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The auxin-inducible degradation system enables conditional PERIOD protein depletion in the nervous system of *Drosophila melanogaster*

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Abstract

Tools that allow inducible and reversible depletion of target proteins are critical for biological studies. The plant-derived auxin-inducible degradation system (AID) enables the degradation of target proteins tagged with the AID motif. This system has been recently employed in mammalian cells as well as in *C. elegans* and *Drosophila*. To test the utility of the AID approach in the nervous system, we used circadian locomotor rhythms as a model and applied the AID method to temporally and spatially degrade PERIOD (PER), a critical pacemaker protein in *Drosophila*. We found that the *period* locus can be efficiently tagged with the AID motif by CRISPR/Cas9-based genome editing without disrupting PER function. Moreover, we demonstrated that the AID system could be used to induce rapid and efficient protein degradation in the nervous system as shown by effects on circadian and sleep behaviors. Furthermore, the protein degradation by AID was rapidly reversible after auxin removal. Together, our results show that the AID system provides a powerful tool for behavior studies in *Drosophila*.

Graphical Abstract

Tools that allow rapid temporal and spatial depletion of target proteins are critical for biological studies. The auxin-inducible degradation system (AID) has been recently developed to enable the degradation of target proteins tagged with the AID motif. Here we used the *Drosophila* circadian locomotor rhythms as a proof of principle to test the potential use of the AID system in the nervous system. We report that the AID system can be used to elicit rapid and reversible degradation of PERIOD protein in the *Drosophila* nervous system.

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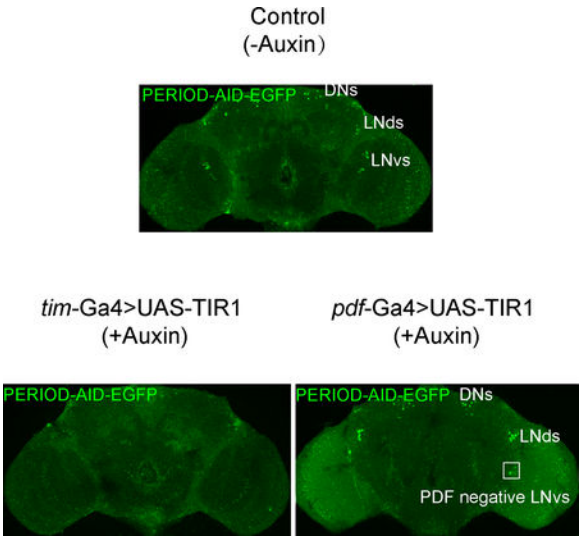
Author contributions

Y.Z. conceived the project. Y.Z. and W.C. designed the experiments. W. C. and M. W performed the experiments and analysis. W. C. and Y. Z. wrote the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.



Keywords

auxin-inducible degradation; CRISPR/Cas9; *Drosophila melanogaster*; PERIOD; circadian rhythm

Introduction

Various experimental tools for precise spatial and temporal control of gene expression have been developed that allow interrogation of gene function in living organisms. Temporally restricted genetic manipulations are critical for biological studies, and several strategies for temporally controlling gene expression have been developed. Some that depend on temperature, chemical ligand, or light triggers have been adapted for use in *Drosophila* [1–6]. Often, these methods cause side effects. For example, elevated temperatures may result in unwanted physiological changes [7, 8]. In addition, the most frequently used techniques in *Drosophila* to deplete gene products in specific stages and tissues have been achieved through RNA interference (RNAi) techniques, FLP-mediated excision of FRT flanked exon techniques, or CRISPR/Cas9-mediated deletion [9–12], approaches that manipulate protein expression at the level of genome or the transcriptome, meaning that they are indirect and sometimes irreversible. Additionally, there are often longer time frames for protein depletion than the process under investigation [13]. Therefore, techniques that directly enable rapid protein depletion in a controllable (inducible and reversible) manner are particularly desirable as this would allow the study of protein function at organismal level.

Recently, a variety of tools have been successfully applied to induce direct and specific protein degradation. Among these methods, the plant-derived auxin-inducible degradation (AID) system [14–17] is a promising tool for the rapid and conditional control of protein clearance. The AID method has been successfully applied to mammalian cells as well as *C. elegans* and *Drosophila* [18–22]. Using the AID system, proteins are tagged with the AID degron peptide. Upon addition of auxin, the AID-tagged protein is recruited to an engineered E3 ubiquitin ligase SCF complex, containing the TIR1 F-box protein from plants, which binds target proteins only in the presence of the plant hormone auxin [14]. Expression of

TIR1 in non-plant cells is sufficient to result in the formation of the SCF complex, which then binds AID-tagged proteins in an auxin-dependent manner, leading to polyubiquitination and degradation via the proteasome [14]. In *Drosophila*, the AID system has been successfully employed in somatic and germline tissues at various stages of development [20, 22]. Given that the central nervous system (CNS) is generally protected by a blood brain barrier, the functionality of the AID system within the CNS cannot be deduced automatically from the previously published AID system tests. Here we used circadian locomotor rhythms as a model to evaluate utility of the AID method for behavioral studies in *Drosophila*.

The circadian rhythm is a roughly 24-hour cycle in the physiological processes of organisms from fungi to mammals. Endogenous molecular oscillators, built into the circadian neuronal network, generate these circadian rhythms. In *Drosophila*, a network of approximately 150 clock neurons comprises the circadian clock, and robust rhythmic expression of clock genes is observed in these cells [23–25]. The clock neuron network in the brain consists of three groups of dorsal neurons (DN1s, DN2s, and DN3s), three groups of lateral neurons (large LNvs, small LNvs, and LNds), and lateral posterior neurons (LPNs) [23–25]. The neuropeptide PIGMENT DISPERSING FACTOR (PDF), the main neuromodulator of the circadian neuronal network, is expressed in the LNvs [23]. The core clock mechanism consists of negative transcription translation feedback loops. The CLOCK/CYCLE (CLK/CYC) heterodimer acts as a transcription factor to initiate rhythmic transcription of hundreds of clock-controlled genes, such as *period* and *timeless* [23]. At a certain concentration, PERIOD (PER) dimerizes with TIMELESS (TIM) and inhibits the transcription activity of the CLK/CYC. The PER protein, encoded by the first gene known to control behavior of any kind, is an essential component of the circadian clock and is well characterized in *Drosophila* [23, 26–28].

Here we used the circadian locomotor behavior as a model to test the potential applicability of the AID method in the nervous system. By tagging the *period* locus with the AID motif and feeding flies a low concentration of auxin, we were able to temporally and spatially deplete PER and thus modulate circadian behavior rhythms. These results demonstrate that the AID system will be a useful functional tool for behavior and neuroscience studies.

Results

Design strategy for the AID system in the nervous system of *Drosophila*

To examine whether the plant-derived AID system is functional in the nervous system of *Drosophila*, we chose *tim*-Gal4, a pan-clock tissue driver [29], and the *period* locus to target in the circadian rhythm system (Fig. 1A). We first applied CRISPR/Cas9-mediated homologous recombination to fuse the AID motif together with an EGFP tag into the *period* locus after the region encoding the C terminus of the protein. Two highly specific genomic target sites (period-gRNA1 and period-gRNA2) flanking the stop codon of the *period* gene were selected using the online flyCRISPR Optimal Target Finder Tool [30] (Fig. 1B). The targeting sequences were cloned into a plasmid under the transcriptional control of the U6 promoter to generate targeting chiRNAs. For homology-directed repair we used a double-stranded DNA plasmid donor with two homology arms of approximately 1 Kb (Fig. 1B). The two protospacer adjacent motifs (PAM) of targeting sites on homology arms were

mutated to prevent cutting by Cas9 after homology-directed repair events (Fig. 1C). chiRNAs and donor plasmids were mixed and injected into nos-Cas9 fly embryos. PCR and sequencing results demonstrated a high percentage of positive KI integration in P0 flies (13 out of 23; 56.5%) (Fig. 1C). Thus, the endogenous *period* locus was site-specifically and efficiently edited with CRISPR/Cas9.

Expression and subcellular localization of the AID- and EGFP-tagged PER protein in adult brains

To determine the expression pattern of the AID-EGFP-fused PER protein, we used anti-GFP and anti-PER antibodies to detect EGFP and the PER protein in *period*-AID-EGFP homozygous flies. We entrained flies under regular light:dark (LD) cycles and dissected fly brains at circadian time 1 (CT1) under the first day of constant darkness, a time of high PER abundance. The expression pattern of EGFP was very similar to that of endogenous PER in the fly brain (Fig. 2A, B). The subcellular localization of the tagged PER protein (as revealed by GFP staining) in the circadian neurons was the same as that of the wild-type PER (Fig. 2C, D). Thus, the AID-EGFP-tagged PER is expressed with the same pattern as the endogenous PER.

PER fused with AID-EGFP at the C terminal is fully functional

We next evaluated whether the fusion of AID-EGFP alters PER function. Under 12:12 h LD conditions, *Drosophila* gradually increase their circadian locomotor activity in advance of the lights-on and lights-off signals and exhibit peaks of activity during dawn and dusk, a phenomenon termed anticipation [23, 24, 31, 32]. The per^{01} mutant flies lose morning and evening anticipation [33–35](Fig. 3A). Both morning and evening anticipation were normal in *period*-AID-EGFP flies (Fig. 3B–C). Moreover, the *period*-AID-EGFP allele was able to rescue the anticipatory behavior in female per^{01} flies (Fig. 3D–H). *Drosophila* sleep is also under circadian regulation, and per^{01} mutants have disrupted rhythms of sleep [24]. The circadian rhythm and sleep patterns of wild-type and *period*-AID-EGFP flies were comparable (Table 1 and Fig. 3I). Thus, these circadian and sleep behavior results indicate that the modification of the *period* allele by fusion to the sequence encoding AID-EGFP had no obvious effects on PER function.

PER abundance oscillates in circadian neurons and peaks during the late night/early day [36, 37]. To determine whether *period*-AID-EGFP flies show normal PER oscillation, we co-stained the brains for GFP and with PER antiserum. We found that the oscillating expression of GFP was comparable to PER in the circadian neuronal groups that we examined (Fig. 4A, B). Indeed, the oscillation of tagged PER in circadian neurons was similar with previous studies [38–40]. Furthermore, circadian clocks have been shown to operate in many peripheral tissues, such as the Malpighian tubules [41]. We observed AID-EGFP-labeled PER-positive cells in the Malpighian tubules, which also exhibit oscillations of both GFP and PER signals (Fig. 4C, D). Taken together, these data indicate that the *period*-AID-EGFP allele encodes a tagged-PER protein that functions normally.

Endogenous PER protein can be conditionally depleted by the AID system

To test whether the AID system can be used to induce PER degradation, we used the GAL4-UAS system to express the TIR1 protein in all circadian tissues from the *tim*-Gal4 driver. When fed flies 3-indole acetic acid sodium salt (IAA) from two different suppliers to induce PER degradation, most of the flies died within two days (data not shown). We then tried the auxin analog 1-naphthaleneacetic acid (1-NAA, hereafter referred to as auxin), and observed no toxicity. We then fed flies with different concentrations of auxin (0, 25, 100, 400 mM). The *period*-AID-EGFP flies with TIR overexpression fed auxin lost morning and evening anticipation under LD condition and became arrhythmic under DD condition (Fig. 5A, B and Table 1). These behavior defects were observed at both concentrations of auxin for 25 and 100 mM, except the flies fed with 400 mM auxin led to decrease of rhythmicity even in control flies (Table 1). These circadian phenotypes upon auxin treatment in the TIR-expressing flies were identical to those of per^{01} mutant flies [26, 33–35]. Moreover, we found that auxin-treated *y,sc,v,per*-AID-EGFP/Y;*tim*-Gal4/+;UAS-TIR1/+ (AID-TG4-TIR, experimental) flies also had sleep defects similar to per^{01} mutants [24] (Fig. 5C). These behavior defects indicated that the molecular clock is disrupted in the *period*-AID-EGFP flies with TIR overexpression fed auxin, presumably due to PER degradation.

To confirm PER is degraded by the AID system, we dissected fly brains at the CT0 and stained with both GFP and PER antibodies. As expected, the level of the PER protein was drastically decreased in the circadian neurons in which the TIR1 protein was expressed, even at the low concentration of auxin (Fig. 6A). In fact, the PER signal was too low to be detected (Fig. 6B). The staining with antibodies against another pacemaker protein VRILLE as a marker (anti-VRI) confirmed that the absence of PER signal is not caused by the loss of circadian neurons (Fig. 6C). To further understand the kinetics of PER degradation upon auxin feeding, we fed flies 25mM auxin at ZT12 and examine PER abundance after 4 hours, 8 hours and 12 hours (Fig.7A). PER abundance started to increase after light off in the control flies and reached peak around late night. Interestingly, we found that AID-mediated PER degradation occurred rapidly within 8 hours after auxin feeding, indicating that the function of auxin in the CNS is highly efficient (Fig. 7A–B).

PER expression in the PDF-positive LNvs specifically controls the morning activity, whereas expression of PER in other circadian neurons, including LNds, controls the evening activity [33, 34]. Therefore, to further confirm the specificity of the AID system, we used *pdf*-GAL4 to express TIR and depleted PER only in PDF-positive LNvs. Upon auxin treatment, arrhythmic behavior under DD was observed in these flies (Fig. 8A and Table 1). Interestingly, under LD, the auxin treatment only disrupted the morning anticipation but not the evening anticipation in these flies, indicating that PER was not affected in the evening neurons. Imaging confirmed that PER protein was only absent in PDF-positive LNvs of the *y,sc,v,per*-AID-EGFP/Y;*pdf*-Gal4/+;UAS-TIR1/+ (AID-PG4-TIR) flies treated with auxin (Fig. 8B–F).

In a control experiment, we analyzed *period*-AID-EGFP/Y;;UAS-TIR1/+ flies, which do not express GAL4. These flies should not express TIR1. In these flies, auxin treatment caused no substantial locomotor activity or sleep behavior defects (Fig. 8B–E). This result indicates that the observed behavior defect is a direct consequence of the TIR1-mediated PER

depletion and is not due to a side effect of the auxin treatment. Therefore, the AID system can be specially applied to induce protein depletion in the nervous system enabling behavior or other physiology studies in the absence of a single protein.

The AID-mediated protein degradation in the nervous system is reversible

Finally, to determine how quickly AID-mediated PER degradation causes a phenotype, flies fed normal food were transferred to food supplemented with auxin, while flies were first treated with auxin and then switched to normal food. In the control flies, the behavior was normal throughout the experiment as were control flies continuously fed with normal food or 25 mM auxin-supplemented food (Fig. 9). By contrast, we found that AID-mediated PER degradation caused a phenotype in the treatment groups within 12 hours even in flies fed a low concentration of auxin (Fig. 10A).

To determine the reversibility of the AID system, flies fed with 25 mM auxin for 3 days and then with normal food for 3 days were subjected to behavior analysis. In the AID-TG4-TIR flies, one day after auxin removal, the suppression decreased, and after 36 hours behavior was normal (Fig. 10B), demonstrating that the AID system acts in a reversible manner. AID-TG4-TIR flies continuously fed with normal food or 25 mM auxin food were used as positive controls (Fig. 10C, D).

Discussion

Recently, several different tools have been successfully applied to induce direct protein degradation. A common strategy utilizes the UBR1 E3 ligase pathway and N-end rule [42, 43]. Some strategies, such as tobacco virus protease (TEV) protease-mediated cleavage, the GFP nanobody coupled with a degradation signal (deGradFP), the photo-inactivation by FlAsH-FALI, the ligand-inducible destabilizing domain (DD) fused protein degradation, and the temperature-sensitive intein-mediated protein splicing, have been adapted to *Drosophila* [44–49]. Limitations are associated with these tools when applied for behavior studies. For example, temperature can cause unwanted physiological changes and have significant impacts on fly behavior that may prevent detection of the phenotype of interest [7, 8]. The reactive oxygen species generated by FlAsH-FALI might also have side effects. Although TEV and deGradFP act at the protein level, neither are particularly fast nor are they readily reversible. Most importantly, none of these tools have been employed in the nervous system. The AID system of plants is a small-molecule-inducible protein degradation system that has enabled rapid, conditional protein depletion in many organisms including *Drosophila* [18–22]. Here, we report for the first time that the AID system can induce rapid and reversible protein degradation in the nervous system enabling behavioral studies in *Drosophila*. Our analyses demonstrate that the AID method allows reversible conditional protein degradation with precise temporal control.

Here we targeted sites in the *Drosophila* endogenous *period* locus using the CRISPR/Cas9 system resulting in flies that express PER with AID-EGPF at the C terminus with very high efficiency. We found that the PER protein fused with the 287-amino acid AID-EGFP is fully functional at molecular and behavior levels. In addition, the fly line *period*-AID-EGFP we

generated here will serve as an alternative in experiments that require detections of PER. The anti-PER antibody is not commercially available but GFP antibodies are.

Normal neuronal function requires well-balanced extracellular ions, nutrients, and metabolic homeostasis. Therefore, all organisms equipped with a complex nervous system have developed the so-called blood-brain barrier (BBB), an evolutionarily ancient structure that provides direct support and protection of the nervous system [50]. The auxin analog employed here must cross the BBB and readily penetrate the membranes of neurons in the fly brain. Synthetic auxin analogs 1-NAA and IAA activate the AID system with similar efficiencies and induce Vasa depletion in *vasa*-AID-EGFP flies [20]. Here, we observed toxicity with IAA. 1-NAA worked well in the adult flies at the relatively low concentration applied. Actually even at low concentration, 1-NAA rapidly triggers the degradation of PER in circadian neurons in less than 8 hours. As 1-NAA will likely cross the BBB in other organisms, the AID system is a promising tool for functional study of nervous systems in organisms in addition to the fly.

Materials and methods

Drosophila strains, plasmid construction, and transgenesis

tim-Gal4 and *pdf*-Gal4 flies were used in previous study [31]. UAS-OsTIR1 (BL76123) and *vasa*-AID-EGFP (BL76126) flies were obtained from Bloomington Stock Center.

To generate the *period*-AID-EGFP fly line, the donor was constructed via the following overlap PCR steps. First, homology sequences were PCR amplified from genomic DNA of the *yw* fly strain using PrimeSTAR Max DNA polymerase (TaKaRa, R045, Kusatsu, Shiga, Japan). The AID-EGFP sequence was amplified from a *vasa*-AID-EGFP fly. To prevent cutting of the repair template, guide RNA recognition sites were eliminated on the plasmid. The following oligonucleotides were used: 5arm-f: 5’-ACATAAACCTCTGGCCACCGTTCT-3’ 5arm-r: 5’-GATCTTTAGGACCTCCGCCGTCGCCGTGCTGTGTCTGGTCCTC-3’ AID-EGFP-f: 5’-CGGCGGAGGTCCTAAAGATCCAGCCAAACCTCC-3’ AID-EGFP-r: 5’-TCAGCAGCAACTGCGGGTGTCATTACTTGTACAGCTCGTCCATGCC-3’ 3arm-f: 5’-ACACCCGCAGTTGCTGCTGACC-3’ 3arm-r: 5’-CAAGCTGCTCATCCTGCCCATTG-3’

Subsequently, 5arm, AID-EGFP, and 3arm fragments were mixed and amplified using 5arm-f and 3arm-r primer pairs. Finally, the PCR product of 5arm-AID-EGFP-3arm was cloned into the pCR-Blunt vector (ThermoFisher, k275020, Waltham, MA, USA) through blunt-end cloning. For chiRNAs construction, the two target site sequences were cloned into the pU6-BbsI-chiRNA plasmid (Addgene, #45946, Cambridge, MA, USA) as previously described [30] using the following oligonucleotides: PAM1-sense: 5’-CTTCGACCAGACACAGCACGGGGAT-3’ PAM1-antisense: 5’-AAACATCCCCGTGCTGTGTCTGGTC-3’ PAM2-sense: 5’-CTTCGTCAGCAGCAACTGCGGGTG-3’ PAM2-antisense: 5’-AAACCACCCGCAGTTGCTGCTGAC-3’

The donor and pU6-BbsI-chiRNA plasmids were mixed and injected into embryos of the nos-Cas9 attp2 fly line (Rainbow Transgenic Flies, Inc., Camarillo, CA, USA).

Screen for integration

After injection, a single F0 fly was collected and crossed with an FM7 balancer fly. In the F1 generations, 10–15 larval or pupa flies from the single cross were pooled together for genome extraction. The following primers for partial EGFP fragment amplification were used: EGFP-f: 5'-ctggtcgagctggacggcgacg-3' EGFP-r: 5'-cacgaactccagcaggaccatg-3'

For the EGFP PCR positive single cross, 10–20 F1 flies were used to do single crossing with the FM7 balancer fly. When eggs were present in the food, the genome of a single F1 fly was extracted and amplified again using EGFP-f and EGFP-r primers. The following primers that flank the two homologous arms plus the primers in the AID-EGFP were used for amplification and sequencing: F1: 5'-CTCCTCCGTGGGCAGTTCCACG-3' R1: 5'-aacagctcctcgcccttgctcac-3' F2: 5'-ctgctgcccgacaaccactacct-3' R2: 5'-GGATTCACCAAGGACCTCACCAGG-3'

Immunostaining

Adult flies (3–5 days old) were collected and fixed at room temperature for 1 h in 4% formaldehyde diluted in PBT (PBS with 0.1% Trition X-100). Then, the brains were dissected in PBT at indicated time points and fixed in 4% formaldehyde for another 20 min at room temperature. The brains were rinsed and washed with PBT three times (15 min each) and then were blocked in 10% normal goat serum in PBT for 2 h at room temperature. Then, the brains were incubated with primary antibody overnight at 4 ℃. Indicated dilutions of the following primary antibodies were made in PBT: rabbit anti-PER (1:1500, a gift from Michael Rosbash, Brandeis University) and mouse anti-GFP (1:400, from ThermoFish, #15379). After three 15-min washes, brains were incubated with secondary goat antibodies overnight at 4 ℃, followed by extensive washes. Finally, the brains were mounted in the Mounting Medium (Vector Laboratories, #H-1000, Burlingame, CA, USA). Imaging was performed on a Leica Confocal Microscope SP8 system. Confocal images were obtained at an optical section thickness of 1–2 μm and were analyzed with Image J.

Locomotor behavior assay

All the flies were raised on cornmeal/agar medium at 25 ℃ under a 12h:12 h LD cycle. Unless noted otherwise, male flies were monitored in a 12 h:12 h LD cycle at 25 ℃ for 3–4 days, followed by 6–7 days in DD using Trikinetics Drosophila Activity Monitors. Activity records were collected in 1-min bins and analyzed using Faas (Fly activity analysis suite) software developed by M. Boudinot and derived from the Brandeis Rhythm Package (http://hawk.bcm.tmc.edu). Circadian periods were determined by periodogram analysis [51]. A signal-processing toolbox was used to plot flyplot and sleep patterns [52]. The morning and evening anticipation were determined by assaying of the locomotor activity [53]. Statistical analyses were performed with two-tailed Student's t-test.

Treatment with auxin and auxin analogs

The auxin 3-indole acetic acid sodium salt (IAA; Abcam, ab146403, Cambridge, MA, USA and Millipore Sigma, I5148, Burlington, MA, USA) stock solutions of 125 or 500 mM were prepared in water. The 1-naphthaleneacetic acid (1-NAA; Millipore Sigma, N0640, Burlington, MA, USA) stock solutions of 125, 500 mM or 2 M were prepared in absolute ethyl alcohol. Then the stock solution was mixed with sucrose agar food (5% sucrose and 2% agar) for behavior assay. Sucrose agar food has been used for circadian rhythms and sleep assay in *Drosophila*. It is easy to make and can last long (for weeks) during the behavior assay.

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Abbreviations

1-NAA	1-naphthaleneacetic acid
AID	auxin-inducible degradation
BBB	blood-brain barrier
chiRNA	chimeric single guide RNA
CRISPR	clustered regularly interspaced short palindromic repeats
gRNA	guide RNA
CNS	central nervous system
CT	circadian time
EGFP	enhanced green fluorescent protein
FlAsH-FALI	FlAsH-mediated fluorescein-assisted light inactivation
FLP	flippase
FRT	flippase recognition target
IAA	3-indole acetic acid sodium salt
LD	light:dark
DD	dark:dark
PG4	*pdf*-Gal4
SCF	SKP1/CUL1/F-box

TEV	tobacco etch virus
TG4	*tim*-Gal4
TIR1	transport inhibitor response 1
UTR	untranslated region
ZT	zeitgeber time

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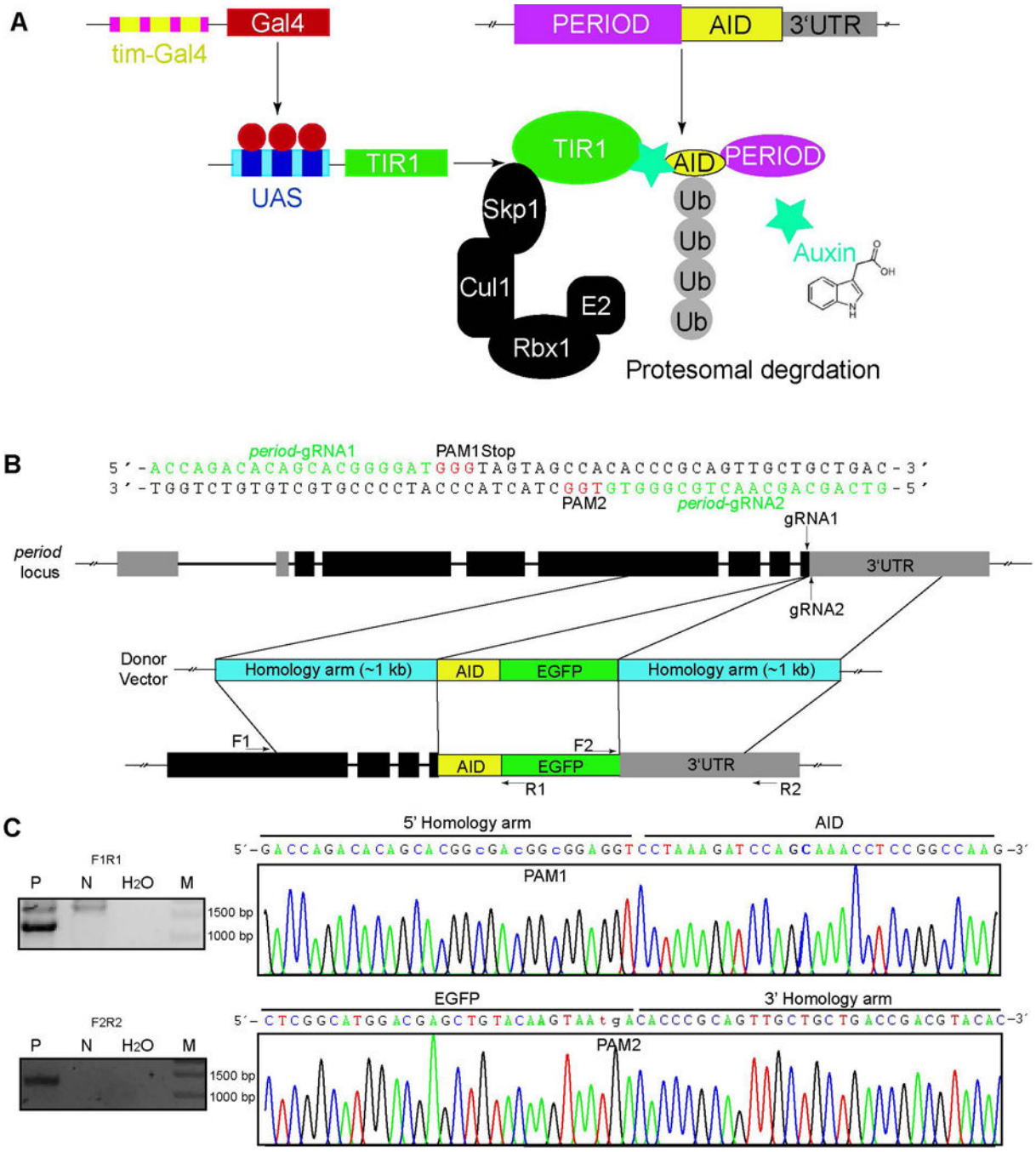

Figure 1. Generation of the *period*-AID-EGFP allele by CRISPR/Cas9-induced homologous recombination.

(A) A schematic of AID in the circadian rhythm system. The Gal4-UAS system is used to introduce TIR1 from rice (OsTIR1), which includes an F box that allows integration into an SCF ubiquitin ligase complex together with the endogenous *Drosophila* proteins. The endogenous PER protein is fused with the AID (PER-AID). In the presence of auxin, PER-AID is recruited to OsTIR1, resulting in its polyubiquitinylation and proteasomal degradation. (B) Strategy for tagging the *period* gene with AID-EGFP. The two target sites located in the last exon and downstream of the 3'UTR are shown. The circle donor vector

containing two homology arms was designed to insert the AID-EGFP tag before the stop codon of *period*. (C) PCR analysis of 5' and 3' integration of AID-EGFP and representative sequencing results of the *period*-AID-EGFP allele. P, positive (*y,sc,v,period*-AID-EGFP), N, negative (*y,sc,v*), M, maker.

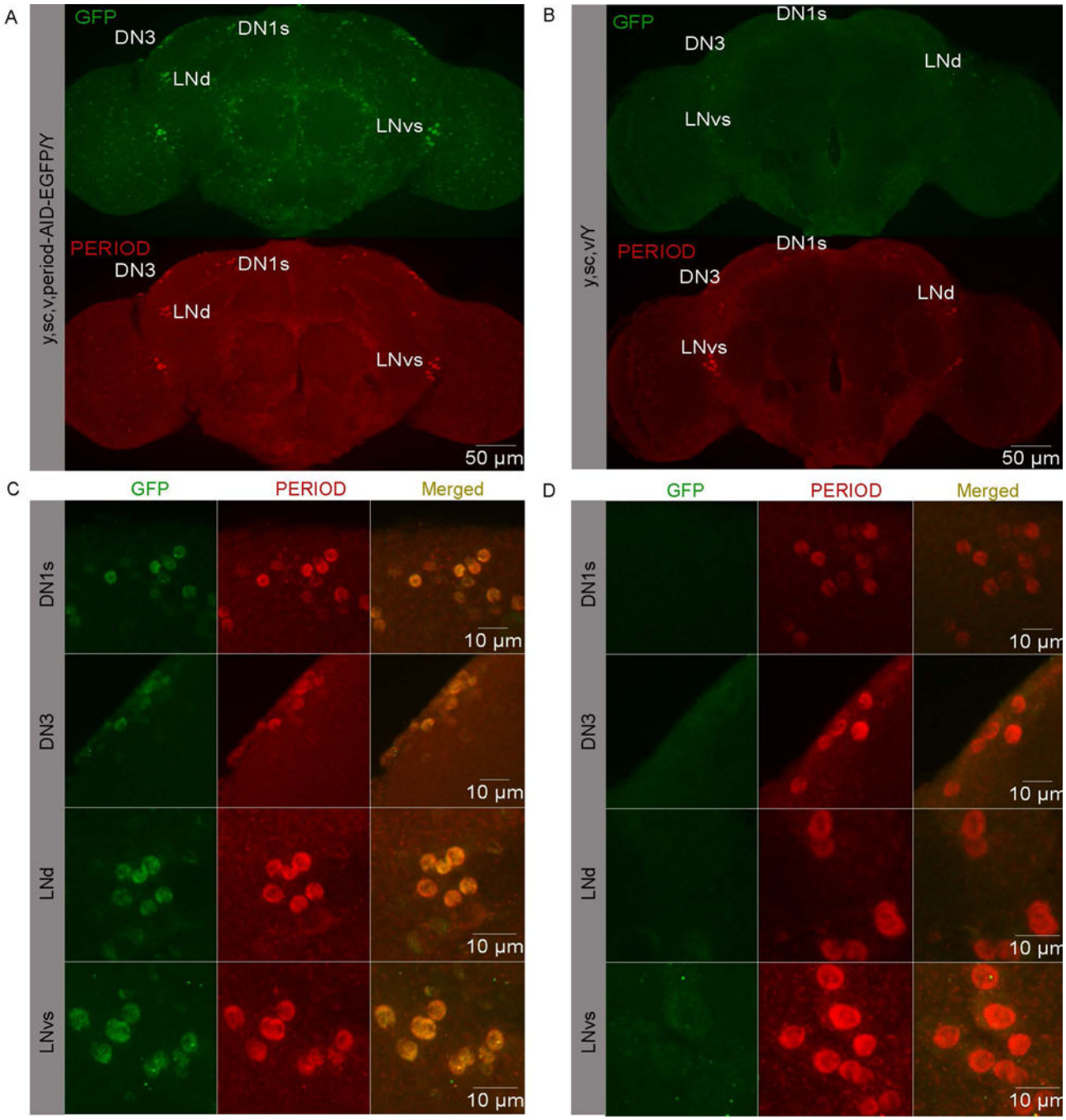

Figure 2. Expression pattern of the AID-EGFP-tagged PER protein in *period*-AID-EGFP and control fly brains.

(A-B) Whole adult brains of A) *period*-AID-EGFP and B) control flies stained with anti-GFP and anti-PER at CT1. (C-D) Specific circadian neurons stained with anti-GFP and anti-PER in C) *period*-AID-EGFP and D) control flies. LNd, dorsal lateral neuron; lLNv, large ventral LN; sLNv, small ventral LN. DN, dorsal neuron.

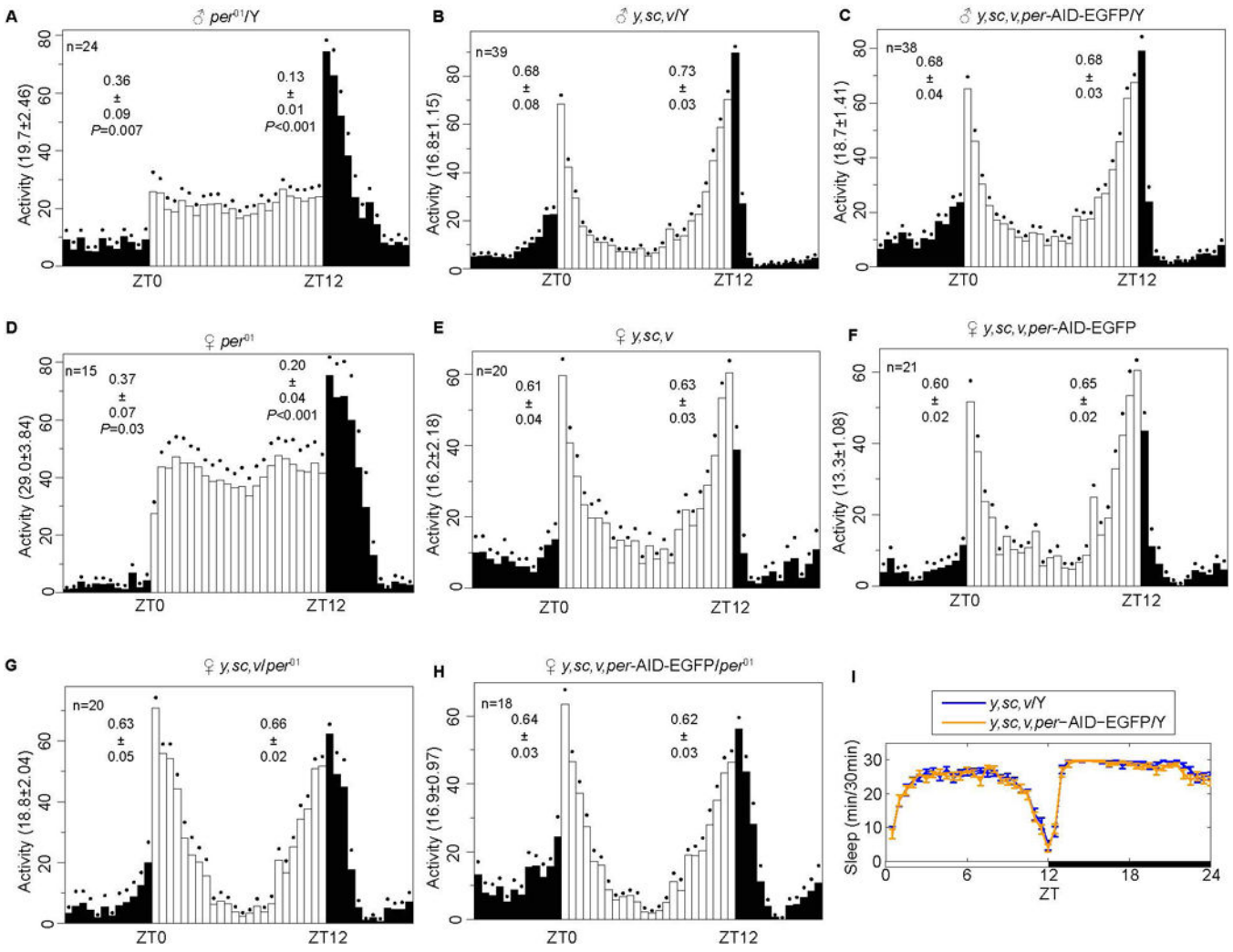

Figure 3. Locomotor and sleep behaviors of *period*-AID-EGFP flies are normal.

(A-H) Average activities for A) male *per*^{01} flies, B) male controls, C) male *y,sc,v,period*-AID-EGFP flies, D) female *per*^{01} flies, E) female controls, F) female *y,sc,v,period*-AID-EGFP flies, G) female *y,sc,v/per*^{01} flies and H) female *y,sc,v,period*-AID-EGFP/*per*^{01} flies during 12 h of light and 12 h of dark at 25 ℃. (I) Sleep patterns of male *y,sc,v,period*-AID-EGFP /Y and control flies. White bars represent day, black bars represent night, Zeitgeber time (ZT) is indicated on the *x* axes. The dots above the bars indicate SEM. The total numbers of each genotype are shown in the figure. The morning and evening anticipation signals are shown as means ± SEM. A Student's two-tailed t-test was employed to determine significance compared to controls (y,sc,v/Y or y,sc,v). *P* values are shown in the figures.

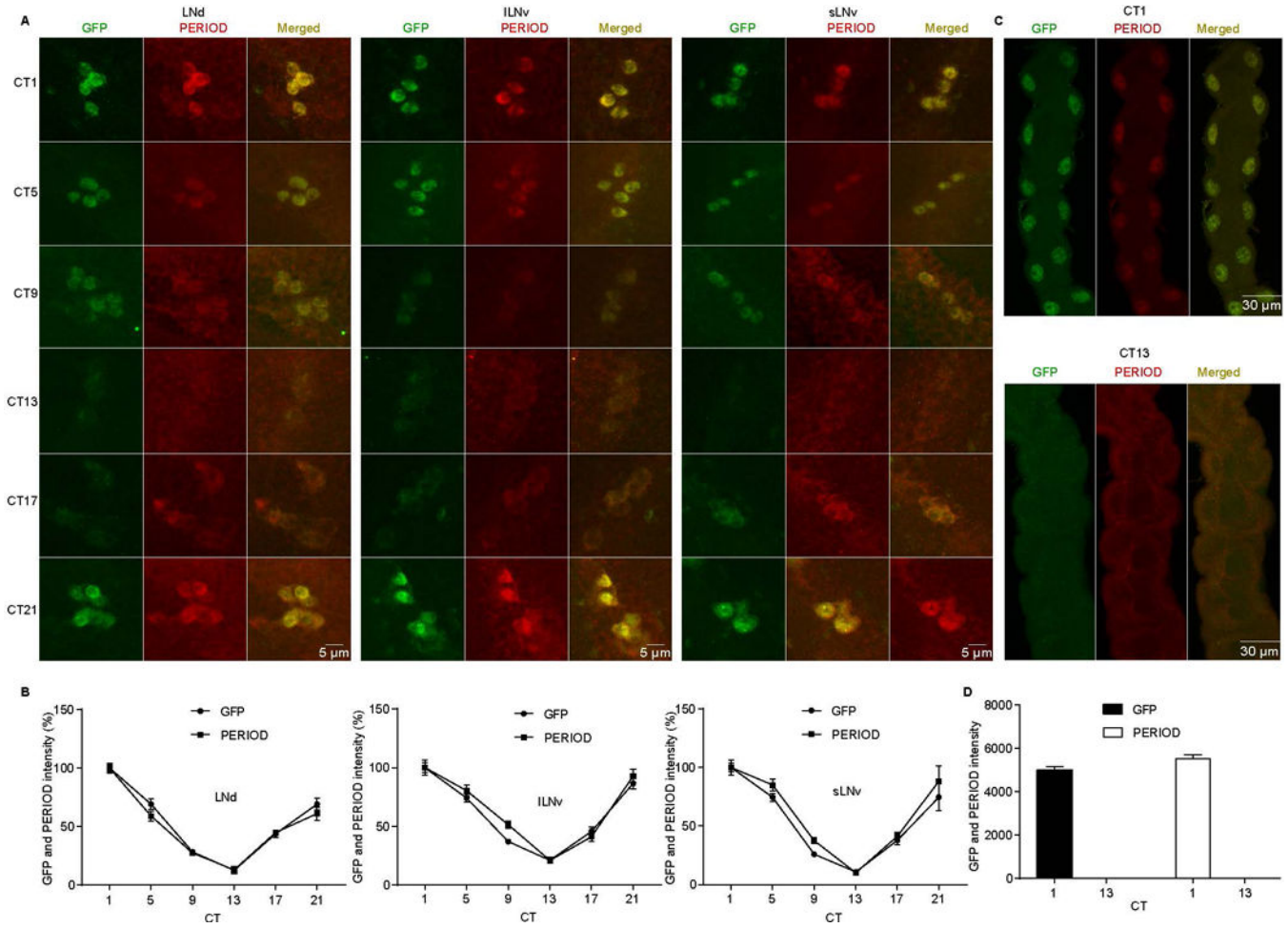

Figure 4. *period*-AID-EGFP flies functional normally on the molecular level.

(A) Oscillation of GFP and PER in *period*-AID-EGFP flies. Adult brains were dissected at CT 1, 5, 9, 13, 17, and 21 at DD1 and circadian neurons were immunostained with anti-GFP and anti-PER. (B) Quantification of GFP and PER average intensity in each clock cell group (n=10). Signal was normalized to the value at CT1 which was set as 100%. CT is indicated on the *x* axes. (C) Malpighian tubules from *period*-AID-EGFP flies collected at the indicated circadian time on DD1 were immunostained with anti-GFP and anti-PER. (D) Quantification of GFP and PER intensity in Malpighian tubules (n=8). CT is indicated on the *x* axes. LNd, dorsal lateral neuron; lLNv, large ventral LN; sLNv, small ventral LN.

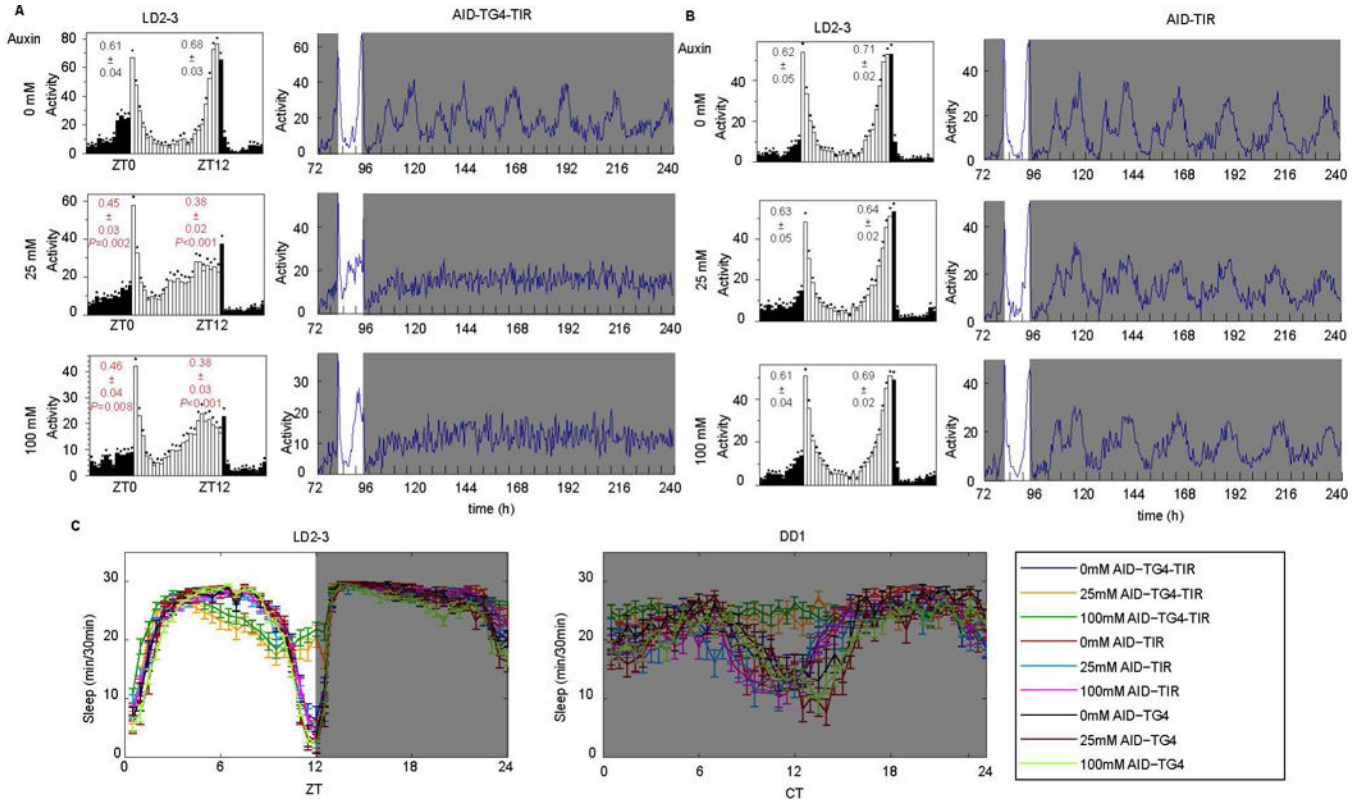

Figure 5. The AID system enables behavioral studies in nervous system of adult *Drosophila*.

(A-B) The averaged activity profiles during LD and actograms throughout the behavioral analyses of A) AID-TG4-TIR flies and B) controls fed with indicated concentrations of auxin. The morning and evening anticipation signals are shown as means ± SEM. A Student's two-tailed t-test was employed to determine significance of the difference between treatment and control groups. *P* values are shown in the figures. (C) Sleep patterns of the AID-TG4-TIR and control flies fed with auxin.

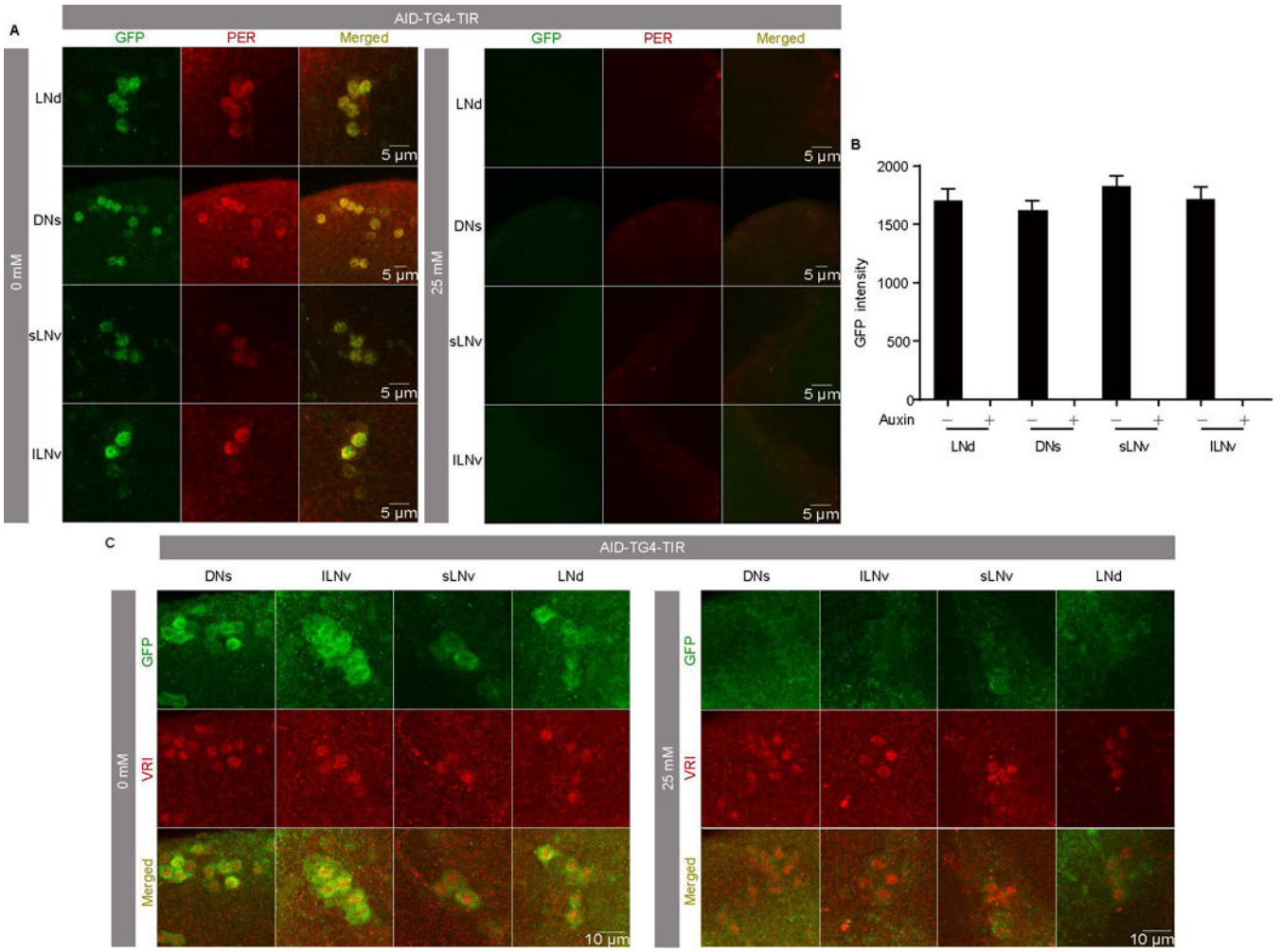

Figure 6. The AID system enables degradation of proteins in nervous system of adult *Drosophila*.

(A) Representative LNd, DN1, and LNvs stained with GFP and PER at CT1 to confirm degradation of PER in AID-TG4-TIR flies after 0 mM and 25 mM auxin treatments. (B) GFP intensities from analyses of AID-TG4-TIR flies after 0 mM (n=8) and 25 mM (n=10) auxin treatments. Flies were fed with auxin for 3 days and then transferred to constant dark (DD) condition. Samples were collected for staining on the first day of DD at CT1. (C) Representative LNd, DNs, and LNvs stained with GFP and VRI at ZT18 after the third day with or without auxin treatment to confirm the normality of circadian neurons (n=8).

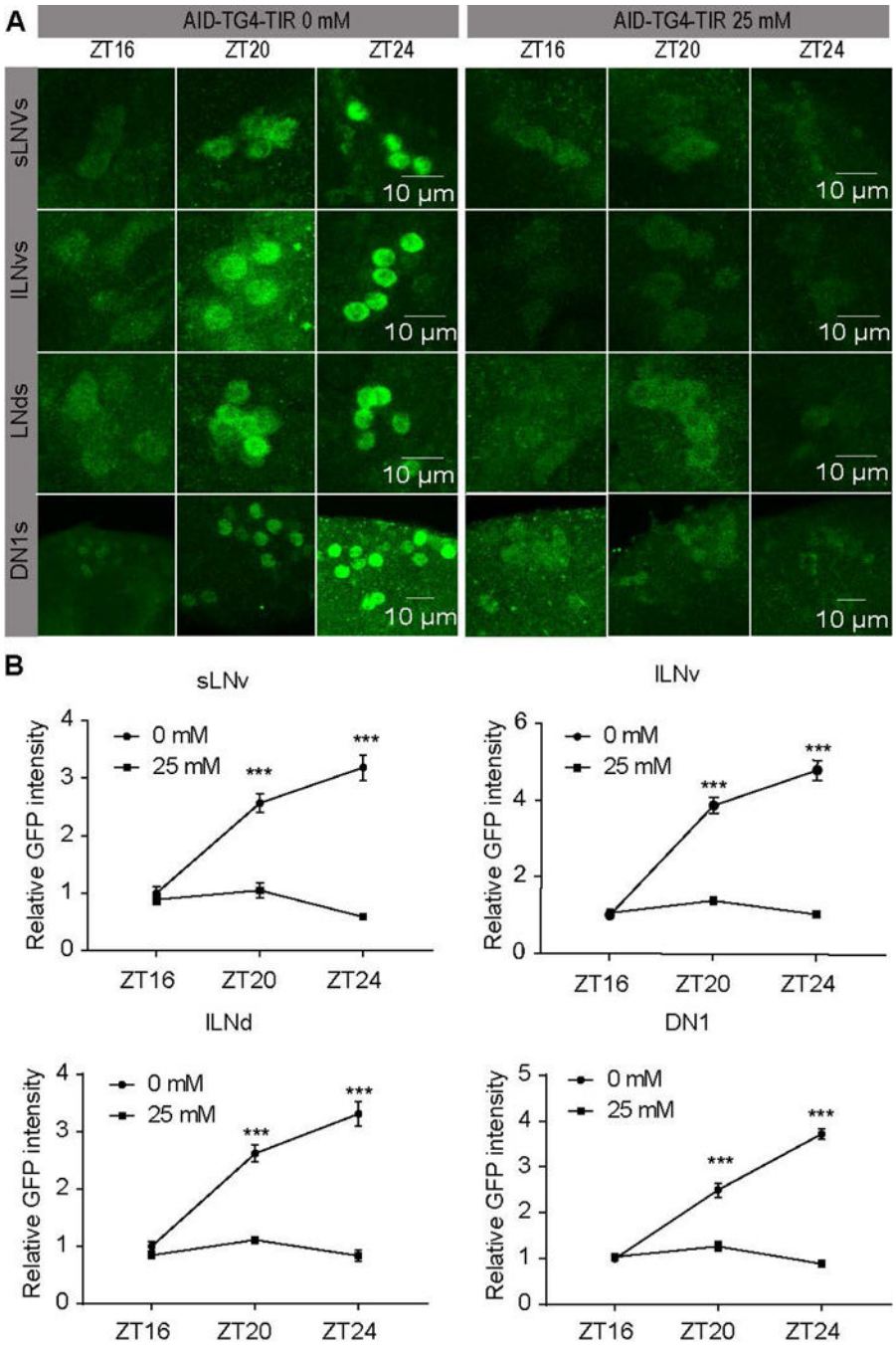

Figure 7. Rapid AID mediated degradation of PER indicated by a kinetic assay.

(A) AID-TG4-TIR flies were feed with 0 mM or 25 mM auxin from ZT12, and then dissection for staining with anti-GFP at ZT16 (n=8), ZT20 (n=10), ZT24 (n=10). Representative LNd, DN1, and LNvs were shown. (B) GFP intensities from analyses of (A). Each time point was normalized to the ZT16 of flies without auxin feeding. The GFP intensity was shown as mean ± SEM. A Student's two-tailed t-test was employed to determine significance compared to controls and treatments at the same time point. *** $P<0.001$

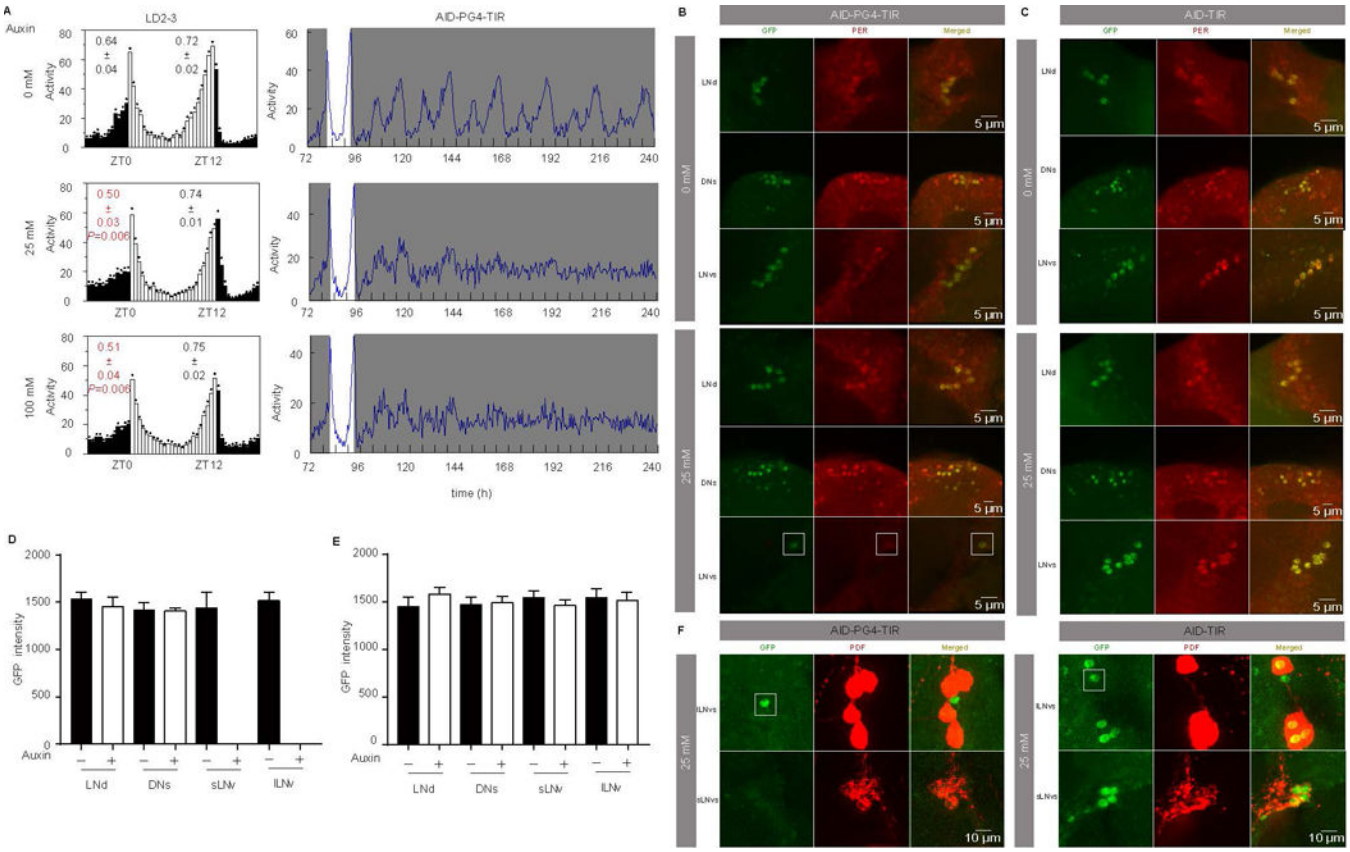

Figure 8. The AID system does not cause non-specific degradation of proteins in nervous system of adult *Drosophila*.

(A) Averaged activity profiles during LD and actograms throughout the behavioral analyses of AID-PG4-TIR flies treated with 0, 25, or 100 mM auxin. (B-C) Representative LNd, DN1, and LNvs stained with GFP and PER at CT1 to confirm degradation of PER in AID-PG4-TIR flies and control flies after 0 mM and 25 mM auxin treatments. (D-E) GFP intensities from analyses of AID-PG4-TIR flies and control flies after 0 mM (n=8) and 25 mM (n=10) auxin treatments. Flies were fed with auxin for 3 days and then transferred to constant dark (DD) condition. Samples were collected and stained on the first day of DD at CT1. Note that in AID-PG4-TIR flies treated with 25 mM auxin, there was only one GFP-positive neuron in the LNvs, which is a PDF-negative neuron, indicated with a box. The morning and evening anticipation signals are shown as means ± SEM. A Student's two-tailed t-test was employed to determine significance of the difference between treatment and control groups. *P* values are shown in the figures. (F) AID-PG4-TIR or control flies treated with 25 mM auxin for three days and then stained with GFP and PDF at CT1 to confirm that the remain neuron is exactly the PDF negative LNv (n=8).

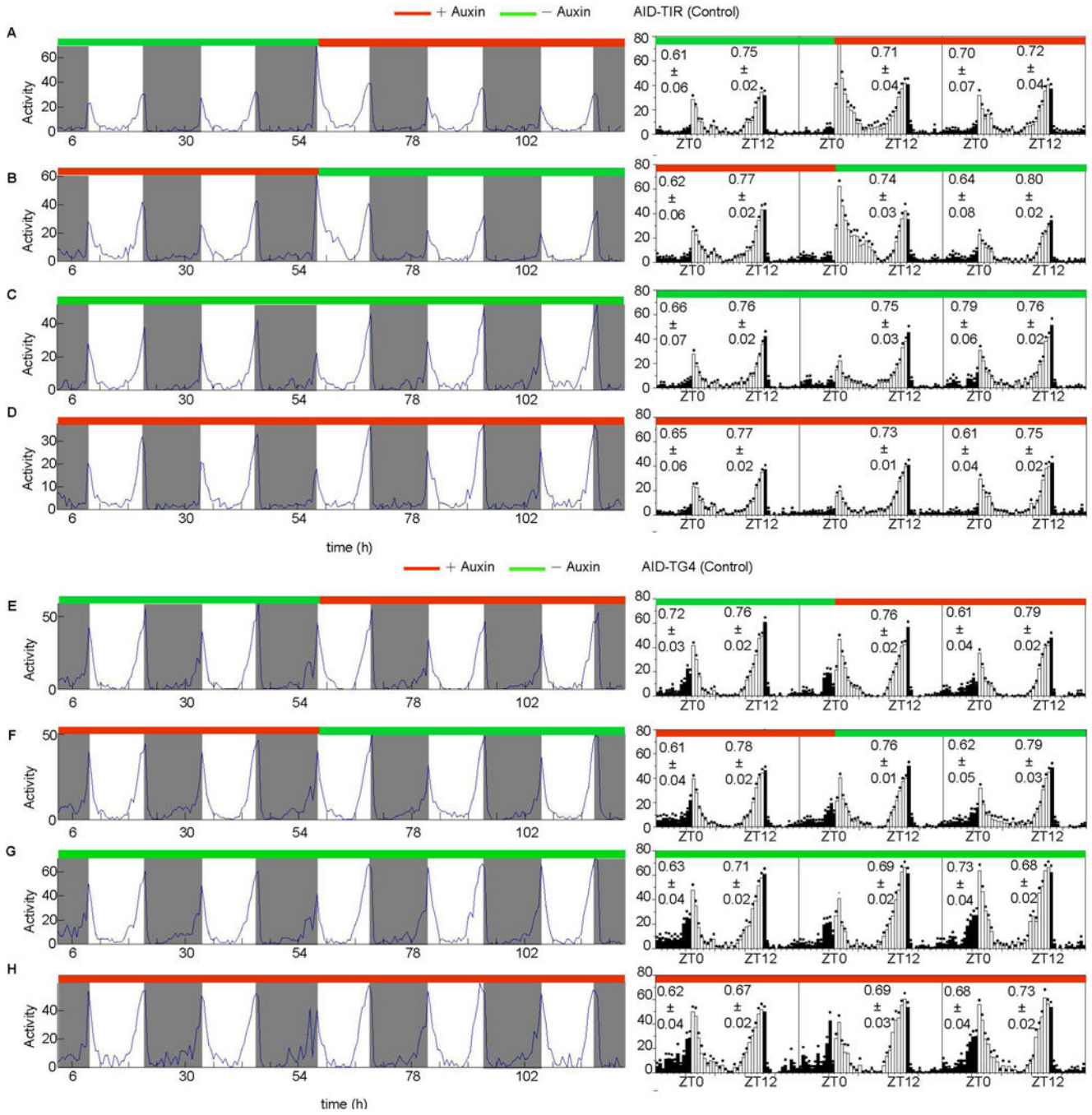

Figure 9. Control fly behavior is not altered by auxin.

(A-D) Behaviors of *y,sc,v,per*-AID-EGFP/+; ;UAS-TIR1/+ control flies A) fed normal food for 3 days then transferred to 25 mM auxin-supplemented food for an additional 3 days (n=45), B) fed 25 mM auxin-supplemented food for 3 days then transferred to normal food for an additional 3 days (n=42), C) fed normal food for the study period (n=51), and D) fed 25 mM auxin-supplemented food for the study period (n=50). (E-H) Behaviors of *y,sc,v,per*-AID-EGFP/+; *tim*-Gal4/+ control flies E) fed normal food for 3 days then transferred to 25 mM auxin-supplemented food for an additional 3 days (n=32), F) fed 25 mM auxin-

supplemented food for 3 days then transferred to normal food for an additional 3 days (n=48), G) fed normal food for the study period (n=32), and H) fed 25 mM auxin-supplemented food for the study period (n=40). Averaged activity profiles during the entire assay period are shown in the left panels. Average locomotor activity plots of a day before and a day after food transfer are shown in the right panels. The morning and evening anticipation signals are plotted as means ± SEM.

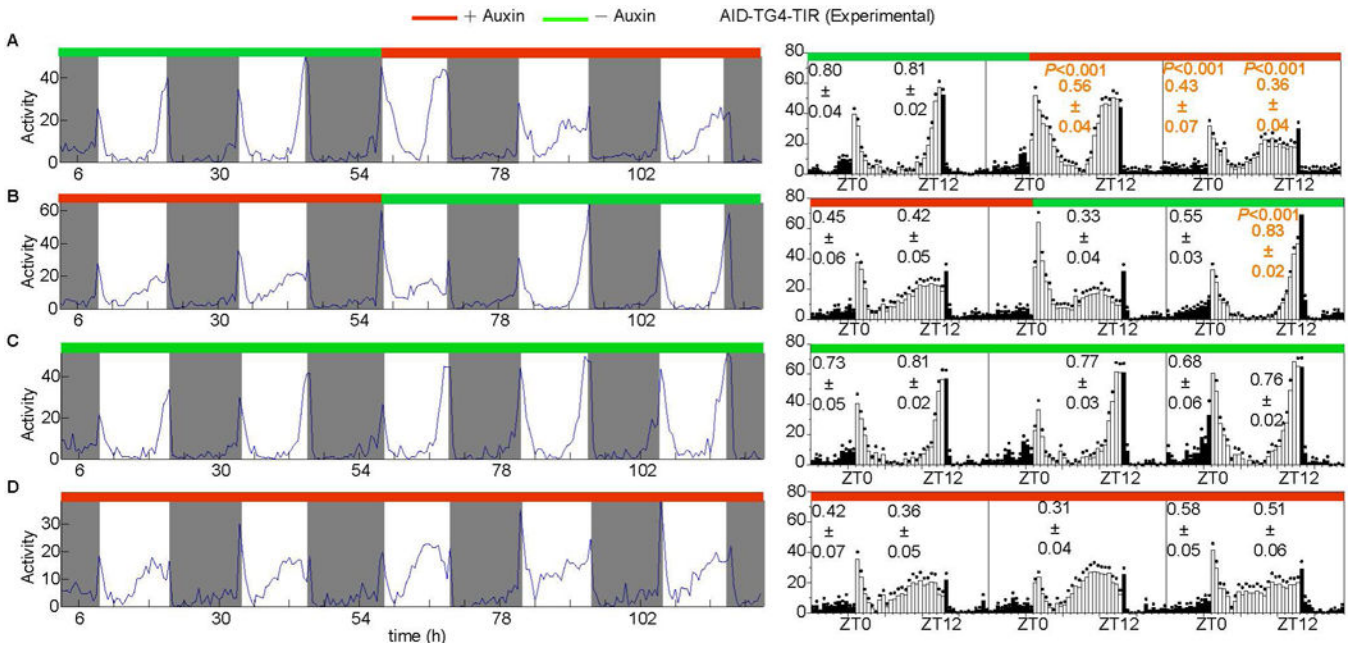

Figure 10. Reversible depletion of the AID-EGFP-tagged PER protein by the AID system.

Behaviors of AID-TG4-TIR flies A) fed normal food for 3 days then transferred to 25 mM auxin-supplemented food for an additional 3 days (n=49), B) fed 25 mM auxin-supplemented food for 3 days then transferred to normal food for an additional 3 days (n=32), C) fed normal food for the study period (n=42), and D) fed 25 mM auxin-supplemented food for the study period (n=50). Averaged activity profiles during the entire assay period are shown in the left panels. Average locomotor activity plots of a day before and a day after food transfer are shown in the right panels. The morning and evening anticipation signals are plotted as means ± SEM. A Student's two-tailed t-test was employed to compare the significance of the difference between the day before and after food transfer. *P* values are shown.

Table 1.

Locomotor activity of flies.

Auxin (mM)	Genotypes	Tau	Power	Rhythmic flies	Total flies	% Rhythmic
	y,sc,v/Y	24.43±0.21	95.40±9.94	12	12	100
	y,sc,v,per-AID-EGFP/Y	24.73±0.21	107.20±9.67	18	18	100
0	AID-TG4-TIR[1]	24.26±0.07	88.07 ±5.92	35	43	81
25	AID-TG4-TIR	23.53±0.80	98.33 ±28.00	4	45	9
100	AID-TG4-TIR	23.77±0.67	38.27 ±12.21	3	47	6
400	AID-TG4-TIR	24.1	115.8	1	41	2.5
0	AID-TIR[2]	24.14±0.08	114.90 ±6.07	34	38	89
25	AID-TIR	24.03±0.06	80.86 ±6.39	35	37	95
100	AID-TIR	24.28±0.06	88.00 ±4.89	43	44	98
400	AID-TIR	24.42±0.13	65.80±7.05	26	18	76
0	AID-TG4[3]	24.81±0.06	97.78 ±5.54	37	38	97
25	AID-TG4	24.88±0.06	92.92 ±5.59	31	39	79
100	AID-TG4	24.96±0.05	77.37 ±4.65	45	56	80
400	AID-TG4	25.20±0.08	76.52±6.07	23	29	79
0	TG4-TIR[4]	24.59±0.12	88.00 ±4.99	38	40	95
25	TG4-TIR	25.13±0.20	84.74 ±4.61	38	47	81
100	TG4-TIR	25.65±0.60	69.67 ±5.59	26	43	60
400	TG4-TIR	25.11±0.24	55.54±6.17	21	40	53
0	TIR/+[5]	24.71±0.17	107.80 ±5.46	41	42	98
25	TIR/+	25.08±0.20	83.13 ±5.85	36	40	90
100	TIR/+	24.77±0.16	78.00 ±4.48	43	44	98
400	TIR/+	24.87±0.20	80.29±5.09	29	36	81
0	TG4/+[6]	24.97±0.07	93.12 ±6.01	24	26	92
25	TG4/+	25.08±0.20	91.54 ±6.42	24	27	89
100	TG4/+	25.11±0.09	80.82 ±6.74	28	38	74
400	TG4/+	25.25±0.14	75.02±6.89	12	15	80
0	AID-PG4-TIR[7]	24.13±0.06	124.30±7.73	36	38	95
25	AID-PG4-TIR	24.62±0.30	38.46±2.44	9	34	26
100	AID-PG4-TIR	24.10±0.29	36.78±9.95	4	40	10
400	AID-PG4-TIR	24.5	25.7	1	17	6
0	AID-PG4[8]	24.63±0.06	124.60±7.36	22	24	92
25	AID-PG4	24.70±0.13	88.73±8.59	18	20	90
100	AID-PG4	24.88±0.17	79.56±8.56	10	10	100
400	AID-PG4	25.14±0.11	85.91±6.52	9	10	90

Auxin (mM)	Genotypes	Tau	Power	Rhythmic flies	Total flies	% Rhythmic
0	PG4-TIR[9]	24.91±0.19	107.80±6.37	36	38	95
25	PG4-TIR	25.46±0.24	78.56±5.43	28	38	74
100	PG4-TIR	24.84±0.20	75.18±7.07	24	38	63
400	PG4-TIR	24.94±0.24	77.20±5.31	15	26	58
0	PG4/+[10]	25.16±0.26	110.60±9.86	17	18	94
25	PG4/+	25.34±0.17	101.40±7.13	18	19	95
100	PG4/+	25.15±0.13	76.38±8.90	11	12	92
400	PG4/+	25.44±0.67	86.01±8.17	12	12	100

[1] AID-TG4-TIR: *y,sc,v,per*-AID-EGFP/Y; *tim*-Gal4/+; UAS-TIR1/+

[2] AID-TIR: *y,sc,v,per*-AID-EGFP/Y;; UAS-TIR1/+

[3] AID-TG4: *y,sc,v,per*-AID-EGFP/Y; *tim*-Gal4/+

[4] TG4-TIR: *y,sc,v* /Y; *tim*-Gal4/+; UAS-TIR1/+

[5] TIR: *y,sc,v* /Y;; UAS-TIR1/+

[6] TG4: *y,sc,v* /Y; *tim*-Gal4/+

[7] AID-PG4-TIR: *y,sc,v,per*-AID-EGFP/Y; *pdf*-Gal4/+; UAS-TIR1/+

[8] AID-PG4: *y,sc,v,per*-AID-EGFP/Y; *pdf*-Gal4/+

[9] PG4-TIR: *y,sc,v* /Y; *pdf*-Gal4/+; UAS-TIR1/+

[10] PG4: *y,sc,v* /Y; *pdf*-Gal4/+