



Drosophila CrebB is a Substrate of the Nonsense-Mediated mRNA Decay Pathway that Sustains Circadian Behaviors

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Post-transcriptional regulation underlies the circadian control of gene expression and animal behaviors. However, the role of mRNA surveillance via the nonsense-mediated mRNA decay (NMD) pathway in circadian rhythms remains elusive. Here, we report that *Drosophila* NMD pathway acts in a subset of circadian pacemaker neurons to maintain robust 24 h rhythms of free-running locomotor activity. RNA interference-mediated depletion of key NMD factors in *timeless*-expressing clock cells decreased the amplitude of circadian locomotor behaviors. Transgenic manipulation of the NMD pathway in clock neurons expressing a neuropeptide PIGMENT-DISPERSING FACTOR (PDF) was sufficient to dampen or lengthen free-running locomotor rhythms. Confocal imaging of a transgenic NMD reporter revealed that arrhythmic *Clock* mutants exhibited stronger NMD activity in PDF-expressing neurons than wild-type. We further found that hypomorphic mutations in *Suppressor with morphogenetic effect on genitalia 5* (*Smg5*) or *Smg6* impaired circadian behaviors. These NMD mutants normally developed PDF-expressing clock neurons and displayed daily oscillations in the transcript levels of core clock genes. By contrast, the loss of *Smg5* or *Smg6* function affected the relative transcript levels of *cAMP response element-binding protein B* (*CrebB*) in an isoform-specific manner. Moreover, the overexpression of a transcriptional repressor form of *CrebB* rescued free-running locomotor rhythms in *Smg5*-depleted flies. These data demonstrate that

CrebB is a rate-limiting substrate of the genetic NMD pathway important for the behavioral output of circadian clocks in *Drosophila*.

Keywords: circadian rhythms, *CrebB*, *Drosophila*, nonsense-mediated mRNA decay

INTRODUCTION

Organisms have endogenous circadian clocks that maintain daily rhythmic activities despite environmental alterations. The circadian clock system consists of three main components: an input pathway, a central oscillator, and an output pathway (Agostino et al., 2011). The input pathway transmits external timing information (e.g., light or temperature cycles) to the brain. In the central oscillator, the timing information cues 24 h rhythm generators (e.g., circadian gene expression and/or neural activities) to govern circadian output pathways. The output circuitry is comprised of downstream genes and neurons that relay clock signals to overt behaviors and physiology, such as locomotor activity and metabolism.

The central molecular oscillators in *Drosophila* run in a network of approximately 150 pacemaker neurons, which are organized into several groups (Beckwith et al., 2015;

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Peschel et al., 2011). The cell-autonomous molecular clocks comprise transcription-translation feedback loops (TTFLs) of circadian transcription factors that are conserved among fruit flies and other species. The first TTFL in *Drosophila* is driven by PERIOD (PER) and TIMELESS (TIM) (Konopka et al., 1971; Sehgal et al., 1994), which accumulate in the cytoplasm as mRNA levels of *per* and *tim* increase throughout the day, reaching the maximum level in the early evening. TIM stabilizes PER (Price et al., 1995; Suri et al., 1999; Voss-hall et al., 1994), and the PER-TIM heterodimer complex enters the nucleus at around midnight where it inhibits the CLOCK (CLK) and CYCLE (CYC) complex, the transcriptional activator of *per* and *tim* (Allada et al., 1998; Rutila et al., 1998). This negative autoregulatory feedback is sustained until TIM is degraded by the photoreceptor CRYPTOCHROME (CRY) in the early morning (Emery et al., 1998; Stanewsky et al., 1998). The heterodimeric CLK-CYC complex drives additional TTFLs by binding E-box sequences in genomic loci of several clock genes and promoting their transcription, such as *PAR-domain protein 1* (*Pdp1*), encoding an activator of *Clk* transcription (Cyran et al., 2003); *vri* (*vri*), encoding a repressor of *Clk* transcription (Blau et al., 1999), and *clockwork orange* (*cwo*), encoding a transcriptional repressor that competes with the CLK-CYC complex for E-box binding (Kadener et al., 2007; Lim, Chung, et al., 2007; Matsumoto et al., 2007; Richier et al., 2008; Zhou et al., 2016).

Although the central oscillators running endogenous clocks have been thoroughly studied at the transcription level, the significance of their post-transcriptional control has begun to emerge (Brunner et al., 2006; Kojima et al., 2011; Lim et al., 2013). Nonsense-mediated mRNA decay (NMD) is a post-transcriptional regulatory mechanism via which mRNAs with premature translation termination codons (PTCs) are recognized and removed. The precise mechanisms that initiate the assembly of NMD effectors differ very little among species (Behm-Ansmant et al., 2007; Eulalio et al., 2007; Muhlemann, 2008; Popp et al., 2014). The conventional model involves an exon junction complex (EJC) that is removed by ribosomes in translation of normal transcript. However, PTCs generated from DNA defect, transcription errors, or alternative splicing, cause the ribosomes to stall upstream of EJC. The distance between the ribosomes and EJC triggers the recruitment of a NMD complex, which consists of a number of UP-FRAMESHIFT (UPF) and SUPPRESSOR WITH MORPHOGENETIC EFFECT ON GENITALIA (SMG) proteins. UPF1 is the core RNA helicase that composes the complex together with UPF2 and UPF3 and is phosphorylated by SMG1, a phosphoinositide-3-kinase-related protein kinase. Dephosphorylation of UPF1 is facilitated by SMG5/7 or SMG6 nucleases.

While it has been shown that NMD regulates 5–10% of the transcriptome (Chan et al., 2007; Mendell et al., 2004; Yepiskoposyan et al., 2011), only a few target genes implicated in circadian rhythms have been identified. In *Arabidopsis*, splice variants of the genes encoding *timing of cab expression 1* and *early flowering 3* are degraded via NMD (Kwon et al., 2014). Other clock-regulated glycine-rich RNA-binding proteins, *GRP7* and *CCR1*, form a feedback loop in which unproductive splicing of each transcript is coupled to

NMD (Schoning et al., 2007; 2008). Alternative splice variants of a Jumonji C domain-containing gene, *JMJC5*, in *Medicago truncatula* also show NMD sensitivity, though the strength of the sensitivity varies depending on the tissue and other exogenous conditions (Shen et al., 2016). *Neurospora crassa* has a shortened periodicity as a result of *Upf1* mutation (Morgan et al., 1997), and NMD was recently found to regulate its major clock gene, *frequency*, for the transcriptional control of *white collar* genes (Aronson et al., 1994; Cheng et al., 2001; Mellow et al., 2001; Wu et al., 2017).

There is abundant experimental evidence implicating ATF/CREB (activating transcription factor/cAMP response element-binding protein) and its transcriptional coactivators in the generation of behavioral rhythms through cell-autonomous molecular clocks (Belvin et al., 1999; Ginty et al., 1993; Kim et al., 2016; Koyanagi et al., 2011; Lee et al., 2010; Lim et al., 2007; O'Neill et al., 2008; Obrietan et al., 1999; Scheving et al., 1998; Sun et al., 2015; Travnickova-Bendova et al., 2002). However, few studies have demonstrated the regulatory mechanism of ATF/CREB expression for clock function (Hendricks et al., 2001; Kako et al., 1996; Maurer et al., 2016; Williams et al., 2001). In this study, we provide strong genetic evidence that NMD acts in *Drosophila* circadian pacemaker neurons to sustain circadian locomotor behaviors and identify *CrebB* as a rate-limiting target of the clock-relevant NMD.

MATERIALS AND METHODS

Fly stocks

All flies were reared at 25°C and 60% humidity on a 12 h light/dark (LD) cycle and with standard cornmeal-yeast food. The wild-type controls were *iso31* (Bloomington *Drosophila* Stock Center #5905). *Smg5* RNAi (#31090, JF01554), *Smg5*^{e04233} (#25167), PBac{IT.GAL4}*Smg5*^{-1409-G4} (#65708, *Smg5*-GAL4), *Df(2L)BSC345* (#24369, *Smg5* deficiency), *Df(3R)BSC494* (#24998, *Smg6* deficiency), UAS-*Upf2.A* (#60579), UAS-*Upf2.FVRR* (#60583), UAS-*Upf2.E801R* (#60581), UAS-*CrebB-a* (#9232), and UAS-*CrebB-c* (#7219) were all from the Bloomington *Drosophila* Stock Center. *Smg6*^{RNAi#1} (6369R-1) and *Smg6*^{RNAi#2} (6369R-3) were from Fly Stocks of National Institute of Genetics, Japan. *Smg6*^{2a} and UAS-*nlsDsRed2::SV40-3'UTR* were kindly provided by Dr. Metzstein (Frizzell et al., 2012), and UAS-*Upf1*^{D45B} was provided by Dr. Singh, who received it from the original generator, Dr. Palumbo (Micale et al., 2009). *Pdf*-GeneSwitch-GAL4 was a gift from Dr. Ceriani (Depetris-Chauvin et al., 2011), and UAS-*CrebB-b* was from Dr. Davis (Yu et al., 2006). The UAS-*Smg6*-V5 transgenic line was made by cloning the full cDNA of *Smg6* at the EcoRI and XhoI sites of the 8.5 kb pUAST-*AttB*-V5 vector.

Fly behavioral assay

The food for behavioral testing was 5% sucrose (31365-1201; Junsei Chemical Co.) and 2% agar (24440-1201; Junsei Chemical Co.). *Pdf*-GeneSwitch was activated by feeding the flies food containing 62.5 μM RU486 (H110-01; Sigma-Aldrich) dissolved in ethanol. The vehicle control was ethanol. The circadian rhythms of the flies were analyzed

with the *Drosophila* Activity Monitoring system (TriKinetics) using MATLAB-based ClockLab (Actimetrics). The rhythm peak was calculated from the first to the seventh dark/dark (DD) cycle with a confidence interval of 0.05. The power of rhythmicity was calculated by subtracting the value of “significance” from the value of “power.” Morning and evening anticipation indices were calculated using an Excel-based Counting Macro (Pfeiffenberger et al., 2010), as follows: morning index = total activity 3 h prior to lights on/total activity 6 h prior to lights on – 0.5; evening index = total activity 3 h prior to lights off/total activity 6 h prior to lights off – 0.5.

Immunofluorescence assay

Flies were entrained to the LD cycle for at least 72 h before dissection between Zeitgeber time 2 (ZT2) and ZT3 for PDF staining or DsRed detection. Briefly, a 30 min fixation in 3.7% formaldehyde and washes in phosphate-buffered saline with 0.5% Tween 20 were followed by two nights of primary antibody staining, as described previously (Lim et al., 2011; Park et al., 2014), with mouse anti-PDF (a-PDF C7-s; Developmental Studies Hybridoma Bank) at a dilution of 1:600. The secondary antibodies were either Alexa Fluor 488 (A21202; Invitrogen) or Alexa Fluor 594 (A21203; Invitrogen) donkey anti-mouse IgG. The samples were scanned at a pixel resolution of 1,024 × 1,024 with an LSM780 confocal microscope (Carl Zeiss) using LSM image browser ZEN software (Carl Zeiss).

RNA isolation and quantitative real-time PCR

Flies were entrained to a 12 h LD cycle for at least 72 h before they were harvested at 4 h intervals (at ZT3, ZT7, ZT11, ZT15, ZT19, and ZT23). The heads of 10–30 flies per genotype were fresh frozen at -80°C. RNA was extracted with TRIzol (15596018; Ambion), digested with RQ1 DNase to remove genomic DNA, and purified with phenol-chloroform-isoamyl alcohol (25:24:1, M3803; Sigma-Aldrich). Purified RNA was reverse transcribed with M-MLV reverse transcriptase (M1705; Promega) and random primers (C1181; Promega). Quantitative PCR was performed with the synthesized cDNA using Prime Q-Mastermix (9200; Genetbio). Real-time PCR results were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). Relative mRNA levels were normalized to the peak value of the respective control and then calculated as a percentage of the peak value (set to 100%).

RESULTS

Smg5 depletion in circadian pacemaker neurons dampens circadian locomotor rhythm

To identify post-transcriptional regulators with circadian clock function, we conducted a behavioral screen of transgenic flies harboring RNAi against genes encoding individual components of processing bodies (Decker et al., 2012; Zheng et al., 2011). We carried out a primary genetic screen using a *tim*-GAL4 line that drives the overexpression of each RNAi transgene in all clock-relevant cells expressing *tim* and depletes its target gene expression. Any phenotype in circadian behaviors was then confirmed using a *Pdf*-GAL4 driver

that limits the RNAi expression to PDF-expressing neurons. PDF is a neuropeptide expressed in small and large ventral lateral neurons (s-LNvs and l-LNvs, respectively) of the adult fly brain and is crucial for synchronizing clock phases among different groups of circadian pacemaker neurons (Lin et al., 2004; Peng et al., 2003). After entrainment to LD cycles, wild-type flies display robust 24 h rhythms of their locomotor activity in free-running constant dark (DD) conditions. Abnormalities in circadian rhythms are thus observed as an altered periodicity or rhythm amplitude of DD behaviors compared with that of wild-type flies.

Of the approximately 40 post-transcriptional regulatory genes, the RNAi-mediated depletion of several affected circadian locomotor behaviors (Fig. 1A). In particular, depletion of a NMD-relevant gene, *Smg5*, in either *tim*-expressing cells or PDF-expressing neurons dampened circadian locomotor rhythms, whereas the inhibition of *tim*-GAL4 activity by PDF-specific GAL80 (Stoleru et al., 2004) partially suppressed the *Smg5* RNAi phenotype (Fig. 1B, Supplementary Table S1). We confirmed that *tim*-GAL4-driven expression of *Smg5* RNAi resulted in *Smg5* mRNA levels in adult heads that were ~60% of wild-type levels (data not shown). By contrast, transgenic flies displayed normal rhythmicity when *Smg5* was depleted in other brain regions by *repo*-GAL4 (glial cells), *dilp2* (*Drosophila insulin-like peptide 2*)-GAL4 (*pars intercerebralis*), or *cry13*-GAL4 (*cry*-expressing cells).

Genetic manipulation of the NMD pathway in PDF-expressing neurons alters behavioral rhythms

We next examined if other NMD components similarly influence circadian behaviors. Indeed, transgenic depletion of each NMD factor (e.g., *Upf1*, *Upf2*, *Upf3*, *Smg1*, or *Smg6*) in *tim*-expressing cells caused behavioral arrhythmicity comparable to that observed with *Smg5* depletion (Fig. 1C, Supplementary Table S2). Furthermore, the individual overexpression of these NMD factors (e.g., *Upf1*, *Upf2*, *Smg5*, or *Smg6*) in *tim*-expressing cells disrupted free-running locomotor rhythms (Supplementary Table S3), indicating that a specific dosage of NMD activity is required for clock function. RNA helicase UPF1 is a key component of the NMD machinery, whereas UPF2 and UPF3 act as adaptors/recruiters of the complex. Transgenic overexpression of *Upf2*.E801R, which disrupts UPF2-UPF3 interaction but rescues lethality in the *Upf2* mutant (Avery et al., 2011), induced arrhythmicity in circadian behaviors comparable to the overexpression of wild-type *Upf2*. By contrast, transgenic overexpression of *Upf2*.FVRR, which disrupts UPF1-UPF2 interaction and cannot rescue lethality in the *Upf2* mutant, negligibly affected circadian behaviors. The UPF1-UPF2 interaction might thus be limiting for clock function in *Drosophila*, as it is important for NMD and adult fly viability. Additionally, we observed that the overexpression of UPF1^{D45B}, a dominant-negative form of UPF1 (Micale et al., 2009), in PDF-expressing neurons was necessary and sufficient to lengthen the free-running periods in locomotor rhythms by >1 h (Fig. 1D, Supplementary Table S4). These results document the importance of the NMD pathway in PDF-expressing neurons for sustaining circadian behaviors.

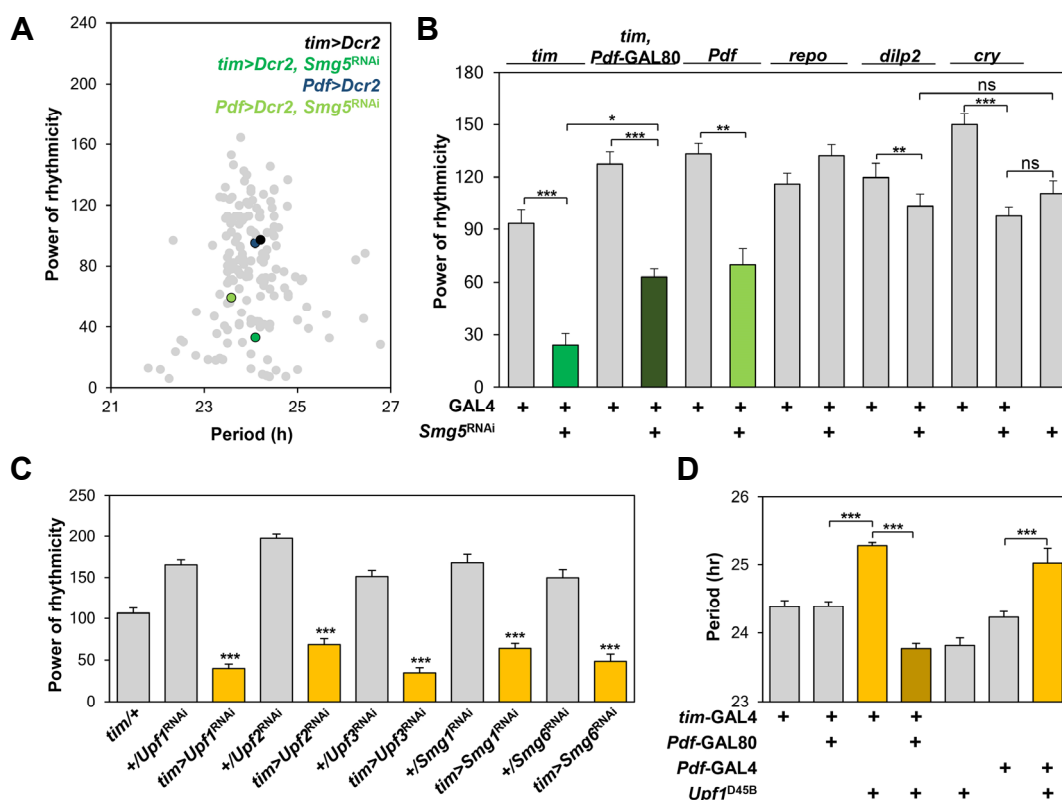


Fig. 1. Genetic suppression of NMD in circadian pacemaker neurons disrupts free-running locomotor rhythms. (A) Results of a genetic screen of transgenic lines harboring RNAi against ~40 post-transcriptional regulators that constitute processing bodies. The RNAi transgenes were individually overexpressed along with the RNAi-enhancing Dicer-2 (*Dcr2*) in all *tim*-expressing clock cells (*tim > Dcr2*) or in PDF-expressing pacemaker neurons (*Pdf > Dcr2*). Circadian periodicity (*x* axis) and the power of rhythmicity (*y* axis) in free-running locomotor activity were calculated in individual flies and averaged for each RNAi line (gray dots). *Smg5^{RNAi}* flies and their controls are indicated by the colored dots. (B) Powers of rhythmicity seen in *Smg5*-depleted flies by different GAL4 drivers (shown at top). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by one-way ANOVA with Tukey's *post hoc* test. Error bars indicate SEMs (*n* = 18-57). (C) Depletion of each NMD factor in *tim*-expressing clock cells significantly decreases behavioral rhythmicity. ****p* < 0.001 versus heterozygous *tim*-GAL4 or RNAi controls by one-way ANOVA. Error bars indicate SEMs (*n* = 20-47). (D) Overexpression of the dominant-negative UPF1^{D45B} transgene in PDF-expressing neurons was necessary and sufficient to lengthen the periodicity of circadian locomotor rhythms. *Pdf*-GAL80 represses the UPF1^{D45B} overexpression in PDF-expressing neurons among other *tim*-expressing cells. ****p* < 0.001 by one-way ANOVA, Tukey's *post hoc* test. Error bars indicate SEMs (*n* = 19-57).

NMD activity in PDF-expressing neurons does not affect neural development but is elevated by *Clock* mutation

To validate that PDF-expressing neurons express NMD-relevant genes and display NMD activity, we performed imaging analyses using confocal microscopy. We first employed an enhancer-trapping line for the *Smg5* locus (PBac[IT.GAL4]*Smg5^{1409-G4}*) and visualized its enhancer activity in the adult fly brain by driving the expression of a fluorescent reporter protein. In this way, we indirectly detected *Smg5* expression in PDF-expressing s-LNvs and l-LNvs as well as in cells over a broad region of the fly brain (Fig. 2A). To further assess NMD activity in PDF-expressing neurons, we utilized a transgenic RNA reporter harboring the SV40 3' untranslated region (UTR)(UAS-*nlsDsRed*::SV40-3'UTR) (Metzstein et al., 2006). The SV40 3' UTR is a known target of NMD activity; therefore, the relative expression levels of the DsRed reporter protein are inversely proportional to endog-

enous NMD activity (Metzstein et al., 2006). We established transgenic flies expressing the NMD reporter in PDF-expressing neurons and compared their levels of DsRed expression among different genetic backgrounds. As expected, confocal imaging revealed that DsRed signals were higher in weak hypomorphic mutants of *Upf2* (*Upf2^{2-8A}*) than in the wild type, validating the functionality and sensitivity of the NMD reporter in PDF-expressing neurons (Fig. 2C). Importantly, we found that the NMD reporter protein was expressed at very low levels in both s-LNvs and l-LNvs of arrhythmic *Clk^{Jrk}* mutants (Fig. 2D). These data suggest that NMD activity in PDF-expressing neurons is suppressed by wild-type *Clk*. Since the *Clk^{Jrk}* allele generates *Clk* transcripts harboring NMD-inducible PTCs, we cannot rule out the other possibility that the *Clk^{Jrk}* mutation potentiates the overall activity of the NMD pathway to promote the decay of its own transcripts.

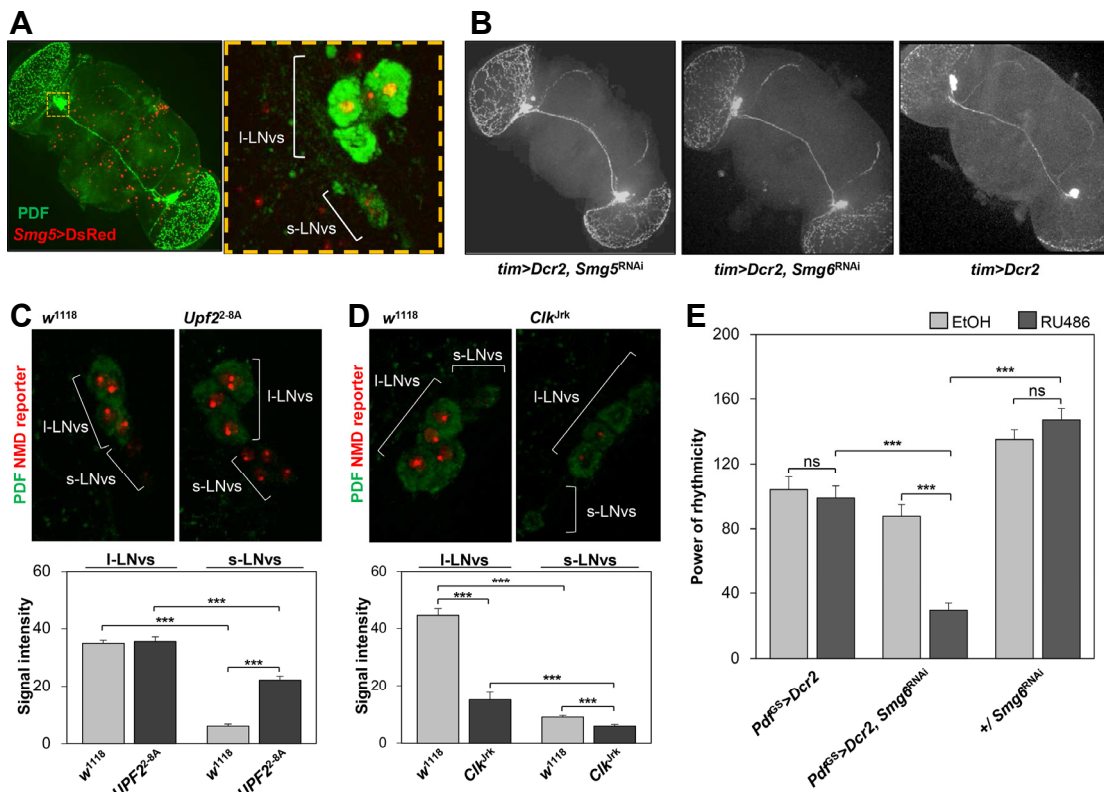


Fig. 2. NMD activity in PDF-expressing neurons does not affect their neural development but is elevated by *Clock* mutation. (A) Expression of DsRed by an enhancer-trapping transgene from an *Smg5* locus in the brain of an adult fly (*Smg5* > DsRed); both I- and s-LNvs were immunostained with an anti-PDF antibody (green). Yellow dashed box in the left panel indicates the region shown at higher magnification on the right. (B) Representative confocal images of whole-mount brains stained with the anti-PDF antibody. All posterior optic tracts from I-LNvs and dorsal projections from s-LNvs in transgenic flies depleted of *Smg5* or *Smg6* are intact compared with those in control flies (*tim* > *Dcr2*). (C, D) Representative images (top) and quantitation (bottom) showing that the *Pdf* > *nlsDsRed::SV40-3'UTR* signal is increased in s-LNvs of *Upf22-8A* flies and decreased in both I- and s-LNvs of *Clk^{Irk}* flies compared with that in the wild type *w¹¹¹⁸*. ****p* < 0.001 by Student's *t* tests. Error bars indicate SEMs (*n* > 16). (E) Conditional depletion of *Smg6* from PDF-expressing neurons dampened DD locomotor rhythms. EtOH (ethanol) indicates a vehicle control for the oral administration of 62.5 μ M RU486 during the behavioral tests. ****p* < 0.001 by one-way ANOVA, Bonferroni's *post hoc* test. Error bars indicate SEMs (*n* = 10-57).

Developmental defects in PDF-expressing neurons and their projections could be responsible for the abnormal circadian behaviors observed in some clock mutants. Although the *Smg5* or *Smg6* RNAi flies showed behavioral arrhythmicity, the cell bodies and axonal projections of their PDF-expressing neurons were intact (Fig. 2B). To further exclude developmental effects of NMD on circadian behaviors, we conditionally depleted *Smg6* expression in PDF neurons by *Pdf^{GeneSwitch}-Gal4* (Depetris-Chauvin et al., 2011). In this experimental condition, *Smg6* RNAi expression was suppressed during development but oral administration of RU486 to adult flies induced GeneSwitch-Gal4 activity and the RNAi expression. The adult-specific depletion of *Smg6* was sufficient to dampen the rhythmicity in circadian locomotor behaviors, consistent with the behavioral phenotypes caused by constitutive depletion (Fig. 2E, Supplementary Table S5). Collectively, these data suggest that the effects of the NMD pathway are not developmental but they maintain behavioral circadian rhythms in adult flies.

Hypomorphic mutations in *Smg5* or *Smg6* weaken morning anticipation and free-running locomotor rhythms

To elucidate the molecular mechanisms underlying NMD effects on circadian behaviors, we established mutant flies hypomorphic for either of the two NMD factors and analyzed their behavioral circadian phenotypes. The *Smg5^{e04233}* mutant allele harbors a piggyBac insertion in the 5' UTR of the *Smg5* locus (Fig. 3A) and reduces *Smg5* mRNA levels to 57% of the wild-type (data not shown). The *Smg6^{2a}* mutant allele possesses an ethyl methanesulfonate-induced missense mutation in the PiT N-terminal (PIN) domain (Fig. 3E) (Frizzell et al., 2012). This catalytic domain for nuclease activity is conserved between SMG5 and SMG6 proteins, implicating both factors in mRNA degradation at the last step of NMD pathway.

During the LD cycle, wild-type flies gradually increase their locomotor activity prior to the light transitions as a part of clock-dependent anticipatory activities to lights-on (i.e., morning anticipation) or lights-off (i.e., evening anticipation).

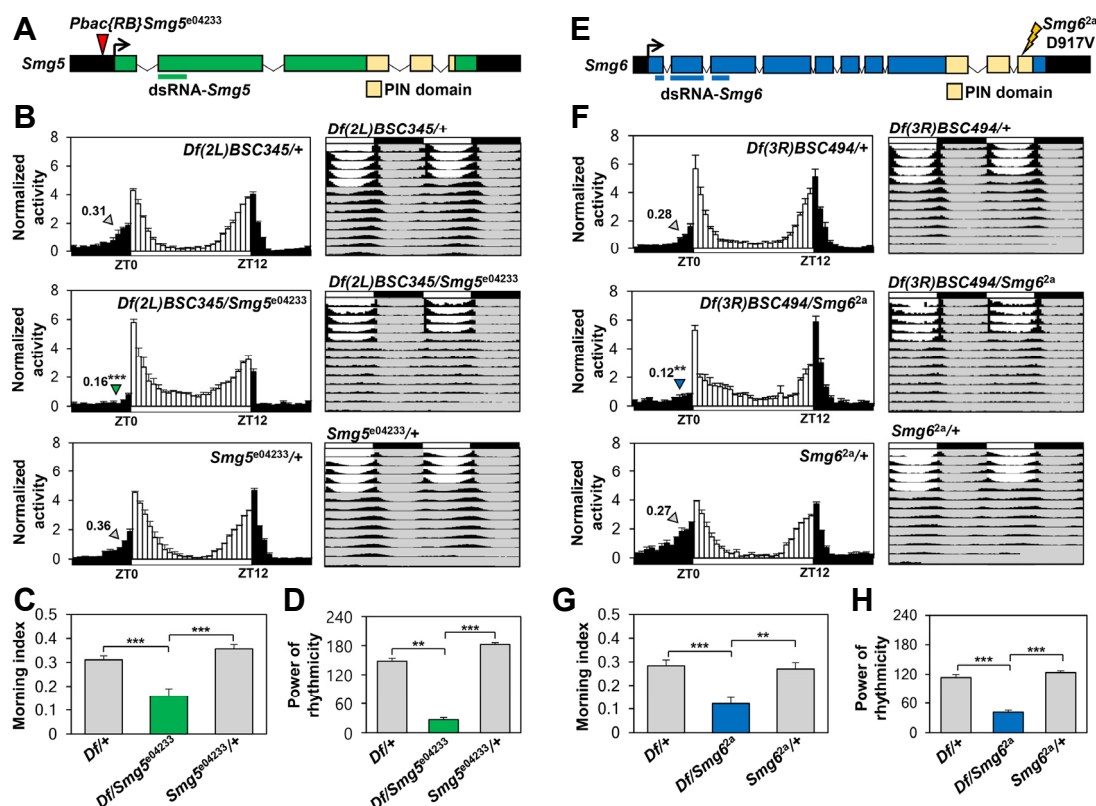


Fig. 3. Hypomorphic mutations in *Smg5* or *Smg6* dampen morning anticipation and free-running locomotor rhythms. (A) Schematic diagram of the *Smg5* locus. Green, yellow, and black boxes correspond to coding regions, the PIN domain, and untranslated regions of *Smg5* transcript, respectively. The green line indicates the coding region targeted by *Smg5* RNAi; the red arrowhead depicts the piggy-Bac insertion site in the 5' UTR of the *Pbac{RB}Smg5^{e04233}* allele. (B, F) Normalized activity profiles from three LD cycles were averaged per genotype. Averaged morning index values are shown above arrowheads. Error bars indicate SEMs ($n = 51-159$ for *Smg5* mutants; $n = 45-156$ for *Smg6* mutants). $**p < 0.01$, $***p < 0.001$ compared with either heterozygous control by one-way ANOVA with Tukey's *post hoc* test. White/black bars, LD cycles. Averaged actograms throughout the behavioral assessments were double plotted ($n = 37-58$ for *Smg5* mutants; $n = 17-50$ for *Smg6* mutants that remained alive after five LD and seven DD cycles). (C, G) Trans-heterozygous mutations in *Smg5* or *Smg6* decreased activity anticipatory to lights-on in LD cycles to lights-on in LD cycles. $**p < 0.01$, $***p < 0.001$ compared with either heterozygous control by one-way ANOVA with Tukey's *post hoc* test. Error bars indicate SEMs ($n = 51-159$ for *Smg5* mutants; $n = 45-156$ for *Smg6* mutants). (D, H) Trans-heterozygous mutations in *Smg5* or *Smg6* decreased the power of rhythmicity in DD locomotor behaviors. $**p < 0.01$, $***p < 0.001$ by one-way ANOVA with Tukey's *post hoc* test. Error bars indicate SEMs ($n = 37-58$ for *Smg5* mutants; $n = 17-50$ for *Smg6* mutants). (E) Schematic diagram of the *Smg6* locus. Blue, yellow, and black boxes correspond to coding regions, the PIN domain with nuclease activity, and untranslated regions of *Smg6* transcript, respectively. Blue lines indicate coding regions targeted by *Smg6* RNAi. *Smg6^{2a}* allele has an aspartate-to-valine missense mutation in the indicated residue of the PIN domain.

By contrast, *Smg5* mutants trans-heterozygous for *Smg5^{e04233}* over the chromosomal deficiency of the entire *Smg5* locus (i.e., *Df(2L)BSC345*) showed significantly weaker morning anticipation than either of the heterozygous controls (Figs. 3B and 3C). *Smg5* mutants also exhibited less robust DD locomotor rhythms than their heterozygous controls (Figs. 3B and 3D, Supplementary Table S6). Dampened morning anticipation in LD cycles and low-amplitude rhythms in DD locomotor behaviors were similarly observed in mutants trans-heterozygous for *Smg6^{2a}* and chromosomal deficiency of the *Smg6* locus (i.e., *Df(3R)BSC494*) (Figs. 3F-3H, Supplementary Table S7). These behavioral phenotypes in NMD mutants are thus consistent with their RNAi phenotypes.

Daily oscillations in levels of clock-relevant mRNAs are comparable between *Smg6* mutants and heterozygous controls

PDF-expressing s-LNvs are a well-established neural locus important for morning anticipation and DD locomotor rhythms (Grima et al., 2004; Stoleru et al., 2004). However, we did not detect any gross defects in the cell bodies or axonal projections of these neurons in either *Smg5* or *Smg6* mutants (Fig. 4A). We reasoned that aberrant NMD of a specific mRNA substrate, but not neuronal defects, might underlie the observed circadian phenotypes. Such targets are expected to have elevated mRNA levels in NMD mutants due to the inefficient degradation of their transcripts, as has

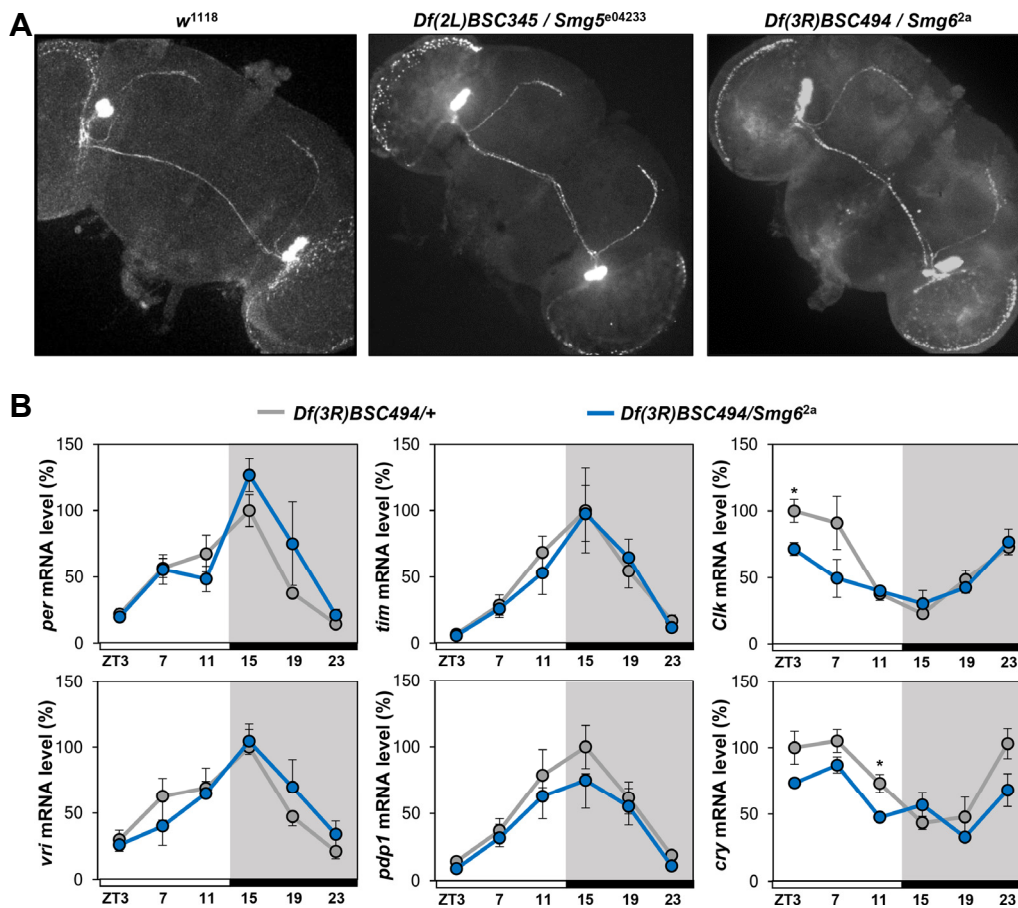


Fig. 4. PDF-expressing neuron development and daily oscillations in clock-relevant mRNA levels are comparable between *Smg6* mutants and heterozygous control. (A) Representative confocal images of whole-mount brains stained with anti-PDF antibody. All posterior optic tracts from l-LNvs and dorsal projections from s-LNvs appear intact. (B) Quantitative analyses of daily rhythmic expression of clock-relevant transcripts in *Smg6* mutants. Adult flies were collected at the indicated time points in LD cycles (lights on at Zeitgeber time zero [ZT0]; lights off at ZT12). Total RNAs were purified from head extracts, and the level of each clock-relevant transcript relative to that of *cyc* was quantified by real-time PCR. The *y* axis indicates the percent expression relative to the peak value in the heterozygous control (set as 100%). Data represent averages \pm SEMs ($n = 3$). $*p < 0.05$ by Student's *t* test. White/black bars, LD cycles.

been observed for other NMD targets (Chapin et al., 2014; Johansson et al., 2007). Since it is technically difficult to quantify transcript levels in PDF-expressing neurons, we examined the mRNA levels of clock-relevant genes in *Smg6* mutant heads. This analysis proved no significant differences between *Smg6* mutants and heterozygous controls in the circadian expression of core clock transcripts (e.g., *per*, *tim*, *vri*, and *Pdp1*) (Fig. 4B). *Smg6* mutation rather reduced *Clk* or *cry* expression only at a specific time of the day in LD cycles.

CrebB is a rate-limiting target of NMD to support robust behavioral rhythms

Mammalian ATF/CREB family genes are known targets of NMD (Hatano et al., 2013; Mendell et al., 2004). Moreover, a number of studies link ATF/CREB-dependent transcription with circadian clocks (Belvin et al., 1999; Ginty et al., 1993; Kim et al., 2016; Koyanagi et al., 2011; Lee et al., 2010; Lim et al., 2007; O'Neill et al., 2008; Obrietan et al., 1999;

Scheving et al., 1998; Sun et al., 2015; Travnickova-Bendova et al., 2002). Accordingly, we examined the expression of the *Drosophila* ATF/CREB homolog, *CrebB*, in *Smg6* mutants. The *CrebB* locus encodes several isoforms that are generated by alternative splicing and differentially include PTCs (Fig. 5A). A quantitative exon-specific analysis of these *CrebB* isoforms revealed that the levels of *CrebB* transcripts lacking exon 6 (*CrebB*- Δ ex6) were higher in *Smg6* mutant heads than in those of the heterozygous controls. By contrast, the expression of *CrebB* transcripts containing exon 4 (*CrebB*-ex4) was lower in *Smg6* mutants. The relative abundance of *CrebB*- Δ ex6 and *CrebB*-ex4 was similarly affected by *Smg5* mutation (Fig. 5B), suggesting that specific isoforms of *CrebB* are bona fide targets of *Drosophila* NMD. In addition, modest but opposing effects of *Smg5* and *Smg6* on *CrebB*-RM levels may indicate their non-redundant roles in the post-transcriptional expression of this specific *CrebB* isoform.

To functionally validate that NMD-dependent regulation of

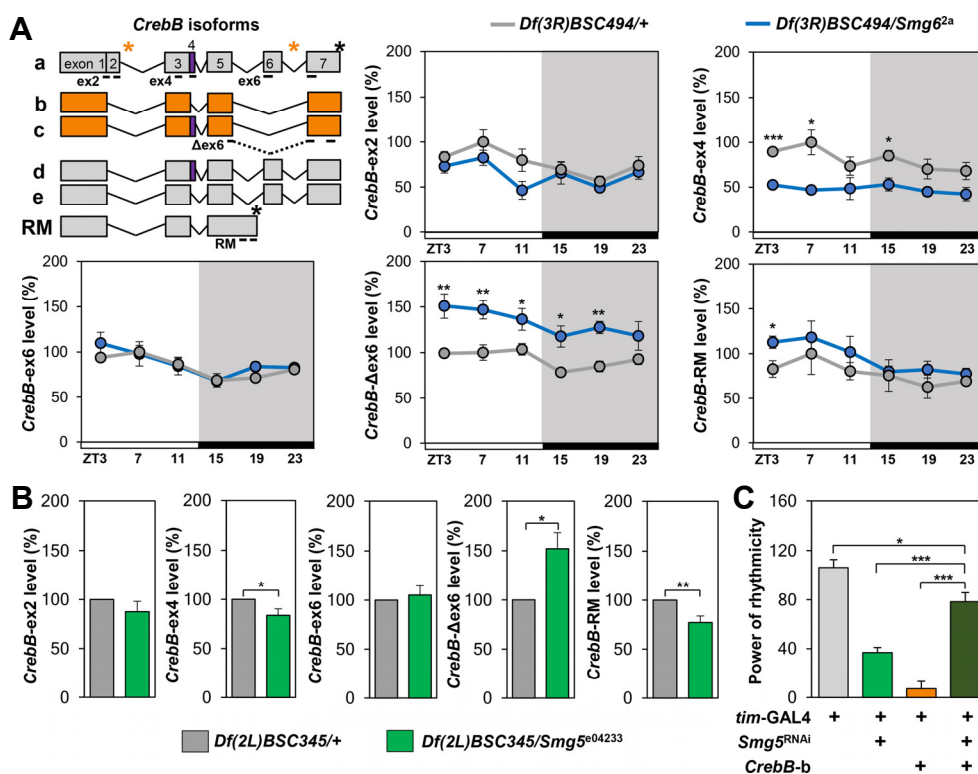


Fig. 5. *CrebB* is a rate-limiting target of NMD to support robust behavioral rhythms. (A) Schematic diagram of *CrebB* isoforms. Black bars indicate approximate annealing regions of exon-specific primers. Transcripts including either exon 4 or exon 6 were detected using the ex4 or ex6 primer sets, respectively. Transcripts including either exon 4 or exon 6 were detected using the Δ ex6 primer set and designated collectively as *CrebB- Δ ex6*. Exon 2 has not been annotated by FlyBase, but we designed the ex2 primer set based on the previously predicted sequence (Yin et al., 1995b). Black asterisks indicate authentic stop codons in *CrebB* transcripts; orange asterisks mark potential PTCs. Quantitative transcript analyses of *CrebB* isoforms in *Smg6* mutants [*Df(3R)BSC494/Smg6^{2a}*, blue lines] and the heterozygous controls [*Df(3R)BSC494/+*, gray lines] were performed as described for Fig. 4B. Data represent averages \pm SEMs ($n = 3$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Student's *t* tests. White/black bars, LD cycles. (B) Quantitative transcript analyses of *CrebB* isoforms in *Smg5* mutants [*Df(2L)BSC345/Smg5^{e04233}*, green bars] and heterozygous control [*Df(2L)BSC345/+*, gray bars]. Data represent averages \pm SEMs ($n = 3-6$). $*p < 0.05$, $**p < 0.01$ by Student's *t* tests. (C) Overexpression of *CrebB-b* isoform rescues disrupted locomotor rhythms in *Smg5*-depleted flies. Power of rhythmicity in DD locomotor activity was significantly different in flies with *Smg5* depletion and *CrebB-b* overexpression ($p < 0.001$, by two-way ANOVA). $*p < 0.05$, $***p < 0.001$ by Sidak's multiple comparison test. Error bars indicate SEMs ($n = 30-65$).

CrebB is responsible for sustaining circadian behaviors, we transgenically overexpressed the *CrebB-b* isoform (Yu et al., 2006) in *Smg5* RNAi or control flies and examined the effect on DD locomotor rhythms. Overexpression of the *CrebB-b* isoform rescued free-running locomotor rhythms in *Smg5*-depleted flies while it strongly dampened circadian behaviors in control flies (Fig. 5C, Supplementary Table S8). These results suggest that *CrebB* is rate-limiting for the NMD pathway to sustain high-amplitude behavioral rhythms.

DISCUSSION

NMD is one of the major RNA quality control mechanisms for degrading nonfunctional transcripts with PTCs and ensuring the translation of error-free mRNAs. In addition, NMD monitors the stability of normal mRNAs by targeting their selective *cis*-elements, such as upstream open-reading

frames, long 3' UTRs, introns in 3' UTRs, and frame-shifting signals (Alonso, 2005; Chang et al., 2007; Hug et al., 2016; Nickless et al., 2017). It has been previously shown that NMD contributes to the circadian control of gene expression, though only a few clock genes have been identified as direct targets (Filichkin et al., 2014; Hatano et al., 2013; Kwon et al., 2014; Mendell et al., 2004; Shaul, 2015; Wu et al., 2017). In this report, we show that NMD is necessary to sustain circadian behaviors in *Drosophila* and localize this control to PDF-expressing pacemaker neurons. We further provide molecular and genetic evidence that NMD targets specific isoforms of *CrebB* to sustain circadian locomotor rhythms.

Daily rhythmic oscillations in cAMP levels are observed in a diversity of species (Eckel-Mahan et al., 2008; Fukuhara et al., 2004; O'Neill et al., 2008; Palacios-Munoz et al., 2018) and linked to the circadian clocks in part via the ATF/CREB family

of transcriptional regulators (Ginty et al., 1993; Koyanagi et al., 2011; Obrietan et al., 1999; Scheving et al., 1998). In mammals, these transcription factors promote *per* transcription (Shimizu et al., 2007; Travnickova-Bendova et al., 2002), and the CLOCK-ARNTL (BMAL) heterodimer in mice negatively regulates ATF5 expression (Lemos et al., 2007). In *Drosophila*, CRE-dependent transcription exhibits both ultradian and circadian rhythms while these rhythms are disrupted by *CrebB* mutation (Belvin et al., 1999). As expected, transcriptional coactivators of the ATF/CREB family members, such as CREB-binding protein or CREB-regulated transcriptional coactivator support circadian rhythms in *Drosophila* and mammals via either CLK-CYC- or CREB-dependent transcription of circadian clock genes (Hosoda et al., 2009; Hung et al., 2007; Kim et al., 2016; Lee et al., 2010; Lim et al., 2007; Sakamoto et al., 2013).

Our genetic analyses revealed that the loss of NMD function potentially dampened circadian locomotor rhythms without impairing the circadian expression of clock genes. Thus, the NMD-dependent clock function maps to the clock output pathway, which relays the timing information from molecular clocks (i.e., daily rhythmic gene expression) to behavioral rhythms. Given the isoform-specific effects of NMD on *CrebB* expression, we reason that NMD tunes the balance of *CrebB* isoforms with transcriptional activator or repressor function rather than silencing the overall activity of *CrebB*. There are two potential PTCs in *CrebB* transcripts: one in the intron after exon 2 and the other after exon 6. *CrebB* transcripts including exons 4 and 6 encode a transcriptional activator if their translation starts from the second start codon after the PTC following exon 2 (Fropf et al., 2013; Tubon et al., 2013; Yin et al., 1995a; Yin et al., 1995b). On the other hand, *CrebB* transcripts without exons 2, 4, or 6, such as *CrebB-b*, encode a transcriptional repressor (Davis et al., 1996; Fropf et al., 2013; Yin et al., 1994; Yin et al., 1995b) while the transcriptional activities of other splice variants have not been characterized (Fropf et al., 2013; Perazzona et al., 2004). Since our transcript analysis in LD cycles did not show any strong time-of-the-day-effects of *Smg6* on the relative levels of *CrebB-Δex6* and *CrebB-ex4*, it is less likely that NMD controls *CrebB* expression in a light- or circadian clock-dependent manner. As reported in a number of species (Barberan-Soler et al., 2008; Lewis et al., 2003; Tabrez et al., 2017; Traunmuller et al., 2014), we speculate that NMD is constitutively coupled to the alternative splicing of *CrebB* for the differential expression of *CrebB* isoforms, thereby sustaining robust behavioral rhythms in constant dark.

The transcriptional activity of two mammalian ATF/CREB homologs (i.e., ATF4 and ATF5) is implicated in circadian gene expression (Koyanagi et al., 2011; Lemos et al., 2007), and their transcript stability is regulated by NMD (Hatano et al., 2013; Mendell et al., 2004). Accordingly, we propose that NMD-dependent regulation of ATF/CREB expression is evolutionarily conserved among diverse species and represents an ancestral clock mechanism that sustains circadian rhythms. Future studies will elucidate the isoform-specific regulation of *CrebB* by NMD in *Drosophila* and whether there is feedback from the circadian clocks to control NMD activity.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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