# Dibutyl phthalate disrupts conserved circadian rhythm in Drosophila and human cells

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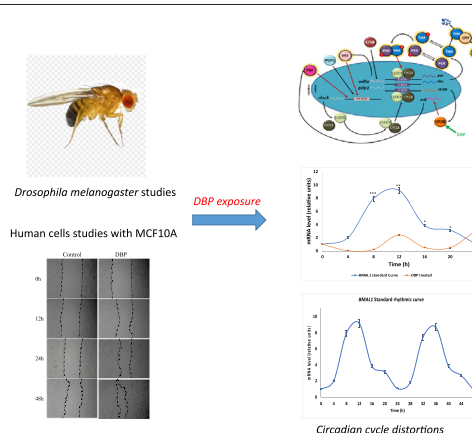[b] Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, Gdańsk, Poland
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## HIGHLIGHTS

- DBP disrupts circadian regulation genes at transcriptional level.
- DBP affects circadian pattern, but does not affect eye morphology.
- It is of great significance to extend environmental pollutants exposure studies with circadian rhythm research.

## GRAPHICAL ABSTRACT



*Drosophila melanogaster* studies

Human cells studies with MCF10A

DBP exposure

*Circadian cycle distortions*

## ARTICLE INFO

## ABSTRACT

People are constantly exposed to phthalates, due to their common use in the production of plastics, pharmaceuticals, cosmetics and skin care products. The ability of phthalates to disrupt endocrine signaling, leading to developmental, reproductive and metabolic defects, has been studied, yet how phthalates interfere with these biological functions is still unclear. To uncover DBP interacting molecular pathways, we raised *Drosophila melanogaster* on food containing dibutyl phthalate (DBP) at various concentrations. Whole transcriptome analysis of adult *Drosophila* reveals that DBP exposure throughout development disrupts the expression of genes central to circadian rhythm regulation, including increased expression of *vrille* (*vri*, human *NFIL3*), *timeless* (*tim*, human *TIMELESS*) and *period* (*per*, human *PER3*), with decreased expression of *Pigment-dispersing factor* (*Pdf*). DBP exposure also alters the expression of the evolutionarily conserved nuclear receptor *Hormone receptor-like in 38* (*Hr38*, human *NR4A2*), which is known to regulate *Pdf* expression. Furthermore, behavioral assays determined that exposing *Drosophila* to DBP throughout development modifies the circadian rhythm of adults. Although DBP inhibits the expression of signaling systems regulating vision, including *Rh5* and *Rh6*, two light-sensing G-protein coupled receptors involved in the daily resetting of circadian rhythm, it does not influence eye development. Circadian rhythm genes are well conserved from flies to humans; therefore, we tested the effect of DBP exposure on human breast cells (MCF10A) and demonstrate that, similar to the fruit fly model, this exposure disrupts circadian rhythm (*BMAL1* expression) at doses that promote the proliferation and migration

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ability of MCF10A cells. Our results are the first to provide comprehensive evidence that DBP interferes with circadian rhythm in both adult *Drosophila* and human cells, which may help to explain the broad physiological action of phthalates.

## 1. Introduction

Endocrine disrupting chemicals (EDCs), which include plasticizers, are prevalent in the environment and can induce severe deficits in reproduction, development, as well as provoke neural abnormalities (Colborn et al., 1993). Plasticizers, which have been produced for nearly 100 years and are found in a variety of everyday products, do not form covalent bonds with plastics (Bertelsen et al., 2013), which allows them to leach from the plastic into their surroundings. Currently, the annual global production of one subfamily of plasticizers, the phthalates, is over 470 million pounds (Serrano et al., 2014). The use of dibutyl phthalate (DBP) has been widespread in the perfume, cosmetic, shampoo and medical device industries.

Even though the use of DBP in cosmetic products has been forbidden in Europe since 2005, its persistence in the environment, including drinking water, means there is continued long-term human exposure through inhalation, oral, and dermal uptake (Dominguez-Morueco et al., 2014; Santana et al., 2014). Although, many epidemiological studies have associated circulating levels of phthalates and their metabolites with various health problems, including infertility and polycystic ovary syndrome (PCOS), precocious puberty, hormone dependent tumors, and several metabolic disorders (Huang et al., 2014; Kim and Park, 2014; Lind et al., 2012a; Lind et al., 2012b; Seidlova-Wuttke et al., 2005; Sun et al., 2014), none of these studies found a clear molecular link between phthalate exposure and these health risks.

In humans, uptake of toxic compounds is not only from direct inhalation and ingestion, but also from bioaccumulation in the food chain, such as the migration from packaging into food and water (Dopico-Garcia et al., 2007; Wagner and Oehlmann, 2009). Analyses keep being developed and an increasing number of research centers undertake efforts to determine the content of DBP in versatile matrices. For example, in China the DBP content in soil samples has been detected at levels ranging from 2.75–29.4 mg/kg (Xu et al., 2008); in the study of Gao et al. (Gao et al., 2019), it was shown that DBP content in wheat tissues is elevated (when compared to stem, leaves and roots). This all means that not only higher organisms, but also plants and invertebrates are constantly exposed to relatively high doses of phthalates affecting their feeding, sleeping, breeding rates.

The circadian rhythm is a ubiquitous biological phenomenon, found in organisms from prokaryotes to higher eukaryotes. These rhythms depend on self-sustaining molecular transcriptional/post-translational feedback loops (TTFLs) (Darlington et al., 1998; Guo et al., 2016; Rosbash et al., 2007). At the organismal level, periodic expression of circadian system proteins drives behavioral and physiological rhythms. In fact, there is evidence that more than 40% of all protein coding genes show circadian rhythm-dependent transcription (Cedernaes et al., 2018). In mammals, the central clock is located in neural networks of the hypothalamic suprachiasmatic nuclei (SCN), which receive photic information from the retina to orchestrate the circadian program (Zhang et al., 2014). The circadian feedback, central to mammalian TTFLs, involves the transcription factors CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like 1), which drive the expression of PERIOD (Per1/2) and TIMELESS (Tim), whose protein products in turn feed-back to inhibit CLOCK and BMAL1 (Mohawk et al., 2012). These four factors regulate thousands of downstream clock-controlled genes, which further regulate the oscillation of tissue-specific physiological and metabolic functions (Reppert and Weaver, 2002).

The *Drosophila* and mammalian circadian clocks are well conserved at the level of homologous genes (Clock, Bmal1, Period, and Timeless) (Kudo et al., 1991). In *Drosophila*, the evolutionarily conserved Clock (CLK) and Cycle (CYC, BMAL1 in humans) proteins promote rhythmic transcription of several key genes in order to drive circadian oscillations. These CLK-CYC regulated genes include *period* (*per*) and *timeless* (*tim*), which after translation, feedback to repress CLK-CYC-regulated transcription (King and Takahashi, 2000). Pigment dispersing factor (PDF), one of the most extensively studied *Drosophila* neuropeptides, is involved in regulating the circadian system (Allada and Chung, 2010; Shafer and Yao, 2014; Talsma et al., 2012; Umezaki et al., 2012; Vecsey et al., 2014; Wulbeck et al., 2008; Yoshii et al., 2009). PDF neurons receive an environmental (light) or circadian signal that induces the release of PDF to signal to PDFR-expressing neurons. PDFR signaling changes internal cAMP levels, leading to the degradation of the circadian regulator Timeless (TIM). This leads to an effect on circadian molecular oscillations through changes in total PER/TIM protein levels. Therefore, PDF exerts a widespread effect on the network and is essential for normal circadian activity patterns in light: dark cycles (LD), as well as for persistent circadian rhythms in constant darkness (DD) (Allada and Chung, 2010; Shafer and Yao, 2014; Talsma et al., 2012; Umezaki et al., 2012; Vecsey et al., 2014; Wulbeck et al., 2008; Yoshii et al., 2009).

*Hr38* is the *Drosophila* orthologue of the mammalian nuclear hormone receptor *NR4A2*, also known as *NURR1*. It was discovered that Hr38 signals downstream of CLK in PDF expressing neurons to inhibit *Pdf* transcription (Mezan et al., 2016; Zhang et al., 2010). In short, PDF feeds back to PDF expressing neurons to inhibit its own expression via a PDFR—CLK—Hr38 pathway. The drosophila model has been very useful to delineate conserved molecular endocrinological mechanisms (Williams et al., 2020) and for sleep studies (Williams et al., 2016a; Williams et al., 2016b).

In order to understand the DBP exposure effects in biological systems, we employed the model organism *Drosophila melanogaster* as our primary experimental model. To do this, we first performed whole transcriptome sequencing using *Drosophila* males exposed to different environmentally relevant concentrations of DBP throughout development. This provided a number of lead candidate genes, many involved in the regulation of circadian rhythm. Subsequently, we performed functional characterization of the effect of DBP exposure on whole animal and human cell lines to better understand how DBP may affect circadian rhythm.

## 2. Materials and methods

### 2.1. Fly strains and maintenance

Wild-type strains *Canton S* and *Oregon R-C* flies, obtained from the Bloomington stock center (Bloomington, Indiana, USA), were crossed together to create the *CSORC* wild-type lab strain. $y^1$ $w^*$; P{Pdf-GAL4.P2.4}2 (*Pdf-GAL4*), $y^1$ $w^*$; P{w[mC] UAS-TrpA} (*UAS-TrpA1*) and $w^*$; P{UAS-Hr38.miRNA}attP16/CyO (*UAS-Hr38RNAi*) were also obtained from the Bloomington stock center (Bloomington, Indiana, USA). Fly stocks were maintained on Jazz-mix *Drosophila* food (Fisher Scientific, Stockholm, Sweden), supplemented with yeast extract (Genesee Scientific, San Diego, California, USA), at 25 °C at 60% humidity, on a 12/12 h light:dark cycle. Due to the fact that the GAL4/UAS system performs better at higher temperatures, flies crossed to the *Pdf-GAL4* driver and controls were raised at 30 °C. All assays were performed at 25 °C. In all

assays, the GAL4 driver and UAS transgenic flies were crossed to $w^{1118}$ flies and their F1 progeny used as controls (Cao et al., 2016; Williams et al., 2014; Williams et al., 2016c).

## 2.2. Dibutyl phthalate feeding experiments

Dibutyl phthalate (DBP, CAS no. 84-74-2, Sigma-Aldrich, Stockholm, Sweden) solution, one ml, was diluted in 50 ml Jazz-mix *Drosophila* food to produce master stock solution. The final concentrations of DBP solutions used in different diets ranged from 45 nM to 450 nM. Equally aged flies maintained on food containing no DBP served as the control group. Mated females were allowed to lay eggs on the food and larvae were raised either on normal lab food or food containing the various concentrations of DBP. Male flies were collected within one day after eclosing and aged on either normal food (controls) or the required DBP diet for 5 to 6 days at 25 °C, 60% humidity, on a 12/12 light:dark cycle before being subjected to the experimental procedures.

## 2.3. Library preparation and sequencing

### 2.3.1. Primary processing of SOLiD RNA-Sseq reads

RNA-seq reads for whole transcriptome were obtained using SOLiD 5500xl paired end sequencing from life technologies. This version produces read length of 75 bp for fragment libraries with the alternative to sequence an additional 35 bp in the reverse direction (paired-end sequencing). The samples were divided into six libraries and since the libraries were molecularly bar-coded the separation of libraries for control and experimental samples was done effortlessly using respective barcodes. The initial quality analysis was performed using a propriety tool 'XSQ Tools package' provided by the life technologies. This package also provides tools for converting files from XSQ to csfasta format and additionally provides qual files containing read quality information. Further analysis was done using the 'Tuxedo suit' (Ghosh and Chan, 2016; Trapnell et al., 2012) composed mainly of three tools TopHat, Cufflinks and CummRbund.

## 2.4. Mapping of RNA-Seq reads using TopHat

TopHat (v2.0.6) incorporates an ultra-high throughput short read aligner Bowtie (version 0.12.7) (Langmead et al., 2009a; Langmead et al., 2009b) as alignment engine. Based on the provided quality control information from the TopHat homepage (http://ccb.jhu.edu/software/tophat/index.shtml), TopHat (v2.0.6) removes a portion of reads and maps the remaining reads to reference genome. Reads were then aligned to the *D. melanogaster* reference genome (build dmel_r5.47_FB2012_05) obtained from flybase using TopHat with the prebuilt bowtie index downloaded from the TopHat homepage (see above website). Transcript assembly and abundance estimation using Cufflinks. The aligned reads were then processed by Cufflinks v2.0.2. Cufflinks tool estimates the relative abundances of transcripts based on how many reads support each transcript, taking into account biases in library preparation protocols and reports it in "fragments per kilobase of transcript per million fragments mapped" or FPKM. Cufflinks constructs a minimum set of transcripts describing the reads in the dataset rather than using existing gene annotation allowing Cufflinks to identify alternative transcription and splicing. However, it should be noted that Cufflinks is dependent on the provided genome annotation and therefore the reported FPKM values relate only to genes described and genes missing in the annotation description file will not be reported.

## 2.5. Differential expression testing using cuffcompare and cuffdiff

Cuffcompare was used to produce combined General Transfer Format (GTF) files of the six libraries. The GTF files were then passed to Cuffdiff along with original alignments obtained with TopHat. Cuffdiff re-estimates the abundance of transcripts listed in GTF files using alignment files. The differential expression is checked for genes, transcripts and isotopes. By tracking changes in the relative abundance of transcripts with a common transcription start site, cuffdiff can identify changes in splicing. Cuffdiff learns how read counts vary for each gene across the replicates and uses these variance estimates to calculate the significance of observed changes in expression. The calculated P and q values (the FDR-adjusted *P* value of the test statistic) from cuffdiff were used to determine significance of differential expression. The significance depends on whether P is greater than the false discovery rate (FDR) after a Benjamini-Hochberg correction (Psarros et al., 2005) for multiple testing.

## 2.6. Immunocytochemistry and imaging

Fly samples were collected 2 h after lights on in the morning. *Drosophila* adult brains were dissected in phosphate-buffered saline (PBS) and then fixed in 4% ice-cold paraformaldehyde (PFA) for 4 h, and subsequently rinsed in PBS for 1 h. Samples were then incubated at 4 °C in primary antibodies diluted in PBS with 0.5% Triton X-100 (PBST) for 2 days. After rinsed in PBST for four times (each time 15 min) at room temperature, the tissues were incubated with secondary antiserum for 2 days at 4 °C. Finally, after a thorough wash in PBST, all samples were then mounted in 80% glycerol with 0.1 M PBS. Images were captured with a Zeiss LSM 710 confocal microscope (Jena, Germany) using 20× or 63× oil immersion objectives. Confocal images were processed Fiji (https://imagej.nih.gov/ij/). The primary antisera rabbit anti-pigment-dispersing hormone (1:3000) were obtained from H. Dircksen, Stockholm, Sweden. The goat anti-rabbit Alexa 546 secondary antisera were used.

## 2.7. RNA extraction

The phenol-chloroform method was used for RNA extraction from flies' tissue samples and MCF10A cells (Chomczynski and Sacchi, 2006), then samples were mixed thoroughly followed by 12,000 *g* centrifugation for 15 min at 4 °C. The aqueous layer, which contained RNA, was separated and transferred into 500 μl of isopropanol (CAS no. 67-63-0, Solveco AB, Rosersberg, Sweden). The precipitation of RNA was done at −20 °C for 1 h or longer and then were centrifuged at 12,000g for 10 min at 4 °C after precipitation. The RNA pellets were washed with 75% ice-cold ethanol for three times and then air dried for 15 min. RNase-free DEPC water was used to re-suspend the RNA samples. RNA concentration was measured using Multiscan GO spectrophotometer (Thermo Scientific, Stockholm, Sweden). cDNA reverse transcription was performed using high-capacity RNA-to-cDNA kit (Applied Biosystems, cat. no. 4387406).

## 2.8. cDNA synthesis

High-capacity RNA to cDNA kit was used for cDNA synthesis (Applied Biosystems, Sweden) and performed accordingly to manufactures instructions.

## 2.9. qRT-PCR

Relative expression levels of housekeeping genes (GADPH, *EF-1*, *Rp49* & *RpL11*) and of the genes of interest were determined with quantitative qRT-PCR. Each reaction vessel, with a total volume of 20 μl, contained 20 mM Tris/HCl pH 9.0, 50 mM KCl, 4 mM MgCl$_2$, 0.2 mM dNTP, DMSO (1:20) and SYBR Green (1:50,000). Template concentration was 5 ng/μl and the concentration of each primer was 2 pmol/L. Primers were designed with Beacon Designer (Premier Biosoft, Palo Alto, CA, USA) using the SYBR Green settings. All qPCR experiments were performed in triplicates; for each primer pair, a negative control with DEPC water was included on each test-plate. Amplifications were performed with 0.02 μg/ml Taq DNA polymerase (Biotools, Madrid,

Spain) under the following conditions: *Drosophila*: Initial denaturation at 95 °C for 3 min, 50 cycles of denaturing at 95 °C for 15 s, annealing at 52–63 °C for 15 s and extension at 72 °C for 30 s. *MCF10A Cells*: Initial denaturation at 95 °C for 3 min, 45 cycles of denaturing at 95 °C for 15 s, annealing at 62 °C for 15 s and extension at 72 °C for 30 s. Analysis of qPCR data was performed using MyIQ 1.0 software (Bio-Rad, Solna, Sweden) as previously reported (Lindblom et al., 2006). Primer efficiencies were calculated using LinRegPCR (Ramakers et al., 2003) and samples were corrected for differences in primer efficiencies. Double delta Ct was calculated and referred to as the relative expression level of each gene in different groups. For sequences of primers used for qPCR please refer to electronic supplementary file.

### 2.10. Cell lines and culture procedures

The MCF10A human mammary epithelial cell line is a widely used in vitro model for studying breast cell function and transformation, which was donated by Professor Anna-Karin Olsson from Department of Medical Biochemistry and Microbiology (IMBIM), Uppsala Biomedical Center (BMC), Sweden. MCF10A cells were maintained as a monolayer in 100 mm$^2$ tissue culture plastic flasks containing 12 mL of growth medium, trypsinized (0.25%) and split 1:4 every 3 days. Complete growth medium consisted of Dulbecco's Modifies Eagle Medium with F-12 (DMEM/F-12) supplemented with horse serum (5%), hydrocortisone (0.5 mg/mL), CT (100 ng/mL), insulin (10 mg/mL), EGF (20 ng/mL), and 5 mL P/S. Cell cultures were maintained at 37 °C and 5% $CO_2$ in a humidified incubator.

### 2.11. Exposure of MCF10A cells to DBP

Based on the data for DBP toxicity to humans in European chemical agency (ECHA (https://echa.europa.eu/brief-profile/-/briefprofile/100.001.416), the recommended No- or Minimal Effect Level (DN(*M*)EL) are: 1) Long-term DERMAL Exposure: (DNEL) 70 μg/kg bw/day; 2) Long-term ORAL Exposure: (DNEL) 7 μg/kg bw/day, for instance, for a person with 70 kg weight, the converted concentrations are DERMAL Exposure 251.5 nM ($7 \times 10^{-4}$% w/v) per day and ORAL Exposure 25.15 nM ($7 \times 10^{-5}$% w/v) per day, respectively. Thus, in order to test the DBP exposure effects on human cells, we chose a concentration gradient around the DN(*M*)EL.

MCF10A cells were trypsinized and resuspended in growth medium, plated in 96-well tissue culture plates ($2 \times 10^4$ cells/well), and allowed to attach for 24 h in a 5% $CO_2$ humidified incubator at 37 °C. After 24 h, the cells were treated with different concentrations of DBP (0.1 nM, 1 nM, 10n M, 0.5 μM, 1 μM, 10 μM, 25 μM, 50 μM, 100 μM, 250 μM and 500 μM) dissolved in DMSO and assay medium (growth medium without the horse serum and EGF). The cells were then incubated for 24, 48 and 72 h. All experiments were repeated three times. Details on the cell viability and cell migration assays are given in electronic supplementary file.

### 2.12. Circadian rhythm assay

#### 2.12.1. Drosophila melanogaster

All the flies used in circadian rhythm assays were continuously raised in vials containing the corresponding DBP food, from eclosion and aged for 5 days. For performing the circadian assay, the Drosophila Activity Monitoring System (DAMS) was employed. Each individual fly was placed in a capillary tube, which was then monitored by the DAMS at 25 °C, 60% humidity. The capillary tube had, on one end, the same DBP-spiked food as described in the feeding procedure, and on the other end was blocked by a cotton plug after the fly was put into it. Thirty-two flies for each test group were used. When the DAMS slot with the capillary tube was plugged in, the beam breakings of the fly were recorded per minute and referred as the fly's activity. For examining the rhythmicity, the flies were allowed to acclimate to the capillary

overnight and then followed by three constant dark:dark cycles. After the constant dark period, the cycle returned back to 12 h/12 h light: dark to assess if they were able to reset their rhythmicity. For the *Pdf-GAL4>UAS-TrpA1* flies, the ambient temperature was 20 °C to inhibit TrpA1. Only for the rescue assay, the temperature was set at 30 °C between ZT3 and ZT7 to activate TrpA1 channel and eventually trigger the activation of PDF neurons. All data, except the habituation period, were analyzed and plotted by DAMFileScan and MATLAB based tool kit-S.C.A.M.P.

#### 2.12.2. Cell lines

Details on MCF10A cells studies with DBP are given in the electronic supplementary file.

### 2.13. Statistical analysis

Mean and standard errors from all replicates of each experiment were calculated. All analysis was performed with GraphPad Prism 4, and ANOVA was used with appropriate post hoc analysis for multiple comparisons (refer to figure legends for which analysis was performed for each assay).

## 3. Results

### 3.1. DBP exposure effects in Drosophila system

To understand how the DBP influences biological systems, we raised wild-type *Drosophila* on food containing 45 nM DBP from hatching until the adults were 5–7 days old. Males were then collected before performing whole transcriptome sequencing. By mapping the *Drosophila* transcriptome to the reference genome obtained from flybase (build dmel_r5.47_FB2012_05), 15,147 transcripts were identified, including expressed genes (mRNA), miRNA, snRNA, snoRNA and tRNA. In this experiment, 215 genes were differentially expressed, taking into consideration the 'false discovery rate' and correcting for it using Benjamini-Hochberg correction method.

After performing pair-wise comparisons between all sequenced conditions, 171 differentially expressed genes remained significant (Supplementary Table 1). Using the metabolic and biological functions categories in Flybase, we categorized the differentially expressed genes by function. Besides genes with unknown function, the largest groups were "Other biological functions", which contain many genes involved in regulating ATP production, and "Response to stimulus", most of which were involved in the signal response to light stimuli. The next largest group was "Nervous system processes", which mostly contained genes involved in vision or pheromone signaling. Finally, there was a group, "Behavior", which contained genes involved in the regulation of circadian rhythm. Interestingly, two of the Rhodopsin receptors in the "Nervous system processes" group, *Rhodopsin 5* (*Rh5*) and *Rhodopsin 6* (*Rh6*), are also involved in the regulation of circadian rhythm (Hanai et al., 2008; Rodriguez Moncalvo and Campos, 2005).

Next, using STRING we identified all interacting clusters between the genes listed in Supplementary Table 1. STRING identified five clusters of gene networks (Clusters 1a and 1b, 2, 3 and 4) (Fig. 1A and B). Cluster 1a consisted of genes involved in light sensing and signaling, including Rhodopsin receptors (*Rh3*, *Rh4*, *Rh5* and *Rh6*), as well as two Arrestins involved in Rhodopsin GPCR recycling (*Arr1* and *Arr2*) (Fig. 1A and B). Furthermore, *ninaD* - a CD36-like scavenger receptor involved in carotenoid transport necessary for proper vision, was also found in this cluster (Fig. 1A and B). The expression of all genes in this cluster was inhibited by exposure to DBP (Fig. 1B). Genes in Cluster 1b were also involved in the visual transduction process. This included G-proteins (*Gaq*, *Gβ76C*, *Gγ30A* and *CG30054*), most of which are known to signal downstream of Rhodopsin GPCRs in visual transduction. Moreover, two kinases known to be involved in regulating visual transduction *inaC* (a Protine Kinase C) and *ninaC* (a Serine-Threonine Protein Kinase)
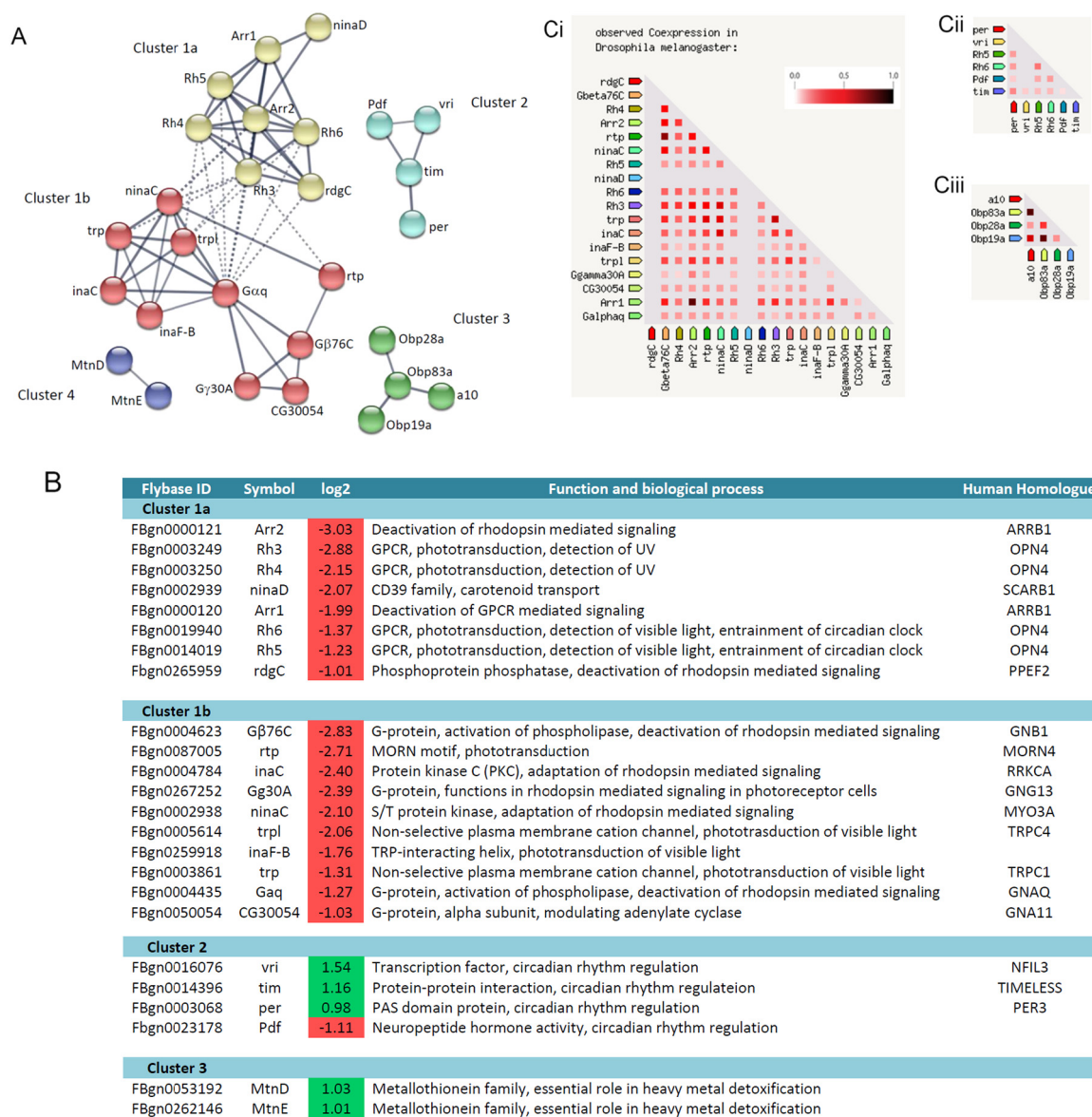
**Fig. 1.** DBP regulates conserved vision and circadian rhythm pathways. (A) String was used to find clusters of interacting genes in the DBP sequencing data. Four clusters were found, including Cluster 1 (consisting of 1a Rhodopsin molecules, their regulators and a retinol transporter; 1b, consisting of molecules that signal downstream of Rhodopsin in vision), Cluster 2 consists of molecules that are direct regulators of circadian rhythm. Cluster 3 includes only pheromone binding proteins. Cluster 4 consists of two metal binding proteins. (B) Whole transcriptome data for the four clusters of genes from DBP fed versus controls 5–7-day old whole males. Interestingly all genes involved in regulating vision are inhibited by DBP exposure during development. (C) String program was used to determine expression overlap for either (Ci) clusters 1a and 1b, (Cii) Cluster 2 or (Ciii) Cluster 3.

were also found in this cluster (Fig. 1A and B). Finally, two cation channels, *trp* and *trpl*, involved in visual signal transduction were in this cluster, (Fig. 1A and B). As with Cluster 1a, all genes in Cluster 1b were downregulated by exposure to DBP (Fig. 1B). Cluster 2 contained four genes involved in the regulation of circadian rhythm, including *vrille* (*vri*), *timeless* (*tim*), *period* (*per*) and *Pigment-dispersing factor* (*Pdf*) (Fig. 1A and B). Looking through the significantly up or down regulated genes identified two other genes involved in regulating circadian rhythm, *Glutamic acid decarboxylase 1* (*Gad1*) and *cryptochrome* (*cry*), which fell below the log2 cutoff of 1.0 to be included in Supplementary Table 1. All together, this made eight genes regulated by DBP exposure that were directly connected to the regulation of circadian rhythm (*Rh5, Rh6, vri, tim, per, Pdf, Gad1* and *cry*).

To validate if genes in the various clusters were interacting, we used the STRING expression function to look for overlaps. For this assay we put Cluster 1a and 1b together to make a supercluster. Of all the genes in this supercluster, only *ninaD* failed to overlap with any of the other genes (Fig. 1Ci). This is not surprising since unlike all the other genes

in the supercluster, which are directly involve in visual signal transduction, *ninaD* is necessary to transport carotenoid, indispensable to produce retinol for the proper function of light-sensing Rhodopsin receptors (Wang et al., 2007). In Cluster2, all of the genes overlapped with at least one other gene, and in Cluster 3 all of the genes overlapped (Fig. 1Cii and Ciii).

### 3.2. DBP exposure disrupts circadian rhythm

To verify the genetic impacts that we observed in the transcript levels, we tested the possible effects on circadian rhythm. To uncover any possible DBP exposure effects on *Drosophila* circadian rhythm we used a concentration gradient (0 nM, 45 nM, 135 nM or 450 nM) to expose flies and then collected the F1 flies after eclosion. To test for effects on circadian rhythm the flies were first maintained in constant darkness for 72 h, then switched to a normal 12:12 light:dark cycle for 72 h. This experimental protocol can evaluate both rhythmic maintenance and circadian resetting. Although, the circadian oscillation of control flies

maintained in constant darkness were weakened (Fig. 2A), males raised on 45 nM, 135 nM or 450 nM DBP were not able to maintain any semblance of normal circadian rhythm when left in constant darkness (Fig. 2B–D). In flies maintained on 135 nM or 450 nM DBP, the siesta, a dip in activity between Zeitgeber times 4 to 8, is absent (Fig. 2C and D). Interestingly, once the flies were switched to a normal 12/12 h light:dark cycle, the circadian rhythm at all DBP concentrations returned to normal, which indicated that DBP might not impinge on the circadian reset.

It was previously published that loss of rhodopsin expression in the eye can lead to retinal denegation (Wang and Montell, 2007). Although the DBP exposed flies were able to reset their rhythmicity, it does not exclude the possibility that DBP could disrupt eye development and cause circadian arrhythmicity. Therefore, we decided to perform transmission electron microscopy to look at the rhabdomeres in adult eyes, and compare those from males raised on normal food with those from males raised on food containing 135 nM DBP (Fig. 2E–H). To do this, the flies were maintained on a normal 12/12 light:dark cycle for the first four days after eclosion, but in order to stress the visual system all flies were kept in constant light for the last 24 h (day 5). When comparing eyes from control males (Fig. 2E and F) with those from males constantly maintained on DBP-containing food (Fig. 2G and H), it became obvious that, even though the expression of many genes involved in visual transductions, including four rhodopsin receptors, were significantly inhibited
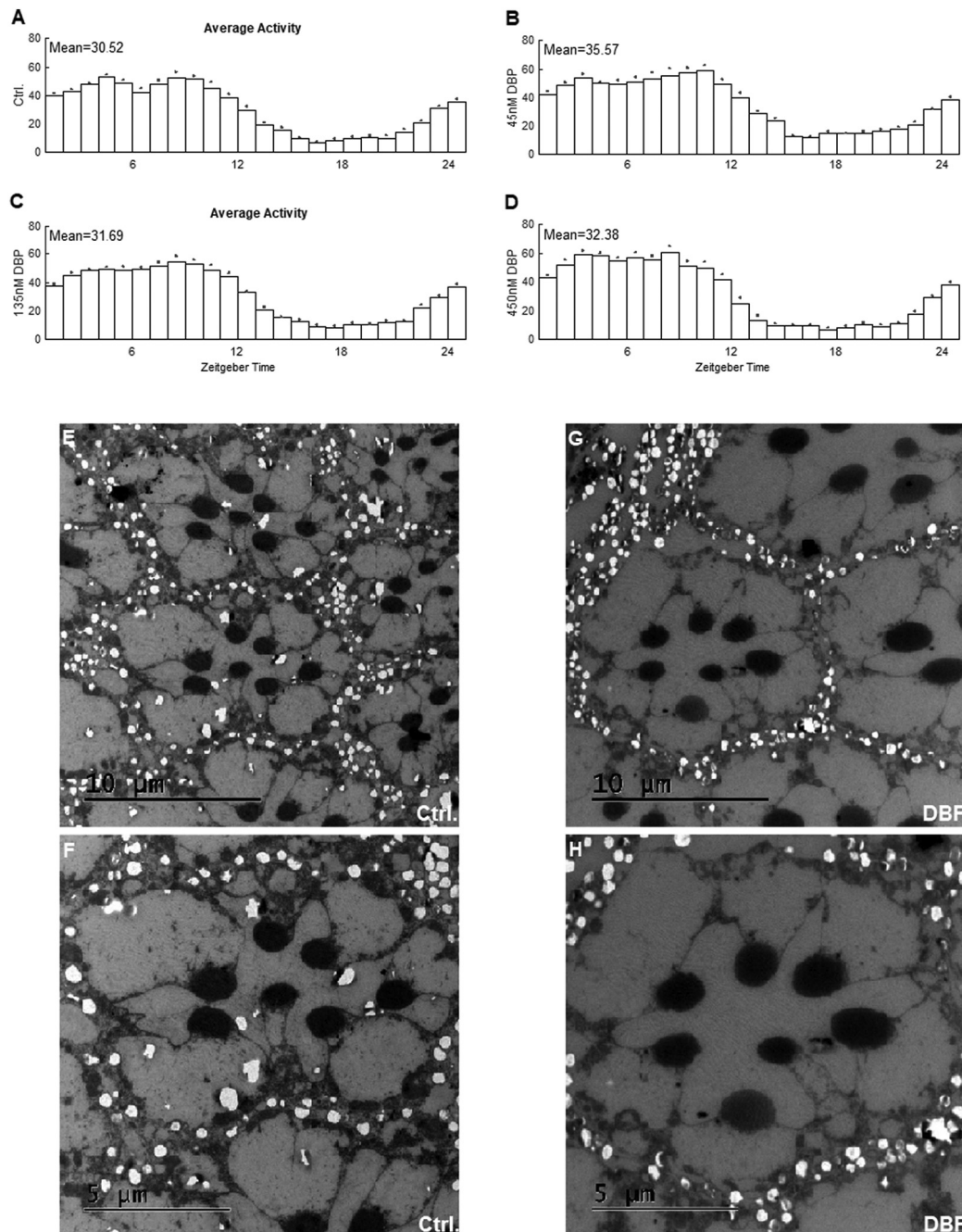


**Fig. 2.** DBP affects circadian pattern, but does not affect eye morphology. The average activity of DBP fed wild-type flies in constant darkness was accessed. Although the activity pattern was weakened, the control fed flies (A) were able to maintain their activity for at least 72 h. However, the 45 nM (B), 135 nM (C) and 45 0 nM (D) DBP fed flies lost their rhythmicity, which was shown as missing of siesta period. Additionally, transmission electron microscopy (TEM) of sectioned adult *Drosophila* eyes was shown. Flies were raised on either control (E and F) or 135 nM DBP containing food (G and H). Flies were aged 4 days in normal 12:12 light:dark conditions after emergence as adults from the pupa case. They were then raised in constant light for 24 h before being collected for TEM. When the eyes of control and experimental flies are compared, there are no striking differences. At least 10 flies for each condition were compared.

by DBP, this had no effect on eye morphology (compare Fig. 2E with G, or F with H).

### 3.3. DBP exposure affects clock gene expression

It is well known that the clock genes oscillate over a 24-hour period. Our whole transcriptome sequencing data hinted at a potential circadian disruption by DBP. Therefore, to understand how DBP may disrupt circadian rhythm, and confirm our sequencing results, we used quantitative RT-PCR (qPCR) to carefully look at the expression levels of key circadian regulatory genes. The wild type flies were raised on varying

concentrations of DBP and collected at different times of day to see how DBP affected the expression levels of the circadian regulatory genes. Interestingly, DBP had a strong effect on both *Pdf* and *cry* at Zeitgeber Time (ZT) 2 (Fig. 3A and B, $P < 0.05$ for all comparison expect 45 nM DBP fed *cry* vs. control). Other than ZT2, DBP exposure affected *pdf* expression at ZT6 and ZT14 while *cry* expression was also inhibited as ZT9. Expression levels of the other three genes tested (*tim*, *per* and *vri*) were also significantly affected (Fig. 3D–E). *Tim* was upregulated mainly at the late ZT times. At ZT15 and ZT18, *tim* expression was significantly increased in most DBP concentrations (Fig. 3C). *Per* expression was strongly influenced at ZT2 and ZT15 (Fig. 3D). Specially, *per* had
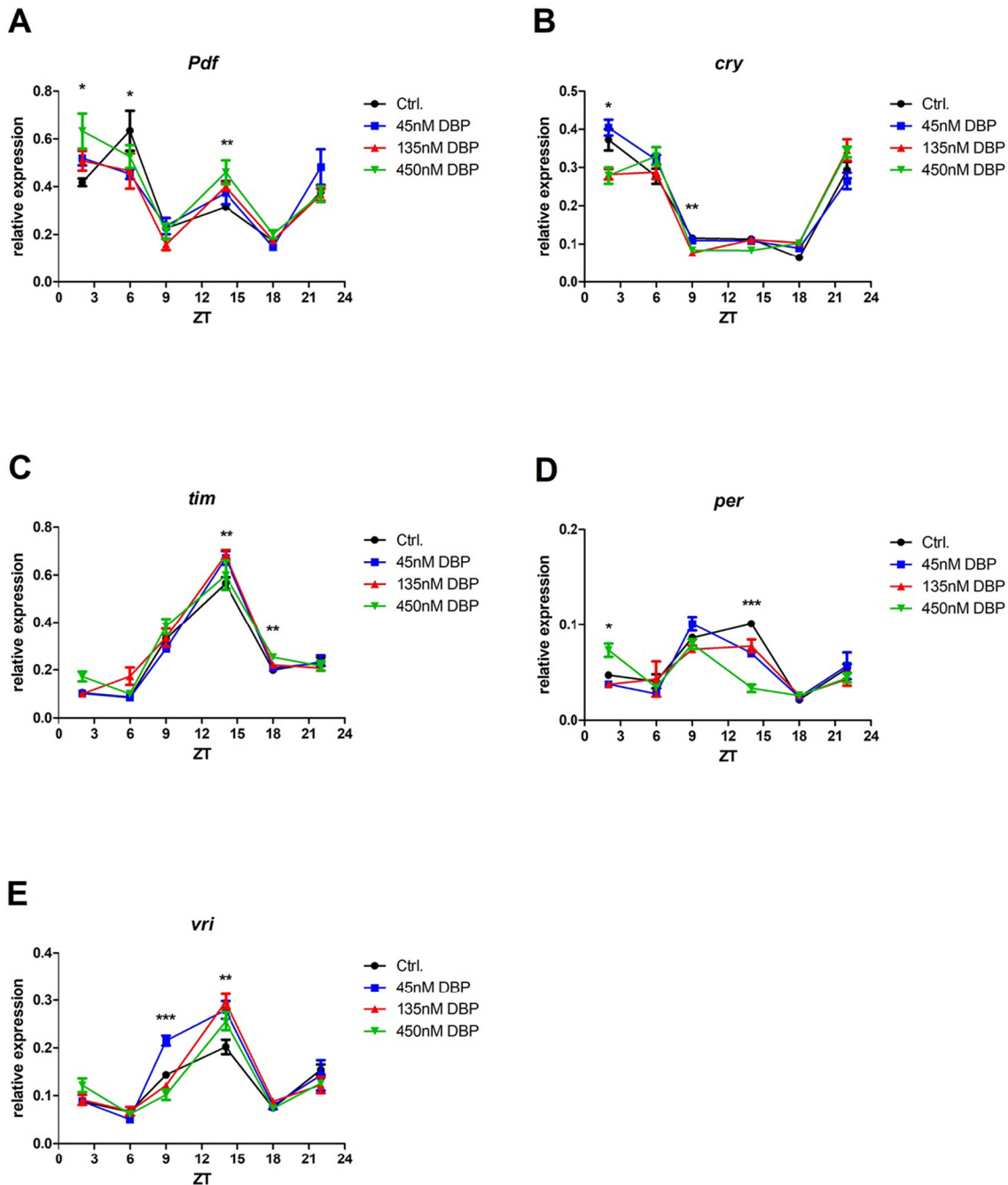


**Fig. 3.** DBP disrupts circadian regulation genes at transcriptional level. Relative expression levels of circadian rhythm genes in 5–7-day old adult males raised on food containing various concentrations of DBP. *Pdf* (A), *cry* (B), *tim* (C), *per* (D) and *vri* (E). The qPCRs were performed at least twice with more than 6 replicates in each feeding group. Error bars indicate SEM. ($n =$ 25 males head per treatment; $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ compared to normal fed controls, detailed comparison was described in the *results* part, one-way ANOVA with Tukey's post hoc test for multiple comparisons).

elevated in 450 nM DBP fed flies at ZT2 ($P < 0.05$) and notably diminished in all concentrations at ZT15 ($P < 0.001$). *Vri* was upregulated at ZT9 and ZT15 in males raised on food containing 45 nM DBP (Fig. 3F).

### 3.4. Pdf and Hr38 as the key molecules respond to DBP exposure

Since the DBP exposed flies were able to reset their biological clock, we hypothesized the circadian disruption during the daytime may be caused by a disruption in *pdf*-related signaling. To understand if DBP exposure disrupted circadian rhythm by affecting PDF neurons, we expressed a temperature sensitive cation channel TrpA1 specifically in PDF neurons (*Pdf-GAL4>UAS-TrpA1*) and maintained the flies at 30 °C from ZT3 to ZT7 to activate the neurons. For both control and experimental crosses, flies were fed on either normal food or food that contained 135 nM DBP. Due to the fact that an increase in ambient temperature would elevate the general activity of flies, the circadian pattern from ZT0 to ZT3 was omitted. Obviously, the average activity of all groups was increased from ZT4 (Fig. 4). When normal fed and DBP fed control crosses were compared, the circadian pattern was shown to be influenced by DBP exposure (compare Fig. 4A and D). However, activation of PDF neurons during the siesta period rescued the circadian arrhythmicity in *Pdf-Gal4>UAS-TrpA1* DBP fed flies (indicated by arrows, ref. to Fig. 4F, compare to Fig. 4D).

Phthalates have been shown to interact with specific nuclear hormone receptors, such as estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and androgen receptor (AR) (Boberg et al., 2008; Kruger et al., 2008; Nakai et al., 1999; Wojtowicz et al., 2017). Intriguingly, two nuclear hormone receptors are known to be involved in the regulation of circadian rhythm in *Drosophila*, Ecdysone-induced protein 75B (E75B, Human NR1D2) and Hormone receptor-like in 38 (Hr38, Human NR4A2). E75B is involved in regulating the expression of the *vri* gene, while it was reported that *Hr38* is involved in a feedback loop that inhibits the expression of *Pdf* (Fig. 5A) (Kumar et al., 2014; Mezan et al., 2016). To understand if DBP could be disrupting circadian rhythm via these two nuclear hormone receptors, we first used qPCR to carefully look at the expression levels of *Hr38* and *E75B* in males raised on food containing 135 nM DBP. As performed previously, flies were raised on food containing DBP and collected at different times of day to see how DBP affected the expression levels of the nuclear hormone receptors. Interestingly, DBP had a strong effect *Hr38* expression at

ZT6 and ZT15, where *Hr38* expression was significantly decreased at ZT6 and significantly increased at ZT15, when compared to control males (Fig. 5B). DBP had no significant influence on the expression levels of *E75B* (Fig. 5C).

To understand if DBP exposure disrupted circadian rhythm by interacting with *Hr38*, we expressed *Hr38* RNAi specifically in PDF neurons (*Pdf-GAL4>UAS-Hr38* RNAi) and either raised the flies on normal food or food containing 135 nM DBP. Similar to what we observed with wild-type *CSORC* flies, when raised in constant darkness, unlike normal fed controls, control flies (*w1118>Pdf-GAL4* and *w1118>UAS-Hr38* RNAi flies) raised on DBP-containing food were not able to maintain a proper circadian rhythm, which was shown as the failure to anticipate daytime (Fig. 5D–E). Not surprisingly, loss of *Hr38* in PDF neurons was sufficient to disrupt circadian rhythm, even in flies raised on normal food (Fig. 5F). Interestingly, raising these flies on DBP containing food did not disrupt circadian rhythm further, indicating that DBP may be interacting with *Hr38*, either directly or indirectly, to disrupt circadian rhythm (Fig. 5I).

The PDF peptide is one of the main signals in the clock circuit in *Drosophila*, and it is produced by a small set of lateral clock neurons (s-LNv and l-LNv) (Renn et al., 1999). Thus, we used immunohistochemistry to test if DBP exposure effects PDF protein levels in *Drosophila*. To do so, wild-type *CSORC* flies were raised either on control food or food containing DBP at a concentration of 135 nM. Then 6–7 days old flies were harvested 2 h after lights on. The PDF neurons and their branches were examined by immunolabeling (Fig. 6). A representative image of PDF l-LNv neurons from control fed and 135 nM DBP fed flies is presented in Fig. 6A. We found that DBP exposure significantly increased PDF protein levels in the l-LNv neurons (Fig. 6), indicating that DBP exposure may influence the release of PDF peptides from these neurons.

### 3.5. DBP exposure effects in human cells

In the last decades, the effect of DBP on cancer cells was studied extensively, especially in prostate, lung and breast cancer (Chen and Chien, 2014; Saillenfait et al., 2008; Turner et al., 2013), and DBP exposure also inhibits tamoxifen-induced apoptosis in human breast cancer cells (MCF-7) (Kim et al., 2004), suggesting that the environmental pollutant DBP plays a role in the occurrence and development of cancer. Interestingly, there have been suggestions that the modern lifestyle (such
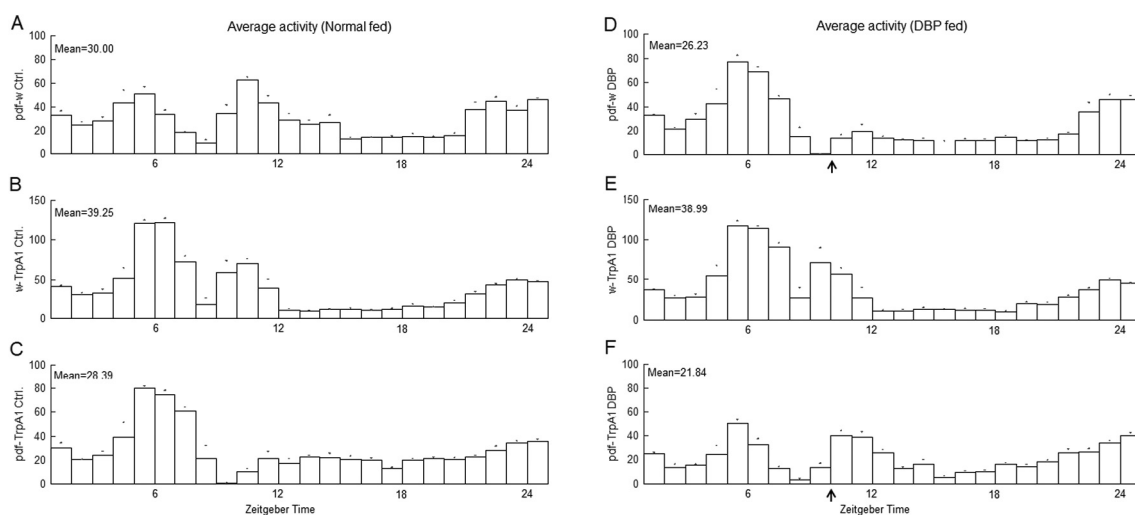


**Fig. 4.** Activating Pdf neurons rescues the DBP induced circadian phenotype. Two genetic control crosses, *Pdf-GAL4>w1118* (A and D) and *w1118>UAS-TrpA1* (B and E), were included. The experimental cross flies, *Pdf-GAL4>UAS-TrpA1* (C and F), overexpressed the temperature sensitive TrpA1 channel in PDF neurons and enabled us to manipulate the activity of Pdf positive neurons by increasing the ambient temperature. Males from all three crosses were raised on either normal (A–C) or on food containing 135 nM DBP (D–F). To activate the TrpA1 channel, and thus hyper-activate the Pdf neurons, flies were maintained at 30 °C between ZT3 to ZT7 every day. This is most easily observed that the evening peak was reduced in (D), compared to (A), which further demonstrated the circadian disruption by DBP exposure. Of note, although the evening peak was reduced in the control fed *Pdf-TrpA1* flies (C) and this phenomenon might result from hyper-activation of Pdf neurons, the DBP-fed *Pdf-TrpA1* flies rescued their circadian pattern (F). (Activities of three constant dark days were averaged from individual flies. N = At least 32 flies. *Pdf-w: Pdf-GAL4>w1118*; *w-TrpA1: w1118>UAS-TrpA1*; *pdf-TrpA1: Pdf-GAL4>UAS-TrpA1*).
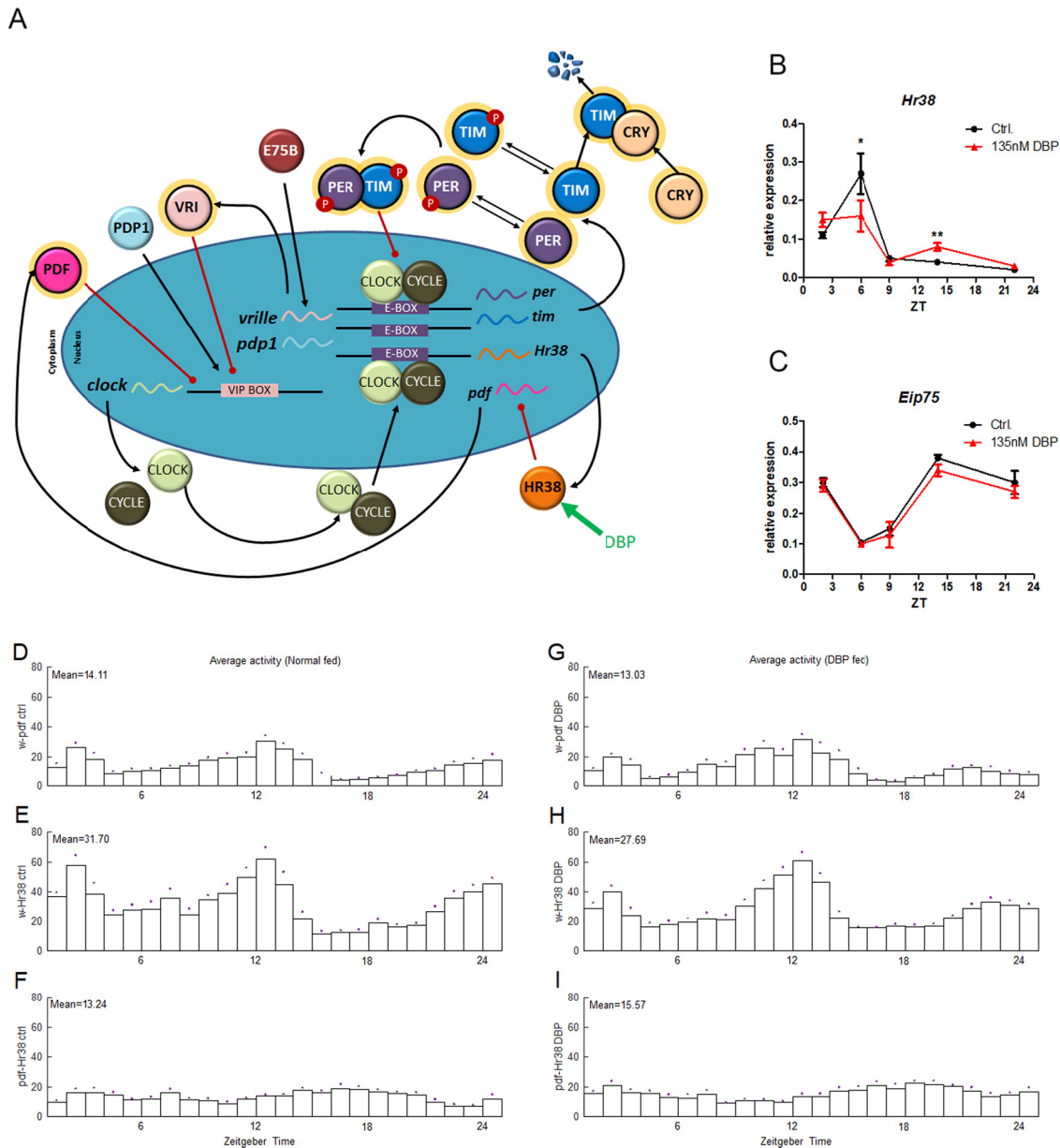
**Fig. 5.** DBP signals through Hr38 to regulate circadian rhythm. (A) Schematic diagram of circadian rhythm regulation in flies. Proteins with yellow circles around them indicate those influenced by DBP exposure in our whole transcriptome sequencing. Relative expression levels of circadian rhythm genes in 5–7-day old adult males raised on food containing 135 nM DBP, Hr38 (B) and E75B (C). The qPCRs were performed at least twice with more than 6 replicates in each feeding group. Error bars indicate SEM. (n = 25 male heads per treatment; *$P < 0.05$ and **$P < 0.01$ compared to normal fed controls, one-way ANOVA with Tukey's post hoc test for multiple comparisons). (D–I) Average activity of male flies from two genetic control group. w1118>Pdf-GAL4 (D and G) and w1118>UAS-Hr38 RNAi (E and H), as well as the experimental group, Pdf-GAL4>w1118 RNAi (F and I) were accessed. For all crosses, male flies were fed on either normal (D–F) or 135 nM DBP food (G-I). (Activities of three constant dark days were averaged from individual flies. $N =$ At least 32 flies. w-Pdf: w1118>Pdf-GAL4; w-Hr38: w1118>UAS-Hr38 RNAi; pdf-Hr38: Pdf-GAL4>UAS-Hr38 RNAi).

as night-shift, repeated bouts of jet lag, etc.) may influence the onset and progression of breast cancer, and epidemiological studies of chronic shift workers suggest that there is a correlation between circadian disruption and breast cancer susceptibility. Evidence also shows that disrupted expression of circadian genes (leading to circadian rhythm disruption) enhances tumor formation in circadian mutant animal models (Papagiannakopoulos et al., 2016) and also promotes breast cancer (Blakeman et al., 2016). The circadian clock controls diverse cellular processes, including cell cycle and DNA damage repair (Shafi and Knudsen, 2019); molecular links that further support the notion disruption of circadian regulation promotes cancer phenotypes. These studies suggest that changes of circadian rhythm (such as clock genes mutation) in humans influence the occurrence and progression of cancer especially for breast cancer. Moreover, humans exposed to air pollution in

circadian manner are reported to have increased risk for pulmonary and cardiovascular events (Haberzettl, 2018).

Therefore, the above evidences motivated us to further study the effects of DBP exposure on the regulation of circadian rhythms in human cells, and also the DBP-induced circadian rhythms changes' effects on cancer such as breast cancer. Human breast cell lines MCF10A is a commonly used line to study circadian rhythm because MCF10A shows self-rhythmic profiles of both clock gene *BMAL1* and *PER2*. Thus, we chose MCF10A cells to do the following tests.

*3.6. DBP exposure affects MCF10A cells proliferation and migration ability*

In order to find the appropriate concentrations for studying circadian rhythm, we looked at proliferation and migration because DBP is

**A**



**B**
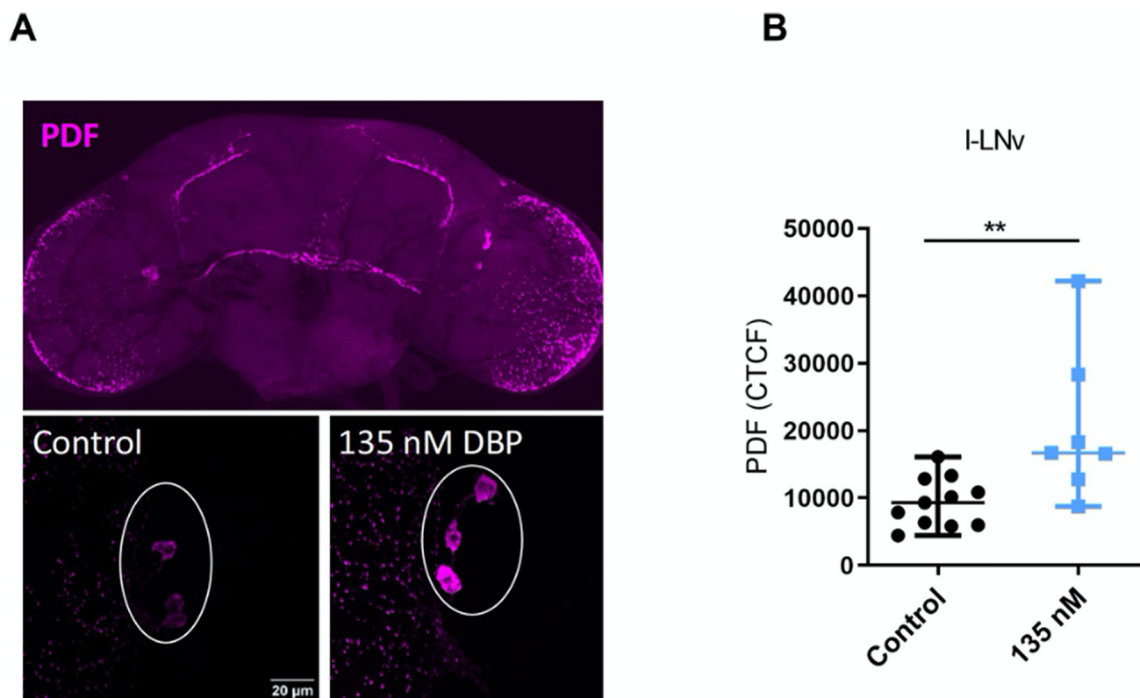


**Fig. 6.** Effects of DBP on PDF signaling. A. PDF neurons and their branches were shown by anti-PDF staining. Represent image of I-LNVs from flies fed with control food or food containing 135 nM DBP. B. Compared to normally fed flies, the PDF levels increased in the I-LNVs of flies fed food with DBP at 135 nM. Error bars indicate SEM. ($n = 7$–10 males from three replicated per treatment; **$P < 0.01$ compared to normal fed controls, unpaired Students' $t$-test).

known to influence these phenotypes in human cancer cells (Chen and Chien, 2014; Saillenfait et al., 2008; Turner et al., 2013), we chose a concentration gradient (0.1 nM, 1 nM, 10 nM, 0.5 μM, 1 μM, 10 μM, 25 μM, 50 μM, 100 μM, 250 μM and 500 μM) to tested the cell proliferation ability (cell viability assay), in order to get the most affective DBP concentration on MCF10A cells. Our results show that DBP-induced cell proliferation is time and concentration dependent (Fig. 7A). Exposure to DBP at low-doses from 1 nM to 50 μM for 72 h significantly increased the cell viability. In contrast, exposure to concentrations equals to 100 μM or higher decreased cell viability at time points 48 h and 72 h (Fig. 7A). Results also show that MCF10A cells exposed to 10 μM significantly increased the cell viability at all time-points. At 72 h, exposure to lower-concentrations, showed significantly higher cell viability rate compared to controls, suggesting an increase in cell proliferation ability. Our results demonstrate that as the exposure time increases, DBP demonstrated a higher MCF10A cells growth rate compared to controls at lower concentrations, especially at 10 μM, which is in good agreement with previous studies showing that perfluorooctanesulfonic acid (PFOS)-induced cell proliferation is dependent on the time and concentration as 10 μM PFOS significantly increased the cell numbers at 24 h, 48 h and 72 h exposure (Pierozan and Karlsson, 2018). Then, to examine the involvement of 10 μM DBP on MCF10A cells migration, we performed a wound-healing migration assay, and the results showed that DBP treatment at 10 μM significantly promoted cell migration of MCF10A cells (Fig. 7B). Our results demonstrated that 10 μM DBP can significantly induce MCF10A cell transformation and thus we used this concentration for the following test.

### 3.7. DBP exposure disrupts circadian rhythm in MCF10A cells

To understand the DBP exposure effects on human breast cell lines' circadian rhythm, we analyzed the clock gene *BMAL1*'s temporal mRNA expression of MCF10A cells, and entrained cell cultures using the well-known serum shock method (Balsalobre et al., 1998; Nagoshi et al., 2004). We measured the expression level of the clock gene *BMAL1* using RT-qPCR in MCF10A cells. Our results show that the *BMAL1* gene exhibits distinctive rhythmicity expression profiles in MCF10A, with a period of around 24 h (see Supplementary Fig. 1) which is also in good agreement with previous results (Rossetti et al., 2012; Xiang et al., 2012).

Then, after 10 μM DBP treatment in MCF10A cells, total RNA was extracted every 4 h up to 24 h and *BMAL1* mRNA expression analyzed by RT-qPCR. As shown in Fig. 8, within 24 h under DBP treatment, clock gene *BMAL1* in MCF10A cells show a significant alteration in the amplitude of its rhythm. Unlike serum shocked MCF10A cells without DBP treatment, the effects of DBP on *BMAL1* gene expression happened very rapidly and the mRNA levels remained generally suppressed nearly over the entire 24 h period. Moreover, following DBP treatment, *BMAL1* gene exhibited a particular rhythmicity profile which suggests a phase shift comparing with the normal MCF10A cells (Fig. 8). Our results suggest that exposure of 10 μM DBP induces a circadian rhythm disruption in human cells, but further investigations is needed to understand the nature of this effect.

## 4. Discussion

Exposure to DBP throughout development caused a significant change in the transcript number of 171 genes in adult *Drosophila* males. Surprisingly, one of the largest groups of transcripts with significant copy number changes are well documented to be involved in the regulation of circadian rhythm. We confirmed the changes by using quantitative real-time PCR for a subset of these circadian rhythm genes. Using behavioral assays, we confirmed that DBP exposure disrupts circadian rhythm in *Drosophila* adults. Furthermore, we demonstrated that the conserved hormone receptor Hr38, which in turn regulates *Pdf* expression (Mezan et al., 2016), is required for DBP circadian disruption in adult flies. Significantly, similar to our fly model, DBP exposure disrupts circadian rhythm in human breast cells.

The genes affected by DBP are well documented to be involved in the regulation of circadian rhythm and most are highly conserved between flies and mammals, including in *vrille* (*vri*, human *NFIL3*), *timeless* (*tim*, human *TIMELESS*) and *period* (*per*, human *PER3*) (see Fig. 1). In

**A**



**B**
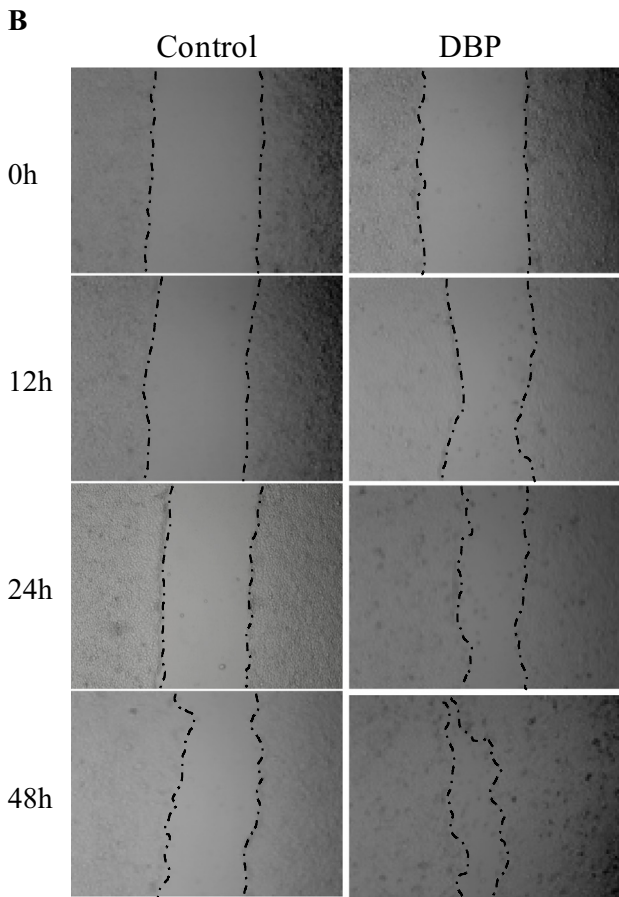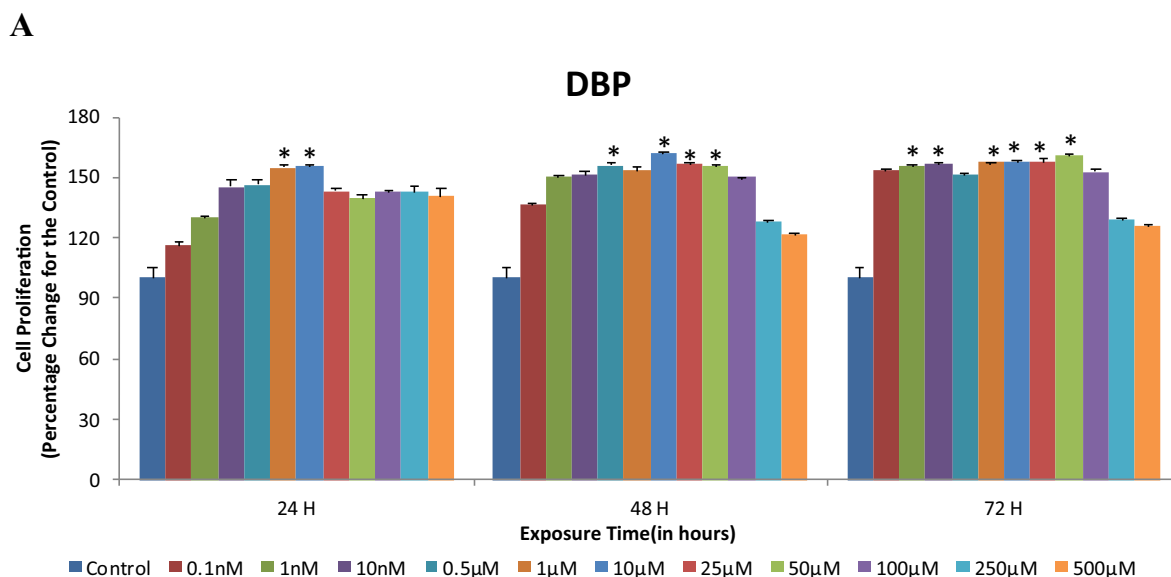


**Fig. 7.** Effects of DBP on the MCF10A cells' (A) proliferation and (B) migration ability. (A) The cells were exposed to 0.1 nM–500 μM DBP for 24, 48 and 72 h. Values represent mean ± SD from three independent experiments. Statistically significant differences from control are indicated as follows: *$P < 0.05$ and One-way ANOVA with Tukey's post hoc test. Error bars = SEM. (B) The cells were exposed to DBP for 0, 12, 24 and 48 h, respectively.

*Drosophila* and mammals, the CLK and CYC (BMAL1 in humans) transcription factors induce the transcription of *per* and *tim*, whose proteins act as a negative feedback to inhibit the activity of the CLK-CYC dimer (see Fig. 5A) (reviewed in Tataroglu and Emery, 2014). The *Drosophila* PDF neuropeptide is released from light-sensitive PDF positive cells (known as M cells) and signals to cells that express the PDF receptor (PDFR), known as E cells, which regulates the expression of TIM protein in the E cells (Hassaneen et al., 2011; Seluzicki et al., 2014). In flies, this signaling system is a major contributor to circadian timekeeping under normal light:dark conditions. Furthermore, a change in the transcription
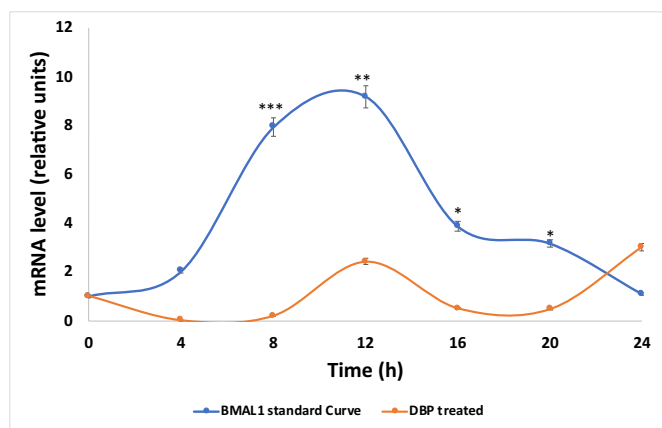
**Fig. 8.** DBP exposure disrupt circadian rhythm in MCF10A cells. Temporal expression of *BMAL1* gene in breast cell line MCF10A after DBP treatment. The graph depicts the level of expression of *BMAL1* at 4 h intervals over 24 h after 2 h serum shock entrainment and DBP treatment at 10 μM. Data points (means of three biological replicates) were normalized using GAPDH relative to the first time point ($t = 0$). Statistically significant differences from control are indicated as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ and One-way ANOVA with Tukey's post hoc test. Error bars = SEM.

timing of *Pdf* would disrupt the interaction between M and E cells, which are necessary to set the morning and evening activity patterns (Hassaneen et al., 2011; Seluzicki et al., 2014). Therefore, to understand if the ability of DBP to disrupt circadian rhythm depended on signaling from PDF neurons, we overexpressed the heat sensitive cation channel TrpA1 specifically in PDF neurons. TrpA1 is involved in heat nociception and is activated at temperatures over 26 °C (Hwang et al., 2012). PDF normally functions as an arousal signal, and when we hyperactivate the PDF neurons during the time where the flies are normally less active - the siesta period - there is a significant increase in activity. Although, raising the flies on DBP did not block this arousal due to increased PDF signaling, overall activity was severely reduced. This would fit with our initial results that indicated DBP exposure inhibited *Pdf* transcription. It also fits with the qPCR data showing that in DBP fed flies *Pdf* transcript was increased at ZT2, and reduced at ZT6. This could indicate a general shift in *Pdf* transcription due to DBP exposure, which in turn disrupts PDF signaling.

It was also reported that once released from M cells, PDF signals back to these same cells through PDFR to activate CLK, which in turn induces transcription of the nuclear hormone receptor *Hr38* (Hassaneen et al., 2011; Mezan et al., 2016; Seluzicki et al., 2014). Hr38 then signals to inhibit the transcription of *Pdf*, which inhibits signaling to the E cells (Mezan et al., 2016). In short, if DBP bypasses the need for CLK in the regulation of *Hr38* transcription, or activates *Hr38* inappropriately, this would be sufficient to disrupt normal circadian rhythm circuitry. In support of an earlier report showing that Hr38 inhibited the transcription of *Pdf* (Mezan et al., 2016), when we expressed *Hr38* RNAi specifically in *Pdf* neurons, circadian rhythm was disrupted. Feeding these flies with DBP did not exacerbate the loss of *Hr38* phenotype, indicating that DBP may require Hr38 to disrupt circadian rhythm. Furthermore, we determined that *Hr38* expression was reduced in the constant presence of DBP, demonstrating that DBP exposure was sufficient to disrupt the normal transcriptional profile of *Hr38*. Even though our initial whole transcriptome sequencing analysis indicated that DBP exposure inhibited *Pdf* expression, when we performed a more detailed study of *Pdf* expression using qPCR at different ZT times, we saw that in the morning *Pdf* expression was increased at ZT2, and then at ZT6 its expression was inhibited in flies raised on DBP containing food, when compared to controls. One possibility is the disruption of *Hr38* transcription, caused by DBP exposure, induces a *Pdf* transcriptional phase-shift and this in turn disrupts normal circadian rhythm. Therefore, we demonstrate that DBP exposure disrupts normal circadian rhythm and that activating one specific set of circadian regulatory neurons, the PDF neurons, can rescue the DBP circadian rhythm defect.

The vasoactive intestinal protein (VIP) in mammals is analogous to PDF in *Drosophila* (Vosko et al., 2007). Several studies have shown a critical role for the neuropeptide VIP in synchronizing and supporting rhythmicity by acting at the cellular and/or circuit levels (Aton and Herzog, 2005; Harmar et al., 2002). VIP neurons are required for normal circadian rhythmicity which is produced by approximately 10% of SCN neurons, and at cellular level VIP and PDF both contribute to molecular oscillations, as well as to the synchronization of diverse pacemakers (Harmar et al., 2002; Lin et al., 2004; Peng et al., 2003). Meanwhile, in *Drosophila*, *Hr38* is the sole homolog of mammalian *NR4A2* (Adhikari et al., 2019), and evidence shows that NR4A2 regulates VIP expression levels by transactivating the VIP promoter through NR4A2-responsive cis elements. Furthermore, in vivo studies demonstrated that loss of NR4A2 function results in diminished VIP mRNA levels within the developing midbrain (Luo et al., 2007). In addition, exposure to di-(2-ethylhexyl) phthalate (DEHP), structurally similar to DBP, disrupts the expression of *NR4A2* through a MAPK signaling pathway (Lee et al., 2017). It was also reported that NR4A2 can be induced by metabolites of the DEHP metabolite mono-(2-ethylhexyl) phthalate (MEHP) (Noda et al., 2007). All the above evidence suggests the circadian rhythm in flies and mammals have a high conservation in output physiology.

The circadian system is responsible for cell-signaling processes during a period of 24 h and disruption of circadian rhythm is associated with the transcriptional and post-transcriptional changes in normal and tumorigenic tissues. In this system, *BMAL1* plays a critical role in the functioning of the molecular clock, which is necessary for regulation of circadian rhythms within cells. In 2007, the International Agency for Research on Cancer classified "shiftwork that involves circadian disruption" as probably carcinogenic to humans. Circadian clocks can be disrupted through irregular shift work, repeated bouts of jet lag and ageing. Desynchronization of circadian rhythmicity was implicated in several pathologic conditions, including tumorigenesis and progression of cancer. In human, circadian clocks regulate the rhythmic expression of numerous genes and disrupted expression of circadian genes (leading to circadian rhythm disruption) can promote cancer. Therefore, it is of great significance to understand the risk of environmental pollutant DBP exposure on circadian rhythm in human cells. In our study, by testing DBP exposure effects on MCF10A cells, we found that DBP exposure disrupted the expression levels of circadian regulation gene *BMAL1*, resulting circadian rhythm disruption. Also, MCF10A cells exposed to DBP demonstrated a higher growth rate compared to control cells.

## 5. Conclusions

In conclusion, our findings are the first to show that dibutyl phthalate, an environmental xenobiotic pollutant, is able to disrupt normal circadian rhythm circuits. Our results using the *Drosophila* system and human cells suggest that it is highly relevant to study the effects of environmental pollutants on circadian rhythm regulation. Due to the ubiquitous exposure of modern society to xenobiotics, and the broad range of secondary effects that may be related to circadian rhythm disturbance, understanding the risks of environmental chemical exposure on the regulation of circadian rhythm are of high societal value. Therefore, further studies regarding the effects of phthalates on circadian rhythm in vivo and in vitro are highly warranted.

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**CRediT authorship contribution statement**

**Wen Liu:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Hao Cao:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing,

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