A Screening of UNF Targets Identifies *Rnb*, a Novel Regulator of *Drosophila* Circadian Rhythms

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Behavioral circadian rhythms are controlled by multioscillator networks comprising functionally different subgroups of clock neurons. Studies have demonstrated that molecular clocks in the fruit fly *Drosophila melanogaster* are regulated differently in clock neuron subclasses to support their specific functions (Lee et al., 2016; Top et al., 2016). The nuclear receptor *unfulfilled* (*unf*) represents a regulatory node that provides the small ventral lateral neurons (s-LNvs) unique characteristics as the master pacemaker (Beuchle et al., 2012). We previously showed that UNF interacts with the s-LNv molecular clocks by regulating transcription of the core clock gene *period* (*per*) (Jaumouillé et al., 2015). To gain more insight into the mechanisms by which UNF contributes to the functioning of the circadian master pacemaker, we identified UNF target genes using chromatin immunoprecipitation. Our data demonstrate that a previously uncharacterized gene *CG7837*, which we termed *R and B* (*Rnb*), acts downstream of UNF to regulate the function of the s-LNvs as the master circadian pacemaker. Mutations and LNv-targeted adult-restricted knockdown of *Rnb* impair locomotor rhythms. RNB localizes to the nucleus, and its loss-of-function blunts the molecular rhythms and output rhythms of the s-LNvs, particularly the circadian rhythms in PDF accumulation and axonal arbor remodeling. These results establish a second pathway by which UNF interacts with the molecular clocks in the s-LNvs and highlight the mechanistic differences in the molecular clockwork within the pacemaker circuit.

Key words: behavior; chromatin immunoprecipitation; circadian rhythms; *Drosophila*; molecular mechanism; pacemaker neurons

Significance Statement

Circadian behavior is generated by a pacemaker circuit comprising diverse classes of pacemaker neurons, each of which contains a molecular clock. In addition to the anatomical and functional diversity, recent studies have shown the mechanistic differences in the molecular clockwork among the pacemaker neurons in *Drosophila*. Here, we identified the molecular characteristics distinguishing the s-LNvs, the master pacemaker of the locomotor rhythms, from other clock neuron subtypes. We demonstrated that a newly identified gene *Rnb* is an s-LNv-specific regulator of the molecular clock and essential for the generation of circadian locomotor behavior. Our results provide additional evidence to the emerging view that the differential regulation of the molecular clocks underlies the functional differences among the pacemaker neuron subgroups.

Introduction

Circadian rhythms enable organisms to anticipate and adapt to rhythmic daily and seasonal environmental changes. The molec-

ular clocks, which largely rely on transcriptional/translational feedback loops (TTFLs), generate 24 h rhythms in eukaryotes (Mohawk et al., 2012; Hardin and Panda, 2013). In *Drosophila*, the periodic transcription of *period* (*per*) and *timeless* (*tim*) by the CLOCK (CLK)/CYCLE (CYC) heterodimer and feedback inhibition of CLK/CYC-dependent transcription by the PER/TIM complex constitute the principal TTFL of the molecular clock.

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Figure 1. *CG7837 (Rnb)* is a direct target of UNF. **A**, Binding of CLK and UNF on the *Tpr2*, *CG7837 (Rnb)*, and *gol* genes is shown as examples of the ChIP results. CLK binding data were obtained from Abuzzi et al. (2011). **B**, UNF target genes were knocked down by RNAi using *Pdf-GAL4* and tested for locomotor behavior. The RNAi lines listed in Table 2 from top to bottom correspond to the *x*-axis from left to right. **C**, Relative *Rnb* mRNA levels normalized to the *Ef1 β* mRNA levels were analyzed using qPCR in the heads of *w¹¹¹⁸* and *unf^{1/20001}* mutant flies. *Rnb* levels were significantly reduced in the *unf^{1/20001}* flies. *** $p < 0.001$ (Student's *t* test). **D**, RNAseq of the LNVs isolated at two time points in DD3 revealed the expression of *Rnb* transcripts in the LNVs. Data represent the average RPKM (reads per kilobase of transcript per million mapped reads) of two independent samples $\pm$ SD. The transcripts of *unf*, *Pdf*, and clock genes, but not the glia-specific gene *reverse polarity* (*repo*), were identified, reflecting the LNV specificity of the sequenced libraries.

CLK/CYC also activates transcription of *pdp-1* and *vri* (*vri*), and PDP-1 and VRI proteins regulate transcription of *Clk*, thereby forming a stabilizing loop (Hardin, 2011). In addition, post-transcriptional and post-translational regulation play important roles in generating 24 h rhythms (Lim and Allada, 2013).

Drosophila circadian locomotor behavior is organized by a neural circuit composed of anatomically and functionally diverse groups of clock-containing neurons. The morning and evening activity bouts under light-dark (LD) cycles are controlled by the small ventral lateral neurons (s-LNVs; M-oscillator) and the E-oscillator group, including the dorsal lateral neurons (LNDs) and the fifth s-LNV (Peschel and Helfrich-Forster, 2011). Free-running behavioral rhythms in constant darkness (DD) are pre-

dominantly driven by the M-oscillator at least within a certain range of periods (Stoleru et al., 2005; Yao and Shafer, 2014). A subgroup of dorsal neurons, DN1s, relay output signals from the M-oscillator to locomotor output circuit to generate morning activity peaks and contribute to the free-running locomotor rhythms (Cavanaugh et al., 2014). DN1s also integrate environmental information and modulate the operation of M- and E-oscillators in driving morning and evening peaks (Zhang et al., 2010a, b).

Gene expression differences among clock neurons underlie their functional differences (Kula-Eversole et al., 2010; Nagoshi et al., 2010). Most notably, the neuropeptide pigment-dispersing factor (PDF), which is expressed only in the s- and l-LNVs, is

Table 1. UNF binding peaks^a

Chromatin	Start	End	Annotation	ZT14 rank	ZT14 <i>p</i>	ZT2 rank	ZT2 <i>p</i>	CLK BD
X	8036801	8040825	Smox-promoter, CG17982-promoter, CG2263-promoter, CG17982-exon, CG15336-promoter, CG2129-exon, CG2129-promoter, CG2263-exon	1	3.3E-29	18	3.3E-11	
3R	20075140	20079908	mask-exon, CG5706-promoter, jar-intron, jar-exon, CG5706-intron, CG5706-exon	2	3.3E-25			
3R	3299277	3301524	CG1142-exon, snRNA:7SK-promoter, snRNA:7SK-exon, CG1142-promoter, snoRNA:MeU5-C46-promoter	3	9.7E-25			
2L	16491360	16493309	Tpr2-exon, Tpr2-promoter, Tpr2-intron	4	9.0E-24	4	2.8E-15	Tpr2
2R	18958854	18960385	l(2)k09913-exon	5	1.4E-23	11	3.6E-12	
2R	9317001	9320635	Ser8-promoter, CG30065-exon, CG42321-exon, CG42321-intron, CG30065-promoter, CG42321-promoter	6	5.5E-21			
3R	25623780	25625727	CG7837-exon, lce-exon, CG7837-intron, lce-promoter, CG7837-promoter	7	8.0E-20	3	6.2E-17	
2R	15301421	15303508	snoRNA:hts-a-exon, snoRNA:hts-a-promoter, hts-intron	8	2.9E-18	6	1.3E-13	
2R	9317424	9320594	Ser8-promoter, CG42321-intron, CG30065-promoter, CG42321-promoter			1	2.8E-17	
3R	3299796	3301424	snRNA:7SK-promoter, snRNA:7SK-exon, CG1142-promoter, snoRNA:MeU5-C46-promoter			2	4.2E-17	
2R	20962653	20965558	gol-intron, gol-exon	9	1.2E-16	8	1.1E-12	gol
3R	4493289	4495665	CG8036-exon, CG8036-intron, CG8036-promoter	10	1.7E-16	19	4.5E-11	
2R	2058366	2060021	EcR-promoter, Cyp6w1-promoter	11	2.6E-16	7	9.0E-13	EcR
3L	14666572	14668637	CG43121-promoter, CG4914-intron, ome-intron, CG4914-exon	12	4.1E-16			
3R	8862579	8864238	CG11668-exon, CG11668-intron, CG11668-promoter, ry-intron, ry-exon	13	1.1E-15			
3R	18619187	18620688	CG7029-exon	14	1.8E-15	5	5.8E-14	
2R	13278911	13280451	Sip1-promoter	15	2.3E-15	16	1.4E-11	
2L	20873588	20875696	sky-exon, sky-intron	16	4.4E-15			
X	2587801	2588943	CG2650-exon, CG2658-exon, CG2650-promoter	17	4.7E-15			
2R	17269291	17271559	CG10795-exon, CG10795-intron	18	5.6E-14			
3R	22772208	22774253	CG14252-exon, CG18472-intron, CG18472-exon, CG18472-promoter, CG14252-intron	19	7.3E-14	40	6.3E-08	
3R	13444545	13446800	CG14324-promoter, CG14326-promoter, CG14326-exon, CG14326-intron	20	3.4E-13			
3L	5789797	5791159	Tektin-C-promoter	21	4.7E-13	39	6.2E-08	
3L	18610853	18612096	CG13380-promoter, CG13380-exon, CG4174-exon, not-exon, CG4174-promoter	22	6.4E-13	23	2.3E-09	
X	5417756	5419200	snoRNA:Psi285-3342-promoter, snoRNA:Psi285-3342-exon	23	1.4E-12	9	1.7E-12	
3L	441077	442721	klar-intron, klar-exon, klar-promoter	24	2.4E-12			
3L	3095106	3097041	Cht7-intron	25	4.1E-12	10	2.5E-12	
3R	13443098	13444388	CG14326-promoter, CG14324-exon			13	9.3E-12	
X	1676738	1677935	Adar-exon, Adar-intron, CG42666-intron			14	9.8E-12	
3R	20076127	20078482	CG5706-promoter, CG5706-intron, CG5706-exon			15	1.2E-11	
3R	5039548	5040831	pum-intron, pum-exon	26	1.4E-11	12	5.2E-12	
3L	14666655	14668094	CG43121-promoter, ome-intron, CG4914-exon			17	1.5E-11	
2R	12106837	12109098	mrj-intron	27	1.5E-11	29	8.4E-09	
2R	4555166	4557028	Acs1-intron, Acs1-promoter	28	1.9E-11			
3R	19555340	19556770	CG10365-exon, RNaseMRP:RNA-exon, CG10365-intron, RNaseMRP:RNA-promoter	29	2.2E-11	70	2.7E-06	
2L	3172537	3173744	CG34406-exon, CG31698-intron, CG31698-exon, CG34406-intron, CG15404-promoter, CG34406-promoter	30	3.2E-11	30	8.7E-09	
3L	22839989	22841597	CR43426-promoter	31	3.4E-11			
2R	7539150	7540664	CG34228-intron, Roc2-intron, Roc2-exon, CG34228-exon, CG34228-promoter, CG9003-promoter, CG13198-promoter	32	4.1E-11			Roc2
3R	13442719	13444544	CG14324-intron, CG14324-promoter, CG14326-promoter, CG14324-exon	33	6.2E-11			
2R	13491220	13493358	lack-exon, CG10936-exon, CG10936-intron	34	8.0E-11			
2R	14664507	14665831	CG15093-promoter	35	1.8E-10	34	2.6E-08	CG15093

(Table continued)

Table 1. Continued

Chromatin	Start	End	Annotation	ZT14 rank	ZT14 <i>p</i>	ZT2 rank	ZT2 <i>p</i>	CLK BD
3R	2954340	2957636	CG31493-promoter, CR42875-intron, CR42875-exon, CG31493-intron, CG31248-intron, CG31493-exon, CG10068-promoter, CG31248-exon, CG31248-promoter	36	4.6E-10			
2R	11747802	11749162	Gpo-1-exon, Gpo-1-intron, Gpo-1-promoter	37	6.7E-10			Gpo-1
X	8105175	8106920	sdt-intron	51	1.2E-08	20	6.8E-10	sdt
3R	17490794	17492282	DNApol- α 180-exon, CG15497-exon, CG15497-promoter, CG15497-intron	38	7.0E-10			
2R	5480924	5482611	CG1902-exon, clos-exon, CG30338-intron, clos-intron, CG1902-promoter, CG30338-exon, CG1902-intron, CG30338-promoter	39	7.1E-10			
3L	17393439	17394974	noe-promoter, blot-exon, noe-exon, blot-intron	40	8.1E-10			
2R	2039462	2040973	EcR-exon, EcR-intron	41	1.1E-09	26	2.9E-09	EcR
3R	2644498	2646006	7SLRNA:CR42652-exon, 7SLRNA:CR32864-exon	45	4.2E-09	21	1.1E-09	
3L	17393439	17394858	noe-promoter, noe-exon, blot-exon, blot-intron			22	1.4E-09	
3R	5154054	5155329	CG8861-intron, CG8861-promoter	42	2.1E-09			
3L	19807399	19808572	cyc-intron, cyc-exon, Cyp305a1-promoter	65	8.2E-08	24	2.4E-09	
2R	13503641	13504878	snoRNA:Or-aca4-exon, CG10936-intron, snoRNA:Or-aca4-promoter			25	2.5E-09	
2L	13892279	13893601	cenG1A-promoter, cenG1A-intron	43	2.5E-09			cenG1A
2R	8221206	8224776	CG34021-intron, Den1-promoter, CG8490-exon, garz-exon, CG34021-promoter, CG8490-intron, CG34021-exon, CG8490-promoter	44	2.7E-09			

^aUNF binding peaks analyzed using anti-UNF ChIP-chip assays are ordered by rank. CLK BD, CLK target gene.

essential for the role of the M-oscillator in generating morning activity peak and for driving free-running behavior (Renn et al., 1999). Recent studies also revealed that TTFL is regulated differently in clock neuron subtypes, suggesting the presence of regulatory mechanisms specific for the s-LNV master pacemaker neurons (Lee et al., 2016; Top et al., 2016). Consistently, the *unfulfilled* (*unf*; *DHR51*) gene encoding a nuclear receptor is selectively expressed in the LNVs within the clock neuron subgroups and is necessary for the functioning of the s-LNVs as the master pacemaker (Beuchle et al., 2012). UNF acts together with the second nuclear receptor E75, a homolog of mammalian REV-ERB α/β , to enhance *per* transcription, thereby reinforcing the master pacemaker TTFL (Jaumouillé et al., 2015).

To better understand the mechanisms by which UNF contributes to the functioning of the M-oscillator, here we identify UNF direct targets using chromatin immunoprecipitation (ChIP). The behavioral screening of UNF target genes in conjunction with functional assays reveals that a previously uncharacterized gene *CG7837*, which we name *Rnb*, acts downstream of UNF to regulate the molecular clockwork of the s-LNVs. By demonstrating the second pathway through which UNF regulates molecular clockwork specifically in the s-LNVs, these results highlight the unexpected diversity of the molecular clock makeup among circadian pacemaker neurons.

Materials and Methods

Fly rearing, crosses, and strains. *Drosophila melanogaster* were reared on standard cornmeal/agar medium supplemented with yeast in 12 h/12 h LD cycles at 25°C, unless otherwise mentioned. *UAS-RNAi* lines were obtained from the Vienna *Drosophila* Resource Center (VDRC). *D52H-GAL4* (Aso et al., 2009), *unf^{Δ1}*, and *unf^{Δ001}* (Beuchle et al., 2012) were previously described. The following lines were obtained from the Bloomington *Drosophila* Stock Center: *CG7837⁰⁴⁶¹⁶*, *CG7837⁰⁵⁴⁰⁰* and *w¹¹¹⁸*; *Df(3R)BSC547* (dubbed *Df*).

Conditional knockdown or overexpression experiments were performed by combining *GAL4* (*Pdf-GAL4* or *gal1118*) and *tubulin-GAL80^{ts}* with the *UAS-transgene* and shifting the temperature of fly rearing be-

tween 18°C (permissive temperature for *GAL80^{ts}*) and 29°C (restrictive temperature for *GAL80^{ts}*) (McGuire et al., 2004). To express *UAS-transgenes* only during development, the flies were crossed and reared at 29°C until eclosion, and the emerged flies were immediately transferred to and maintained at 18°C for the following experiments. To express *UAS-transgenes* only during adulthood, the flies were crossed and reared at 18°C, and the emerged flies were immediately transferred to 29°C.

To generate the *UAS-Rnb::Venus* construct, the *Rnb* coding sequence without the stop codon was PCR-amplified from the LD3331 clone (Berkeley *Drosophila* Genome Project) and cloned into the pBID-UASC-GV vector (Wang et al., 2012) using the Gateway Cloning System (Invitrogen). *UAS-Rnb::Venus* construct was integrated into the attP40 landing site on the second chromosome by phiC31-mediated site-directed transgenesis. Embryo injections were conducted at BestGene.

Behavioral assays. The locomotor behavior was conducted as previously described (Beuchle et al., 2012) and analyzed using FaasX software (Blanchardon et al., 2001). Briefly, male flies were first entrained in 12 h/12 h LD cycles for 4 d, followed by the recording in DD for 8–10 d. The flies with a power >20 and a width >2.5 according to the χ^2 periodogram analysis were defined as rhythmic. The significance threshold was set to 5%. The χ^2 test was used to compare the rhythmicity of the flies, and the Student's *t* test (two-tailed) was used to compare the free-running period.

Immunostaining, microscopy, and quantification. The immunostaining of adult fly brains with anti-PER and anti-PDF antibodies was performed as previously described (Shafer et al., 2002). RNB::VENUS and mCD8::VENUS were immunostained using anti-GFP antibodies (Invitrogen rabbit anti-GFP #6455) in a dilution 1:500. Briefly, the dissected brains were fixed in 4% PFA at room temperature for 15 min, followed by washing three times for 10 min in PBT (PBS, 0.5% Triton X-100). Antibodies were diluted in PBT, and primary antibody staining was performed overnight.

Images were captured using a Leica SP5 confocal microscope. A minimum of 10 brain hemispheres was subjected to quantification using ImageJ software (National Institutes of Health). Anti-PER signal was quantified as previously described (Beuchle et al., 2012). PDF immunoreactivity in the s-LNV cell bodies and axonal termini was quantified from the sum projections of the confocal Z stacks. The region of interest (ROI) for the axonal termini (from the tip of the s-LNV dorsal termini until

Table 2. Free-running locomotor rhythms of the flies expressing RNAs against UNF targets in the LNvs^a

Gene	Rank	VDR	ID	%R	Period ± SEM (h)	Power ± SEM	n
<i>Pdf-GAL4/+</i>	—	—	—	96.8	24.1 ± 0.08	126.1 ± 6.63	31
<i>Pdf > miR-unf</i>	—	—	—	22.6	23.6 ± 0.20	47.9 ± 6.52	31
CG15336	1	102385	KK	96.7	24.5 ± 0.05	133.2 ± 5.86	29
CG17982	1	33035	GD	86.7	24.1 ± 0.05	118.1 ± 5.58	31
CG2262	1	105687	KK	93.5	24.4 ± 0.04	125.1 ± 4.64	31
CG2263	1	33515	GD	81.3	24.8 ± 0.06	122.7 ± 5.29	28
CG33106	2	103411	KK	89.7	24.4 ± 0.04	122.8 ± 4.96	31
CG5695	2	108221	KK	100	24.4 ± 0.04	133.5 ± 4.18	31
CG5706	2	42046	GD	90.3	24.9 ± 0.06	118.4 ± 6.19	28
CG4599	4	107813	KK	100	25.2 ± 0.11	110.9 ± 6.51	31
CG3082	5	102477	KK	90	25.2 ± 0.08	120.4 ± 6.03	32
CG30065	6	102347	KK	93.8	24.1 ± 0.06	123.3 ± 6.19	10
CG4812	6	104534	KK	96.9	24.1 ± 0.05	134.4 ± 3.87	15
CG42321	6	107000	KK	86.7	23.8 ± 0.07	98.3 ± 5.98	30
CG7837	7	23099	GD	96.8	25.6 ± 0.03	139.8 ± 4.88	32
CG7788	7	28065	GD	93.1	24.2 ± 0.10	86.4 ± 5.54	29
CG9325	8	29102	GD	100	23.8 ± 0.05	180.0 ± 2.93	32
CG8036	9	105633	KK	100	25.2 ± 0.06	130.1 ± 6.50	31
CG2679	12	107822	KK	96.9	25.2 ± 0.06	132.3 ± 5.22	32
CG1765	14	37059	GD	100	23.9 ± 0.06	190.0 ± 4.31	31
CG42280	15	8369	GD	96.8	24.3 ± 0.07	115.4 ± 6.33	26
CG11668	16	105073	KK	100	25.0 ± 0.05	135.9 ± 5.00	31
CG7029	17	107658	KK	100	24.6 ± 0.05	152.7 ± 6.51	31
CG9339	20	108736	KK	90.3	24.6 ± 0.08	90.3 ± 6.80	30
CG2650	21	105930	KK	96.9	25.3 ± 0.08	113.9 ± 5.73	31
CG10795	22	110153	KK	90.3	23.6 ± 0.05	102.1 ± 5.04	31
		8833	GD	100	23.9 ± 0.09	119.2 ± 6.30	28
CG14324	26	102574	KK	92	24.7 ± 0.08	120.6 ± 4.98	25
		5897	GD	93.8	23.7 ± 0.06	98.1 ± 4.31	32
CG9755	37	45815	GD	93.8	24.3 ± 0.06	106.6 ± 5.26	32
CG12598	39	105612	KK	96.6	24.7 ± 0.06	107.5 ± 5.57	29
		7764	GD	93.8	23.7 ± 0.06	121.4 ± 7.68	32
CG42666	39	103667	KK	96.8	23.8 ± 0.07	106.7 ± 4.77	31
		38827	GD	100	24.0 ± 0.08	96.0 ± 6.18	31
CG13198	50	104553	KK	93.8	23.5 ± 0.03	104.8 ± 7.46	32
		44080	GD	100	24.2 ± 0.07	121.5 ± 3.79	32
CG8998	50	100629	GD	100	24.0 ± 0.09	119.9 ± 4.87	28
CG9003	50	23482	GD	100	24.0 ± 0.07	110.7 ± 3.99	32
CG8727	66	11765	GD	100	25.0 ± 0.04	185.4 ± 3.71	28
CG15404	80	102242	KK	87.5	24.3 ± 0.07	78.5 ± 5.03	32
		23313	GD	93.8	24.0 ± 0.08	101.1 ± 5.10	32
CG31698	80	110231	GD	93.8	24.1 ± 0.08	119.3 ± 4.86	32
CG2652	83	33542	GD	84.6	24.6 ± 0.09	93.7 ± 7.23	30
CG2655	83	104381	KK	96.7	24.8 ± 0.06	126.8 ± 6.42	30
CG30122	84	106984	KK	96.2	23.6 ± 0.06	107.4 ± 7.41	26
CG33724	84	100994	KK	100	23.7 ± 0.10	91.3 ± 6.06	29
		48814	GD	96.9	24.7 ± 0.05	98.9 ± 3.96	32
CG8817	108	13082	GD	100	23.7 ± 0.05	190.0 ± 2.54	32

^aRank, rank in the UNF ChIP. When the gene is bound to UNF at both ZT2 and ZT14, the higher rank is shown. n, Number of flies; %R, % of rhythmic flies.

where the terminal arbors first branch) was specified with the polygon selection tool, and the intensity sum within each ROI was measured. To quantify s-LNv axonal spread, LNvs projections were visualized by expressing mCD8::Venus in the LNvs. The ROI for the s-LNv axonal termini was determined manually as described above and the area of the ROI was measured.

ChIP-chip and data analysis. *w¹¹¹⁸* flies were entrained in LD for 3 d and collected at ZT2 and ZT14. ChIP using rabbit anti-UNF antibodies (Lin et al., 2009) were performed and hybridized to the GeneChip *Drosophila* Tiling 2.0R Arrays (Affymetrix) as previously described (Menet et al., 2010). Two independent ChIP-chip experiments were performed at both time points. To assess the UNF ChIP signals outside MB, a ChIP-chip assay was performed with the *D52H-Gal4*, *UAS-miR UNF* flies (Aso et al., 2009) harvested at ZT2. ChIP-chip data analysis was performed as previously described (Abruzzi et al., 2011) using the MAT algorithm

(Johnson et al., 2006). The ChIP peaks were visualized using the Integrated Genome Browser.

RNA analysis. Total RNA was isolated from adult fly heads using Trizol (Invitrogen) following the manufacturer's instructions. The RNA was reverse-transcribed using oligo(dT) primers, and the resulting cDNAs were quantified using real-time qPCR as previously described (Nagoshi et al., 2010). The mRNA levels of *Rnb* were normalized to those of *elongation factor 1β* (*Ef1β*).

Cell sorting and RNA sequencing (RNAseq). Manual cell sorting protocol was modified from the previously described method (Nagoshi et al., 2010; Abruzzi et al., 2015). Flies carrying *Pdf-GAL4* and *tubulin-GAL80^{ts}* were combined with *UAS-nls::GFP* to mark s-LNvs and l-LNvs and reared at 18°C. Emerged flies were transferred to 29°C and entrained for 3 d in 12 h light/12 h dark (LD), followed by 3 d in constant darkness (DD). Flies were collected at CT2 and CT14. Cells were dissociated from

Table 3. Free-running locomotor rhythms^a

Temperature	Genotype	Period ± SEM (h)	Power ± SEM	n	%R
25°C	<i>Pdf-GAL4, UAS-dcr2/+</i>	24.5 ± 0.08	101.8 ± 9.42	32	62.5
	<i>Pdf-GAL4, UAS-dcr2/UAS-Rnb RNAi²³⁰⁹⁹</i>	26.6 ± 0.09***	108.9 ± 7.04	29	79.3
	<i>tim-GAL4/+</i>	24.4 ± 0.05	119.2 ± 5.91	26	92.3
	<i>tim-GAL4/+; UAS-Rnb RNAi²³⁰⁹⁹</i>	26.3 ± 0.04***	136.9 ± 4.68	32	100
	<i>tub-GAL4/+</i>	23.7 ± 0.04	156.8 ± 8.4	62	96.7
	<i>tub-GAL4/UAS-Rnb RNAi²³⁰⁹⁹</i>	25.2 ± 0.07***	98.5 ± 8.5	40	80.0*
	<i>Pdf-Gal4/+</i>	24.0 ± 0.06	84.2 ± 5.0	61	95.0
	<i>UAS-Rnb::Venus/+</i>	23.6 ± 0.04	94.3 ± 6.2	57	96.5
	<i>Pdf-GAL4, UAS-Rnb::Venus</i>	24.1 ± 0.07	78.9 ± 5.7	61	85.3
	<i>UAS-Rnb RNAi²²⁵⁷³/+</i>	23.9 ± 0.06	122.4 ± 9.8	63	87.3
	<i>Pdf-GAL4/UAS-Rnb RNAi²²⁵⁷³</i>	24.5 ± 0.02****	174.9 ± 4.9	122	96.7
	<i>w¹¹¹⁸</i>	23.7 ± 0.17	111.5 ± 7.20	63	93.6
	<i>Rnb^{f04616}/+</i>	24.0 ± 0.21	59.9 ± 3.11	64	70.3***
	<i>Rnb^{f04616}</i>	25.2 ± 0.76	18.8 ± 2.25	89	14.6***
	<i>Rnb^{f04616}/Df</i>	23.5 ± 0.21	80.5 ± 36.99	16	18.8***
	<i>Rnb^{f05400}/+</i>	23.8 ± 0.9	60.2 ± 2.59	64	67.2***
	<i>Rnb^{f05400}</i>	25.8 ± 0.76*	22.6 ± 3.17	90	17.8***
	<i>Rnb^{f05400}/Df</i>	23.2 ± 0.2	64.5 ± 10.53	63	23.8***
	<i>Rnb^{f04616}/Rnb^{f05400}</i>	23.3 ± 0.1	111.8 ± 23.9	31	35.5***
	<i>Df/+</i>	23.4 ± 0.1	74.9 ± 12.20	10	50.0***
	<i>w¹¹¹⁸</i>	23.7 ± 0.08	147.1 ± 9.69	32	84
	<i>Pdf-GAL4/+; tub-GAL80ts/+</i>	24.3 ± 0.06	175.7 ± 6.40	62	95.2
	<i>gal1118/tub-GAL80ts</i>	23.5 ± 0.04	134.0 ± 6.89	58	89.7
	<i>UAS-Rnb RNAi²³⁰⁹⁹/Pdf-GAL4; tub-GAL80ts/+</i>	26.0 ± 0.07***	109.8 ± 6.90	31	100
	<i>UAS-Rnb RNAi²³⁰⁹⁹/+; gal1118/tub-GAL80ts/+</i>	25.4 ± 0.19***	87.3 ± 7.46	28	85.7
	<i>UAS-miR-unf/+; gal1118/tub-GAL80ts</i>	25.4 ± 0.15***	127.5 ± 5.70	87	97.7
	<i>UAS-miR-unf/UAS-Rnb RNAi²³⁰⁹⁹; gal1118/tub-GAL80ts</i>	26.1 ± 0.11***	118.6 ± 5.15	61	93.4
18°C → 29°C	<i>Pdf-GAL4/+; tub-GAL80ts/+</i>	24.1 ± 0.06	94.1 ± 6.93	62	80.6
Adult-only	<i>UAS-Rnb RNAi²³⁰⁹⁹/Pdf-GAL4; tub-GAL80ts/+</i>	24.4 ± 0.08**	93.3 ± 7.33	31	93.5
29°C → 18°C					
Developmental					

^an, Number of flies; %R, % of rhythmic flies. Rhythmicity was compared with the driver control or with control genotypes (*w¹¹¹⁸* for *Rnb* mutants) by a χ^2 test.

Periods were compared with the controls (Student's *t* test): **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

~70 brains as previously described (Nagoshi et al., 2010), except brains were treated with papain for 25 min. After trituration in 300 μ l SM^{active} medium containing 50 μ M AP5, 20 μ M DNQX, 0.1 μ M TTX, the cell suspension was filtered through a sterile 70 μ m membrane and distributed to 3 wells of 3 two-well chamber glass slides (LabTek II, Nunc) kept on ice. The second well of each slide contained ice to cool the cells while we sorted GFP-positive cells on a fluorescence microscope equipped with a motorized stage and a needle holder (CellSorter Company for Innovations). Cells were picked manually using capillaries with 50 μ m tip diameter. Collected cells were released in a clean part of the chamber and immediately picked up again, greatly reducing the amount of debris, before cells were transferred to a fresh slide in 100 μ l medium spread in a 1 × 1 cm GeneFrame (ThermoFisher Scientific). Cells were reselected for 2 rounds and finally transferred using a new capillary with 30 μ m tip diameter to 100 μ l lysis buffer (Absolutely RNA Nanoprep Kit; Agilent). Cells of multiple collections were pooled if needed to obtain ~200 cells per sample, and total RNA was extracted according to manufacturer's instructions. RNA was converted to cDNA and amplified using the SMART-Seq4 Kit (Takara Bio). Libraries were generated using NexteraXT (Illumina) and sequenced on an Illumina HiSeq 2500 or 4000.

Results

Identification of UNF direct targets

We identified UNF direct targets by Chromatin-IP tiling array (ChIP-chip) using anti-UNF antibodies (Lin et al., 2009). Because UNF levels cycle in the s-LNVs with a peak around ZT2 (2 h after light-on in LD cycles) (Beuchle et al., 2012), ChIP assays were performed using adult heads harvested at ZT2 or ZT14 in LD. Fifty-five significant binding sites above a stringent *p* < 10^{−7} cutoff were identified. Nine UNF binding sites were found within 1 kb of the previously identified CLK-binding peaks (Abruzzi et al., 2011), suggesting that UNF regulates the transcription of

some CLK/CYC target genes (Fig. 1A). More UNF binding peaks were identified at ZT14 than at ZT2, which is the opposite trend to the UNF accumulation in the LNVs. This finding suggests that UNF is more transcriptionally active and has a higher turnover rate at ZT14 than at ZT2, consistent with the previous UNF ChIP results in S2 cells (Jaumouillé et al., 2015) (Table 1).

UNF is expressed in the 9 PDF-positive LNVs and in the mushroom body (MB), which consists of ~2000 neurons per hemisphere (Lin et al., 2009; Sung et al., 2009). No significant binding peaks (>5% false discovery rate, *p* < 10^{−7}) were identified in the flies with MB-specific *unf* knockdown with *D52H-Gal4* (Aso et al., 2009). Therefore, not surprisingly, the ChIP data largely reflect the UNF targets in the MB. Some of the targets may be shared between MB neurons and LNVs, but the LNVs-specific sites were undetectable in this assay. This explains why no UNF-binding peaks above background levels were detected in the *per* gene (Jaumouillé et al., 2015).

CG7837 (*Rnb*) is a target of UNF and controls behavioral rhythms in the s-LNV master pacemaker neurons

To examine whether any of the UNF direct target genes is involved in the clockwork or output mechanism of the LNVs, we next performed a behavior screen for locomotor activity, in which candidate genes were constitutively knocked down in the LNVs with *Pdf-GAL4*. We selected 39 UNF target genes for screening, considering the rank and the expression levels of their transcripts in the s-LNVs (Kula-Eversole et al., 2010). For each candidate gene, one or two *UAS-RNAi* lines from the VDRC libraries were used, avoiding those with off-target sites. Fly loco-

Figure 2. *Rnb* is required in adult LNvs for the generation of high-amplitude molecular rhythms and 24 h locomotor rhythms. **A, B**, *Rnb* was conditionally knocked down in the LNvs by expressing *UAS-Rnb RNAi* with *Pdf-GAL4, tubulin-GAL80^{ts}*, and shifting the temperature between 18°C and 29°C. Top panels, Group activities in LD, in which the black and white columns represent the activities in the night and day, respectively. Bottom panels, Double-plotted actograms show the average locomotor activities of groups of flies in LD (white background) (Figure legend continues.)

motor behavior was assayed in standard 12 h/12 h LD cycles followed by DD at 25°C.

None of the knockdowns significantly reduced the free-running rhythm strength; however, several knockdowns altered the period length (Table 2; Fig. 1B). We focused subsequent studies on the *CG7837* gene, as its knockdown resulted in the longest period among the genes tested (25.6 ± 0.03 h with VDRC RNAi line #23099). Coexpression of *dicer 2* (*dcr2*) with *CG7837* RNAi to increase RNAi efficacy further lengthened the period by ~ 1 h (26.6 ± 0.09 h). *CG7837* knockdown in pan-clock neurons by *tim-GAL4* also resulted in long-period rhythms. In addition, knockdown of *CG7837* in the LNVs with a second RNAi line (VDRC #22573) also lengthened the period (24.5 ± 0.03 h) (Table 3). We used VDRC #23099 line for subsequent studies as it knocks down *CG7837* more efficiently than the line #22573 (Fig. 3A).

Furthermore, *CG7837* mRNA levels were significantly reduced in the heads of the trans-heterozygous combination of two *unf* hypomorphic mutants, *unf^{z1/20001}* (Bates et al., 2010) (Fig. 1C), indicating that UNF positively regulates *CG7837* gene expression. RNAseq of isolated LNVs verified that both *unf* and *CG7837* are expressed in the LNVs (Fig. 1D). Together with the fact that period lengthening by LNV-targeted *CG7837* knockdown is similar to the phenotype of UNF adult-specific knockdown in the LNVs (Beuchle et al., 2012), these results suggest that *CG7837* acts downstream of UNF in the LNVs and contributes to the regulation of circadian behavior.

CG7837 encodes a 975-amino acid long protein of unknown function containing N-terminal Armadillo (ARM)-repeats and a C-terminal BTB/POZ domain. *CG7837* mRNA is broadly and nonrhythmically expressed in the brain (Kula-Eversole et al., 2010; Nagoshi et al., 2010). Because of its role in the rhythms and the structure containing Armadillo and BTB, we named this gene *R* and *B* (*Rnb*).

Rnb acts downstream of UNF in adulthood in the s-LNVs

UNF is required for the functional development of the s-LNVs and for the functioning of adult s-LNVs as the master circadian pacemaker (Beuchle et al., 2012). To determine whether *Rnb* acts downstream of UNF during development or in adulthood, we conditionally knocked down *Rnb* in the LNVs using a combination of the temperature-dependent repressor of GAL4 (*tubulin-Gal80^{ts}*) and an LNV-specific GAL4 driver (*Pdf-GAL4* or *gal1118-GAL4*) (McGuire et al., 2004). *Rnb* RNAi expression was silenced when flies were maintained at the permissive temperature of GAL80^{ts} (18°C) and induced at the GAL80^{ts} restrictive temperature (29°C). Unlike the *unf* developmental knockdown, *Rnb* knockdown during development only mildly lengthened free-running behavioral period of adult flies without affecting the rhythm strength (Table 3; Fig. 2A). In contrast, the adult-restricted knockdown of *Rnb* in the LNVs lengthened the free-running pe-

riod by ~ 2 h (Table 3; Fig. 2B). LD behavior was not affected by either of the *Rnb* knockdowns (Fig. 2A, B).

PER rhythms in the s-LNVs were delayed in DD by the LNV-targeted adult-specific knockdown of *Rnb* (Fig. 2C, D). The rhythms in the DN1s were also impaired non-cell-autonomously, consistent with previous findings that s-LNVs control DN1 rhythms (Nitabach et al., 2006; Zhang et al., 2010a; Beuchle et al., 2012; Jaumouillé et al., 2015) (Fig. 2D). These cellular and behavioral phenotypes associated with the *Rnb* adult-restricted knockdown in the LNVs are reminiscent of those caused by the *unf* adult-specific knockdown (Beuchle et al., 2012).

Double knockdown of *Rnb* and *unf* in adult LNVs further lengthened the long-period rhythms caused by single knockdowns (*Rnb* single knockdown vs double knockdown with *gal1118*, $p < 0.05$; *unf* single vs double knockdown with *gal1118*, $p < 0.001$, *t* test) (Table 3). This additive effect likely reflects the combination of partial knockdown (Fig. 3A) and the fact that UNF has more functions than controlling *Rnb* expression, as evident from the ChIP results (Table 1). Together, these results indicate that *Rnb* mediates at least part of the role of UNF in adult s-LNVs in the generation of 24 h molecular rhythms and circadian behavioral rhythms.

Rnb is required for the generation of molecular rhythms in the s-LNVs

To learn more about the role of *Rnb* in circadian behavioral control, we assayed the behavior of two *Rnb* mutants, *Rnb^{f04616}* and *Rnb^{f05400}*. Both alleles were caused by a transposon insertion and severely hypomorphic (Fig. 3A). Heterozygous and homozygous mutants of both alleles, and the trans-heterozygotes and hemizygous flies showed significantly reduced free-running locomotor rhythmicity and reduced morning anticipation (Table 3; Fig. 3B). These results confirmed that *Rnb* is required for normal circadian locomotor behavior. *Rnb* mRNA levels in heterozygous flies were $< 50\%$ of the wild-type levels (Fig. 3A), which is consistent with the reduced locomotor rhythmicity of the heterozygotes and hemizygotes and indicates that *Rnb* is a haploinsufficient gene. The ubiquitous knockdown of *Rnb* with *UAS-Rnb* RNAi²³⁰⁹⁹ driven by *tubulin-GAL4*, which reduces *Rnb* mRNA levels in fly heads by $\sim 65\%$ (Fig. 3A), lengthened the period and reduced behavioral rhythmicity (Table 3). Because *Rnb* knockdown in all clock neurons by a pan-clock neuron driver *tim-GAL4* also lengthened the period but did not affect the rhythm strength (Table 3), *Rnb* expressed in nonclock cells likely contributes to reinforcing behavioral rhythm strength.

We next examined whether the *Rnb* mutation affects the functioning of clock neurons by immunostaining PER and PDF throughout the subjective day. PER levels were markedly reduced during the peak time of PER accumulation (CT22–CT2), and the rhythms were dampened in the s-LNVs of *Rnb* homozygous mutants on DD3. In the LNDs and DN1s, PER oscillations were phase-advanced in *Rnb* mutants, suggesting a slight shortening of free-running rhythms in these cells (Fig. 3C). Considering that mutations more strongly reduce *Rnb* mRNA levels than RNAi (Fig. 3A), the cellular phenotypes in the s-LNVs were comparable between RNAi and mutations and confirm that the loss of *Rnb* expression disrupts the molecular clockwork in the s-LNVs. The rhythms of the LND and DN1s were differently affected between *Rnb* mutations and RNAi (Figs. 2D, 3C). This effect likely reflects the fact that s-LNV clocks were more severely disrupted by *Rnb* mutations than by RNAi. Consequently, the communication between the s-LNVs and other clock neurons was more profoundly

←

(Figure legend continued.) and DD (gray background). **A**, Locomotor behavior following *Rnb* knockdown during development. **B**, Locomotor behavior of the flies with adult-restricted *Rnb* knockdown. **C**, **D**, *Rnb* was knocked down in the LNVs only during adulthood, and the PER levels in the clock neurons were analyzed on DD3. **C**, Representative confocal z-stack images of the s-LNVs stained for PER (green) and PDF (magenta). Scale bar, 10 μ m. **D**, Quantification of the PER staining intensity. PER rhythms are delayed in the s-LNVs of the *Rnb* knocked down flies compared with control flies (*Pdf-GAL4*, *tub-GAL80^{ts}*), and the rhythms in DN1s are impaired. Significant differences in PER levels between the two genotypes at several time points: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t* test). CT, Circadian time (i.e., subjective time in constant darkness).

Figure 3. *Rnb* mutation impairs s-LNV clocks and circadian locomotor behavior. **A**, Left, *Rnb* gene, mutants, and the *Rnb* RNAi target site. Right, *Rnb* mRNA levels at ZT14 in the heads of *Rnb* mutants, knockdowns (*tubulin-GAL4* driving *UAS-Rnb RNAi* #23099 or #22573), and controls relative to those of *w*¹¹¹⁸ were analyzed using qPCR. **p* < 0.05, ***p* < 0.01 (Student's *t* test). **B**, Cartesian plots for group average activities of *w*¹¹¹⁸ and *Rnb* mutant flies in LD and DD. **C**, PER levels of *w*¹¹¹⁸ and *Rnb* mutant flies on DD3. PER rhythms were advanced ~4 h in both *Rnb*^{f04616} (magenta) and *Rnb*^{f05400} (blue) mutants in all three cell types, with a profound reduction in rhythm amplitude in the s-LNVs. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (Student's *t* test).

Figure 4. *Rnb* is required for the output rhythms of the s-LNvs. **A**, Left, Representative images of anti-PDF signals in the s-LNv dorsal termini in *Rnb* mutants and *w¹¹¹⁸* on DD3. Right, Quantification of PDF levels on DD3 in the s-LNv axon termini. The rhythms of PDF signals in the axon termini of *Rnb* mutants are dampened compared with those in *w¹¹¹⁸*. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t* test). **B**, **C**, s-LNv axonal arborization in *w¹¹¹⁸* and *Rnb* mutants was analyzed by expressing *UAS-mCD8::Venus* with *Pdf-GAL4* and staining with anti-GFP antibodies at two time points in LD. **B**, Representative confocal z-stack images with the ROIs for quantification of dorsal terminal spread. **C**, Mean area covered by the axonal arborization $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ (Student's *t* test). **D**, Quantification of s-LNv axonal arborization in the flies with adult-restricted LNV-targeted *Rnb* knockdown analyzed at six time points on DD3. ** $p < 0.01$, **** $p < 0.0001$. **E**, Expression of *GFP* or *Rnb::Venus* was driven by *Pdf-GAL4* in wild-type flies and analyzed by anti-GFP/PDF staining at ZT2. Images were captured (Figure legend continues.)

To gain insight into the mechanisms by which *Rnb* contributes to the functioning of the s-LNVs, we sought to examine the subcellular localization of RNB protein. To this end, we drove the expression of *Rnb::Venus* fusion gene in the LNVs by *Pdf-GAL4*. These flies showed normal locomotor rhythms (Table 1). Whereas UAS-GFP driven by *Pdf-GAL4* was present throughout the cell body and projections of the s-LNVs, by using the same

microscopy setting, the RNB::VENUS signal was detected almost exclusively in the cell body with enrichment in the nucleus (Fig. 4E). RNB has a theoretical molecular weight of 110.7 kDa; and, particularly as a fusion with VENUS (~27 kDa), it is unlikely to passively diffuse into the nucleus (Wang and Brattain, 2007). Although overexpression of tagged protein may not fully recapitulate the subcellular localization of endogenous protein, these results at least suggest that RNB can be actively transported into the nucleus to execute its function in the molecular clock.

Recent studies have shown the existence of the clock regulatory machineries that distinguish s-LNVs master pacemaker neurons from other clock neuron subpopulations (Lee et al., 2016; Top et al., 2016). In this study, to gain mechanistic insight into the differential regulation of the molecular clock, we analyzed the downstream pathway of UNF, an s-LNV-specific molecular clock regulator (Beuchle et al., 2012; Jaumouillé et al., 2015). We identified *Rnb* as a direct target of UNF and demonstrated that *Rnb* acts downstream of UNF in the s-LNVs to control molecular clockwork and behavioral output. These results establish that UNF interacts with the core TTFL in the s-LNVs via at least two pathways: regulation of *per* transcription (Jaumouillé et al., 2015) and the upregulation of *Rnb* expression (Fig. 5). Together, these results corroborate the emerging view that molecular clocks are regulated differently within the pacemaker circuit. This is accomplished by the cell-type specific genetic and biochemical environments, underpinning the functional differences among the pacemaker neuron subgroups.

UNF ChIP-chip experiments identified 55 high-confidence binding sites, of which 9 sites were cotargets of CLK (Table 1). CLK is expressed in all clock-containing cells, whereas UNF expression is restricted to the LNVs; thus, these sites may not be cobound by CLK and UNF within the LNVs. Notably, one of the

(Figure legend continued.) using the same microscopy setting for both genotypes. Representative images of the s-LNVs (top) and high-magnification views of the s-LNV cell bodies (bottom) stained for GFP or VENUS (green) and PDF (magenta).

CLK/UNF cotargets, *Ecdysone receptor* (*EcR*) is an upstream regulator of the nuclear receptor E75 (Gauhar et al., 2009). Because E75 and UNF coregulate *per* transcription in the s-LNvs to reinforce TTFL (Jaumouillé et al., 2015), coregulation of *EcR* expression by CLK and UNF would add an additional feedback loop in the s-LNvs clockwork. However, the knockdown of the *EcR* (*CG1765*) gene by *Pdf-GAL4* did not alter behavioral rhythms, and this possibility remains an open question. Increasing RNAi efficacy by coexpressing *dcr2* and *EcR* gain-of-function experiments would provide a more clear-cut answer to this question.

Rnb loss-of-function mutations dampen the molecular rhythms, reduce PDF levels, blunt PDF accumulation rhythms, and perturb rhythms in axonal remodeling in the s-LNvs. Concomitantly, *Rnb* mutants have reduced morning anticipation in LD and are arrhythmic in DD (Figs. 3, 4). Adult-specific LNv-targeted *Rnb* knockdown disrupts s-LNv molecular clocks, dampens s-LNv axonal remodeling rhythms, and lengthens free-running rhythms (Figs. 2, 4D). It is noteworthy that PDF levels at the s-LNv dorsal terminal appeared moderately reduced by *unf* knockdown in the LNvs; however, the reduction was statistically nonsignificant (data not shown). This result probably reflects insufficient decrease of *Rnb* level but is nevertheless consistent with UNF being an upstream regulator of *Rnb*. In addition, although *Rnb* knockdown in developing or adult LNvs did not impair morning anticipatory behavior (Fig. 2A,B), probably also due to insufficient knockdown (Fig. 3A), these results together indicate that *Rnb* expression within the s-LNvs during adulthood is required for the pacemaking role of the s-LNvs.

RNB contains an ARM-repeat and a BTB/POZ domain. The BTB/POZ domain mediates protein–protein interaction and is often found in transcriptional regulators (Collins et al., 2001). Indeed, a recent study using an elegant assay in S2 cells showed that RNB has transcriptional cofactor activity (Stampfel et al., 2015). Nuclear localization of overexpressed RNB::VENUS (Fig. 4E) suggests that RNB can be actively transported to the nucleus and supports the possibility that RNB is involved in the transcriptional control of the molecular clock in the pacemaker neurons.

ARM-repeat proteins are critical members of the Wnt/Wingless signaling pathway, which controls a wide range of developmental and cell biological processes (Willert and Jones, 2006; Bejsovec, 2013). Interestingly, RNB was identified as a potential regulator of the Wnt/Wingless signaling pathway in two prior genetic screens (DasGupta et al., 2005; Gersten et al., 2014). Intriguingly, several members of the Wnt/Wingless pathway are essential regulators of the fly TTFL (Rosato et al., 2006). Therefore, RNB might also function at the interface between the Wnt/Wingless signaling pathway and the molecular clock in the generation of molecular rhythms and output of the s-LNvs (Fig. 5). Our study provides an entry point to future research projects aimed at the detailed molecular dissection of the mechanisms by which RNB affects circadian functions in the s-LNv master pacemaker neurons.

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