Transcriptional Regulation via Nuclear Receptor Crosstalk Required for the *Drosophila* Circadian Clock

**Highlights**

- Nuclear receptors E75 and UNF are expressed in the fly circadian pacemaker neurons
- Flies lacking E75 and UNF show no locomotor rhythms
- E75 and UNF collaborate to enhance CLK/CYC-mediated transcription of *period*

**Authors**

Edouard Jaumouillé, Pedro Machado Almeida, ..., Rafael Koch, Emi Nagoshi

**Correspondence**

emi.nagoshi@unige.ch

**In Brief**

Nuclear receptors REV-ERB α and REV-ERB β are components of the mammalian circadian clock. Jaumouillé et al. report that E75, the fly homolog of REV-ERB α and REV-ERB β, and the nuclear receptor UNF are essential for the fly molecular clock, showcasing the importance of transcriptional control via nuclear receptors in circadian clocks across species.
Transcriptional Regulation via Nuclear Receptor Crosstalk Required for the Drosophila Circadian Clock

Edouard Jaumouillé,1,2,4 Pedro Machado Almeida,2,4 Patrick Stähli,1 Rafael Koch,2 and Emi Nagoshi2,3,*

1Institute of Cell Biology, University of Bern, Baltzerstrasse 4, 3012 Bern, Switzerland
2Department of Genetics and Evolution, Sciences III, University of Geneva, 30 Quai Ernest-Ansermet, 1211 Geneva 4, Switzerland
3PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan
4Co-first author
*Correspondence: emi.nagoshi@unige.ch
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SUMMARY

Circadian clocks in large part rely on transcriptional feedback loops. At the core of the clock machinery, the transcriptional activators CLOCK/BMAL1 (in mammals) and CLOCK/CYCLE (CLK/CYC) (in Drosophila) drive the expression of the period (per) family genes. The PER-containing complexes inhibit the activity of CLOCK/BMAL1 or CLK/CYC, thereby forming a negative feedback loop [1]. In mammals, the ROR and REV-ERB family nuclear receptors add positive and negative transcriptional regulation to this core negative feedback loop to ensure the generation of robust circadian molecular oscillation [2]. Despite the overall similarities between mammalian and Drosophila clocks, whether comparable mechanisms via nuclear receptors are required for the Drosophila clock remains unknown. We show here that the nuclear receptor E75, the fly homolog of s-LNvs, and adult LNvs [14, 15]. The E75 targets include Eip75B, and E75C [10], all of which share a large part of the C-terminal domain [9], thereby forming a negative feedback loop [1]. In mammals, the ROR and REV-ERB family nuclear receptors add positive and negative transcriptional regulation to this core negative feedback loop to ensure the generation of robust circadian molecular oscillation [2]. Despite the overall similarities between mammalian and Drosophila clocks, whether comparable mechanisms via nuclear receptors are required for the Drosophila clock remains unknown. We show here that the nuclear receptor E75, the fly homolog of REV-ERBα and REV-ERBβ, and the NR2E3 subfamily nuclear receptor UNF are components of the molecular clocks in the Drosophila pacemaker neurons. In vivo assays in conjunction with the in vitro experiments demonstrate that E75 and UNF bind to per regulatory sequences and act together to enhance the CLK/CYC-mediated transcription of the per gene, thereby completing the core transcriptional feedback loop necessary for the free-running clockwork. Our results identify a missing link in the Drosophila clock and highlight the significance of the transcriptional regulation via nuclear receptors in metazoan circadian clocks.

RESULTS

The Nuclear Receptor E75 Is Required for the Development and the Free-Running Clocks of the s-LNvs

Drosophila circadian locomotor rhythms are generated by subsets of clock-containing neurons in the brain. Under light-dark (LD) cycles, the small ventral lateral neurons (s-LNvs; M oscillators) and a small subset of lateral and dorsal neurons named E oscillators drive morning and evening activity peaks. In constant darkness (DD), the s-LNvs control the synchrony of clock neurons and drive behavioral rhythms, thus serving as the master pacemakers [3–5]. Nuclear receptors are ligand-dependent transcription factors that regulate diverse biological processes [6]. A number of nuclear receptors are known to play key roles in the molecular clock and its output pathways in mammals [2]. With the exception of unfilled (unf; DHR51), which is required for the pacemaker function of the s-LNvs [7], whether nuclear receptors provide important regulatory points in Drosophila circadian rhythms remains unclear.

To test the requirements of the nuclear receptors in Drosophila circadian rhythms, we sought to knock down each of the 18 nuclear receptor genes in the s-LNvs. We chose to use recently generated UAS-microRNAs (miRNAs) targeting fly nuclear receptors because each UAS line polycistronically expresses two independent miRNAs, which permit the efficient silencing of the target with a minimum off-target effect [8, 9]. We expressed UAS-miRNAs with the LNV-specific GAL4 driver, Pdf-GAL4 or Gal1118, and analyzed the locomotor activities of the flies in LD and DD. Consistent with our previous results [7], the knockdown of unf rendered flies arrhythmic in DD. Additionally, the knockdown of E75 (also known as ecdysone-induced protein 75, Eip75B) in the LNvs led to a similarly high proportion of arrhythmicity in DD (Figures S1A and S1B). The knockdown of seven-up (svp) or estrogen-related receptor (ERR) had a moderate effect on free-running rhythms (Table 1).

The E75 gene produces three isoforms of E75, named E75A, E75B, and E75C [10], all of which share a large part of the C-terminal region, including the ligand-binding domain, but differ in their N terminus structures. Despite these differences, all the isoforms are functionally redundant to some extent in ecdysone-induced developmental processes [11]. E75 isoform redundancy is at least partly explained by the fact that all three isoforms, including E75B (which lacks DNA-binding domain), can heterodimerize with the DHR3 nuclear receptor and repress its transcriptional activity [12, 13]. Our previous RNA analysis from isolated LNvs showed that all E75 isoforms are expressed in both larval and adult LNvs [14, 15]. The UAS-miRNAs against E75 targets two sequences in the common C-terminal domain [9], thereby enabling the knockdown of all the isoforms. Expression of
UAS-E75 miRNAs under the control of ubiquitously expressed tubulin-GAL4 driver caused near 100% embryonic lethality, which recapitulates the lethal phenotype of the E75 null mutants lacking all isoforms [11]. By contrast, expression of UAS-RNAi targeting E75 from the Vienna Drosophila Resource Center (VDRC) collection with tubulin-GAL4 had no effect on viability (Figure S2A). LNv-targeted expression of E75 miRNAs rendered flies arrhythmic, whereas expression of either of the two VDRC UAS-RNAi lines had no effect on the locomotor rhythms (Figure S2B). These results indicate that UAS-miRNAs efficiently silences the E75 gene and confirm that behavioral arrhythmia is specifically caused by the reduction of the E75 expression in the LNvs. E75 is a homolog of mammalian REV-ERB α and REV-ERB β, which are important transcriptional regulators contributing to the molecular clockwork and the clock output [16–18]. Intrigued by the high proportion of arrhythmia in the knockdown and the relevance to the circadian rhythms in mammals, we further investigated how E75 contributes to the behavioral rhythm generation in the LNvs. First, we overexpressed E75A, the isoform containing both DNA-binding and ligand-binding domains, in the LNvs. These flies showed no differences in locomotor behavior compared with the control (Figures S1A and S1B). Thus, loss of function, but not overexpression, of E75 in the LNvs impairs the free-running locomotor rhythms.

We next examined the effect of E75 loss of function in developing and adult LNvs separately via stage-specific knockdown using a combination of UAS-E75 miRNAs, Pdf-GAL4, and a temperature-sensitive GAL80 expressed under the control of the tubulin promoter (tub-GAL80°C) [19]. To analyze the role of E75 in the developing LNvs, we raised these flies until eclosion at 29°C (the restrictive temperature of GAL80°C), enabling the Pdf-E75 miRNAs expression. The eclosed flies were transferred to 18°C (the permissive temperature of GAL80°C) to stop the expression of E75 miRNAs and subsequently tested for locomotor activity under this condition. Strikingly, 100% of these flies were arrhythmic in DD. The rhythms in LD were unaffected (Figures 1A and 1B). This is consistent with the results of the E75 constitutive knockdown performed at 25°C. By staining for PDF and PER, we found that all the PDF-positive s-LNvs were undetectable, whereas the l-LNvs and the PDF-negative fifth s-LNv were normal in number and morphology (Figure 1C). Although this does not exclude the possibility that the s-LNvs are still present, it nevertheless implies that at least multiple genetic programs are severely impaired in the s-LNvs. These results indicate that E75 is critically required for the development of the s-LNvs, which are the master pacemaker neurons for free-running rhythms; therefore, E75 gene silencing during development renders adult flies arrhythmic in DD.

To determine whether E75 in adult LNvs contributes to the generation of locomotor rhythms, we next knocked down E75 only during adulthood by raising the flies carrying UAS-E75

### Table 1. Effects of the Nuclear Receptor Knockdown on the Free-Running Locomotor Rhythms

| Tested NR | Genotype | Period ± SEM | Power ± SEM | n   | R (%) | χ² (a) | χ² (b) |
|-----------|----------|--------------|-------------|-----|-------|--------|--------|
| Control   | W1118    | 23.9 ± 0.07  | 202.2 ± 14.37 | 32  | 93.8  |        |        |
| Control (gai1118) | Gal1118-GAL4/+ | 24.1 ± 0.06  | 271.6 ± 16.13 | 32  | 93.8  |        |        |
| Control (Pdf-GAL4) | Pdf-GAL4/+ | 24 ± 0.08   | 158.7 ± 7.2 | 32  | 91.7  |        |        |
| Control (E75-miRNAs) | UAS-E75-miRNAs/+;Gal1118-GAL4/+ | 23.8 ± 0.07  | 146.9 ± 6.4 | 32  | 96.9  |        |        |
| DHR51 (unf) (gai1118) | UAS-DHR51-miRNAs/+;Gal1118-GAL4/+ | 23.5 ± 0.22  | 73.2 ± 15.95 | 30  | 16.7  | ***    |        |
| DHR51 (unf) (Pdf-GAL4) | Pdf-GAL4/UAS-DHR51-miRNAs | 22.8 ± 0    | 63.2 ± 0 | 31  | 3.2   |        |        |
| DHR4      | UAS-DHR4-miRNAs/+;Gal1118-GAL4/+ | 24.9 ± 0.04  | 231.3 ± 10.85 | 32  | 100   |        |        |
| DHR96     | UAS-DHR96-miRNAs/+;Gal1118-GAL4/+ | 24.5 ± 0.04  | 241.6 ± 13.38 | 32  | 96.9  |        |        |
| DHR3      | UAS-DHR3-miRNAs/+;Gal1118-GAL4/+ | 24.2 ± 0.06  | 221.2 ± 14.03 | 32  | 81.3  |        |        |
| E75       | UAS-E75-miRNAs/+;Gal1118-GAL4/+ | 23.2 ± 0.17  | 149 ± 29.62 | 17  | 17.6  | ** *** |        |
| DHR38     | UAS-DHR38-miRNAs/+;Gal1118-GAL4/+ | 24.7 ± 0.04  | 221.8 ± 13.04 | 32  | 100   |        |        |
| FTZ       | Pdf-GAL4/UAS-FTZ-miRNAs | 24 ± 0.05   | 236.8 ± 14.83 | 27  | 92.6  |        |        |
| svp       | Pdf-GAL4/UAS-svp-miRNAs | 23.7 ± 0.04  | 275.6 ± 14.23 | 27  | 59.3  | *      |        |
| E78       | Pdf-GAL4/UAS-E78-miRNAs | 24.1 ± 0.06  | 248.9 ± 10.84 | 22  | 86.4  |        |        |
| usp       | Pdf-GAL4/UAS-usp-miRNAs | 23.8 ± 0.04  | 258.4 ± 13.88 | 32  | 87.5  |        |        |
| NHP4      | Pdf-GAL4/UAS-NHP4-miRNAs | 24.3 ± 0.07  | 220 ± 16 | 22  | 81.8  |        |        |
| DHR78     | Pdf-GAL4/UAS-DHR78-miRNAs | 24 ± 0.06   | 252.8 ± 20.62 | 22  | 90.9  |        |        |
| EcR       | Pdf-GAL4/UAS-EcR-miRNAs | 23.7 ± 0.07  | 216.4 ± 15.65 | 31  | 96.8  |        |        |
| DSF       | Pdf-GAL4/UAS-DSF-miRNAs | 24.5 ± 0.07  | 242.9 ± 11.65 | 32  | 96.9  |        |        |
| DHR83     | Pdf-GAL4/UAS-DHR83-miRNAs | 24.2 ± 0.06  | 262.3 ± 13.73 | 31  | 90.3  |        |        |
| ERR       | Pdf-GAL4/UAS-ERR-miRNAs | 24 ± 0.08   | 199.2 ± 13.71 | 30  | 53.3  | *      |        |
| tll       | Pdf-GAL4/UAS-tll-miRNAs | 25.1 ± 0.2   | 123.6 ± 13.05 | 31  | 77.4  |        |        |
| DHR39     | Pdf-GAL4/UAS-DHR39-miRNAs | 23.9 ± 0.05  | 249.2 ± 15.16 | 32  | 68.8  |        |        |

n, number of flies analyzed; R (%), percent of flies that display rhythms; χ² (a), chi-square test knockdown versus GAL4; χ² (b), knockdown versus UAS. *p < 0.05, **p < 0.025, ***p < 0.001. See also Figure S1.
**Figure 1. Conditional E75 Knockdown in the LNvs**

(A–F) Locomotor activity and the morphology of the lateral neurons of the flies with LNv-targeted E75 developmental knockdown (A–C) or adult-only knockdown (D–F).

(B and D) Top: average LD activity. White and black columns represent daytime and nighttime activity, respectively. Bottom: double-plotted actograms for average DD activity. White and gray bars indicate the subjective day and night.

(C and F) Anti-PDF (magenta) and PER (green) staining of the brains at circadian time (CT)0 on DD3. Blue arrowheads indicate dorsal projections of the s-LNvs. Red, yellow, white, and magenta arrowheads indicate the cell bodies of l-LNvs, s-LNvs, fifth s-LNv, and LNds, respectively. Scale bars represent 25 μm.

### Table 1: Summary of Knockdown Experiments

| Condition | Genotype | Period ± SEM | Power ± SEM | n  | %R  |
|-----------|----------|--------------|-------------|----|-----|
| **Developmental Knockdown** | | | | | |
| control (driver) | Pd-GAL4/+;tub-GAL80°/+ | 24.1±0.1 | 106.7±8.1 | 32 | 90.6 |
| control (UAS) | UAS-E75-miR/+ | 23.8±0.1 | 71.3±9.4 | 28 | 75.0 |
| E75 developmental KD | Pd-GAL4/UAS-E75-miR; tub-GAL80°/+ | - | - | 29 | 0*** |

***p<0.001, chi-square test driver versus KD, UAS versus KD.

| Condition | Genotype | Period ± SEM | Power ± SEM | n  | %R  |
|-----------|----------|--------------|-------------|----|-----|
| **Adult-only Knockdown** | | | | | |
| control (driver) | Pd-GAL4/+;tub-GAL80°/+ | 24.1±0.1 | 160.3±8.0 | 27 | 92.6 |
| control (UAS) | UAS-E75-miR/+ | 23.5±0.1 | 159.0±11.5 | 27 | 81.5 |
| E75 adult KD | Pd-GAL4/UAS-E75-miR; tub-GAL80°/+ | 27.3±0.3*** | 87.2±7.2 | 31 | 90.3 |

*** p<0.0001, t-test knockdown versus driver.
miRNAs, Pdf-GAL4, and tub-GAL80\textsuperscript{ts} at 18°C until eclosion and incubating the adult flies at 29°C. The adult-specific LNv-targeted E75 knockdown lengthened the free-running period by over 3 hr (Figures 1D and 1E). By staining brains at six time points on the third day in DD (DD3) with anti-PDF/PER double staining, we found that both s-LNvs and l-LNvs were present and morphologically normal in the adult-specific E75 knockdown flies (Figure 1F), but PER levels and oscillations were substantially reduced in the s-LNvs in DD (Figures 2A and 2D). These results indicate that E75 is indispensable for robust free-running molecular rhythms in adult s-LNvs. In contrast, PER rhythms in the dorsal lateral neurons (LNds) were unaffected by this manipulation (Figures 2B and 2D). In the dorsal neurons 1 (DN1s), the phase of PER rhythms was approximately 12 hr delayed in the knockdown flies compared with the control, indicating that E75 knockdown in adult LNvs non-cell-autonomously slowed down the clocks in the DN1s by approximately 4 hr per day (Figures 2C and 2D).

The Nuclear Receptors E75 and UNF Act Together to Enhance CLK/CYC-Mediated Transcription of \textit{period}

Similar to E75, the nuclear receptor \textit{unf}; DHR51 is expressed in the LNvs throughout development and adulthood and is required for the proper functioning of the s-LNvs as the circadian master pacemakers [7, 15]. Unf knockdown in developing LNvs does not affect the gross structure of the LNVs but leads to the complete disruption of free-running clocks in adult s-LNvs. Unf downregulation in adult LNVs dampens their molecular oscillation in DD, resulting in long-period free-running rhythms [7]. Thus, the loss of function of \textit{unf} in adult LNVs partially phenocopies that of E75. This further suggests that UNF and E75 might function in the same pathway controlling the molecular clocks in the s-LNvs. We next addressed this possibility by testing the genetic interaction between \textit{unf} and E75 in adult LNVs. Interestingly, \textit{unf} and E75 double knockdown in adult LNVs rendered flies arrhythmic in DD. Overexpression of E75 did not alter the lengthened free-running period in the \textit{unf} knockdown flies (Figures S3A and S3B). Because behavioral rhythms were normal when double knockdown flies were kept at 18°C throughout developmental
and adult stages, behavioral arrhythmia in the adult-specific knockdown flies was not caused by leaky miRNA expression during development (Figure S3C). These results indicate that UNF and E75 genetically interact in the pathway controlling the molecular clockwork in the s-LNvs, but E75 is unlikely to be downstream of UNF.

To understand the mechanisms by which E75 controls molecular clockwork in the s-LNvs and how UNF takes part in this process, we sought to identify the E75 downstream target genes. Because the adult-specific E75 knockdown in the LNvs not only diminishes the molecular oscillation but also dramatically reduces PER levels (Figure 2A and 2D), we speculated that E75 controls the transcription of the gene(s) directly involved in the molecular oscillation. Because the DNA-binding domains of E75 and mammalian REV-ERBα and REV-ERBβ are highly conserved, we used the well-characterized REV-ERBα and REV-ERBβ binding sequences to find potential E75 binding sites in Drosophila core clock genes. REV-ERBα/β is known to bind to the ROR element (ROKE) ([A/T][A/T][A/T][A/G]GGTCA) as a monomer [25]. Interestingly, a consensus RORE was found in the first intron of the per gene. Moreover, the RORE is located near the CLK/CYC binding peaks identified in [26] (Figure 3C). These bioinformatic analyses predict that E75 could modulate the CLK/CYC-mediated transcription of per. To test this possibility, we performed transactivation assays in Schneider 2 (S2) cells, which permit the analysis of transcriptional regulation in a non-cycling context. We transiently transfected S2 cells with varying combinations of Clk, E75A, and unf expression constructs and analyzed the endogenous per and tim mRNA levels by qPCR (Figure 3A). In agreement with a previous study [27], because cyc is expressed in S2 cells, the expression of Clk alone significantly upregulated the per and tim RNA levels. Unf or E75 alone or together but without Clk had no effect on the per and tim RNA levels. When either unf or E75 was co-expressed with Clk, we observed a small but non-significant increase of per mRNA levels compared with Clk transfection alone. Strikingly, the addition of unf and E75 together to Clk dose dependently increased per mRNA levels by at least 4-fold. Neither unf nor E75 affected the tim RNA levels when co-expressed with Clk.

To examine the extent to which UNF and E75 transcriptionally control per expression, we generated luciferase reporters to monitor transcription from wild-type per promoter (per-luc) and...
per promoter lacking RORE (delta RORE-luc [dRORE-luc]) in S2 cell co-transcription assay. Expression of unf significantly increased Clk-dependent transcription from both promoters, whereas E75 caused only a slight and non-significant increase. Unf/E75/Clk co-expression yielded higher transcriptional activity than unf/Clk transfection; specifically, the increase of the dRORE-luc activity was statistically significant. Intriguingly, dRORE-luc activity was significantly higher than per-luc in both unf/Clk and unf/E75/Clk co-transfections (Figure 3B). This suggests that transcriptional repression through RORE is involved in the overall transcriptional control of per, but other domains within the reporter construct mediate net transcriptional activation. Compared to the strong upregulation of per mRNA level by unf/ E75/Clk co-transfection (Figure 3A), the difference between unf/ Clk and unf/E75/Clk transfection on per-luc activation was less evident. This probably reflects that regulatory sequences outside the region cloned in the luciferase reporters are also involved in the regulation of per transcription. Together with the data indicating that unf/E75 co-transfection synergistically activates transcription of dRORE-luc, these results suggest that UNF upregulates CLK/ CYC-mediated per transcription and E75 enhances this process, at least via proximal and intronic promoters, except RORE.

To test whether UNF and E75 bind to per regulatory sequences and thereby regulate transcription, we performed chromatin immunoprecipitation (ChIP) in S2 cells transfected with epitope-tagged Clk, unf, and E75 and analyzed the binding by qPCR. As expected, CLK was bound to the per gene regulatory sequences around circadian regulatory sequence (CRS), RORE, and a downstream E-box (E8), reminiscent of prior ChIP studies [28, 28] (Figure S3D). Both UNF and E75 binding patterns were nearly identical to the CLK binding profile. Strikingly, although E75 binding was unaffected by the co-expression of UNF, UNF binding was approximately 3- to 4-fold reduced when co-expressed with E75 (Figure 3C). These data suggest that both UNF and E75 can independently bind to per regulatory sequences, but the presence of E75 increases the turnover of UNF binding. This high turnover of UNF binding is concomitant with higher transcriptional activity (Figure 3D). This model is in concert with the notion that transcriptional activation from highly regulated promoters often requires the turnover of the nuclear receptors and their co-regulators [29]. Taken together with the behavioral results (Figure S3A), these in vitro data suggest that coordinated action of E75 and UNF on per transcription is essential for the molecular clock machinery in the s-LNvs.

**DISCUSSION**

Here, we identified the nuclear receptors E75 and UNF as components of the molecular clocks in the s-LNvs. E75 is the closest homolog of mammalian REV-ERB α and REV-ERB β, which play important roles in the molecular clock feedback loops. In contrast with Rev-Erb α/β, which represses transcription, our results demonstrated that E75 is neither a potent repressor nor a strong activator but potentiates the activation of per transcription by UNF. Despite these mechanistic divergences, the notion that Rev-Erb homologs are integral to the molecular oscillators in both Drosophila and mammals highlights the significance of transcriptional regulations via nuclear receptors in metazoan circadian clocks. Rev-Erb α and Rev-Erb β are rhythmically transcribed by the CLOCK/BMAL1 transcriptional activators, and REV-ERBs periodically repress the transcription of Bma1, thereby forming a feedback loop to ensure robust molecular oscillations of the mammalian clock [17]. A previous study demonstrated that E75 is a cycling target of CLK/CYC in the fly head [26]. Because E75 has three isoforms, we were unable to determine whether any of the isoforms were rhythmically expressed in the LNvs from the RNA profiles of the isolated LNvs [14]. Nonetheless, our results indicate that E75 together with UNF (which is not a CLK/CYC target) reinforces the main loop of the core fly clock composed of CLK/CYC and PER/TIM through a feedforward mechanism, showcasing the mechanistic parallels between fly and mammalian clocks.

E75 has been demonstrated to covalently bind to heme, and its binding appears to stabilize the E75 and facilitates the binding of nitric oxide (NO) and carbon monoxide (CO). The NO/CO binding to E75 modulates the transcriptional activity of its known heterodimeric partner DHR3 [13, 30]. To test whether similar mechanisms are involved in the action of E75 in the s-LNvs, we attempted to disrupt cellular heme metabolism by knocking down the enzymes in the heme biosynthesis pathway, coproporphyrinogen oxidase (Coprox) and protoporphyrinogen oxidase (Ppox), and the key enzyme in the heme degradation pathway, heme oxygenase (Ho). These experiments were inconclusive, as we did not observe any effect on the behavioral rhythms by any knockdown with Pdx-GAL4, and knockdown with Tim-GAL4 was lethal (data not shown).

S2 cell experiments showed that UNF is a transcriptional activator of per, and concurrent expression of E75 and UNF increases the turnover of UNF binding to per regulatory sequences. This high turnover is correlated with higher transcriptional activity (Figure 3). The finding that E75 acts through UNF on transcription is consistent with our in vivo data: (1) depletion of both UNF and E75 in adult LNvs abolishes the behavioral rhythms (Figure S3A); (2) E75 overexpression has no effect on the behavioral rhythms (Figure S1A); and (3) E75 overexpression does not rescue UNF knockdown (Figure S3A). Although unf mRNA levels do not oscillate, UNF protein levels cycle in the s-LNvs, peaking at zeitgeber time (ZT)2 and lowest at ZT14 [17]. Low UNF levels may reflect the degradation as a consequence of higher transcriptional activity. Indeed, per is most actively transcribed around ZT13 [31] when UNF levels are minimum in the s-LNvs. Nonetheless, downregulation and arrhythmia of PER levels in the s-LNvs is most probably not the sole cause of the altered locomotor rhythms in the UNF knockdown, E75 knockdown, and UNF/E75 double knockdown. A recent study showed the implication of E75 in the repression of Clk transcription [32], although our results are not in concordance with this observation probably due to the differences in the agents used for E75 knockdown and the timing of knockdown. Deciphering whether E75 and UNF heterodimerize or bind to adjacent sequences, how they cooperate with CLK/CYC, and whether any ligand is involved in their transcriptional regulation will yield new insights into the diverse mode of nuclear receptor crosstalk and their critical roles in circadian biology.

**EXPERIMENTAL PROCEDURES**

Experimental procedures are detailed in the Supplemental Information.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.04.017.

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