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PERIOD-controlled deadenylation of the *timeless* transcript in the *Drosophila* circadian clock

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The *Drosophila* circadian oscillator relies on a negative transcriptional feedback loop, in which the PERIOD (PER) and TIMELESS (TIM) proteins repress the expression of their own gene by inhibiting the activity of the CLOCK (CLK) and CYCLE (CYC) transcription factors. A series of posttranslational modifications contribute to the oscillations of the PER and TIM proteins but few posttranscriptional mechanisms have been described that affect mRNA stability. Here we report that down-regulation of the POP2 deadenylase, a key component of the CCR4–NOT deadenylation complex, alters behavioral rhythms. Down-regulating POP2 specifically increases TIM protein and *tim* mRNA but not *tim* pre-mRNA, supporting a posttranscriptional role. Indeed, reduced POP2 levels induce a lengthening of *tim* mRNA poly(A) tail. Surprisingly, such effects are lost in *per⁰* mutants, supporting a PER-dependent inhibition of *tim* mRNA deadenylation by POP2. We report a deadenylation mechanism that controls the oscillations of a core clock gene transcript.

circadian rhythms | clock genes | mRNA poly(A) tail | CAF1/POP2 | CCR4–NOT complex

Circadian clocks are present in most living organisms and drive 24-h molecular oscillations to adapt physiological and behavioral functions to day–night cycles. Animal circadian oscillators rely on a transcriptional negative feedback loop where an activation complex induces the expression of its own repressors (1). A key feature of this loop is the slow accumulation of the repressors, which temporally defines active and inactive phases of transcription during a 24-h cycle. In *Drosophila*, the two basic helix–loop–helix PER–ARNT–SIM (bHLH PAS) proteins CLOCK (CLK) and CYCLE (CYC) form the activation complex and the repression complex is made of the PERIOD (PER) and TIMELESS (TIM) proteins (2). Whereas PER is a clear transcriptional inhibitor, TIM appears to be essential for controlling the stability, subcellular localization, and transcriptional activity of its PER partner (3–5). The temporal restriction of PER-dependent transcriptional repression to late night and early day is largely due to a number of posttranslational mechanisms where a series of kinases and phosphatases as well as specific components of the ubiquitin–proteasome pathway target PER and TIM proteins (3, 6–8). In the last few years, components of the translational machinery were added to the repertoire of molecules that control PER cycling (9, 10).

The comparison between circadian transcription and cycling transcripts reveals a strong contribution of posttranscriptional mechanisms to circadianly-controlled gene expression in flies (11–14) and mammals (14–17). A few posttranscriptional mechanisms have been reported to control core clock gene mRNAs in *Drosophila* (9, 18). These include alternative splicing of *per* mRNA, which contributes to the environmental adaptation of the clock, and posttranscriptional control of *Clk* mRNA stability, thus CLK protein levels, in particular through miRNAs (19, 20).

The polyadenylation of eukaryotic mRNAs stabilizes mRNAs and plays a major role in their export and subsequent translation (21, 22). In mammals, circadian control of mRNA poly(A) tail

length affects numerous transcripts and contributes to the oscillations of the corresponding protein levels (23, 24). A key player in regulating poly(A) length is the CCR4–NOT complex (25), which contains two deadenylase components encoded by the *Pop2* (homolog of *Schizosaccharomyces pombe caf1*) and *twin* (homolog of *S. pombe ccr4*) genes in flies (26). In this study, we reveal an example of the regulation of mRNA oscillations of a core clock gene, *timeless*, through the control of the polyadenylation of its mRNA by the POP2 deadenylase. Furthermore, we show that POP2-dependent deadenylation of the *tim* transcript is controlled by PER.

Results

The POP2 Deadenylase Is Required for Behavioral and Molecular Cycling. To isolate new clock components, UAS-RNAi lines from fly stocks of the National Institute of Genetics (NIG-Fly) collection were crossed with flies carrying the *gal4* driver *gal1118*, which mostly targets the neurons expressing the pigment-dispersing factor (PDF) neuropeptide (27). The rest-activity rhythms of about 6,000 *gal1118 > RNAi* flies were tested in constant darkness (DD) after entrainment in light–dark (LD) cycles (28). We observed that down-regulating the *Pop2* gene decreased behavioral rhythmicity and two other nonoverlapping *Pop2* RNAis gave similar effects, indicating that the behavioral defects were a consequence of *Pop2* down-regulation (Fig. 1A and *SI Appendix*, Table S1). Down-regulating *twin*, which encodes the other deadenylase subunit of the CCR4–NOT complex (29), did not affect the behavioral rhythms (*SI Appendix*,

Significance

Circadian oscillators rely on transcriptional negative feedback loops. In *Drosophila*, the key transcriptional repressor PERIOD (PER) slowly accumulates during the night under the control of its partner TIMELESS (TIM). A large number of posttranslational mechanisms regulate PER and TIM stability, but no mechanisms affecting the stability of their transcripts have been described. mRNA stability depends on the length of the poly(A) tail. We show that a deadenylase, POP2, shortens *tim* mRNA poly(A) tail, thus decreasing *tim* mRNA and TIM protein levels. Moreover, POP2 activity on *tim* mRNA appears to be inhibited by PER itself. These results reveal polyadenylation control of a core clock gene transcript and suggest that the repressor of the feedback loop also acts as a posttranscriptional regulator.

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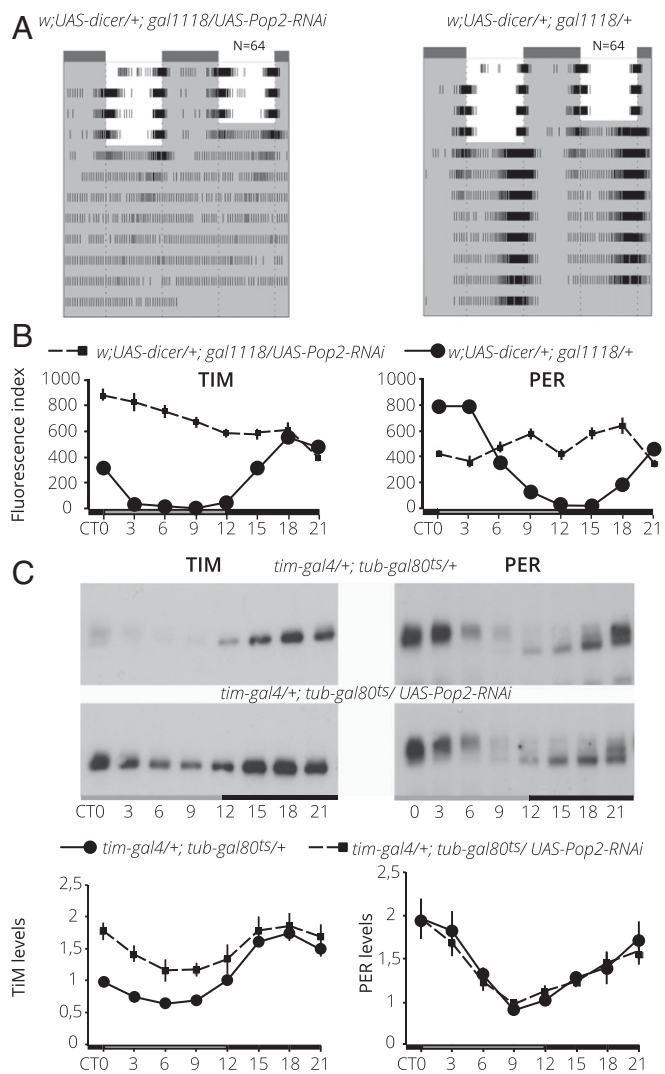


Fig. 1. *Pop2* down-regulation alters behavioral and molecular rhythms. (A) Averaged actograms. White areas correspond to light on and gray areas to darkness. N, number of flies. (B and C) Bars indicate night (black) and subjective day (gray). (B) Anti-PER and anti-TIM immunoreactivity in the s-LNVs. Fluorescence index is given in arbitrary units. Error bars indicate SEM. (C, Top) Anti-PER and anti-TIM Western blots of head extracts. (C, Bottom) Quantification of PER and TIM in Western blots. The results are normalized to the control mean value at CT12, set to 1. Error bars indicate SEM. The difference was significant ($P < 0.0001$) for TIM, nonsignificant (ns) for PER, using a two-way ANOVA of genotype and time (CT0–CT9).

Table S1), suggesting a specific clock function for POP2 within or out of the CCR4–NOT complex. Down-regulation of *Not1*, which encodes the scaffold protein of the complex, strongly damaged the clock neurons (SI Appendix, Fig. S1), preventing us from analyzing its behavioral function.

Oscillations of the clock proteins were analyzed in *Pop2* RNAi (*Pop2R*) flies. We looked at the PDF-expressing small ventral lateral neurons (s-LNVs), which are the key pacemaker neurons for behavioral rhythms in DD (30, 31). PER and TIM cycling were blunted in *gal1118 > Pop2R* flies with a large increase of TIM immunoreactivity and intermediate levels of PER immunoreactivity (Fig. 1B). *Pop2* loss-of-function alleles as well as *Pop2* RNAi expression under the control of the broader *tim-gal4* driver were lethal. Restricting RNAi expression to the adult stage by combining *tim-gal4* with *tub-gal80^{TS}* (hereafter *tim-tub*) allowed us to obtain adult flies that lived for a few days. This was

not sufficient for behavioral analysis but the genotype could thus be used for molecular assays (Materials and Methods). We first looked at PER and TIM in the s-LNVs of *tim-tub > Pop2R* flies (SI Appendix, Fig. S1). The two proteins showed a strong increase of their levels during subjective day (corresponding to light phase during entrainment) but maintained robust oscillations. To better characterize the effect of *Pop2* down-regulation, we analyzed *tim-tub > Pop2R* head extracts. *Pop2* mRNA levels were decreased by 30–40% in these extracts (SI Appendix, Fig. S1). *Pop2* expression being not restricted to *tim-gal4*-expressing cells, the real decrease of mRNA levels in these cells is likely much more pronounced. These experiments first indicated that *Pop2* mRNA levels do not cycle in wild-type flies at DD1 (SI Appendix, Fig. S1). We then analyzed PER and TIM oscillations in *tim-tub > Pop2R* head extracts (Fig. 1C). TIM cycling was strongly affected at DD1 with a twofold increase of protein levels during the subjective day and higher levels persisting at the beginning of the night. In contrast, PER oscillations were unaffected at DD1. We believe that driver-dependent differences in *Pop2* RNAi expression levels in the different cells explain most of these differences, but it is also possible that POP2 has a more prominent role in s-LNVs.

The clearly different effects of *Pop2* RNAi on PER and TIM oscillations in head extracts supported TIM as the primary target of *Pop2* down-regulation. Since TIM protects PER from degradation (32), the large PER increase that was observed in the s-LNVs of *Pop2* down-regulated flies could be a consequence of their very high TIM levels. In contrast to DD, daytime TIM levels were only slightly increased in LD conditions (SI Appendix, Fig. S2), indicating that light could counteract *Pop2* RNAi effects, likely through light-induced TIM degradation (33–35).

POP2 Controls *tim* but Not *per* mRNA Stability. The deadenylase function of POP2 prompted us to analyze *per* and *tim* mRNA oscillations in *Pop2* RNAi flies. We compared mRNA and pre-mRNA levels at DD1. In agreement with the Western blot results for PER and TIM proteins, *tim* mRNA levels but not *per* mRNA levels were increased during subjective day in head extracts (Fig. 2A). For both *per* and *tim*, lower mRNA levels were observed during the subjective night (corresponding to dark phase during entrainment). As a consequence, *tim* mRNA oscillations were almost abolished in *Pop2* down-regulated flies, whereas *per* oscillations persisted but with lower amplitude compared to control flies. A different picture was observed for pre-mRNAs, which also showed lower levels during subjective night but were not affected during subjective day. The comparison between *per* and *tim* mRNA on one hand and between *tim* mRNA and pre-mRNA on the other hand, supported a specific stabilization of *tim* mRNA during subjective day in *Pop2R* flies. During subjective night, the increased TIM protein levels could explain the lower *per* and *tim* transcription, although it is possible that *Pop2* also has a more direct inhibitory effect on *per* and *tim* transcription (36). *Clk* pre-mRNA and mRNA also showed decreased levels, suggesting lower *Clk* transcription (SI Appendix, Fig. S2). We finally analyzed *per* and *tim* mRNA in LD cycles and observed a similar increase of *tim* mRNA during daytime (Fig. 2B), indicating that the low increase of TIM protein during the day in LD was likely a consequence of light-induced TIM degradation. *Pop2* down-regulation thus induces a specific increase of *tim* mRNA levels during daytime in the presence or absence of light.

POP2 Specifically Regulates *tim* mRNA Polyadenylation. We asked whether *Pop2* would specifically control *tim* mRNA polyadenylation. We used a poly(A) tail-length assay (PAT assay, see Materials and Methods) to ask whether down-regulating *Pop2* would change the 3' ends of *per* and *tim* mRNAs. We looked at *per* and *tim* transcripts at circadian time (CT)6, when *tim* mRNA levels are largely increased by *Pop2* down-regulation. A clear lengthening of *tim* mRNA 3' end was observed, whereas *per* mRNA 3' end length remained unchanged (Fig. 3A). *tim* mRNA

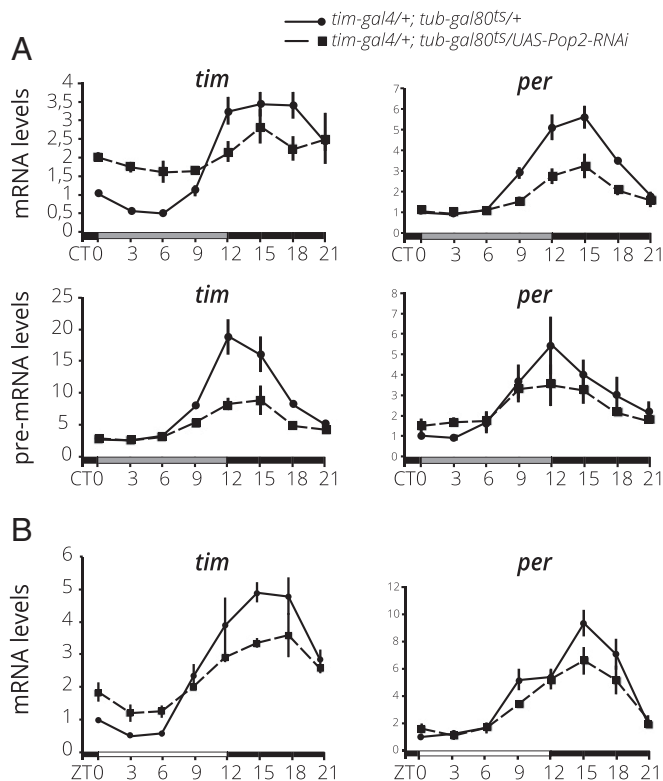


Fig. 2. Posttranscriptional control of *tim* mRNA cycling by *Pop2*. Quantitative RT-PCR analysis of *per* and *tim* mRNA (A and B) and pre-mRNAs (A) in head extracts. Error bar indicate SEM. (A) DD conditions. Average values from at least three independent experiments are normalized to the control mean value at CT0 set to 1. The difference was significant with $P < 0.0001$ for *tim*, ns for *per*, pre-*tim* and pre-*per*, using a two-way ANOVA of genotype and time (CT0–CT6). (B) LD conditions. ZT, zeitgeber time. Bars indicate night (black) and day (white). The difference was significant with $P < 0.001$ for *tim*, ns for *per*, using a two-way ANOVA of genotype and time (ZT0–ZT6).

3' end was then sequenced and no difference was observed in the 3' UTR up to the expected poly(A) start (SI Appendix, Fig. S3). Thus, the PCR products' size change in *tim* > *Pop2R* flies was indeed the consequence of a lengthening of the poly(A) tail and was not due to the use of a more distal polyadenylation site. We then asked whether *tim* poly(A) would show a circadian variation of its length but we could not detect length changes around the clock at DD1 (Fig. 3B, Left). The same experiment was done with *Pop2R* flies. At all time points, *Pop2* down-regulation increased the size of *tim* poly(A) with an almost complete disappearance of the smaller species at CT21–6 (Fig. 3B, Right). This suggested that *tim* poly(A) deadenylation was more sensitive to *Pop2* down-regulation at CT21–6, possibly supporting the idea that POP2 activity would be lower during this time window in wild-type flies. We then used capillary electrophoresis to determine more precisely the effect of POP2 on *tim* mRNA poly(A) at CT6 (Fig. 3C). In a wild-type background, *tim* and *per* mRNA poly(A) tails were short, with *tim* poly(A) (most species below 50 nucleotides) shorter than *per* poly(A) (most species below 150 nucleotides). In contrast, the poly(A) tail of the ACTIN-encoding gene *Act5* was much longer and distributed in discrete species ranging from 0 to 350 nucleotides. In *Pop2* down-regulated flies, *tim* poly(A) length dramatically increased (with a broad peak over 50 nucleotides), whereas the length of *per* and *Act5* mRNA poly(A) tails remained unchanged. We concluded that POP2 is required for keeping the poly(A) tail of *tim* mRNA very short, possibly with a stronger activity at CT9–18 when *tim* transcription is high.

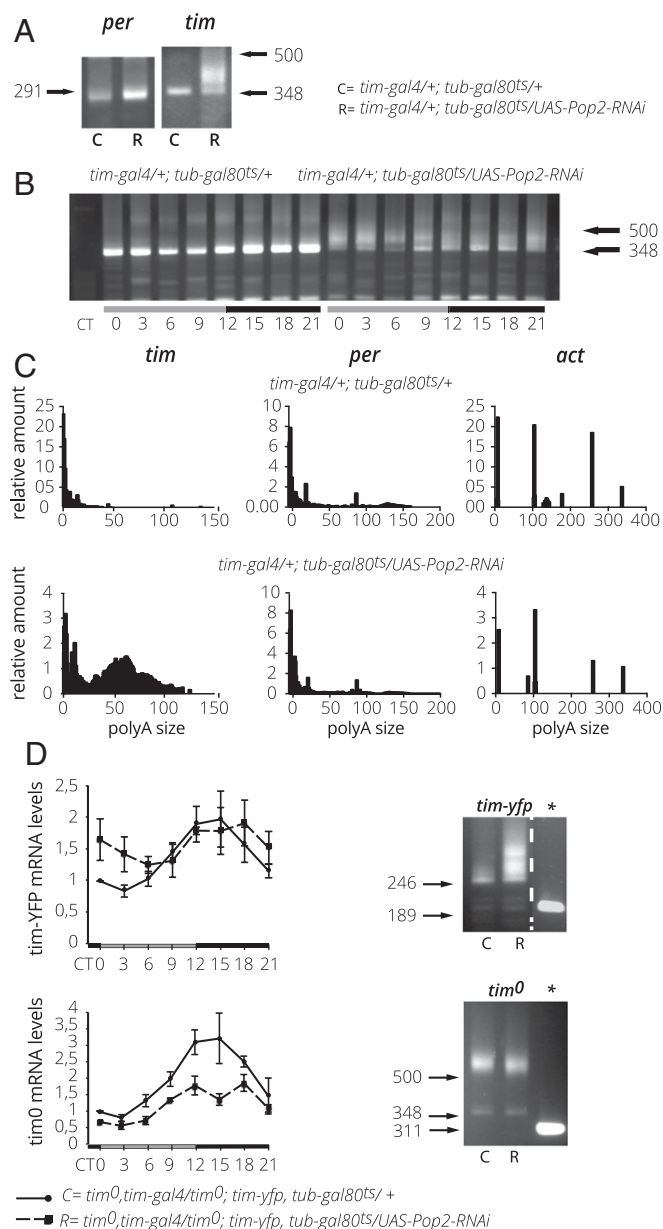


Fig. 3. *Pop2* down-regulation specifically lengthens *tim* mRNA poly(A) tail. (A) Poly(A) tail-length assay of *per* and *tim* mRNA in head extracts. The sizes of the expected PCR products with no poly(A) tail for *per* and *tim* are indicated. The nonquantitative PCR conditions that are used allow to compare mRNA sizes but not levels. (B) Poly(A) tail-length assay of *tim* mRNA. (C) Poly(A) tail-length assay of *per*, *tim*, and *actin* mRNA. FAM-labeled PCR products were analyzed by capillary electrophoresis. The y axis represents the relative amount present at each position of the poly(A) tail that is shown on the x axis from 5' to 3'. (D, Left) Quantitative RT-PCR analysis of *tim-yfp* and *tim⁰* mRNA. Average values from at least three independent experiments are normalized to the control mean value at CT0 set to 1. Error bar indicate SEM. Two-way ANOVA of genotype and time finds a significant difference between controls and *Pop2* RNAi flies for *tim-yfp* with $P = 0.0065$ (CT0–CT6). (D, Right) Poly(A) tail-length assay of *tim-yfp* and *tim⁰* mRNA. The asterisks indicate the controls corresponding to the amplification of gene-specific fragments using specific reverse primers. The size of the expected PCR products with no poly(A) tail for *tim-yfp* and *tim⁰*, as well as the size of the expected gene-specific fragments, are indicated.

The CCR4–NOT deadenylation complex is often recruited by RNA-binding proteins that bind to the 3' UTR of mRNAs (37) and we asked whether the 3' UTR of *tim* mRNA was required for POP2 function. We used the behaviorally rhythmic *tim⁰ tim-yfp* flies (38) that carry a functional *tim* transgene lacking its normal 3' UTR (*SI Appendix, Fig. S3*). As observed for wild-type *tim* mRNA, the *tim-yfp* mRNA showed increased levels during subjective day in *Pop2* down-regulated flies (Fig. 3D). POP2 thus does not require the *tim* mRNA 3' UTR to control *tim* mRNA levels. In contrast, *tim⁰* mRNA levels were not affected by *Pop2* RNAi. Since the *tim⁰* mutant carries a 70-bp deletion in the coding region that induces a frameshift and leads to a truncated protein (39), it is likely that the *tim⁰* mRNA is submitted to the nonsense-mediated mRNA decay (NMD) pathway (40). Analysis of mRNA polyadenylation showed that *Pop2* down-regulation increased the poly(A) tail of *tim-yfp* but not *tim⁰* transcripts (Fig. 3D). Thus, POP2 does not require the 3' UTR sequences to control *tim* mRNA polyadenylation.

POP2-Dependent *tim* mRNA Deadenylation Depends on PER but Not on a Functional Clock. Since *Pop2* down-regulation was sensitive to circadian time, we asked whether the clock could regulate POP2 function in *tim* mRNA deadenylation. In behaviorally arrhythmic *per⁰* mutants, *Pop2* RNAi decreased *Pop2* mRNA levels (*SI Appendix, Fig. S4*), but did not increase *tim* mRNA levels and had little effects on *tim* pre-mRNA levels (Fig. 4A). Indeed, *tim* mRNA poly(A) length was only slightly affected by *Pop2* down-regulation in *per⁰* mutants (Fig. 4B, Right and Fig. 4C). Thus, in the absence of PER, POP2 does not appear to be required to shorten *tim* mRNA poly(A), indicating that PER and POP2 genetically interact to control *tim* mRNA polyadenylation. However, in a *Pop2⁺* background, no *tim* poly(A) tail difference could be observed between *per⁰* mutants or behaviorally arrhythmic flies overexpressing PER and wild-type flies (Fig. 4B, Left). This indicated that PER effects on *tim* poly(A) could be detected only when POP2 function was compromised. One possible mechanism is that PER somehow inhibits POP2-dependent *tim* mRNA deadenylation.

The *per⁰ per^{Δ2-100}* flies express a PER protein lacking the N terminus domain that is important for PER degradation under the control of the SLMB ubiquitin ligase (41). They are behaviorally arrhythmic and show constant levels of phosphorylated PER (41). We previously noticed that we could obtain viable adult flies by growing *tim > UAS-Pop2-RNAi* flies at 18 °C without *tub-gal80^{TS}*, likely because *tim-gal4* expression is reduced at low temperature. We thus applied this protocol (*Materials and Methods*) to the experiments with the *per⁰ per^{Δ2-100}* genetic background. Noncycling TIM levels were lower in *per⁰ per^{Δ2-100}* flies than in *per⁰* controls, but *Pop2* down-regulation increased TIM levels in *per⁰ per^{Δ2-100}* flies (about 2.5-fold), whereas it had no effect in *per⁰* flies (Fig. 4D). The data thus suggested that PER protein, and not a functional clock, makes POP2 sensitive to down-regulation. We then analyzed *tim* mRNA in the same genotypes. Although a 1.6-fold increase of *tim* pre-mRNA levels was observed, *tim* mRNA showed a higher increase (2.3-fold). This posttranscriptional effect was supported by the strong lengthening of *tim* mRNA poly(A) tail in the *per⁰ per^{Δ2-100}* flies expressing *Pop2* RNAi (Fig. 4E). Importantly, *Pop2* mRNA levels were not modified by the PER^{Δ2-100} protein (*SI Appendix, Fig. S4*). Thus, the PER^{Δ2-100} protein is sufficient to make *tim* mRNA deadenylation POP2 dependent. Since PER and PER^{Δ2-100} proteins act in the nucleus, we addressed the subcellular localization of POP2 in clock cells. In the absence of an anti-POP2 antibody working in brain immunolabelings, we expressed a *UAS-Pop2-HA* transgene in the PDF cells and observed that the POP2-HA protein was distributed in both the cytoplasm and the nucleus (*SI Appendix, Fig. S4*). We finally asked whether PER could be part of the POP2-containing *tim* mRNA-binding complex but coimmunoprecipitation experiments did not reveal

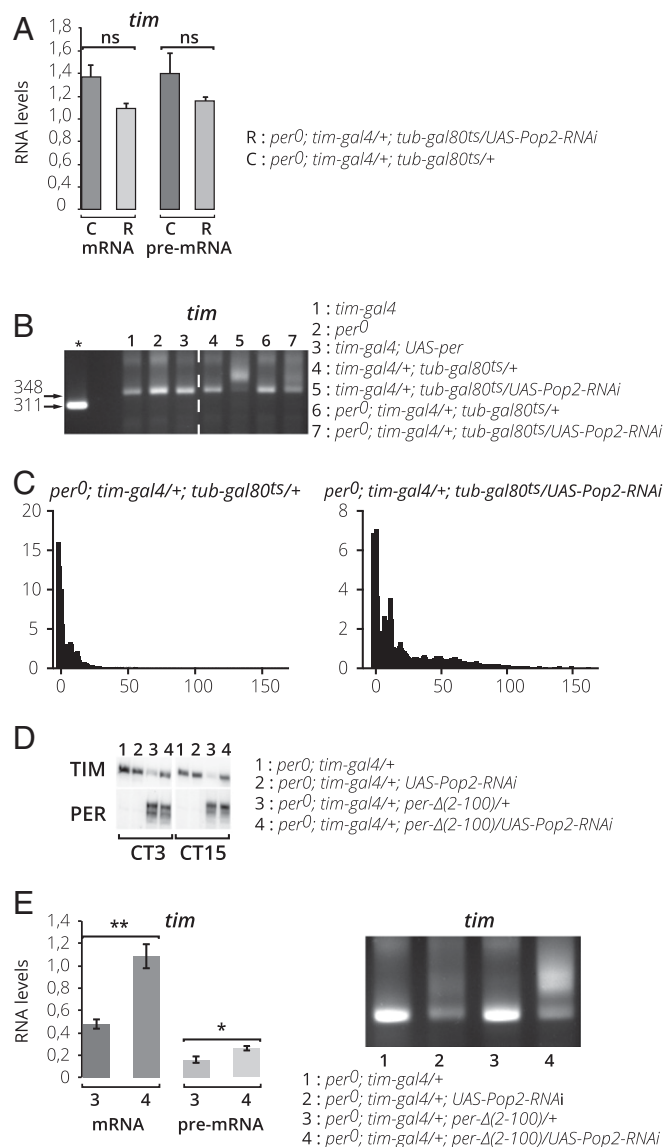


Fig. 4. The effects of Pop2 down-regulation on *tim* transcripts depend on PER. (A and E, Left) Quantitative RT-PCR analysis of *tim* mRNA and pre-mRNA from heads collected at CT3, CT6, CT15, and CT18. Average values were calculated from the four CTs and normalized to the *per⁰* control mean value at CT3 set to 1. Error bars indicate SEM. Unpaired *t* test indicated no significant difference in A, and significant differences in E, with *******P* = 0.0021 for mRNA and ***P** = 0.031 for pre-mRNA. (B and C) Poly(A) tail-length assay of *tim* mRNA. Flies were collected at CT6. (B) The asterisk indicates the control corresponding to the amplification of the gene-specific fragment. The size of the expected PCR products with no poly(A) tail for *tim*, as well as the size of the expected gene-specific fragment, are indicated. (C) The FAM-labeled PCR products were analyzed by capillary electrophoresis. (D) Western blots of head extracts with anti-PER and anti-TIM antibodies. Heads were collected at CT3–CT15. (E, Right) Poly(A) tail-length assay of *tim* mRNA. Flies were collected at CT3.

PER–POP2 interactions, supporting an indirect mechanism. The results thus indicate that PER negatively interacts with POP2-dependent *tim* mRNA deadenylation through a mechanism that does not require PER oscillations or a functional clock.

Discussion

Oscillations of *per* and *tim* mRNAs are a key feature of the *Drosophila* circadian oscillator and largely result from the negative transcriptional feedback loop that operates in clock cells

(2). We show here that a posttranscriptional mechanism strongly contributes to the oscillations of the *tim* mRNA and those of the protein. This control relies on the POP2 deadenylase, which shortens *tim* mRNA poly(A) tail. POP2 function does not affect the *Clk* and *per* transcripts that also display oscillations of their levels, revealing its specific role in the control of *tim* mRNA stability by poly(A) deadenylation. The similar increase in mRNA and protein levels (about twofold in head extracts) in *tim > Pop2R* flies supports the hypothesis that the main consequence of *Pop2* down-regulation is to stabilize the mRNA, as opposed to increasing translation. In contrast to most deadenylase activities (37), POP2 function on *tim* mRNA does not require the *tim* 3' UTR. As expected from studies of the CCR4–NOT complex to which it is known to belong, POP2 does not seem to directly bind *tim* mRNA, but none of the identified adaptor proteins (37) that we could test with mutants or RNAs affected the behavioral rhythms. We could not test the behavioral role of the NOT1 scaffold protein and we thus cannot totally exclude the possibility that POP2 acts in a CCR4–NOT1-independent manner. Interestingly, the amplitude of *Drosophila* behavioral rhythms is influenced by the ME31B RNA helicase which appears to bridge NOT1 and ATAXIN2 (ATX2) to silence gene expression through miRNA (10). This complex acts downstream of the PER/TIM oscillator and is not linked to the control of PER translation (10), which also involves ATX2 (9).

As previously reported (42, 43), *per* and *tim* mRNA peak around CT15 in wild-type flies, whereas the pre-mRNA peak is around CT12. With high temporal resolution, the rising phase of *per* and *tim* mRNA oscillations was shown to be significantly delayed compared with transcription, as measured from run-on experiments, suggesting more active posttranscriptional control in the evening, when transcription is high (11). *Pop2* down-regulation increases *tim* polyadenylation at all circadian times but appears to be less efficient in the evening, suggesting that POP2 activity could be higher in the evening and strongly contribute to the delayed rise of mRNA. In comparison with transcripts such as *actin*, *per*, and *tim*, mRNAs have short poly(A) tails, with *tim* poly(A) length well below 50 nucleotides in average. Short poly(A) tails are associated with mRNA degradation (44), and *tim* mRNA is thus likely unstable, as expected for a cycling transcript (14). Similarly, short half-life transcripts that encode transcription factors of the zebrafish segmentation clock display short poly(A) tails whose size is controlled by the CCR4–NOT1 complex (45). Short poly(A) tails have been recently associated with high translational efficiency (46) and the two effects of short poly(A) (instability and high translation) might be important for keeping high-amplitude *tim* mRNA oscillations.

One of the most intriguing results of this study is the interaction between POP2 activity and PER. In the absence of PER but not in the absence of a functional clock, POP2 is not required for *tim* mRNA deadenylation. A simple hypothesis would be that PER inhibits POP2-dependent *tim* mRNA deadenylation. In the absence of PER, the high deadenylase activity would not be sufficiently decreased by *Pop2* RNAi to induce strong *tim* poly(A) lengthening. However, *per⁰* mutants do not show higher deadenylation than *per⁺* flies in a *Pop2⁺* background. It is possible that the very short *tim* poly(A) might not be detectably shortened by the increased POP2 activity of *per⁰* flies. Surprisingly, we did not observe *tim* poly(A) length cycling in wild-type flies. A more extensive analysis will be required to eventually detect such cycling in wild-type flies, which might be of low amplitude. Alternatively, POP2 activity could not be cycling and would constitutively maintain a short *tim* mRNA poly(A) for short half-life and robust oscillations. Constitutive *per* transgenic expression in *per⁰* mutants restores robust protein oscillations and rhythmic behavior, whereas *tim* constitutive expression is much less efficient to do so (47), suggesting that *tim* mRNA cycling is more important to generate protein oscillations. The finding that PER influences POP2 function also

supports oscillating *tim* poly(A) but the combination of cycling *tim* transcription and cycling PER might just provide a way to keep *tim* poly(A) length constantly short.

How could the PER transcriptional repressor control POP2 activity? Our data indicate that the PER^{Δ2–100} protein does not affect *Pop2* mRNA levels. Although a new transcription-independent role of PER cannot be excluded, different mechanisms have been revealed that link transcription with the control of mRNA polyadenylation and stability (48, 49). Notably, interactions between the transcription machinery and the CCR4–NOT complex indicate that mRNA deadenylation can be regulated at the transcriptional level (50–53), raising the possibility that PER might interfere with POP2 activity through its role as a transcriptional repressor. For example, CBP/p300 acetylates CAF1 to promote its activity (52). Although down-regulation of the CBP fly ortholog *nejire* induces behavioral defects as a consequence of its function in regulating CLK/CYC activity (54), we could not detect changes in *tim* polyadenylation.

Although our data indicate that POP2 is present in the nucleus of clock cells, the absence of PER/POP2 interaction would rather support an indirect mechanism. One interesting possibility is that PER would modify some transcriptionally coupled imprinting mechanism on *tim* mRNA. Such mRNA imprinting mechanisms have been recently described in the control of mRNA polyadenylation by the CCR4–NOT complex (50, 55). In mammalian cells, mRNA degradation is promoted by the YTHDF2 methylation reader protein that recruits CCR4–NOT to deadenylate m⁶A-mRNAs (55). Since *tim* mRNA deadenylation might be higher in the evening, *tim* mRNA methylation (or any other mRNA imprinting) could occur during transcription and be repressed when PER binds to the CLK/CYC transcription complex in the late night. Such an imprinting cycling could thus drive POP2-dependent *tim* mRNA deadenylation and subsequent degradation. Further work will be required to decipher the mechanism by which the PER transcriptional repressor controls the polyadenylation of the *timeless* mRNA.

POP2 might also be involved in the control of a number of cycling transcripts that show weak or no cycling transcription (13). Interestingly, down-regulation of *Neurospora Not1* or *Ccr4* affects the phase of the circadian oscillator, but the Not1 protein interacts with the transcriptional activator WC-1, suggesting that the mechanism might not be related to mRNA polyadenylation (56). In mammals, posttranscriptional mechanisms strongly contribute to mRNA cycling (15, 16) and the circadianly regulated Nocturnin deadenylase generates oscillations of poly(A) length for a large set of mRNAs (23, 24). It will be interesting to investigate whether the CNOT6/7/8 deadenylases of the mammalian CCR4–NOT complex contribute to the oscillations of some of the core clock components.

Materials and Methods

Fly Lines. Fly stocks were maintained on standard cornmeal–yeast–agar medium on 12 h:12 h LD conditions at 25 °C. The genotypes are described in *SI Appendix*. *gal1118 > UAS-Pop2-RNAi* genotypes and corresponding controls were grown and tested at 25 °C; *tim > UAS-Pop2-RNAi tub-gal80^{TS}* genotypes and controls were grown at 18 °C and transferred at 28 °C for testing; and *tim > UAS-Pop2-RNAi* genotypes and controls were grown at 18 °C and transferred at 25 °C for testing.

Behavioral Analysis. Locomotor activity of individual flies was measured with the *Drosophila* activity monitors (TriKinetics) at 25 °C. Young adult males (1–5 d) were first entrained to 12 h:12 h LD cycles for 4 d and then transferred to DD. The activity data analysis was done with the FaasX 1.21 software (neuro-psi.cnrs.fr/spip.php?article298&lang=en), as described in *SI Appendix*.

Brain Immunolabeling. Flies were entrained in LD for 4 d, then transferred to DD and collected during the first DD day at the indicated circadian times for dissection. Immunolabelings were done as previously described (57). See *SI Appendix* for a complete description.

Western Blotting. Flies were entrained in LD for 4 d, then transferred to DD and collected during the first DD day. For each time point, 20–40 flies were collected on dry ice and processed as previously described (28). See *SI Appendix* for a complete description.

Quantitative RT-PCR. Flies were entrained in LD for 4 d, then either collected on the fifth LD day, or transferred to DD and collected during the first DD. For each time point, about 35 flies were collected on dry ice and processed as previously described (28). See *SI Appendix* for a complete description.

Poly(A) Tail-Length Assay. The PAT assay was performed using a poly(A) tail-length assay kit (Affymetrix) according to the manufacturer's protocol, as described in *SI Appendix*. For analysis by capillary electrophoresis (GATC), the PCR was performed using the same gene-specific forward primers except that 5' FAM-labeled primers were used. See *SI Appendix* for a detailed description.

Statistical Analysis. Quantifications of Western blots and quantitative RT-PCR experiments were analyzed by two-way ANOVA and unpaired Student's *t* tests with Prism 7 (GraphPad Software).

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