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# *Nipped-A* regulates the *Drosophila* circadian clock via histone deubiquitination

Bei Bu[1,2,†], Lixia Chen[1,†], Liubin Zheng[1], Weiwei He[1] & Luoying Zhang[1,3,*]

## Abstract

**Psychiatric diseases are often accompanied by circadian disruptions, but the molecular underpinnings remain largely unclear. To address this, we screened genes that have been previously reported to be associated with psychiatric diseases and found that *TRRAP*, a gene associated with schizophrenia, is involved in circadian rhythm regulation. Knocking down *Nipped-A*, the *Drosophila* homolog of human *TRRAP*, leads to lengthened period of locomotor rhythms in flies. Molecular analysis demonstrates that NIPPED-A sets the pace of the clock by increasing the mRNA and protein levels of core clock genes *timeless* (*tim*) and *Par domain protein 1ε* (*Pdp1ε*). Furthermore, we found that NIPPED-A promotes the transcription of *tim* and *Pdp1ε* possibly by facilitating deubiquitination of histone H2B via the deubiquitination module of the transcription co-activator Spt-Ada-Gcn5 acetyltransferase complex. Taken together, these findings reveal a novel role for NIPPED-A in epigenetic regulation of the clock.**

**Keywords** circadian clock; *Drosophila*; histone deubiquitination; NIPPED-A
**Subject Categories** Chromatin, Transcription & Genomics; Neuroscience; Signal Transduction
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## Introduction

A myriad of our behavioral and physiological processes exhibit ~24-h rhythms or circadian rhythms (Dibner, 2019). These rhythms exist in almost all organisms on the earth and are driven by a relatively conserved molecular clockwork consisting of several transcriptional and translational feedback loops (Li & Zhang, 2015). In *Drosophila* and many other animals including humans, these loops center around two transcription factors, CLOCK (CLK) and CYCLE (CYC; Hardin, 2011). As a heterodimer, CLK and CYC activate the transcription of *period* (*per*) and *timeless* (*tim*) via E-box elements in the promoter regions of these two genes. PER and TIM proteins accumulate in the cytoplasm, form a complex, and translocate into the nucleus where they inhibit the transcriptional activities of CLK/CYC, thus repressing their own transcription. PER/TIM undergo a series of post-translational modifications (PTMs) that ultimately lead to their degradation, thus enabling CLK/CYC to activate transcription and start a new cycle. CLK/CYC also activate the transcription of two additional transcription factors, *vrille* (*vri*) and *PAR domain protein 1ε/δ* (*Pdp1ε/δ*), with the former repressing while the latter activating *clk* transcription.

Although generally accepted by the field, this is an over-simplified model of the clock. *In vivo*, DNA wraps around nucleosomes and together they are packaged into chromatin. Chromatin state, determined by histone PTMs, is a key factor regulating transcription. A number of studies in mammals have demonstrated daily oscillations of histone acetylations and methylations which modulate rhythmic circadian gene expression (Papazyan *et al*, 2016). Consistently, it has also been shown in flies that the acetylation of histone H3-K9 and trimethylation of H3-K4 occur in concert with CLK/CYC binding to E-boxes and activation of *per*/*tim* transcription (Taylor & Hardin, 2008). One recent study reported circadian H2B monoubiquitination in the mouse liver which may influence the expression of clock genes (Tamayo *et al*, 2015). Overall, it is largely unclear how these chromatin modifications contribute to clock regulation and ultimately to rhythmic behavior and physiology.

Disruptions of circadian rhythms are associated with many diseases and disorders (Takahashi *et al*, 2008). Psychiatric diseases such as major depressive disorder, bipolar disorder, and schizophrenia make up approximately 20% of all illnesses and about 25% of the population is affected at some point during their lifetime (Zordan & Sandrelli, 2015). Most psychiatric disorders involve circadian disruptions including alterations of sleep/wake cycle, core body temperature rhythms, as well as rhythms in melatonin and cortisol secretion (Jones & Benca, 2015). Moreover, nearly every antidepressant and anti-psychotic medication exerts effects on circadian rhythm, and chronobiological interventions demonstrate efficacy in improving mood-related symptoms (Zhang *et al*, 2014; Jones & Benca, 2015). Although circadian abnormalities have long been hypothesized to play a role in the pathology of various psychiatric

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diseases, how circadian disruptions occur in these illnesses remains elusive (McClung, 2013).

In this study, we screened genes that have been reported to be associated with human psychiatric disorders to identify candidates involved in regulating circadian rhythms in flies. We discovered *Nipped-A*, the fly homolog of *TRRAP* which is associated with schizophrenia, to play a role in regulating the circadian clock (Xu *et al*, 2012). TRRAP/NIPPED-A is an evolutionarily conserved protein known to function as a histone acetyltransferase (HAT) cofactor by facilitating the recruitment of HAT to chromatin to regulate transcription and DNA repair (Murr *et al*, 2007). Here, we demonstrate that knocking down *Nipped-A* leads to lengthened period of locomotor rhythm, likely due to decreased transcription of *tim* and *Pdp1ε*. Furthermore, we found that NIPPED-A is physically associated with *tim* and *Pdp1* loci, facilitating the deubiquitination of H2B by the deubiquitination (DUB) module of the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex which could in turn enhance transcription.

## Results

### Knocking down *Nipped-A* lengthens the period of locomotor rhythm

We initiated a RNAi screen of *Drosophila* homologs of genes reported to be associated with psychiatric conditions in humans to identify genes that are involved in circadian regulation. We knocked down the expression of these candidate genes in all clock cells (including neurons and glial cells) using a *tim*GAL4 driver or mainly in circadian neurons using a *cryptochrome* (*cry*)GAL4-16 driver (Emery *et al*, 1998, 2000), and assessed the effects of these manipulations on fly locomotor rhythm. So far we have tested 24 genes and have identified *Nipped-A*, the *Drosophila* homolog of human *TRRAP*, to be involved in determining the period length of fly locomotor rhythm under constant darkness (DD). Knocking down *Nipped-A* results in roughly 1- to 3-h longer period with 6 RNAi lines (generated from 3 independent RNAi transgenes; Fig 1A–C; Appendix Table S1). When *Nipped-A* is knocked down only in the PIGMENT-DISPERSING FACTOR (PDF)-expressing neurons, which are believed to be the major pacemaker neurons, the period is lengthened by ~1 h in 3 of the RNAi lines (Fig 1D; Appendix Table S1; Renn *et al*, 1999). We also observed significant reduction in the power of the rhythms in some of the lines and have verified that *Nipped-A* mRNA levels are decreased in all RNAi lines used (Appendix Figs S1 and S2, and Table S1).

We employed a CRISPR/Cas9-based transcriptional activation system, flySAM, to over-express *Nipped-A* (Jia *et al*, 2018). This manipulation does not significantly alter period length on WT background, but it rescues the long-period phenotype in *Nipped-A* RNAi flies, validating that the lengthened period is indeed caused by *Nipped-A* deficiency (Fig EV1; Appendix Table S2). As a control, we expressed GFP and did not observe shortening of the period length in *Nipped-A* RNAi flies (Appendix Table S3).

To test whether NIPPED-A functions in the adult circadian system, we used a temperature-sensitive *tubulin* (*tub*)GAL80$^{ts}$ in combination with *cry*GAL4-16 to knock down *Nipped-A* specifically during the adult or developmental stage (McGuire *et al*, 2004). *tub*GAL80$^{ts}$ represses the transcriptional activities of GAL4 at permissive temperature (18°C), and thus, GAL4-driven transcription can only occur under restrictive temperature (29°C). When RNAi is specifically expressed in adults, the period is lengthened by ~1 h (Fig 1E; Appendix Table S1; McGuire *et al*, 2004). On the other hand, when RNAi is expressed exclusively during the developmental stage, the period does not appear to be altered (Fig 1F; Appendix Table S1). We also employed a gene switch system to turn on RNAi expression specifically in the PDF neurons of adult flies a few days before the start of behavioral monitoring (Depetris-Chauvin *et al*, 2011). Similarly, we observed ~1-h longer period in drug-treated flies which have RNAi expression activated, but not in the vehicle-treated controls (Fig 1G and H; Appendix Table S1). Taken together, these results indicate that NIPPED-A is involved in setting the pace of the adult clock.

### NIPPED-A sets the pace of the clock by promoting *tim* and *Pdp1ε* mRNA levels

We next sought to characterize the mechanism of how NIPPED-A regulates the clock by examining the effects of knocking down *Nipped-A* on clock gene expression. *tim* and *Pdp1ε* mRNA levels are significantly reduced in the heads of these flies compared to the control (Fig 2A). We furthered validated the effects by measuring TIM and PDP1ε protein levels and found these to be reduced as well (Fig 2B and C). Moreover, we observed a significant decrease of PER in flies with *Nipped-A* knocked down, whereas *per* mRNA levels are not significantly altered (Fig 2A–C). This is likely caused by the decrease in TIM levels, as TIM is known to stabilize PER (Hardin, 2011). Since DD period is believed to be determined by the PDF-expressing small ventral lateral neurons (s-LNvs), we assessed the expression of core clock proteins in these cells (Stoleru *et al*, 2005). Consistent with changes occurring at the whole-head level, TIM, PDP1ε, and PER protein levels are also reduced in the s-LNvs (Fig 2D and E). We identified a similar role for NIPPED-A in cultured *Drosophila* cell line. Knocking down *Nipped-A* in S2 cells leads to substantial reduction of *tim* and *Pdp1ε* mRNA levels, and to a lesser extent, *vri* mRNA level (Fig EV2).

Based on our findings, we propose that NIPPED-A regulates clock timing by promoting *tim* and *Pdp1ε* mRNA levels. To test this hypothesis, we first over-expressed *tim* in flies with *Nipped-A* knocked down and found this partially rescues the long-period phenotype, whereas over-expressing *tim* in control flies does not affect the period (Fig 3A; Appendix Table S4 and Fig S3A; McDonald *et al*, 2001). On the other hand, knocking down *Nipped-A* in a *tim*$^{01}$ heterozygous mutant background dramatically reduces the power of the rhythm, while knocking down *Nipped-A* in a wild-type (WT) background does not substantially decrease the power (Sehgal *et al*, 1994; Fig 3B; Appendix Table S4). Next, we show that over-expressing *Pdp1ε* in *Nipped-A* RNAi flies completely reverts the lengthened period, whereas over-expressing *Pdp1ε* in WT flies does not alter the period (Fig 3C; Appendix Table S5; Benito *et al*, 2007). We have validated that this manipulation not only increases *Pdp1ε* mRNA level, but also the mRNA levels of *clk*, *per*, and *tim* (Appendix Fig S3B). Heterozygous *Pdp1ε*$^{3135}$ mutation further enhances the long-period

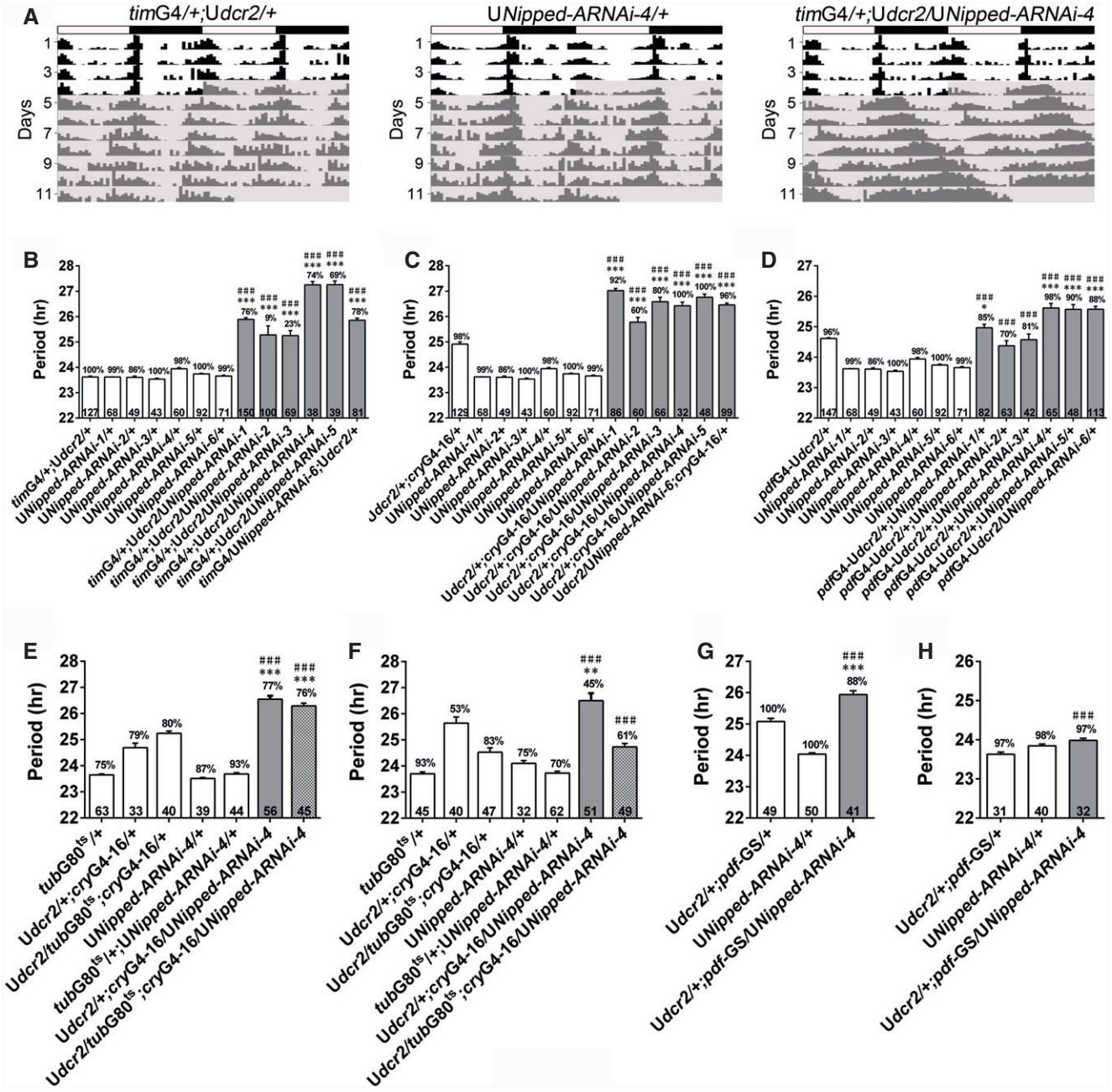



**Figure 1. Knocking down *Nipped-A* lengthens the period of fly locomotor rhythms.**

A Double-plotted representative actograms of the indicated genotypes. Flies are monitored in LD for 4 days and then DD for 7 days. *Dicer2* (*dcr2*) is co-expressed to enhance the effects of RNAi. White boxes indicate the light phase, and black boxes indicate the dark phase. Gray shades indicate DD.

B–D The period of DD locomotor rhythms of flies with *Nipped-A* knocked down and controls.

E, F Using temperature-sensitive GAL80 system to control *Nipped-A* RNAi expression. (E) The period of DD locomotor rhythms of flies raised at 18°C and tested at 29°C. (F) The period of DD locomotor rhythms of flies raised at 29°C and tested at 18°C.

G, H The period of DD locomotor rhythms of flies treated with RU486 to activate the *pdf*G4-geneswitch (*pdf*-GS) driver (G) or vehicle control (H).

Data information: (B–H) Error bars represent standard error of the mean (SEM). Digits on the bars are the number of flies tested. Percentage of rhythmicity is indicated above the bars. Statistical difference is measured using one-way ANOVA, $P < 0.001$, Tukey's multiple comparison test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***/\#\#\#}P < 0.001$, * compared with the GAL4 controls, # compared with the UAS controls. White bar indicates UAS or GAL4 controls. Gray bar indicates flies with *Nipped-A* knocked down. G4, GAL4; U, UAS.

phenotype caused by knocking down *Nipped-A*, but exerts no effect in control flies (Zheng *et al*, 2009; Fig 3D; Appendix Table S5). Since knocking down *Nipped-A* leads to reduced PER protein level, we over-expressed *per* in *Nipped-A* RNAi flies and found this manipulation fully rescues the long-period phenotype, while a $per^{L}$ mutation synergistically enhances

this phenotype (Fig 3E and F; Appendix Table S6 and Fig S3C; Kaneko & Hall, 2000; Konopka & Benzer, 1971). The genetic interactions appear to be relatively specific, as we do not detect prominent interaction between *Nipped-A* and the core clock gene *clock* (*clk*; Appendix Fig S4 and Table S7; Zhao *et al*, 2003). Taken together, these data support a role for NIPPED-A in determining the speed of the molecular clock by promoting *tim* (and consequently PER) and *Pdp1ε* expression.

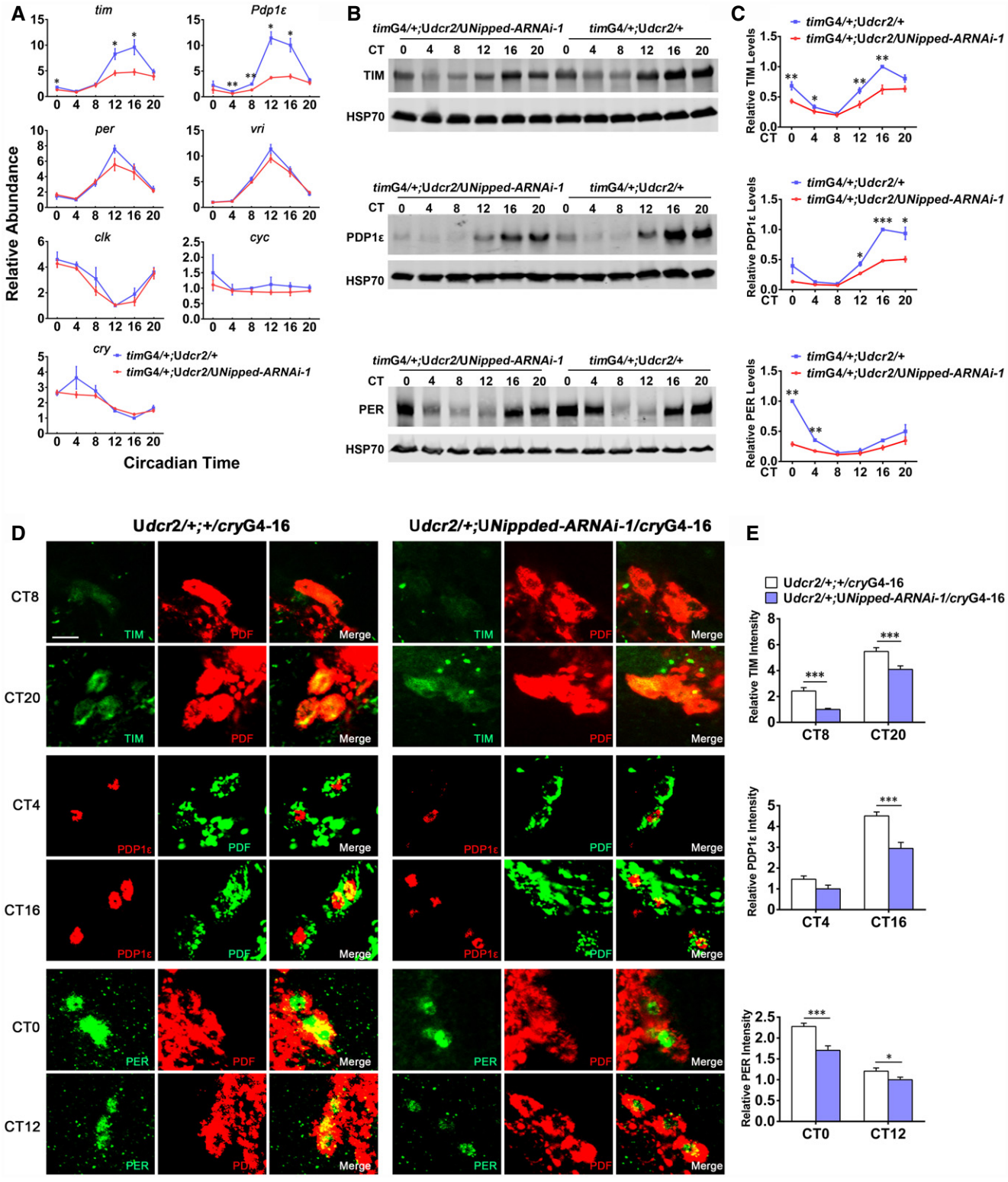



**Figure 2.**

**Figure 2. NIPPED-A acts to increase the mRNA and protein levels of core clock gene *tim* and *Pdp1ε*.**

A Plots of relative mRNA abundance vs. circadian time (CT, CT0 is the time of subjective lights on) for clock genes determined by qRT–PCR in whole-head extracts of *Nipped-A* RNAi (*tim*G4/+;U*dcr2/UNipped-ARNAi-1*/+) and control (*tim*G4/+;U*dcr2*/+) collected on the first day of DD (DD1) (*tim/cry*, $n = 5$; *Pdp1ε/per/clk/cyc*, $n = 3$; *vri*, $n = 6$). For each time series, the value of the lowest time point was set to 1.

B Western blots of proteins from whole-head extracts of *Nipped-A* RNAi and control flies collected on DD1 and probed with TIM, PDP1ε, and PER antibodies.

C Quantification of TIM ($n = 5$), PDP1ε ($n = 3$), and PER ($n = 3$) protein levels of blots in (B). TIM, PDP1ε, and PER protein levels were normalized to that of HSP70. For each time series, the value of the control at the peak time point was set to 1.

D Brains from U*dcr2/+;cry*G4-16/+ and U*dcr2/+;cry*G4-16/U*Nipped-A*RNAi flies collected at CT0, 4, 8, 12, 16, 20 on DD1 were immunostained with TIM (green), PDP1ε (red), PER (green), and PDF (red or green) antisera. The scale bar represents 10 μm.

E Quantification of TIM, PDP1ε, and PER protein levels in the s-LNvs of images in (D) (TIM: CT8: U*dcr2/+;cry*G4-16/+, $n = 89$; U*dcr2/+;cry*G4-16/U*Nipped-A*RNAi, $n = 58$; CT20: U*dcr2*/+;cryG4-16/+, $n = 130$; U*dcr2/+;cry*G4-16/U*Nipped-A*RNAi, $n = 80$; PDP1ε: CT4: U*dcr2*/+;cryG4-16/+, $n = 46$; U*dcr2/+;cry*G4-16/U*Nipped-* RNAi, $n = 26$; CT16: U*dcr2*/+;*cry*G4-16/+, $n = 65$; U*dcr2/+;cry*G4-16/U*Nipped-A*RNAi, $n = 41$; PER: CT0: U*dcr2/+;cry*G4-16/+, $n = 90$; U*dcr2/+;cry*G4-16/U*Nipped-A*RNAi, $n = 58$; CT12: U*dcr2/+;cry*G4-16/+, $n = 86$; U*dcr2/+;cry*G4-16/U*Nipped-A*RNAi, $n = 64$).

Data information: Error bars represent SEM. Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. G4, GAL4; U, UAS.
Source data are available online for this figure.

### NIPPED-A is physically associated with *tim/Pdp1ε* loci and does not exhibit significant oscillation

We next tested whether NIPPED-A functions as an instructive or permissive signal to the clock by assessing its temporal expression pattern. Neither the mRNA nor the protein level of *Nipped-A* displays significant oscillation under light–dark cycles (LD) or DD (Fig 4A and B). We detected binding of NIPPED-A at *tim* and *Pdp1* gene loci that is significantly above background, although the binding does not appear to cycle throughout the day, suggesting that NIPPED-A plays a permissive rather than instructive role in timing the clock (Fig 4C; Appendix Fig S5). As a positive control, we examined the binding of CLK at *tim* and *Pdp1ε* promoters and observed significant changes throughout the day, consistent with previous studies that report cyclic binding of CLK at E-box genes (Appendix Fig S6; Zhou *et al*, 2016). Notably, knocking down *Nipped-A* significantly increases CLK binding at *Pdp1ε* and *per* promoters, while a trend of increase is observed at *tim* promoter. On the other hand, CLK binding at *vri* promoter is not significantly altered. We also observed binding of NIPPED-A at *per* locus although NIPPED-A does not appear to regulate *per* transcription (Fig 4C).

### Knocking down *Nipped-A* reduces *tim/Pdp1ε* pre-mRNA levels and increases H2B ubiquitination at *tim/Pdp1ε* loci

To address how NIPPED-A enhances the mRNA levels of *tim* and *Pdp1ε*, we examined the pre-mRNA levels of these two genes in *Nipped-A* RNAi flies and observed significant reduction, suggesting that knocking down *Nipped-A* down-regulates the transcription of *tim* and *Pdp1ε* (Fig 5A). This is consistent with a role for NIPPED-A/TRAPP in transcriptional activation (Murr *et al*, 2007).

Since NIPPED-A/TRRAP is known to facilitate transcription by recruiting HAT and thus promoting the acetylation of Histone H3 and H4, we measured the effects of knocking down *Nipped-A* on H3 and H4 acetylation status at *tim* and *Pdp1ε* gene loci (Murr *et al*, 2007). In contrary to our expectation, we found there is either no significant change or in some cases even an increase in acetylation (H3K9 and H3K27), which is usually associated with elevated rather than diminished transcription (Fig EV3; Murr *et al*, 2007). This means reduction of *tim* and *Pdp1ε* transcription in *Nipped-A* RNAi flies is probably not due to defects in histone acetylation.

It has been reported that NIPPED-A/TRRAP is a member of the SAGA complex which contains both a HAT and a DUB module, and mutations in TRRAP can impair HAT activity of the SAGA complex (Koutelou *et al*, 2010; Helmlinger, 2012). Since TRRAP serves as a scaffold to interact with both enzymatic modules, we hypothesized that in our case here knocking down *Nipped-A* interferes with the DUB activity of SAGA which in turn impairs transcription, as deubiquitination of histone H2B has been shown to promote transcription (Henry *et al*, 2003; Daniel *et al*, 2004; Wyce *et al*, 2007; Sharov *et al*, 2017). Indeed, we observed significantly enhanced H2B ubiquitination at the promoter and transcriptional start site of *tim* and *Pdp1ε* gene loci in *Nipped-A* RNAi flies, which could account for reduced expression of these two genes (Fig 5B). On the other hand, H2B ubiquitination is not significantly altered at *per* and *clk* loci, which is consistent with the observation that the transcript levels of these two genes are not affected by *Nipped-A* deficiency.

### NIPPED-A functions in synergy with SAGA DUB module to set the pace of the clock

To validate whether H2B deubiquitination by the SAGA complex plays a role in period determination, we knocked down *not*, the deubiquitinase in SAGA complex (Weake *et al*, 2008). The power of the rhythm is significantly reduced when *cry*GAL4-16 is used to drive the expression of two independent RNAi lines, and we have confirmed that *not* mRNA level is indeed decreased (Fig 6A and B, and Appendix Fig S7A and Table S8). At the molecular level, we observed that knocking down *not* leads to significant reduction in the mRNA level of all six core clock genes and significant elevation of H2B ubiquitination at *tim/Pdp1* loci (Fig 6C and D). Over-expressing *not* shortens the long-period phenotype caused by *Nipped-A* deficiency (Fig 6E and F, and Appendix Fig S7B and Table S9). On the other hand, knocking down *not* synergistically enhances the period lengthening effect of *Nipped-A* RNAi, while knocking down *not* alone does not substantially alter the period (Fig 6G; Appendix Table S9). These results indicate that NIPPED-A and NOT function together to regulate the clock.

To further verify that deubiquitination by SAGA is involved in timing the clock, we tested flies lacking *Sgf11*, which is another subunit in the DUB module (Weake *et al*, 2008). Knocking down *Sgf11* leads to significantly reduced power, and the phenotype is quite prominent with one of the RNAi lines but rather mild with the

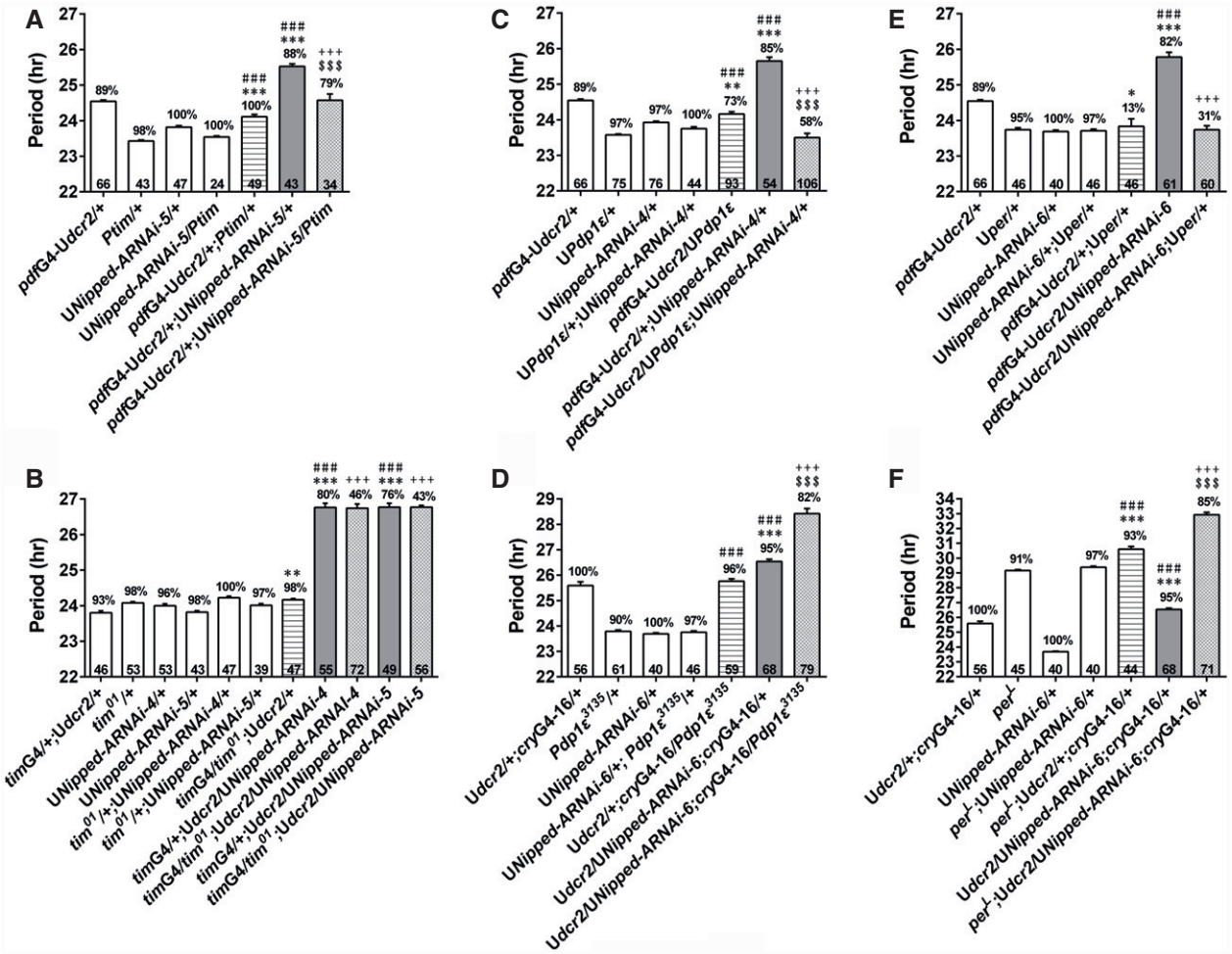



**Figure 3. *Nipped-A* synergistically interacts with *tim*, *Pdp1ε*, and *per* to determine period length.**

A, B The period of DD locomotor rhythm of *Nipped-A* RNAi flies over-expressing *tim* (A) or carrying heterozygous $tim^{01}$ mutation (B). *Ptim* is a *tim* cDNA construct driven by *tim* promoter.

C, D The period of DD locomotor rhythm of *Nipped-A* RNAi flies over-expressing *Pdp1ε* (C) or carrying heterozygous $Pdp1\varepsilon^{3135}$ mutation (D).

E, F The period of DD locomotor rhythm of *Nipped-A* RNAi flies over-expressing *per* (E) or carrying $per^{\perp}$ mutation (F).

Data information: Error bars represent SEM. Digits on the bar are the number of flies tested. Percentage of rhythmicity is indicated above the bars. Statistical difference is measured using one-way ANOVA, $P < 0.001$, Tukey's multiple comparison test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***/\#\#\#/+++/\$\$\$}P < 0.001$, * compared with the G4 control, # compared with the UAS control, + compared with the *Nipped-A* RNAi flies, $ compared with the over-expression or mutant flies. White bar indicates UAS or GAL4 controls. Dashed bar indicates flies with genetically manipulated core clock gene. Gray bar indicates flies with *Nipped-A* knocked down. Checked bar indicates flies with both genetically manipulated core clock gene and *Nipped-A* knocked down. G4, GAL4; U, UAS.

other RNAi line (Fig EV4A–C; Appendix Table S10). There is a significant lengthening of the period with one of the RNAi lines, but the extent of the change is quite small. *Sgf11* mutants also do not show prominent changes in period or power (Fig EV4D; Appendix Table S11; Weake *et al*, 2008). Overall, *Sgf11* deficiency does not appear to result in prominent locomotor rhythm phenotype. However, knocking down or mutating *Sgf11* substantially lengthens the period in *Nipped-A* RNAi flies (Fig EV4E and F; Appendix Table S11). This synergistic interaction between *Nipped-A* and *Sgf11* in determining period length further supports the idea that NIPPED-A acts cooperatively with the DUB module of SAGA to set the pace of the clock, and consistent with the notion that the effects of NIPPED-A on *tim* and *Pdp1ε* are mediated by H2B deubiquitination.

## Discussion

Our study identifies a role for *TRRAP*/*Nipped-A* in setting the pace of the circadian clock in flies. A missense variant (Ile295Phe) and a splice site variant (c.7223.+6) in *TRRAP* have been reported to be associated with schizophrenia in humans, and up to 80% of schizophrenia patients experience circadian/sleep disruptions (Wulff *et al*, 2012; Xu *et al*, 2012; Cosgrave *et al*, 2018). The splice site mutation is predicted to affect the binding of SRP55 splicing factor within a splicing enhancer, and thus could severely disrupt the structure and function of the protein product (Xu *et al*, 2012). The missense variant is located within the Armadillo-like helical motifs at the N terminal of the protein and may affect interactions with binding partners (Sharov *et al*, 2017). Therefore, it is possible these variants result in decreased TRRAP

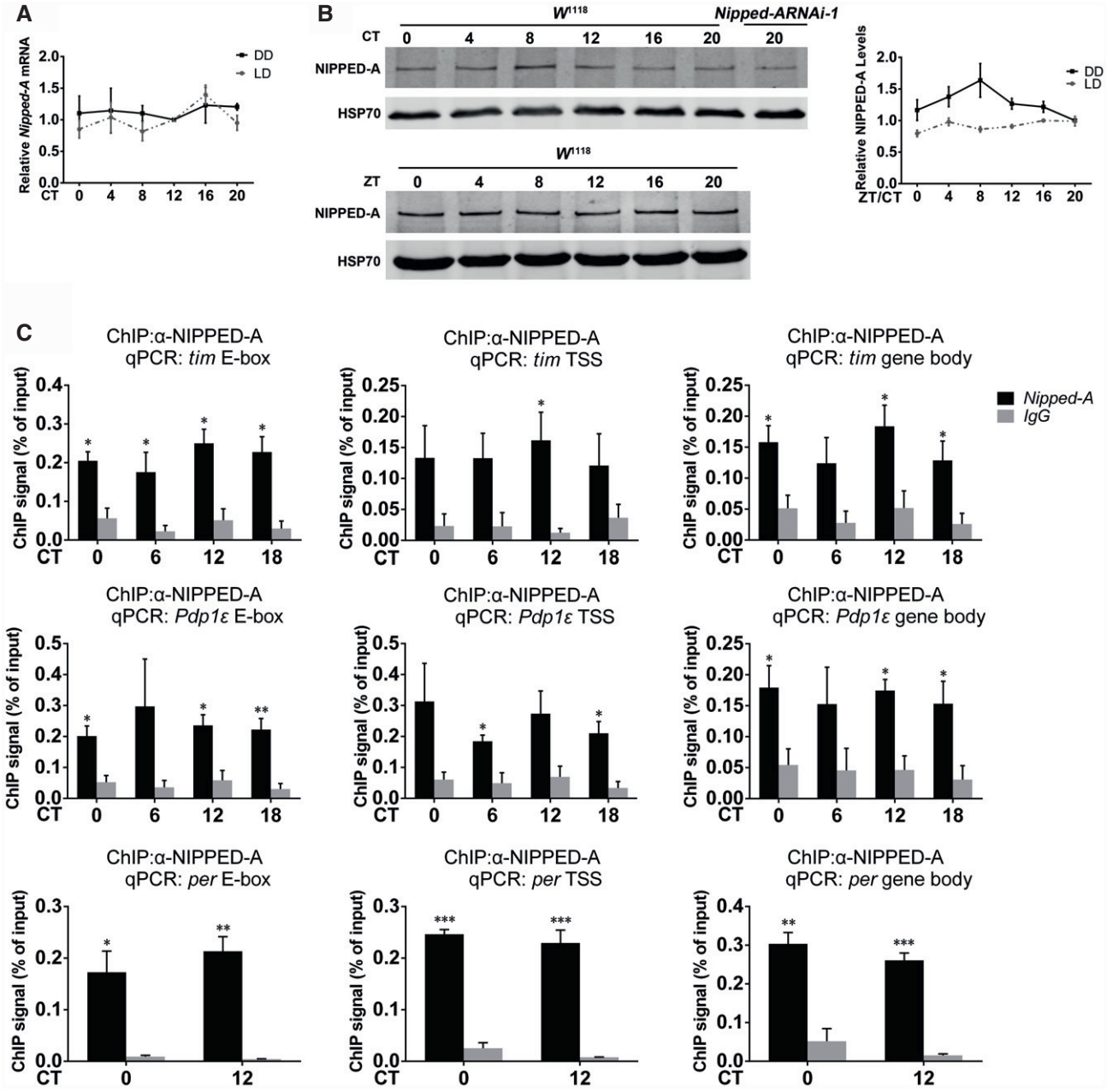



**Figure 4. NIPPED-A is physically associated with *tim* and *Pdp1ε* loci.**

A Plots of relative mRNA abundance of *Nipped-A* in whole-head extracts of $w^{1118}$ flies in LD and DD1 determined by qRT–PCR ($n$ = 3).

B Left panel: Western blots of proteins from whole heads of $w^{1118}$ and *Nipped-A* RNAi flies collected during LD or DD1 and probed with NIPPED-A antibody. Right panel: quantification of NIPPED-A protein levels of blots in the left panel (LD, $n$ = 3; DD1, $n$ = 5). ZT, Zeitgeber time. ZT0 is the time of lights on.

C Chromatin immunoprecipitation (ChIP) assays to detect NIPPED-A binding at E-box, transcription start site (TSS), and gene body of *tim*, *Pdp1ε*, and *per* using $w^{1118}$ flies ($n$ = 3).

Data information: Error bars represent SEM. Two-way ANOVA, and significant effect between NIPPED-A and IgG were found for *tim* E-box ($P < 0.001$), *Pdp1ε* E-box ($P < 0.001$), *per* E-box ($P < 0.001$), *tim* TSS ($P < 0.001$), *Pdp1ε* TSS ($P < 0.001$), *per* TSS ($P < 0.001$), *tim* gene body ($P < 0.001$), *Pdp1ε* gene body, and *per* gene body ($P < 0.001$). Student's $t$-test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. G4, GAL4; U, UAS.
Source data are available online for this figure.

function, which can be mimicked by knocking down *Nipped-A* in flies. Given the conservation of TRRAP protein and the molecular clockwork throughout evolution, we believe TRRAP may carry out similar functions in mammals (Murr *et al*, 2007; Li & Zhang, 2015).

We clearly demonstrate a role for NIPPED-A in circadian period length determination in adults. However, UAS*dcr2/tub*GAL80$^{ts}$*; cry*GAL4-16/UAS*Nipped-ARNAi-4* raised at the permissive temperature and tested at the restrictive temperature showed ~1 h

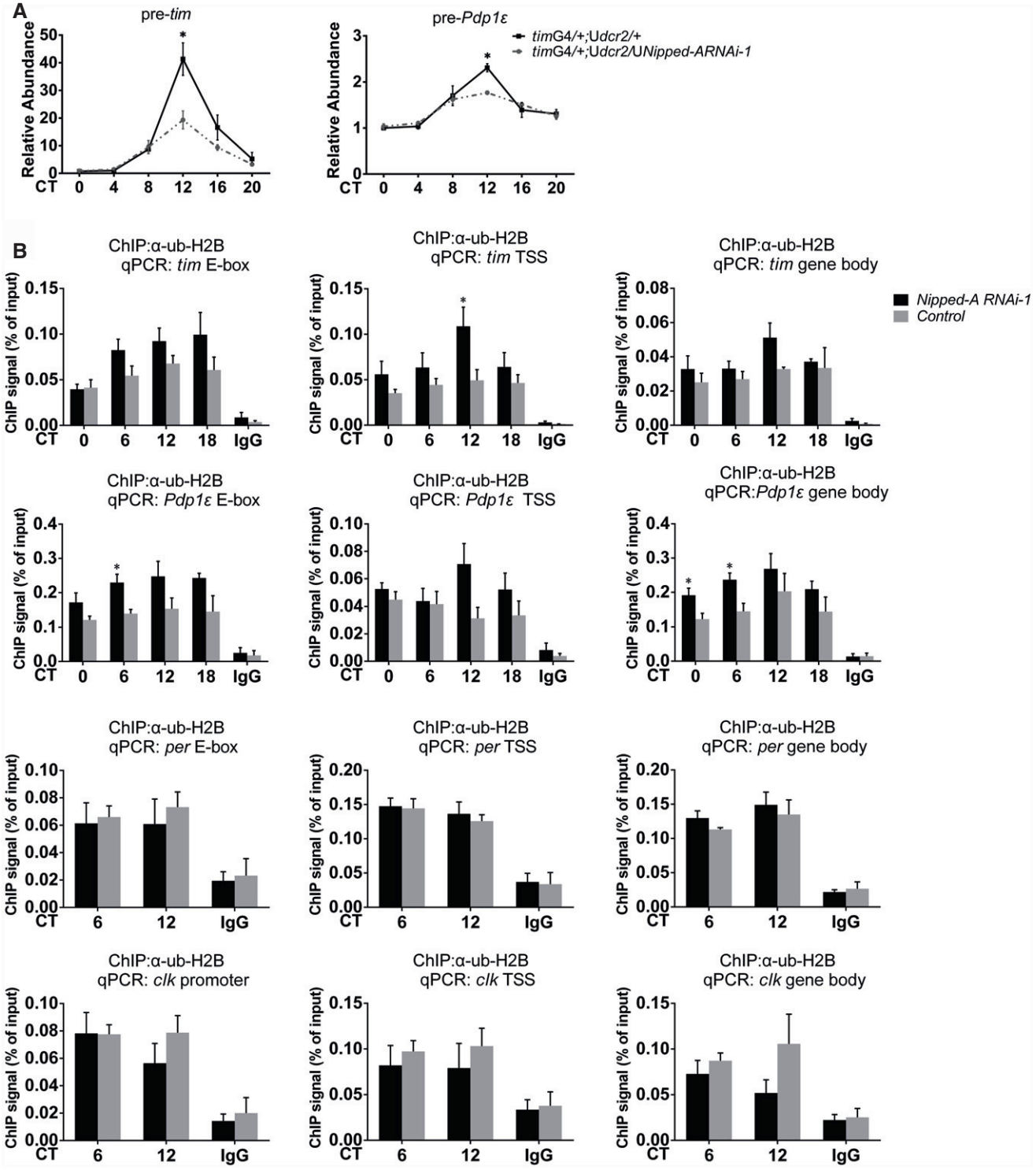
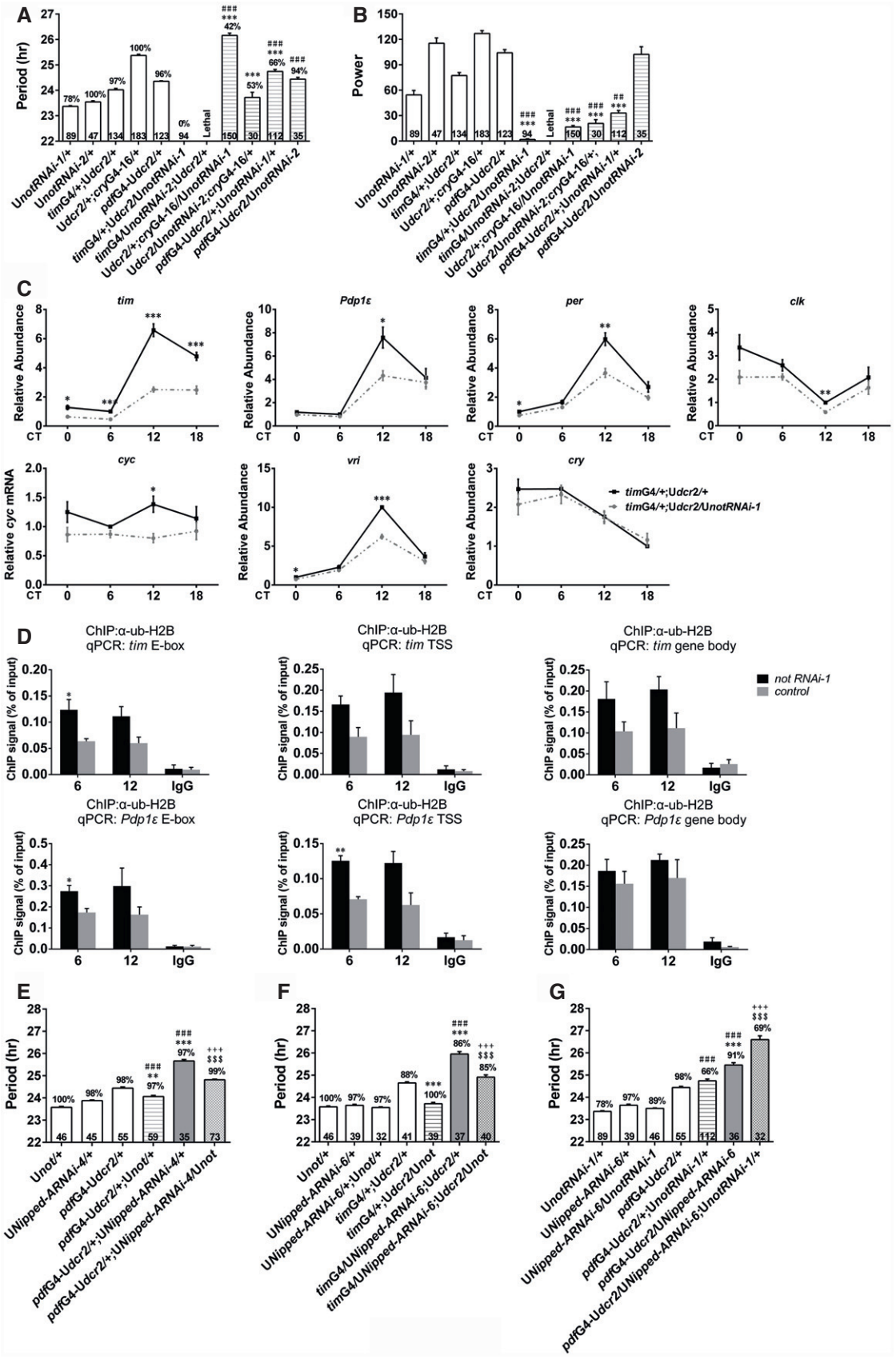



**Figure 5. NIPPED-A promotes the transcription of *tim* and *Pdp1ε* and facilitates H2B deubiquitination.**

A Plots of relative pre-mRNA abundance of *tim* ($n$ = 5) and *Pdp1ε* ($n$ = 3) determined by qRT–PCR in the whole-head extracts of *Nipped-A* RNAi (*timG4/+;Udcr2/UNipped-ARNAi-1*) and control (*timG4/+;Udcr2/+*) flies during DD1.

B ChIP assays to detect ub-H2B binding at E-box, TSS, and gene body of *tim*, *Pdp1ε*, *per, and clk* in *Nipped-A* RNAi (*timG4/+;Udcr2/UNipped-ARNAi-1*) and control (*timG4/+;Udcr2/+*; $n \geq 3$).

Data information: Student's *t*-test, *$P < 0.05$. Two-way ANOVA, and significant effect between ub-H2B and IgG were found for all genomic regions tested ($P < 0.001$). Significant effect of genotypes was found for *tim* E-box ($P < 0.05$), *Pdp1ε* E-box ($P < 0.01$), *tim* TSS ($P < 0.01$), and *Pdp1ε* TSS ($P < 0.001$). G4, GAL4; U, UAS.


A
Period (hr)
B
Power
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tim
Pdp1ε
per
clk
cyc
vri
cry
Relative Abundance
Relative cyc mRNA
CT
timG4/+;Udcr2/+
timG4/+;Udcr2/UnotRNAi-1
D
ChIP:α-ub-H2B
qPCR: tim E-box
qPCR: tim TSS
qPCR: tim gene body
qPCR: Pdp1ε E-box
qPCR: Pdp1ε TSS
qPCR: Pdp1ε gene body
ChIP signal (% of input)
not RNAi-1
control
IgG
E
F
G
Period (hr)
Unot/+
UNipped-ARNAi-4/+
pdfG4-Udcr2/+
pdfG4-Udcr2/+;Unot/+
pdfG4-Udcr2/+;UNipped-ARNAi-4/+
pdfG4-Udcr2/+;UNipped-ARNAi-4/Unot
UNipped-ARNAi-6/+
UNipped-ARNAi-6/+;Unot/+
timG4/+;Udcr2/+
timG4/+;Udcr2/Unot
timG4/UNipped-ARNAi-6;Udcr2/+
timG4/UNipped-ARNAi-6;Udcr2/Unot
UnotRNAi-1/+
UNipped-ARNAi-6/+
UNipped-ARNAi-6/UnotRNAi-1
pdfG4-Udcr2/+
pdfG4-Udcr2/+;UnotRNAi-1/+
pdfG4-Udcr2/UNipped-ARNAi-6
pdfG4-Udcr2/UNipped-ARNAi-6;UnotRNAi-1/+

**Figure 6.**

**Figure 6. *Nipped-A* synergistically interacts with *not* to determine period length.**

A, B The period (A) and power (B) of DD locomotor rhythms of flies with *not* knocked down and controls.

C Plots of relative mRNA abundance vs. CT for clock genes determined by qRT–PCR in whole-head extracts of *not* RNAi (*tim*G4/+;U*dcr2*/U*notRNAi*) and control (*tim*G4/+;U*dcr2*/+) collected on DD1 ($n$ = 5).

D ChIP assays to detect ub-H2B binding at E-box, TSS, and gene body of *tim* and *Pdp1ε* in *not* RNAi (*tim*G4/+;U*dcr2*/U*notRNAi*) and control (*tim*G4/+;U*dcr2*/+; $n$ = 3).

E, F The period of DD locomotor rhythm of flies when knocking down *Nipped-A* and over-expressing *not*.

G The period of DD locomotor rhythm of flies co-expressing *Nipped-A* RNAi and *not* RNAi.

Data information: Error bars represent SEM. (A, B, E–G) Digits on the bar are the number of flies tested. Percentage of rhythmicity is indicated above the bars. Statistical difference is measured using one-way ANOVA, $P < 0.001$, Tukey's multiple comparison test, $^{**}P < 0.01$, $^{***/\#\#\#/+++/\$\$\$}P < 0.001$, * compared with the G4 control, # compared with the UAS control, + compared with the *Nipped-A* RNAi flies, $ compared with the *not* over-expression flies. White bar indicates UAS or GAL4 controls. Dashed bar indicates flies with genetically manipulated *not*. Gray bar indicates flies with *Nipped-A* knocked down. Checked bar indicates flies with both genetically manipulated *not* and *Nipped-A* knocked down. (C, D) Student's $t$-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistical difference is measured using two-way ANOVA, $P < 0.001$, and significant effect of genotypes was found for *tim* E-box ($P < 0.01$), *Pdp1ε* E-box ($P < 0.05$), *tim* TSS ($P < 0.05$), *Pdp1ε* TSS ($P < 0.001$) and *tim* gene body ($P < 0.01$). G4, GAL4; U, UAS.

lengthening of the period (compared to UAS*dcr2/tub*GAL80$^{ts}$*;cry*GAL4-16/+), whereas UAS*dcr2/+;cry*GAL4-16/UAS*Nipped-ARNAi-4* flies in the same experiment showed nearly 2 h longer period (compared to UAS*dcr2/+;cry*GAL4-16/+). This implicates that knocking down *Nipped-A* during development exerts influence on adult period, suggesting a role for NIPPED-A in modulating the development of the clock.

Previous work reported a potential role for NIPPED-A in mediating light resetting effects on the clock possibly by modulating light-induced degradation of the blue light photoreceptor CRYPTOCHROME and subsequently TIM (Sathyanarayanan *et al*, 2008; Adewoye *et al*, 2015). However, the circadian changes we observed here are in the absence of light, and *cry* mutants do not show circadian defects in DD (Stanewsky *et al*, 1998). Therefore, we believe NIPPED-A also functions in the clock under DD and the actions on TIM are likely independent of CRY. In line with this idea, we do not observe significant effects of knocking down *Nipped-A* on *cry* mRNA level. Unfortunately, we were not able to obtain a working CRY antibody to examine whether there is any alteration of CRY protein.

The lengthened period observed in flies with *Nipped-A* knocked down is at least in part due to decrease in TIM and consequently PER. This could lead to a delay in PER accumulation which ultimately slows down the pace of the molecular clockwork (Hardin, 2011). Consistently, over-expressing *tim* or *per* rescues the lengthened period caused by knocking down *Nipped-A*. As for whether alteration in PDP1ε levels can exert effects on the core clock is still an issue of debate (Cyran *et al*, 2003; Benito *et al*, 2007; Lim *et al*, 2007; Zheng *et al*, 2009). PDP1ε is known to activate *clk* transcription; however, in *Nipped-A* RNAi flies, we observe reduced *Pdp1ε* but not *clk* mRNA level. This may be because the remaining PDP1ε is sufficient for maintaining normal *clk* expression. On the other hand, we are able to specifically rescue the period phenotype of *Nipped-A* deficiency by over-expressing *Pdp1ε,* and we have verified that over-expressing *Pdp1ε* enhances *clk* mRNA level. This in turn leads to increased *per* and *tim* expression, thus reverting the long-period phenotype. *Pdp1* and *per* mutations interact with *Nipped-A* to synergistically lengthen the period, whereas *tim* mutation appears to interact with *Nipped-A* to impinge on the power of the rhythm. We reason that the effect of $tim^{01}$/+ on PER may not be strong enough to influence period length, but may act together with *Nipped-A* to affect the power of the rhythm via other mechanisms that are yet unclear.

Multiple studies in yeast have indicated that Tra1, the yeast homolog of TRRAP, interacts with transcriptional activators and plays a crucial role in recruiting the SAGA complex to promoters (Weake & Workman, 2012). CLK and CYC are well-characterized transcriptional activators of *tim* and *Pdp1ε*, binding rhythmically to the E-box elements upstream of these genes (Li & Zhang, 2015). However, here we do not observe rhythmic association of NIPPED-A at *tim* and *Pdp1ε* loci, suggesting that NIPPED-A is not recruited by CLK/CYC. It has been shown that the SAGA complex may also be recruited and/or retained at the promoter region by interacting with the core transcription machinery or histone marks, which could potentially explain the constitutive association of NIPPED-A at *tim* and *Pdp1ε* loci (Weake & Workman, 2012). Another possibility is that CLK/CYC recruits NIPPED-A in clock cells, but this rhythmic recruiting event is masked by constitutive binding in non-clock cells. More sensitive methods will be needed to reveal a potential temporal binding of NIPPED-A to its targets. We found that knocking down *Nipped-A* leads to enhanced (or a trend of enhanced) binding of CLK at *Pdp1ε*, *per,* and *tim* promoters but not at *vri* promoter, which may be due to some compensatory mechanism to make up for the lack of *tim*, *Pdp1ε,* and *per*.

Although TRRAP/NIPPED-A has previously been characterized as a HAT cofactor, our results indicate that NIPPED-A regulates the clock by facilitating H2B deubiquitination specifically at *tim* and *Pdp1ε* loci, thus promoting the transcription of these two genes. NIPPED-A binding is not limited to *tim* and *Pdp1ε* loci, as NIPPED-A also binds to the *per* locus and likely other sites. However, NIPPED-A appears to specifically regulate H2B deubiquitination at *tim*/*Pdp1ε* loci possibly via the SAGA complex. Consistently, it has been shown in yeast that Tra1 only modulates the expression of a subset of the SAGA-dependent genes and Tra1 mutation eliminates the recruitment of SAGA to some genes but not others (Helmlinger *et al*, 2011). Therefore, it is possible that *tim* and *Pdp1ε* transcription are particularly susceptible to NIPPED-A deficiency, whereas the SAGA complex can still be assembled and recruited to the other clock genes.

Knocking down *not* reduces the mRNA level of several core clock genes, including but not limited to *tim* and *Pdp1ε*, which could contribute to the different behavioral phenotypes between *not* RNAi and *Nipped-A* RNAi flies. *Sgf11* RNAi and heterozygous mutant flies do not demonstrate prominent locomotor rhythm phenotypes, even though *Sgf11* mRNA levels are substantially reduced. This is likely because the remaining SGF11 in these flies is sufficient to maintain circadian function. Despite the differences in circadian phenotypes caused by lack of *Nipped-A* vs. *not* or *Sgf11*, both *not* and *Sgf11* deficiency synergistically enhances the long-period phenotype caused by knocking down *Nipped-A*. Therefore, we believe NIPPED-A functions together with the DUB module of SAGA to regulate the clock.

 

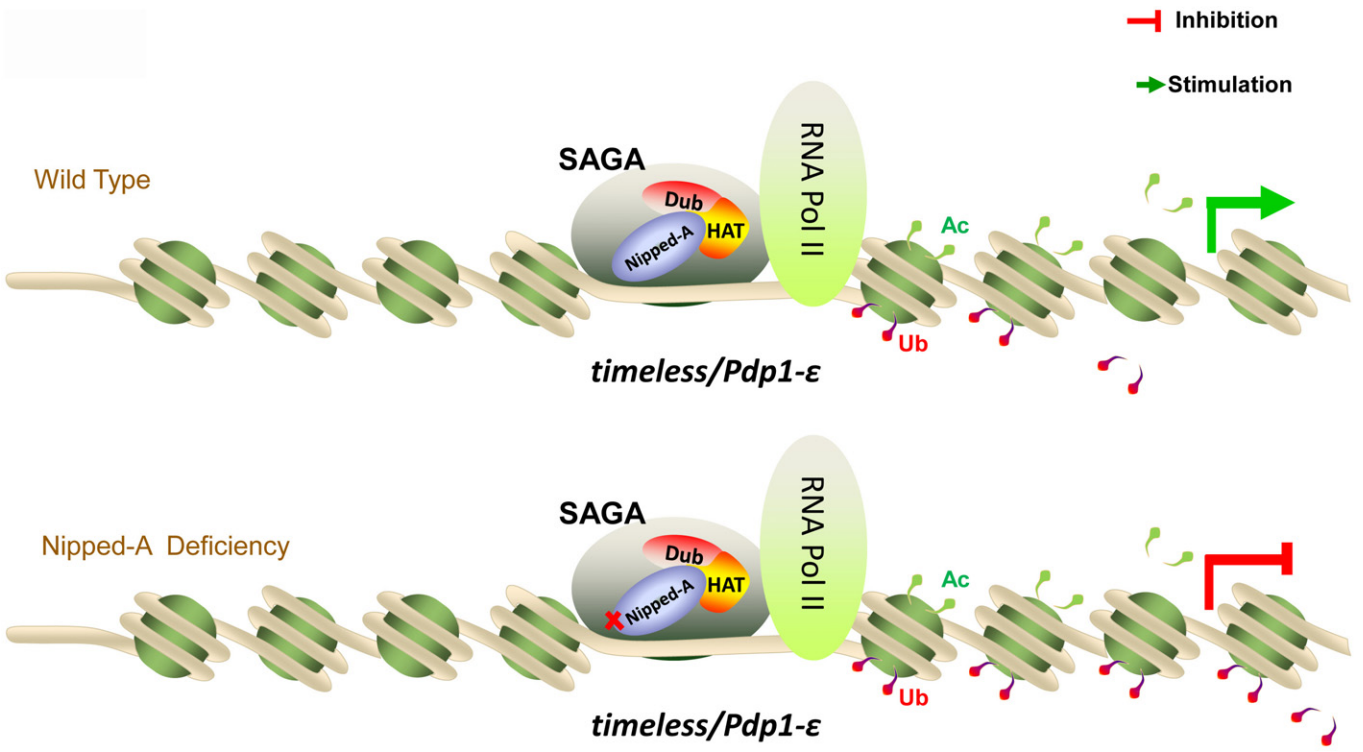


**Figure 7. A model for the role of NIPPED-A in setting the pace of the clock.**
NIPPED-A functions in the SAGA complex and promotes *tim/Pdp1ε* transcription by facilitating deubiquitination of H2B. When NIPPED-A is deficient, ubiquitylation of H2B is enhanced, thus preventing the transcription of *tim* and *Pdp1ε*.

On the other hand, the up-regulated H3K9 and H3K27 acetylation at *tim* and *Pdp1ε* promoter regions may reflect some sort of compensatory mechanism in response to lack of *tim* and *Pdp1ε*. Interestingly, mutation of ATAXIN7, a protein that mediates the association of DUB module with the rest of the SAGA complex, has been shown to induce H3 hyperacetylation, but the genes that are hyperacetylated are transcriptionally down-regulated, similar to what we observed here in *Nipped-A* RNAi flies (Helmlinger *et al*, 2006; Koutelou *et al*, 2010).

Compared to other epigenetic modifications such as histone methylation and acetylation, ubiquitination of histones is much less studied and understood. A previous study in mouse liver demonstrates rhythmic H2B monoubiquitination of circadian E-box genes including *Per1* and *Per2*, which may be regulated by Ddb1-Cullin-4 ubiquitin ligase (Tamayo *et al*, 2015). Reducing H2B ubiquitination in fibroblast culture results in shortened period and enhanced *Per1/2* mRNA levels. The authors propose a role for DDB1-CULLIN-4-mediated H2B monoubiquitination during the transcriptional repression phase of the circadian cycle. Here, we showed that knocking down *Nipped-A* leads to increased H2B ubiquitination accompanied by lengthened period and reduced *tim/Pdp1ε* mRNA levels, which is mediated by the DUB module of the SAGA complex. These results support a role for the SAGA complex and H2B deubiquitination in the transcriptional activation phase of the circadian cycle. However, we did not observe cycling of H2B ubiquitination, which could be masked by the constant levels of H2B ubiquitination in non-clock cells in the fly head.

In summary, we propose a role for NIPPED-A in promoting the transcription of *tim* and *Pdp1ε* by facilitating the deubiquitination of H2B, which adds another layer of epigenetic regulation to the clock (Fig 7). Moreover, environmental factors play critical roles in the etiology of schizophrenia, and epigenetic alterations including histone modifications have been implicated in this process (Thomas, 2017). Our findings here could thus provide a potential explanation for circadian disruptions associated with schizophrenia.

## Materials and Methods

### Fly strains

The following fly strains were used in this study: $w^{1118}$, *yw*, genetic background control lines for the RNAi lines (VDRC:60000, VDRC:60100, BDSC:36303), UAS*Nipped-A*RNAi-1 (THU5747), UAS*Nipped-A*RNAi-2 (THU3524), UAS*Nipped-A*RNAi-3 (THU0698), UAS*Nipped-A*RNAi-4 (VDRC:44781), *Nipped-A*RNAi-5 (VDRC:40789), UAS*Nipped-A* RNAi-6 (VDRC:40790), UAS*GFP*, UAS*not*RNAi-1 (THU3093), UAS*not*RNAi-2 (v45776), UAS*not* (DGRC:206254), UAS*Sgf11*RNAi-1 (v100581), UAS*Sgf11*RNAi-2 (v17166), $Sgf11^{e01308}$ (BDSC:17941), *tim*GAL4, *tim*GAL4;UAS*dcr2*, UAS*dcr2*;*cry*GAL4-16, *pdf*GAL4-UAS*dcr2*, *tub*GAL80$^{ts}$, *pdf*GAL4-geneswitch, $tim^{01}$, *Ptim*, UAS*Pdp1ε*, $Pdp1\varepsilon^{3135}$, UAS*per*-10, $per^{L}$, and UAS*clk*. UASflySAM2.0-*Nipped-A* is generated by Tsinghua Fly Center following previously published methods (Jia *et al*, 2018). sgRNA (GCAGTAAACAT

GCAAATAAG) targeting upstream sequence of *Nipped-A* was cloned into flySAM2.0 vector. The construct was then injected into *y sc v nanos-integrase*; *attP40* embryos.

#### *Drosophila* activity monitoring and behavior analysis

Flies were reared on standard cornmeal–yeast–sucrose medium and kept in LD cycles at 25°C. Three- to four-day-old male flies were used to monitor locomotor activity levels using the *Drosophila* Activity Monitor system (Trikinetics) for 4 days of LD followed by 7 days of DD.

*tub*GAL80$^{ts}$ flies were raised at 18°C/29°C and transferred to 29°C/18°C 4 days before the start of behavioral assays. Behavioral assays are conducted at 29°C/18°C accordingly.

To monitor activities of strains containing the *pdf*G4-geneswitch (*pdf*-GS) driver, flies were raised on standard cornmeal–yeast–sucrose medium until pupation. After eclosion, flies were transferred to tubes with standard food containing RU486 (250 μM) for 2–3 days before monitoring locomotor activity. Food used during behavioral assay also contained 250 μM RU486. Ethanol used to solubilize RU486 was added to the food as vehicle control.

#### Cell culture and transient transfection

*Drosophila* S2 cells were maintained in Schneider's medium (Life Technologies) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Life Technologies). The cells were plated in a 12-well plate ($2 \times 10^5$ cell/well) and incubated for 24 h, and si-*Nipped-A* was transiently transfected using Lipofectamine 2000 Transfection Reagent (Life Technologies) according to the protocol provided by the manufacturer. Cells were collected 48 h after the transfection.

#### RNA extraction and quantitative real-time PCR (qRT–PCR)

Flies were entrained in LD for 3 days, collected on the fourth day of LD or DD1 at the indicated time points (ZT or CT) and frozen immediately on dry ice. Fly heads or S2 cells were isolated and homogenized in TRIzol reagent (Ambion, Life Technologies). Chloroform was subsequently added and centrifuged at 12,000 *g* for 15 min at 4°C. Aqueous top layer was collected, and ethanol was added to precipitate RNA. The precipitates were collected by centrifuging at 12,000 *g* for 10 min at 4°C. RNA pellets were washed with 75% ethanol. After air dry, the pellets were dissolved in RNAse-free water. Contaminating genomic DNA was removed by RQ1 DNase (Promega) digestion, and total RNA was directly amplified with TransScript Green One-Step qRT–PCR SuperMix (TransGen Biotechnology). All qPCRs were carried out on a Step One Plus Real-Time PCR System (Applied Biosystems, Life Technologies). The templates were reverse-transcribed at 45°C for 5 min and denatured at 95°C for 30 s, followed by forty cycles with 5 s at 95°C, 15 s at 60°C, 30 s at 72°C, and 15 s at 75°C for data acquisition. Primers used for expression analysis are listed in Appendix Table S12.

#### Western blot

Flies were entrained in LD for 3 days and collected during LD or DD1 at the indicated time points (ZT or CT) and frozen immediately on dry ice. Heads were separated and homogenized in RIPA buffer (20 mM Tris at pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05 mM EGTA, 10% glycerol, 1% Triton X-100, 0.4% sodium deoxycholate, 0.1% SDS) containing protease inhibitor mixture (Roche) and phosphatase inhibitor mixture (Roche). This homogenate was sonicated 3–5 times for 8 s each time and then centrifuged at 16,200 *g* for 15 min at 4°C to remove cell debris. Supernatant was collected, transferred to new tubes, and centrifuged again at 16,200 *g* for 10 min at 4°C. Supernatant was collected, and protein lysates were prepared in SDS–PAGE loading buffer. Equal amounts of protein were run on 7.5% SDS–PAGE gels and then transferred to nitrocellulose membrane. After incubation with primary antibodies at 4°C overnight, membranes were incubated with secondary antibodies at room temperature for 1 h. The primary antibodies used were rat anti-TIM (1:1,000; a gift from Joanna Chiu), guinea pig anti-PDP1ε (1:5,000; a gift from Paul Hardin), guinea pig anti-PER (1:1,000; a gift from Joanna Chiu), and mouse anti-HSP70 (1:5,000; Sigma). Secondary antibodies used were conjugated either with IRDye 680 or IRDye 800 (LI-COR Biosciences) and incubated at a concentration of 1:1,000. Blots were visualized with Odyssey Infrared Imaging System (LI-COR Biosciences). Antibodies used in this study are listed in Appendix Table S13.

#### Immunostaining

Male flies were entrained for 3 days in LD and collected on DD1. Flies were anesthetized with $CO_2$ and dissected in 3.7% formaldehyde diluted in PBS. After fixing for 30 min at room temperature, the brains were rinsed three times in PBS and incubated in PBS with 1% Triton for 15 min at room temperature. The brains were then incubated with 5% goat serum diluted in PBT (PBS with 0.3% triton) for 1 h at room temperature, followed by overnight incubation of 1:200 rat anti-TIM (a gift from Joanna Chiu), 1:500 rabbit anti-PER (a gift from Michael Rosbash), 1:1,000 guinea pig anti-PDP1ε (a gift from Paul Hardin), and 1:50 mouse anti-PDF (Drosophila Studies Hybridoma Bank) in PBT at 4°C. After 6 × 20 min PBT rinses, the brains were incubated with secondary antibodies: donkey anti-mouse Alexa Fluor-488 (1:1,000, Molecular Probes), donkey anti-mouse Alexa Fluor-594 (1:1,000, Life Technologies), donkey anti-rat Alexa Fluor-488 (1:500, Abcam), donkey anti-guinea pig Alexa Fluor-594 (1:1,000, Jackson ImmunoResearch Laboratories), and donkey anti-rabbit Alexa Fluor-488 (1:1,000, Abcam). After incubating overnight at 4°C, the brains were rinsed 6 × 20 min in PBS, and then mounted and imaged using an Olympus FV1000 confocal microscope with 20× or 60× oil lens (Olympus). Images were acquired using the same settings (power, gain, offset) for each experiment. Antibodies used in this study are listed in Appendix Table S13.

#### Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted following a published protocol (Kwok *et al*, 2015). Flies were entrained in LD at 25°C for 3 days and collected at four time points (CT) on DD1. 600 μl of fly heads were mixed with 2 ml of NEB buffer [10 mM Tris–HCl at pH 8.0, 10 mM NaCl, 0.1 mM EGTA at pH 8.0, 0.5 mM EDTA at pH 8.0, 1 mM DTT, 0.5% tergitol NP-10, 0.5 mM spermidine, 0.15 mM spermine,

 

1× protease inhibitor mixture (Roche), and phosphatase inhibitor mixture (Roche)]. The homogenates were centrifuged at 4,700 *g* for 10 min to remove cell debris. Pellets were resuspended in 1 ml of sucrose gradient in NEB (0.6 ml of 1.6 M sucrose in NEB, 0.4 ml of 0.8 M sucrose in NEB) and centrifuged at 13,800 *g* for 20 min. Pellets were subsequently resuspended in 1 ml of NEB with 1% formaldehyde and crosslinked for 15 min at room temperature with rotation. 150 μl of 1 M glycine was added to stop the crosslinking with rotation for 10 min at room temperature. Pellets were collected by centrifugation at 4,700 *g* for 5 min, washed two times with 1 ml NEB buffer each time, and resuspended in 500 μl of sonication buffer [10 mM Tris–HCl at pH 7.5, 2 mM EDTA, 1% SDS, 0.2% Triton X-100, 0.5 mM spermidine, 0.15 mM spermine, 1× protease inhibitor mixture (Roche), and 1× phosphatase inhibitor mixture (Roche)]. Samples were sonicated 6 × 30 s on 30% of high setting and then centrifuged at 13,800 *g* for 10 min. Supernatants were collected and centrifuged again at 16,200 *g* for 10 min at 4°C. 200 μl of the supernatants were used for IP/IgG, respectively, and 20 μl for input. IP buffer [50 mM Tris–HCl at pH 7.6, 2 mM EDTA, 1% Triton X-100, 0.1% DOC, 150 mM NaCl, 0.5 mM EGTA, 1× protease inhibitor mixture (Roche), and 1× phosphatase inhibitor mixture (Roche)] was added to IP reactions to a total of 800 μl per sample. Antibodies were then added and incubated with rotation overnight at 4°C. Antibodies used for ChIP are as follows: acetyl-histone H3 (Millipore; 06-599), acetyl-histone H3-K9 (Abcam; ab4441), acetyl-histone H3-K14 (Millipore; 07-353), acetyl-histone H3-K27 (Abcam; ab4729), acetyl-histone H4 (Millipore; 06-866), acetyl-histone H4-K16 (Millipore; 07-329), TRRAP (Sigma, PLA0167), ubiquityl-histone H2B (Millipore; 05-1312-I), and mouse/rabbit anti-IgG (Santa Cruz). For each IP/IgG, 50 μl of Protein G or Protein A magnetic beads (Bio-Rad) were washed twice in 1 ml of CW buffer [50 mM Tris–HCl at pH 7.6, 1 mM EDTA, 1% Triton X-100, 0.1% DOC, 0.1% BSA, 0.5 M KCl in PBS, 150 mM NaCl, 0.5 M EGTA, 0.1% SDS, 1× protease inhibitor mixture (Roche), and 1× phosphatase inhibitor mixture (Roche)]. The beads were then added to the samples and incubated for 2 h with rotation at 4°C. After that, beads were captured and washed for 30 min for two times in 1 ml of CW buffer, once in LW buffer (10 mM Tris–HCl at pH 8.0, 0.25 M LiCl, 0.5% NP40, 0.5% DOC, 1 mM EDTA), and once in TE buffer for 10 min. Supernatants were removed, and 150 μl of CE buffer (50 mM Tris–HCl at pH 8.0, 10 mM EDTA, 1% SDS, 1 mM DTT, 0.1 mg/ml proteinase K, 50 mM NaCl, and 0.05 mg/ml RNase A) was added. Equal amount of CE buffer was added to input samples as well. All samples were incubated for 2 h at 37°C. Beads were then removed from IP samples, and supernatants were de-crosslinked overnight at 65°C. DNA was extracted using phenol: chloroform:iso-amyl alcohol (25:24:1) and subjected to qPCR (primers used are listed in Appendix Table S12).Three technical replicates of qPCR were performed for each biological replicate, and at least three biological replicates were performed. Antibodies used in this study are listed in Appendix Table S13.

#### Immunoprecipitation

Flies were entrained in LD at 25°C for 3 days and collected at one time point (CT 12) on DD1. Heads were collected on dry ice, and 1 ml RIPA buffer was added for each reaction. Using a motorized plastic pestle, fly heads were homogenized and then sonicated for 3 × 5 s with 10-s pauses. Debris were centrifuged at 17,000 *g* for 15 min at 4°C. Supernatants were transferred into a new 1.5-ml tube, and pellets were discarded. Samples were centrifuged for another 10 min at 17,000 *g* (4°C), and then, supernatants were collected. Protein concentration was quantified. 50 μl of the supernatant was used as input, and the remaining sample was divided equally into two parts. 7 μg NIPPED-A antibody and IgG were added, respectively. The samples were incubated at 4°C overnight, and 50 μl of Protein A magnetic beads (Bio-Rad) were added followed by incubation for 2 h with rotation at 4°C. Beads were then captured and washed for 10 min for two times in 1 ml RIPA buffer. SDS–PAGE loading buffer was added subsequently. Samples were incubated at 95°C for 10 min. Equal amounts of protein were run on 7.5% SDS–PAGE gels.

#### Quantification and statistical analysis

##### *Behavior analysis*

Analyses of period, power, and rhythmicity during DD were carried out using ClockLab (Actimetrics) software. For DD rhythmicity, rhythmic flies were defined as those with chi-squared power-significance ≥ 10. Period calculations considered all flies with power-significance ≥ 10. One-way ANOVA and Tukey's multiple comparison test (Prism GraphPad) were used to compare the differences between different genotypes.

##### *Immunostaining quantification*

The intensity of TIM, PDP1ε, and PER signals was quantified by ImageJ software. Student's *t*-test (Microsoft Excel) was used to compare the average intensity values between different genotypes.

##### *Quantitative RT–PCR*

*Beta-actin* or *rp49* (for mRNA analysis) and *cbp20* (for pre-mRNA analysis) were used as normalization control. The delta–delta CT method was used for quantification. The value of the control genotype was set to 1. Student's *t*-test (Microsoft Excel) was used to compare the differences between genotypes.

##### *Western blot quantification*

Protein levels of TIM, PDP1ε, PER, NIPPED-A, and HSP70 were quantified by the software image studio for Odyssey (LI-COR Biosciences). TIM/PDP1ε/PER/NIPPED-A levels were normalized to HSP70. The value of the control genotype was set to 1. Student's *t*-test (Microsoft Excel) was used to compare the differences between genotypes.

##### *ChIP assay quantification*

ANOVA (Prism GraphPad) and Student's *t*-test (Microsoft Excel) were used to compare the differences between genotypes and conditions.

**Expanded View** for this article is available online.

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### Author contributions

BB, LC, and LZha designed the experiments; BB, LC, LZhe, and WH conducted the experiments; and LZha wrote the paper.

### Conflict of interest

The authors declare that they have no conflict of interest.

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