Cassiau, A., and Delaunay, F. (2008). The nuclear hormone receptors family round the clock. *Mol. Endocrinol.* **12,** 2573–2582.

Wijnen, H., and Young, M. W. (2006). Interplay of circadian clocks and metabolic rhythms. *Annu. Rev. Genet.* **40,** 409–448.

Yan, J., Wang, H., Liu, Y., and Shao, C. (2008). Analysis of gene regulatory networks in the mammalian circadian rhythm. *PLoS Comput. Biol.* **4,** e1000193.

Yang, X., Downes, M., Yu, R. T., Bookout, A. L., He, W., Straume, M., Mangelsdorf, D. J., and Evans, R. M. (2006). Nuclear receptor expression links the circadian clock to metabolism. *Cell* **126,** 801–810.

Zhang, Y. K., Yeager, R. L., and Klaassen, C. D. (2009). Circadian expression profiles of drug-processing genes and transcription factors in mouse liver. *Drug Metab. Dispos.* **37,** 106–115.

Zheng, X., Koh, K., Sowcik, M., Smith, C. J., Chen, D., Wu, M. N., and Sehgal, A. (2009). An isoform-specific mutant reveals a role of PDP1 epsilon in the circadian oscillator. *J. Neurosci.* **29,** 10920–10927.