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ORIGINAL ARTICLE

Nuclear Envelope Protein MAN1 Regulates the *Drosophila* Circadian Clock *via Period*

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Abstract Almost all organisms exhibit ~24-h rhythms, or circadian rhythms, in a plentitude of biological processes. These rhythms are driven by endogenous molecular clocks consisting of a series of transcriptional and translational feedback loops. Previously, we have shown that the inner nuclear membrane protein MAN1 regulates this clock and thus the locomotor rhythm in flies, but the mechanism remains unclear. Here, we further confirmed the previous findings and found that knocking down *MAN1* in the pacemaker neurons of adult flies is sufficient to lengthen the period of the locomotor rhythm. Molecular analysis revealed that knocking down *MAN1* led to reduced mRNA and protein levels of the core clock gene *period* (*per*), likely by reducing its transcription. Over-expressing *per* rescued the long period phenotype caused by *MAN1* deficiency whereas *per* mutation had an epistatic effect on *MAN1*, indicating that MAN1 sets the pace of the clock by targeting *per*.

Keywords Circadian clock · *Drosophila* · Nuclear envelope · MAN1 · *Period*

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Introduction

Circadian rhythms, or ~24-h rhythms in various behavioral and physiological processes, are a fundamental property of life. These rhythms exist across phylogeny from prokaryotes to humans, and are driven by a relatively conserved molecular clockwork consisting of transcriptional and translational feedback loops [1]. In *Drosophila*, two transcription factors CLOCK (CLK) and CYCLE (CYC) heterodimerize and activate the transcription of *period* (*per*) and *timeless* (*tim*) *via* E-box elements located in the promoter/enhancer regions of these genes [2]. PER and TIM proteins accumulate in the cytoplasm, bind each other, and enter the nucleus where they repress the transcriptional activity of CLK/CYC, thus inhibiting their own transcription and forming the major feedback loop of the clockwork. PER/TIM undergo post-translational modifications that ultimately lead to their degradation. Once they are degraded, CLK/CYC can activate transcription again and start a new cycle. This serves as the basis of the overt 24-h rhythms. CLK/CYC also drive the transcription of two additional transcription factors, *vrille* (*vri*) and *PAR-domain protein 1ε/δ* (*Pdp1ε/δ*). The former represses while the latter activates *clk* transcription, forming accessory loops of the clockwork.

The nuclear envelope (NE) has long been considered simply as a barrier that separates genetic material from the rest of the cell and controls the entry/exit of molecules into/out of the nucleus [3]. However, in the past 20 years or so, emerging studies have demonstrated that NE interacts extensively with chromatin and DNA, influencing the spatial organization of the genome and modulating gene expression [3, 4]. Recent work has reported that circadian-regulated genes are rhythmically recruited to the nuclear periphery where they interact with chromosomal regions

that associate with nuclear lamins (lamin-associated domains) and transcription is attenuated *via* epigenetic modifications [5]. Once they move away from the nuclear envelope, transcription is re-activated and thus cyclic transcription can be achieved. However, the detailed mechanisms underlying this process remain largely unclear. Previously, we found three NE proteins, MAN1, lamin B1, and lamin B receptor, to be involved in determining the circadian period length in both human cell lines and *Drosophila*, but little is known regarding how they exert effects on the clock *in vivo* [6]. Therefore, the goal of this study is to investigate the mechanism underlying how MAN1 regulates the clock in flies.

Materials and Methods

Fly Strains

We used the following fly strains: w^{1118}, *yw*, genetic background control lines for the RNAi lines (VDRC:60100), UAS*MAN1RNAi*-1 (NIG:3167R-1), UAS*MAN1RNAi*-2 (NIG: 3167R-2), UAS*MAN1RNAi*-3 (VDRC:108906), $MAN1^{GS2297}$ [6], *tim*GAL4;UAS*dcr2* [6], *cry*G4-16 [6], UAS*dcr2;cry*-GAL4-16 [6], *cry*G4-39;UAS*dcr2* [6], *pdf*G4-GS [7], UAS*per* [8], per^{L} [9], UAS*clk* [10], and clk^{jrk} [11]. *Dicer2* (*dcr2*) was co-expressed to enhance the effects of RNAi [12].

Drosophila Activity-Monitoring and Behavioral Analysis

Flies were reared on standard cornmeal-yeast-sucrose medium and kept in 12 h light:12 h dark (LD) cycles at 25°C. Male flies 3–4 days old were used to monitor locomotor activity levels using the *Drosophila* Activity Monitor system (Trikinetics, Waltham, MA, USA) for 4 days of LD followed by 7 days of constant darkness (DD). To monitor the locomotor activity of strains containing the *pdf*G4-GeneSwitch (*pdf*-GS) driver, flies were raised on standard cornmeal-yeast-sucrose medium until pupation [7]. After eclosion, flies were transferred to tubes with standard food containing 250 μmol/L RU486 (Sigma-Aldrich, St. Louis, MO, USA) for 2–3 days before monitoring locomotor activity. The food used during behavioral assays also contained 250 μmol/L RU486. Ethanol used to solubilize RU486 was added to the food as vehicle control.

Analyses of period, power, and rhythmicity during DD were carried out using ClockLab software (Trikinetics). For DD rhythmicity, rhythmic flies were defined as those with χ^2 power-significance $\geq$10. Period calculations considered all flies with power-significance $\geq$10. One-way ANOVA and Tukey's multiple comparison test (Prism Graphpad, La Jolla, CA, USA) were used to compare the differences between genotypes.

RNA Extraction and Quantitative Real-Time PCR

Flies were entrained in LD for 3 days, collected on DD1 at the indicated circadian time points (CTs) and frozen immediately on dry ice. Fly heads were isolated and homogenized in TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Chloroform was subsequently added and the mixture centrifuged at 12,000g for 15 min at 4°C. Aqueous top layer was collected and ethanol (Sigma-Aldrich) was added to precipitate RNA. The precipitates were collected by centrifuging at 12,000g for 10 min at 4°C. The RNA pellets were washed with 75% ethanol. After air drying, the pellets were dissolved in RNAse-free water. Contaminating genomic DNA was removed by RQ1 DNase (Promega, Madison, WI, USA) digestion, and total RNA was directly amplified with TransScript Green One-Step qRT-PCR SuperMix (Transgen Biotechnology, Beijing, China). All qPCR reactions were carried out on a Step One Plus Real-Time PCR System (Life Technologies). The templates were reverse-transcribed at 45°C for 5 min, denatured at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C, 15 s at 60°C, 30 s at 72°C, and 15 s at 75°C for data acquisition. The primers used for expression analysis were as follows: *MAN1* forward, 5′-GACTCA CGGGGAAATCTGC-3′ and reverse, 5′-GCAGATTGG GTTCAGAAAGC-3′; *tim* forward, 5′-CTGGGGAGTGA CCATGG-3′ and reverse, 5′-GCTGGAATCGCCACTG-3′; *per* forward, 5′-CAGCAGCAGCCTAATCG-3′ and reverse, 5′-GAGTCGGACACCTTGG-3′; *clk* forward, 5′-TACTG CGTGAGGATATCG-3′ and reverse, 5′-GTTG TTGTTCTGGTTGC-3′; *Pdp1ε* forward, 5′-GAACCCAAG TGTAAAGACAATGCG-3′ and reverse, 5′-CTGGAAAT ACTGCGACAATGTGG-3′; *vri* forward, 5′-TGTT TTTTGCCGCTTCGGTCA-3′ and reverse, 5′-TTACGA CACCAAACGATCGA-3′; *beta-Actin* forward, 5′-CTA ACCTCGCCCTCTCCTCT-3′ and reverse, 5′-GCAGCC AAGTGTGAGTGTGT-3′; *rp49* forward, 5′-TACAGGCC CAAGATCGTGAA-3′ and reverse, 5′-GCACT CTGTTGTCGATACCC-3′; pre-*per* forward, 5′-TAGCTC AACGTTCCCATTCG-3′, and reverse 5′-AGATAATG-CAGCACAGGAAGC-3′; and *Cbp20* forward, 5′-AGATC CACGAGCTCTTCTCC-3′, and reverse 5′-CCGATCGGA CATAGTACTCC-3′.

Beta-actin or *rp49* (for mRNA analysis) and *cbp20* (for pre-mRNA analysis) were used as normalization controls. 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, Seattle, WA, USA) was used to compare the differences between genotypes.

Western Blot

Flies were entrained in LD for 3 days, collected during DD1 at the indicated CTs, and frozen immediately on dry ice. Heads were separated and homogenized in RIPA buffer (20 mmol/L Tris at pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.05 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 0.4% sodium deoxycholate, 0.1% SDS) containing a protease

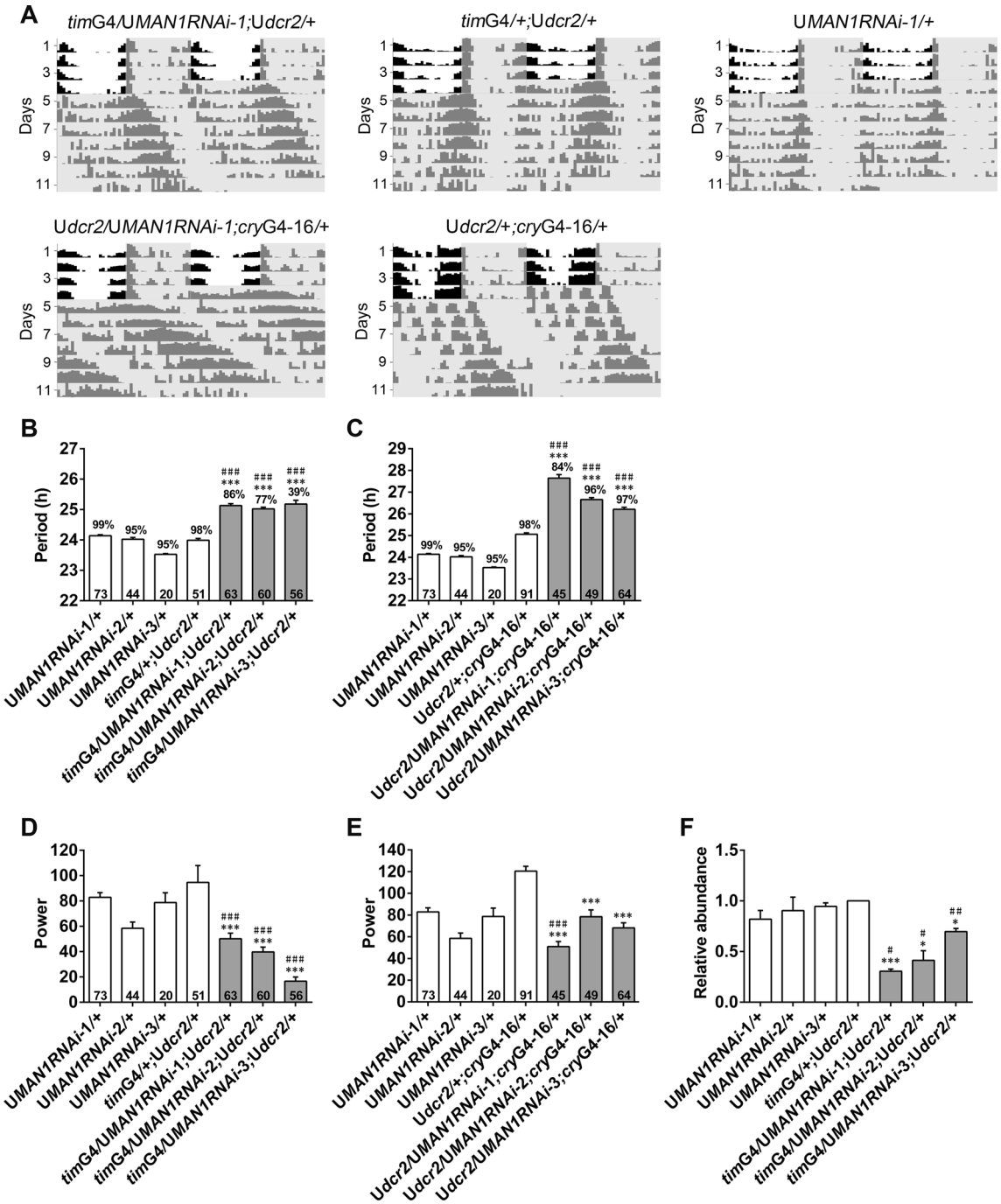


Fig. 1 Knocking down *MAN1* lengthens the period of the locomotor rhythm. **A** Double-plotted representative actograms of the indicated genotypes. Flies were monitored in LD for 4 days and then DD for 7 days. Gray shades indicate dark phases. **B**–**E** The period (**B**, **C**) and power (**D**, **E**) of the DD locomotor rhythm of flies with *MAN1* knocked down and controls. Digits on the bars are the number of flies tested. Percentage of rhythmicity is indicated above the bars ($P < 0.001$, one-way ANOVA; $^{***/\#\#\#}P < 0.001$, Tukey's multiple comparison test). **F** Plots of relative mRNA abundance of *MAN1* in whole head extracts of flies with *MAN1* knocked down and controls determined by qRT-PCR ($n = 3$; $^{*/\#}P < 0.05$, $^{**/\#\#}P < 0.01$, $^{***}P < 0.001$ * *vs* GAL4 controls, $^{\#}$ *vs* UAS controls, Student's *t* test). Error bars represent the standard error of the mean (SEM). G4, GAL4; U, UAS.

inhibitor mixture (Roche, Basel, Switzerland) and phosphatase inhibitor mixture (Roche). This homogenate was sonicated 3 to 5 times for 8 s each time, and then centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris. The supernatant was collected, transferred to new tubes, and centrifuged again at 13,000 rpm for 10 min at 4°C. The 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 membranes. After incubation with primary antibodies at 4°C overnight, the membranes were incubated with secondary antibodies at room temperature for 1 h. The primary antibodies used were rat anti-TIM (1:1000; a gift from Dr. Joanna Chiu, University of California, Davis, CA, USA), guinea pig anti-PER (1:1000; a gift from Dr. Joanna Chiu), and mouse anti-HSP70 (1:5000; Sigma-Aldrich). Secondary antibodies were conjugated with IRDye 800 (LI-COR Biosciences, Lincoln, NE, USA) and incubated at 1:10000. Blots were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Protein levels of TIM, PER, and HSP70 were quantified by the software image studio for Odyssey. TIM and PER levels were normalized to HSP70. The value of the control genotype was set to 1. Student's *t* test (Microsoft Excel, Seattle, WA, USA) was used to compare the differences between genotypes.

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 phosphate-buffered saline (PBS). After fixing for 30 min at room temperature, the brains were rinsed 3 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 with 1:500 rabbit anti-PER (a gift from Dr. Michael Rosbash, Brandeis University, Waltham, MA, USA) and 1:50 mouse anti-PDF (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) in PBT at 4°C. After 6 × 20-min PBT rinses, the brains were incubated with the secondary antibodies donkey anti-mouse AlexaFluor-594 (1:1000; Life Technologies) and donkey anti-rabbit AlexaFluor-488 (1:1000; Abcam, Cambridge, UK). 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 a 60× oil-immersion lens (Olympus, Tokyo, Japan). Images were acquired using the same settings (power, gain, and offset) for each experiment. The intensity of PER signals was quantified by ImageJ software. Student's *t* test (Microsoft Excel) was used to compare the average intensity values between different genotypes.

Results

Knocking Down *MAN1* in Adult Pacemaker Neurons Lengthens the Period of the Locomotor Rhythm

In a previous study, we demonstrated that both knocking down and over-expressing *MAN1* in circadian neurons significantly lengthens the period of the locomotor rhythm under constant darkness (DD) [6]. Here, we used three RNAi lines (including two lines that were not tested in our previous study) to knock down *MAN1* in all clock cells using a *tim*GAL4 driver or mainly in circadian neurons using a *cryptochrome* (*cry*)GAL4-16 driver (Fig. S1) [13, 14]. We found a ~1 to > 2 h longer period of the DD locomotor rhythm in the RNAi flies, accompanied by a modest yet significant reduction in the power of the rhythm (Fig. 1A–E and Table 1). We then verified the knockdown

Table 1 Knocking down *MAN1* lengthens the period of the locomotor rhythm.

Genotype	Period (h) ± SEM	Power ± SEM	%Rhythmic	*n*
*tim*G4/U*MAN1RNAi*-1;U*dcr2*/+	25.13 ± 0.06$^{***\#\#\#}$	50.17 ± 4.39$^{***\#\#\#}$	86	63
*tim*G4/U*MAN1RNAi*-2;U*dcr2*/+	25.02 ± 0.05$^{***\#\#\#}$	39.71 ± 3.88$^{***\#\#\#}$	77	60
*tim*G4/U*MAN1RNAi*-3;U*dcr2*/+	25.18 ± 0.13$^{***\#\#\#}$	16.69 ± 3.24$^{***\#\#\#}$	39	56
*tim*G4/+;U*dcr2*/+	23.99 ± 0.06	94.69 ± 13.26	98	51
U*dcr2*/U*MAN1RNAi*-1;*cry*G4-16/+	27.64 ± 0.16$^{***\#\#\#}$	50.88 ± 4.75$^{***\#\#\#}$	84	45
U*dcr2*/U*MAN1RNAi*-2;*cry*G4-16/+	26.66 ± 0.09$^{***\#\#\#}$	78.41 ± 6.37***	96	49
U*dcr2*/U*MAN1RNAi*-3;*cry*G4-16/+	26.21 ± 0.10$^{***\#\#\#}$	68.20 ± 4.63***	97	64
U*dcr2*/+;*cry*G4-16/+	25.06 ± 0.06	120.49 ± 4.41	98	91
U*MAN1RNAi*-1/+	24.14 ± 0.03	82.89 ± 3.68	99	73
U*MAN1RNAi*-2/+	24.02 ± 0.06	58.46 ± 4.91	95	44
U*MAN1RNAi*-3/+	23.53 ± 0.03	78.75 ± 7.69	95	20

One-way ANOVA, $P < 0.001$, Tukey's multiple comparison test, $^{***}P < 0.001$ *vs* G4 and U*dcr2*; $^{\#\#\#}P < 0.001$ *vs* U*RNAi*. G4, GAL4; U, UAS.

efficiency of the RNAi (Fig. 1F). We also over-expressed *MAN1* using *cry*GAL4-16 and found a > 1 h longer period as well as substantially reduced power of the rhythm, confirming our previous findings (Table S1) [6].

To test whether MAN1 functions in the adult circadian system, we used a gene-switch system to turn on RNAi expression specifically in the pigment-dispersing factor (PDF)-expressing pacemaker neurons of adult flies a few days before the start of behavioral monitoring [7]. We found that the period in drug-treated flies with RNAi expression activated was lengthened by close to 2 h, whereas the period in the vehicle-treated controls was not significantly altered (Fig. 2 and Table 2). Knocking down *MAN1* in the pacemaker neurons of adults modestly reduced the power of the rhythm as well. We also over-expressed *MAN1* in the adult pacemaker neurons but found no significant effect on the period or power (Table S2). Taken together, these results showed that knocking down *MAN1* in the adult pacemaker neurons is sufficient to lengthen the period of the clock. Given that over-expressing *MAN1* in adults did not appear to alter the locomotor rhythm, we suspected that the behavioral phenotypes observed in UAS*MAN1/+;cry*GAL4-16/+ were due to developmental defects caused by over-expression. Therefore, for further investigations we focused on *MAN1RNAi* flies.

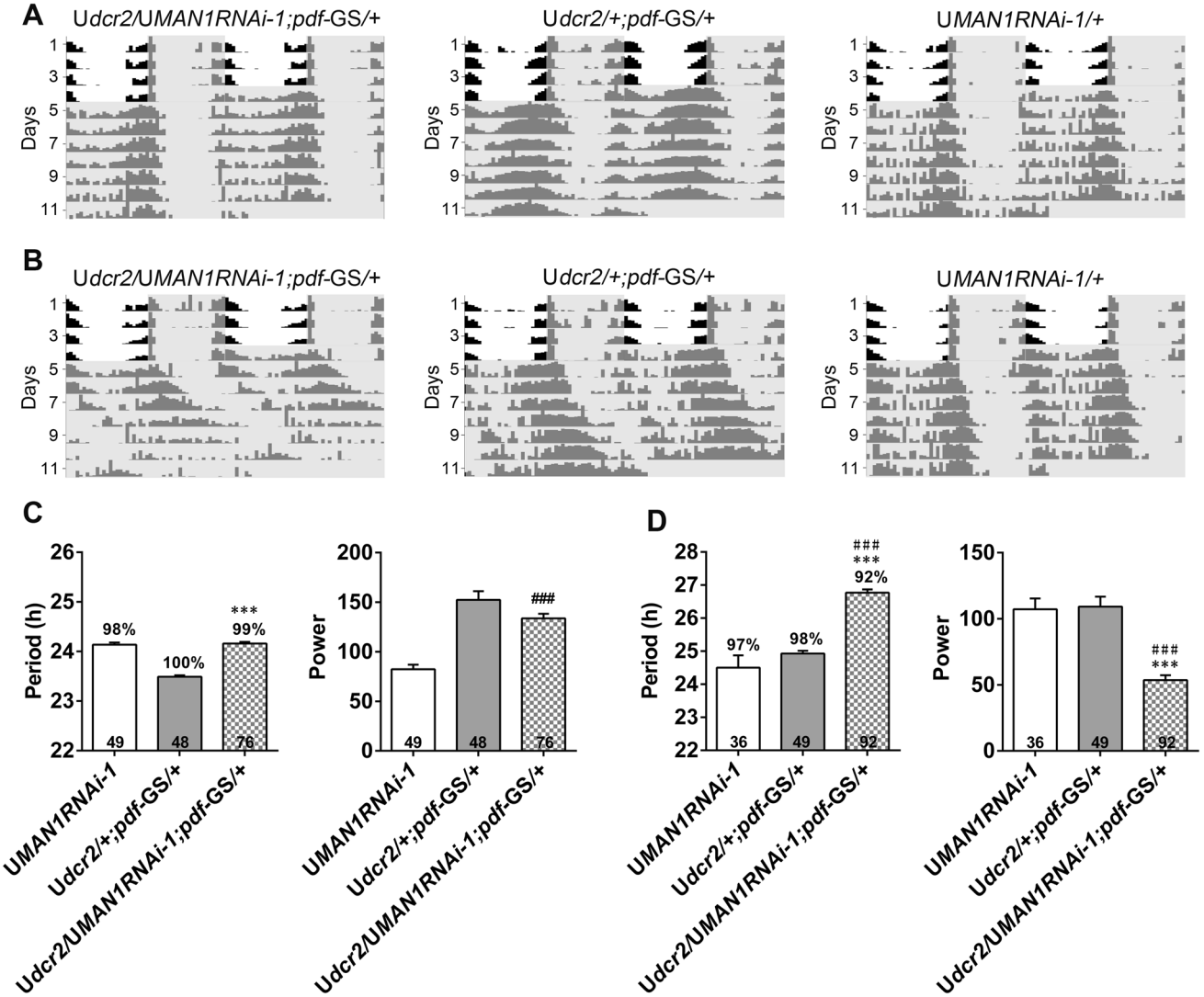


Fig. 2 Knocking down *MAN1* in adults lengthens the period of the locomotor rhythm. **A**, **B** Double-plotted representative actograms of the indicated genotypes. Flies treated with vehicle control (**A**) or RU486 (**B**) to activate the *pdf*G4-geneswitch (*pdf*-GS) driver. **C**, **D** The period and power of DD locomotor rhythms of flies with *MAN1* knocked down and controls. Flies treated with vehicle control (**C**) or RU486 (**D**). Flies are monitored in LD for 4 days and then DD for 7 days. Gray shades indicate dark phases. Error bars represent SEM. Digits on the bars are the number of flies tested. Percentage of rhythmicity is indicated above the bars ($P < 0.001$, one-way ANOVA; ***/###$P < 0.001$, * *vs* GAL4 controls; # *vs* UAS controls, Tukey's multiple comparison test). G4, GAL4; U, UAS.

Table 2 Knocking down *MAN1* in adults lengthens the period of the locomotor rhythm.

Genotype	Period (h) ± SEM	Power ± SEM	%Rhythmic	*n*
Ethanol-treated				
U*dcr2*/+;*pdf*-GS/+	23.49 ± 0.03	152.41 ± 8.75	100	48
U*MAN1RNAi*-1/+	24.14 ± 0.04	82.40 ± 4.53	98	49
U*dcr2*/U*MAN1RNAi*-1;*pdf*-GS/+	24.16 ± 0.03***	133.76 ± 4.63$^{\#\#\#}$	99	76
pdf-GS/+	24.53 ± 0.05	134.92 ± 7.82	100	31
U*MAN1*/+	23.56 ± 0.03	76.89 ± 6.23	96	45
U*MAN1*/+;*pdf*-GS/+	23.50 ± 0.07	150.68 ± 11.94$^{\#\#\#}$	100	11
Drug-treated				
U*dcr2*/+;*pdf*-GS/+	24.93 ± 0.09	109.22 ± 7.52	98	49
U*MAN1RNAi*-1/+	24.50 ± 0.37	107.24 ± 8.09	97	36
U*dcr2*/U*MAN1RNAi*-1;*pdf*-GS/+	26.77 ± 0.10***$^{\#\#\#}$	53.59 ± 3.65***$^{\#\#\#}$	92	92
pdf-GS/+	24.94 ± 0.08	80.53 ± 5.49	96	50
U*MAN1*/+	23.74 ± 0.05	101.28 ± 5.79	98	52
U*MAN1*/+;*pdf*-GS/+	24.92 ± 0.08$^{\#\#\#}$	88.54 ± 5.35	91	87

One-way ANOVA, $P < 0.001$, Tukey's multiple comparison test, ***$P < 0.001$ *vs* G4, $^{\#\#\#}P < 0.001$ *vs* UAS. Drug-treated, flies treated with RU486 after eclosion; ethanol-treated, flies treated with ethanol instead of RU486 after eclosion. G4, GAL4; U, UAS.

Knocking Down *MAN1* Reduces *per* mRNA and Protein Levels

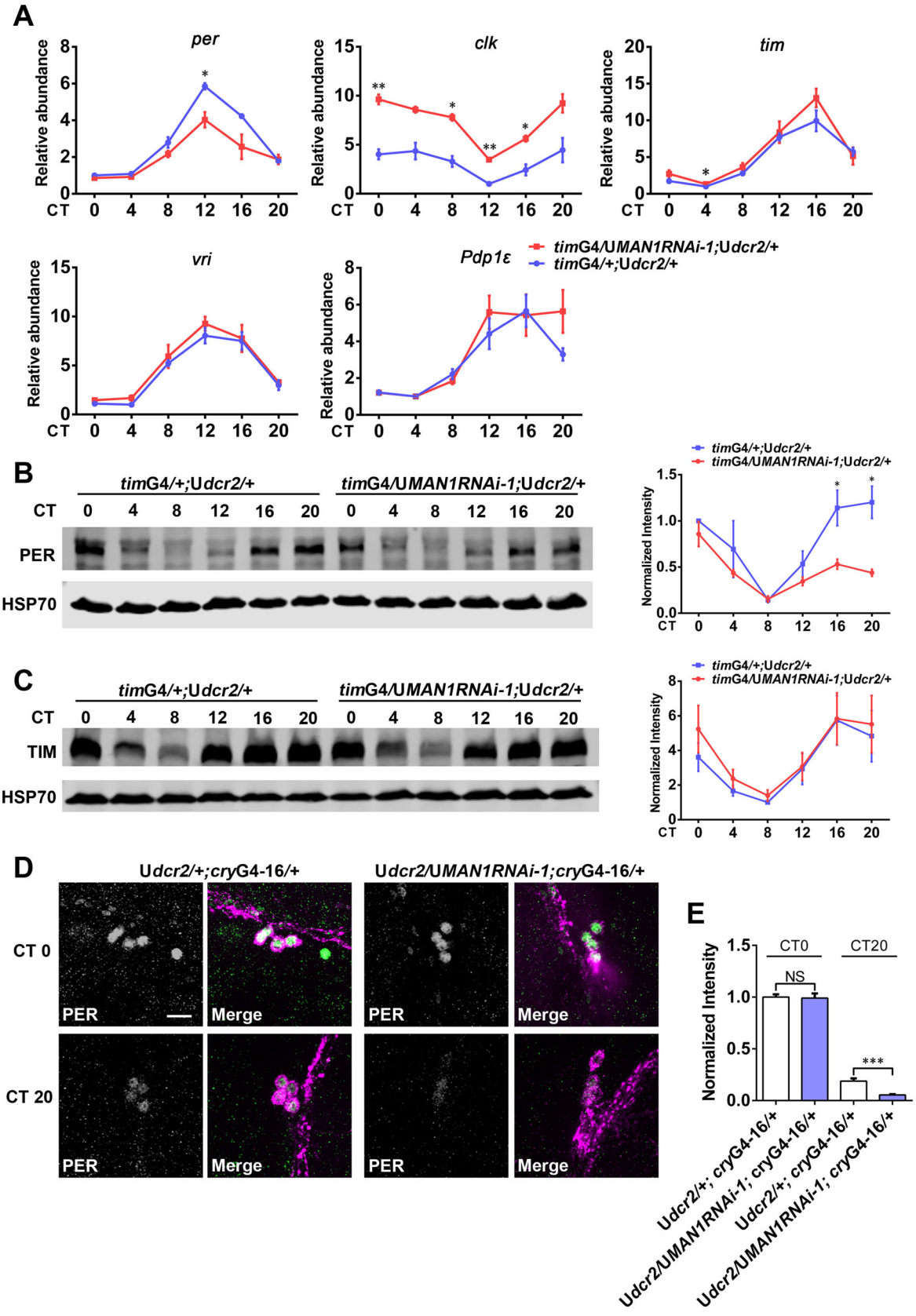
We next sought to characterize the mechanism by which MAN1 sets the pace of the clock by examining the effects of knocking down *MAN1* on clock gene expression. Previously, we showed that silencing *MAN1* reduces the transcription of *BMAL1* in human cell lines but does not alter the mRNA level of *cyc* (the *Drosophila* ortholog of *BMAL1*) in fly heads [6]. Here, we found that knocking down *MAN1* substantially reduced the *per* mRNA level and increased *clk* mRNA level (Fig. 3A). To further confirm this, we measured the protein level of PER and found it to be decreased, consistent with reduction in the mRNA level (Fig. 3B). On the other hand, TIM, the binding partner of PER, was not significantly changed (Fig. 3C) [15]. Because the period of the locomotor rhythm under DD is believed to be determined by the small ventral lateral neurons that express PDF (sLNvs), we assessed PER in these cells and found a significant reduction of the PER protein level at least at Circadian Time 20 (CT20; CT0 was defined as subjective lights-on time) (Fig. 3D,E) [16].

per has an Epistatic Effect on *MAN1* in Period Length Determination

Based on our findings, we proposed that MAN1 times the clock by regulating *per* and/or *clk* mRNA levels. To test this hypothesis, we first over-expressed *per* in flies with *MAN1* knocked down and found that this significantly reverted the long period phenotype, whereas over-expressing *per* in control flies did not significantly affect the period (Fig. 4A, B and Table 3) [8]. On the other hand, knocking down *MAN1* on a per^L mutant background did not lead to further lengthening of the period (Fig. 4C, D and Table 3) [9]. These results demonstrated that when *per* is either over-expressed or mutated, the influence of MAN1 on period length is blocked, indicating an epistatic effect of *per* on *MAN1*. In combination with the molecular changes, we propose that MAN1 sets the pace of the clock via *per*.

We also tested for genetic interactions between *MAN1* and *clk*. Over-expressing *clk* slightly shortened the period, and knocking down *MAN1* in flies over-expressing *clk* did not substantially lengthen the period, indicating that over-expressing *clk* also has an epistatic effect on *MAN1* (Table S3) [10]. Given that CLK activates the transcription of *per*, it is possible this epistatic effect is caused by

Fig. 3 Knocking down *MAN1* reduces the mRNA and protein levels of *per*. **A** Plots of relative mRNA abundance *versus* CT for clock genes determined by qRT-PCR in whole head extracts of *MAN1 RNAi* (*tim*G4/U*MAN1RNAi*;U*dcr2*/+) and control (*tim*G4/+;U*dcr2*/+) flies collected on DD1 ($n \geq 3$). For each time series, the value of the lowest time point was set to 1. **B**, **C** Western blots of proteins from whole head extracts of *MAN1RNAi* and control flies collected on DD1 and probed with PER and TIM antibodies (left). Quantification of PER and TIM protein levels (right, $n = 4$). PER and TIM 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*/+;cryG4-16/+ and U*dcr2*/U*MAN1RNAi*;cryG4-16/+ flies collected at CT 0 and 20 on DD1 immunostained with PER (green) and PDF (red) antisera. Scale bar, 10 μm. **E** Quantification of PER protein levels in the s-LNvs of images as in (D) ($n = 72$–80). Error bars represent SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t* test. G4, GAL4; U, UAS.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Relative abundance
CT 0 4 8 12 16 20
timG4/UMAN1RNAi-1;Udcr2/+
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timG4/+;Udcr2/+
timG4/UMAN1RNAi-1;Udcr2/+
CT 0 4 8 12 16 20
PER
HSP70
Normalized Intensity
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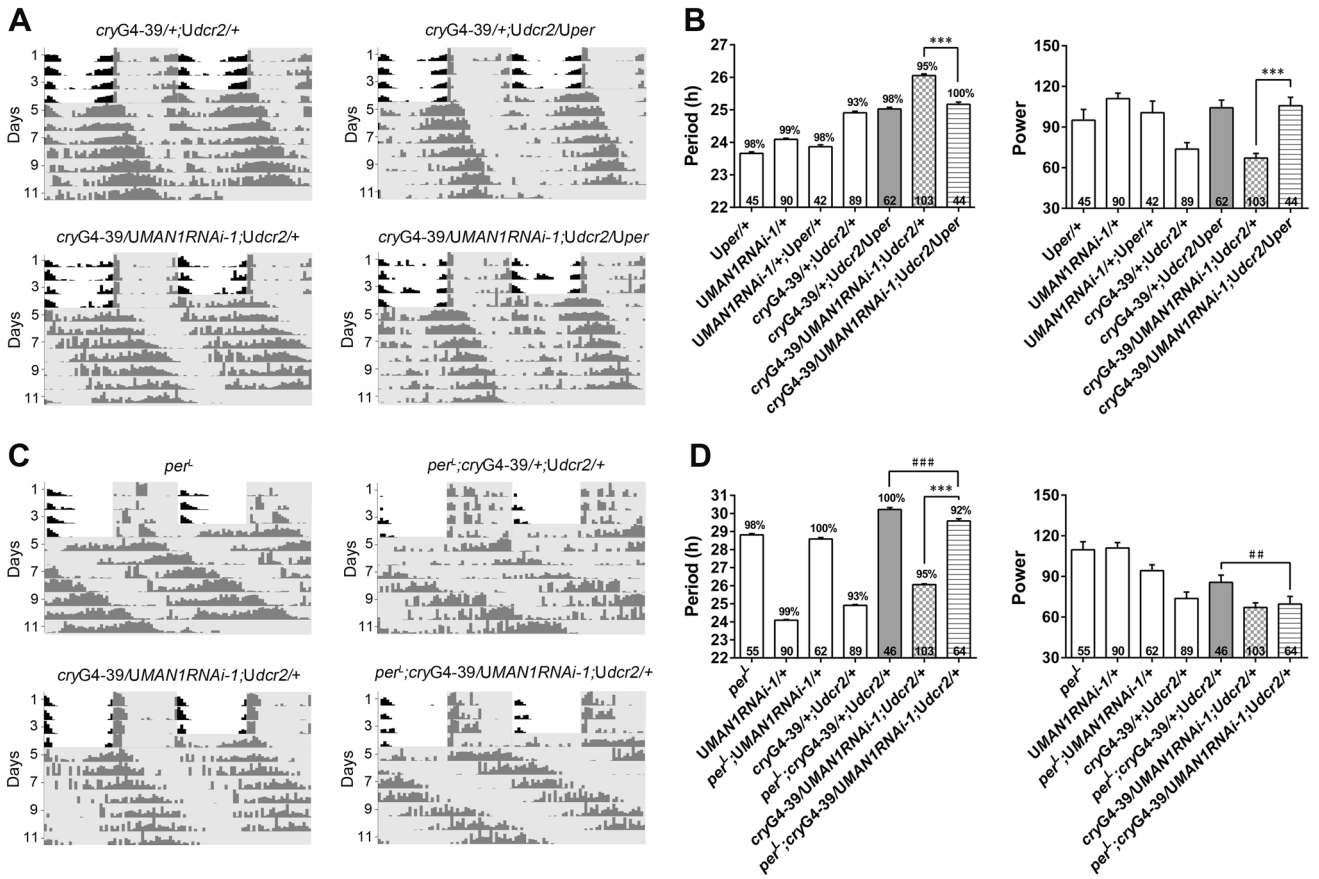

Fig. 4 *per* has an epistatic effect on *MAN1* in period length determination. **A**, **C** Double-plotted representative actograms of the indicated genotypes. Flies were monitored in LD for 4 days and then DD for 7 days. Gray shades indicate dark phases. **B** The period and power of the DD locomotor rhythm of *MAN1RNAi* flies over-expressing *per*. **D** The period and power of the DD locomotor rhythm of *MAN1RNAi* flies carrying the per^L mutation. Error bars represent SEM. Digits on the bar are the number of flies tested. Percentage of rhythmicity is indicated above the bars ($P < 0.001$, one-way ANOVA, $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$, * *vs MAN1-RNAi* flies, # *vs* over-expression or mutant flies, Tukey's multiple comparison test). G4, GAL4; U, UAS.

Table 3 Genetic interaction between *MAN1* and *per*.

Genotype	Period (h) ± SEM	Power ± SEM	%Rhythmic	*n*
U*per*/+	23.66 ± 0.05	95.10 ± 7.92	98	45
U*MAN1RNAi*-1/+	24.09 ± 0.03	111.00 ± 3.99	99	90
U*MAN1RNAi*-1/+;U*per*/+	23.87 ± 0.06	100.63 ± 8.51	98	42
*cry*G4-39/+;U*dcr2*/+	24.91 ± 0.05	73.76 ± 4.68	93	89
*cry*G4-39/+;U*dcr2*/U*per*	25.02 ± 0.05	104.29 ± 5.58	98	62
*cry*G4-39/U*MAN1RNAi*-1;U*dcr2*/+	26.06 ± 0.05$^{+++\dagger\dagger\dagger}$	67.12 ± 3.37†††	95	103
*cry*G4-39/U*MAN1RNAi*-1;U*dcr2*/U*per*	25.17 ± 0.07***	105.72 ± 6.29***	100	44
per^L	28.81 ± 0.07	109.66 ± 5.96	98	55
per^L;U*MAN1RNAi*-1/+	28.59 ± 0.08	94.23 ± 4.37	100	62
per^L;*cry*G4-39/+;U*dcr2*/+	30.22 ± 0.11	85.63 ± 5.37	100	46
per^L;*cry*G4-39/U*MAN1RNAi*-1;U*dcr2*/+	29.58 ± 0.12***$^{\#\#\#}$	69.64 ± 5.58$^{\#\#}$	92	64

One-way ANOVA, $P < 0.001$, Tukey's multiple comparison test, $^{***}P < 0.001$ *vs MAN1RNAi*-1 flies; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* per^L;*cry*G4-39/+;U*dcr2*/+ flies; $^{+++}P < 0.001$ *vs cry*G4-39/+;U*dcr2*/+ flies; $^{\dagger\dagger\dagger}P < 0.001$ *vs* U*MAN1RNAi*-1/+ flies. G4, GAL4; U, UAS.

enhanced *per* [11, 17]. The clk^{jrk}/+ mutation, on the other hand, leads to a very poor rhythm and thus it is not possible to determine whether a genetic interaction exists between clk^{jrk} and *MAN1* (Table S3) [11].

Knocking Down *MAN1* Reduces *per* Pre-mRNA Levels

Since genetic interaction analysis strongly suggested that MAN1 determines the period by regulating *per*, we next addressed how MAN1 acts on *per*. As knocking down *MAN1* decreased the *per* mRNA level, we assayed the pre-mRNA level of *per* in these flies, which reflects the status of *per* transcription. We found that the *per* pre-mRNA level was significantly reduced in *MAN1RNAi* flies, indicating that MAN1 functions to promote *per* transcription (Fig. 5A). Furthermore, to test whether MAN1 serves as a permissive or instructive signal, we examined the temporal expression profile of *MAN1*. We did not find significant changes in *MAN1* mRNA levels throughout the day, demonstrating that MAN1 is not circadian-regulated at the transcription level (Fig. 5B).

Discussion

Previously, we showed that MAN1 binds to and promotes the transcription of the core clock gene *BMAL1* (the mammalian ortholog of *Drosophila cyc*) in human U2OS cells, while over-expressing *MAN1* in flies enhances *cyc* mRNA levels and knocking down *MAN1* does not alter the *cyc* level [6]. We proposed that MAN1 uses a conserved mechanism to regulate the clock by promoting the transcription of *BMAL1*/*cyc*. However, we found that the circadian phenotypes associated with *MAN1* over-expression were likely due to developmental defects rather than a relatively specific and direct effect of MAN1 on the clock, as over-expressing *MAN1* in adult flies did not result in significant changes in circadian behavior. On the other hand, knocking down *MAN1* in adults led to a substantially lengthened period, which means that *MAN1RNAi* flies are an appropriate model in which to study the role of MAN1 in the clock.

Molecular analysis has revealed that knocking down *MAN1* reduces *per* while increasing the *clk* mRNA levels. We confirmed the altered expression of *per* at the protein level and intended to do the same for *clk*, but unfortunately we do not have a CLK antibody that works well. However, we believe MAN1 is more likely to act directly on *per* rather than *clk* to determine period length. First of all, over-expressing *MAN1* leads to a trend of increased *per* mRNA, opposite to the effect of knocking down *MAN1* that we found here [6]. As for *clk*, both over-expressing and knocking down *MAN1* led to elevated levels, implying an indirect modulation. Second, downregulation of PER levels are associated with a lengthened period, similar to the phenotype of *MAN1RNAi*, whereas upregulated *clk* shortens the period [10, 18]. Third, based on our current understanding of how the clock operates, if an increase in *clk* is able to influence the period length, it should lead to an increase of *per*/*tim* transcription and subsequently their mRNA levels [2]. However, we found significantly less *per* mRNA in *MAN1RNAi* flies, which would be unexpected if CLK function is indeed enhanced. What is more, over-expressing *per* specifically rescued the long-period phenotype of *MAN1RNAi*, while per^L mutation had an epistatic effect on *MAN1*. It is also possible that knocking down *MAN1* does not further lengthen the period of per^L flies is the result of a "ceiling effect", but in our opinion this is less likely because we have unpublished results demonstrating that genetically modulating certain genes can lengthen the period of per^L by an additional $\sim$3 h. Therefore, we believe knocking down *MAN1* in the presence of per^L does not reach the limit of the clock timing mechanism, and the failure to further lengthen the period indeed reflects true genetic interactions. Based on these findings, we propose that MAN1 sets the pace of the clock by promoting *per* expression.

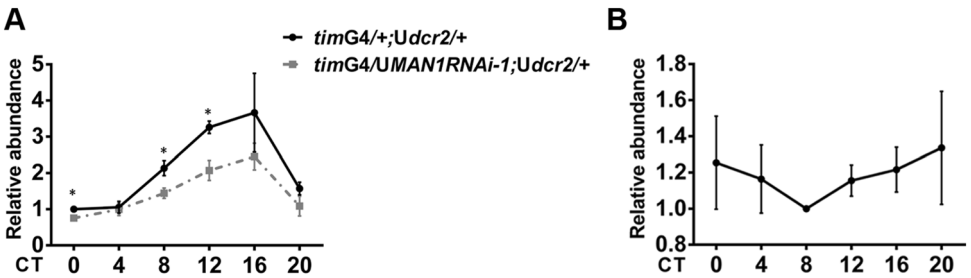

Fig. 5 Knocking down *MAN1* reduces the pre-mRNA level of *per*. **A** Plots of relative pre-mRNA abundance of *per* determined by qRT-PCR in whole head extracts of *MAN1RNAi* (*tim*G4/*MAN1-RNAi;*U*dcr2*/+) and control (*tim*G4/+;U*dcr2*/+) flies during DD1 ($n = 4$; $^*P < 0.05$, Student's t test). **B** Plots of relative mRNA abundance of *MAN1* in whole head extracts of w^{1118} flies in DD1 determined by qRT-PCR ($n = 3$; $P > 0.05$, one-way ANOVA).

Previous studies have demonstrated an important role for MAN1 in transcriptional regulation [19]. In line with this, we found that knocking down *MAN1* reduced *per* transcription, although it remains to be further determined whether MAN1 binds directly to the *per* locus to regulate transcription or indirectly by modulating other factors that in turn control *per* transcription. It has been shown that circadian genes are rhythmically recruited to the NE where they interact with lamin-associated domains and their transcription is attenuated by histone methylation events [5]. Given the role of MAN1 in clock regulation, it is possible that MAN1 participates in modulating transcription when circadian genes such as *per* move to the nuclear periphery.

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Conflict of interest The authors declare no competing interests.

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