

1 **A novel *Smg6* mouse model reveals regulation of circadian period and daily**
2 **CRY2 accumulation through the nonsense-mediated mRNA decay pathway**

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12 **Abstract**

13 Nonsense-mediated mRNA decay (NMD) has been intensively studied as a surveillance
14 pathway that degrades erroneous transcripts arising from mutations or RNA processing errors.
15 While additional roles in controlling regular mRNA stability have emerged, possible functions
16 in mammalian physiology *in vivo* have remained unclear. Here, we report a novel conditional
17 mouse allele that allows converting the NMD effector nuclease SMG6 from wild-type to
18 nuclease domain-mutant protein. We analyzed how NMD downregulation affects the function
19 of the circadian clock, a system known to require rapid mRNA turnover. We uncover strong
20 lengthening of free-running circadian periods for liver and fibroblast clocks, and direct NMD
21 regulation of *Cry2* mRNA, encoding a key transcriptional repressor within the rhythm-
22 generating feedback loop. In the entrained livers of *Smg6* mutant animals we reveal
23 transcriptome-wide alterations in daily mRNA accumulation patterns, altogether expanding the
24 known scope of NMD regulation in mammalian gene expression and physiology.

25 **Introduction**

26 Nonsense-mediated mRNA decay (NMD) functions as an important surveillance pathway to
27 reduce gene expression errors that arise from mutations or mis-splicing and that are
28 recognized due to aberrant translation termination on “premature translation termination
29 codons” (PTCs) (reviewed in ^{1,2}). In mammals, PTCs are defined due to their position relative
30 to an exon-junction complex (EJC), a multiprotein assembly that is deposited on mRNAs
31 during splicing and removed from the transcript by the passage of translating ribosomes.
32 Termination upstream of an EJC identifies the stop codon as aberrant, promoting the
33 formation of an NMD factor complex comprising several UPF (up-frameshift) and SMG
34 (suppressor with morphogenetic effects on genitalia) proteins. Briefly, interactions between
35 UPF1, UPF2 and UPF3 proteins trigger UPF1 phosphorylation by the kinase SMG1.
36 Phosphorylated UPF1 further recruits SMG5, SMG6 and SMG7, which are involved in
37 executing the actual mRNA degradation step. Previous models suggested two distinct,
38 redundant branches for decay involving SMG5-SMG7 (that can recruit general, non-NMD-
39 specific exonucleases) or SMG6 (an NMD-specific endonuclease); recent evidence, however,
40 argues for mechanistic overlap ³, and a linear pathway involving decay “licensing” through
41 SMG5-SMG7 followed by SMG6-mediated endonucleolytic cleavage has been proposed as
42 the main mechanism of how mRNA decay is carried out ⁴.

43 Early transcriptome-wide analyses already noted that in addition to NMD activity on aberrant
44 transcripts, the pathway can be co-opted for the decay of regular, physiological mRNAs as
45 well ⁵. Most of the initially identified NMD-activating features on endogenous transcripts are in
46 line with the above rules for PTC definition - e.g.: introns in 3' untranslated regions (UTRs);
47 translated upstream open reading frames (uORFs) in 5' UTRs; selenocysteine codons that
48 are interpreted as stop codons - yet later studies showed that long 3' UTRs can activate NMD
49 *per se*, in the absence of a downstream splice junction ^{6,7}. The generality of a “3' UTR length
50 rule” has, however, been questioned recently in a nanopore sequencing-based study that
51 (after removing the transcripts from the analysis for which there was evidence for splicing in
52 the 3' UTR) found no predictive value of 3' UTR length for NMD regulation ⁸. Independently of
53 which mechanisms trigger NMD on non-classical NMD substrates, it has been proposed that
54 the expression of up to 20-40% of genes is directly or indirectly affected when NMD is
55 inactivated in mammalian cell lines ^{4,8}, and it is tempting to speculate that the pathway may
56 have extensive functions in regular gene expression control ². It is largely unknown whether
57 this regulatory potential of NMD extends to the intact organ and living organism *in vivo*, and if
58 so, which specific molecular and physiological pathways it controls.

59 Certain physiological processes are particularly reliant on rapid, well-controlled RNA turnover;
60 conceivably, co-opting NMD could thus be especially opportune. In this respect, the circadian

61 clock stands out as an important functional system that controls daily rhythms in transcription,
62 mRNA and protein abundances, affecting thousands of genes across the organism and
63 controlling daily changes in behavior, physiology and metabolism (reviewed in ⁹). In the
64 mammalian body, the circadian system is hierarchically organized with a master clock in the
65 brain's suprachiasmatic nucleus (SCN) that synchronizes peripheral clocks that operate in
66 most cell types and that are responsible for driving cellular rhythmic gene expression
67 programs. Across cell types, clocks have a similar molecular architecture, with a core clock
68 mechanism that generates gene expression oscillations through transcription factors that
69 interact in negative feedback loops. In the main loop, BMAL1:CLOCK (BMAL1:NPAS2 in
70 neurons) function as the main activators and bind to E-box enhancers in their target genes,
71 which include the *Period* (*Per1, 2, 3*) and *Cryptochrome* (*Cry1, 2*) genes. Negative feedback
72 is achieved when PER:CRY complexes translocate to the nucleus and repress their own
73 transcription by inhibiting BMAL1:CLOCK. PER and CRY protein degradation temporally limits
74 the repressive activity, eventually allowing a new cycle to ensue. Conceivably, the rapid decay
75 of *Per* and *Cry* mRNAs is critical for this mechanism - as a means of restricting PER:CRY
76 biosynthesis and availability in time - yet the responsible decay pathways remain poorly
77 investigated. Additional feedback mechanisms (in particular involving nuclear receptors of the
78 REV-ERB/ROR families) interlock with the above main feedback loop and confer both
79 robustness and plasticity to this system (reviewed in ¹⁰). Through the numerous rhythmic
80 transcriptional activities that are generated through this clockwork, rhythmic mRNA production
81 is driven at hundreds to thousands of downstream, clock-controlled genes (CCGs). The
82 stability of CCG transcripts critically determines to what extent their initial transcriptional
83 rhythm is propagated to the mRNA and protein abundance levels. Mechanisms that have been
84 implicated in post-transcriptionally regulating rhythmic mRNAs in mammals include miRNA-
85 mediated mRNA decay ¹¹ and regulated deadenylation ¹². With regard to a possible
86 involvement of NMD, first evidence for roles in the circadian system has been reported from
87 fungi, plants and flies ¹³⁻¹⁵, but how NMD globally shapes rhythmic transcriptomes, let alone in
88 a mammalian organism *in vivo*, is still unknown.

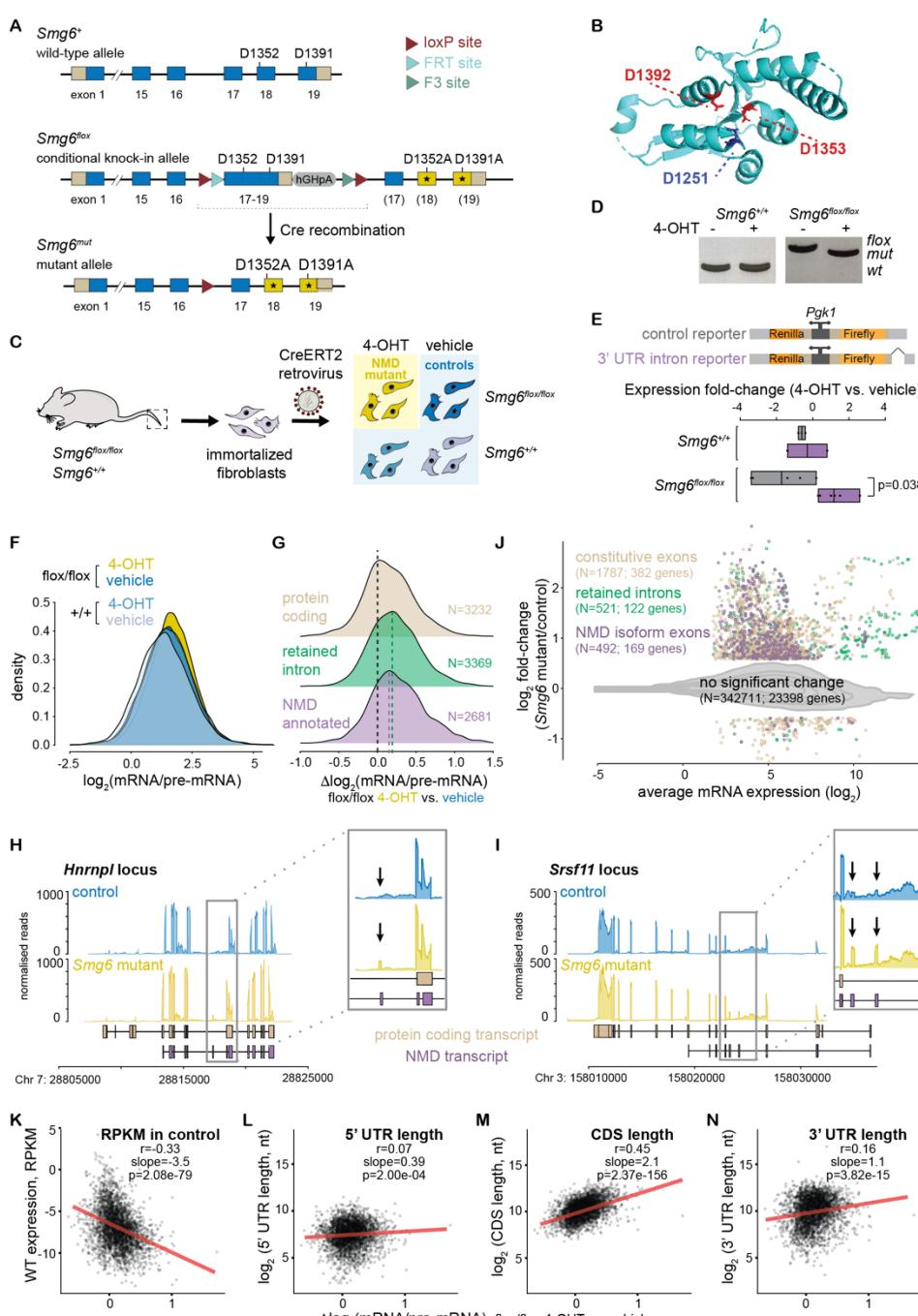
89 In this study, we have comprehensively investigated the role of NMD in the mammalian
90 circadian system *in vivo*. Using a novel conditional NMD loss-of-function mouse model, we
91 uncover that NMD is directly implicated in regulating the circadian period of peripheral clocks.
92 We identify *Cry2* as a direct NMD target and further determine how the hepatic diurnal
93 transcriptome is rewired in the absence of a functional NMD pathway. Our new mouse model
94 and findings on circadian regulation provide important conceptual advances on *in vivo*
95 functions of NMD and reveal a novel mechanism of post-transcriptional gene expression
96 regulation that acts in the mammalian core clock.

97 **Results**

98 **A novel conditional NMD loss-of-function allele based on SMG6 mutated in its nuclease
99 domain**

100 To inactivate NMD *in vivo* we generated mice in which we could conditionally recombine
101 *Smg6*^{fl/fl} to *Smg6*^{mut} (**Fig. 1A**), i.e. from an allele encoding wild-type SMG6 protein to a version
102 specifically point-mutated at two of the three highly conserved aspartic acid (D) residues of
103 the catalytic triade of the protein's PIN (PiT N-terminus) nuclease domain ¹⁶ (**Fig. 1B**). We
104 chose this strategy over a full gene knockout because NMD factors, including SMG6, carry
105 additional functions in telomere and genome stability ¹⁷. These functions have been shown to
106 be selectively maintained by expressing an NMD-inactive SMG6 protein lacking its nuclease
107 domain ¹⁸. We first validated our genetic model in primary tail fibroblasts from homozygous
108 *Smg6*^{fl/fl} and *Smg6*^{+/+} littermate mice that we stably transduced with a retroviral vector
109 expressing tamoxifen-activatable CreERT2 (**Fig. 1C**). *Smg6*^{fl/fl} cells specifically and
110 efficiently recombined to *Smg6*^{mut/mut} by addition of 4-hydroxytamoxifen (4-OHT) to the culture
111 medium (**Fig. 1D**). In these cells, a lentiviral luciferase reporter carrying an intron in its 3' UTR
112 became upregulated, as expected for an inactive NMD pathway (**Fig. 1E**). We next used RNA-
113 seq on 4-OHT-treated and -untreated cells of both genotypes to analyze gene expression
114 changes transcriptome-wide. Our method, based on random priming of rRNA-depleted total
115 RNA, allowed for the quantification of both mRNA (exon-mapping reads) and pre-mRNA
116 abundances (intron-mapping reads), the latter serving as a proxy for gene transcription rates
117 ^{11,19,20}. In analogy to previous studies ^{11,19} we used mRNA/pre-mRNA ratios to estimate mRNA
118 stability changes between NMD-inactive and control cells, and to distinguish them from
119 secondary effects involving altered transcription rates. Our analyses revealed a shift to higher
120 mRNA/pre-mRNA ratios (more stable mRNAs) specifically in NMD-inactive (*Smg6*^{fl/fl} + 4-
121 OHT) cells (**Fig. 1F**). Two transcript groups that were particularly affected, as predicted, were
122 genes with known, annotated NMD-sensitive mRNA isoforms (according to Ensembl
123 annotations) and with retained introns (**Fig. 1G**). Visual inspection of individual examples
124 further validated these findings, as shown for *Hnrnpl* and *Srsf11*, for which a specific up-
125 regulation of NMD isoform-specific exons in the mutants was evident (**Fig. 1H-I**).
126 Transcriptome-wide differential expression analysis at the exon level indicated that apart from
127 NMD-annotated isoforms and retained introns, hundreds of constitutive exons from canonical
128 mRNAs (i.e., without annotated NMD isoforms) increased in abundance under *Smg6* mutant
129 conditions, pointing to widespread NMD regulation of the transcriptome (**Fig. 1J**). We next
130 analyzed if specific transcript features correlated with *Smg6* mutation-dependent changes in
131 mRNA/pre-mRNA ratios. As expected for potential NMD substrates, the transcripts that were
132 most strongly affected were low expressed in control cells (**Fig. 1K**). 5' UTR length (which

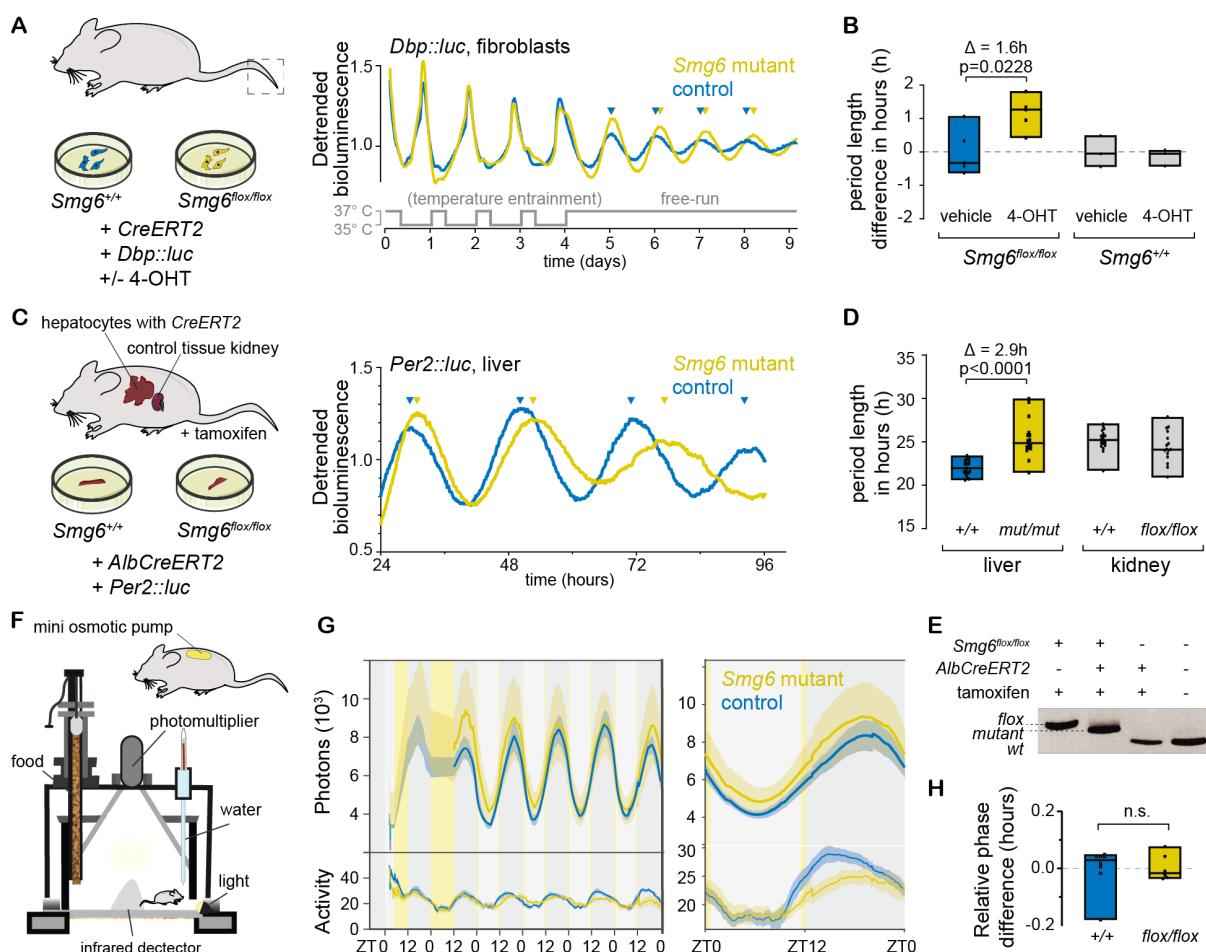
133 correlates with uORF content²¹) was weakly, though significantly, associated with increased
 134 mRNA/pre-mRNA ratios (Fig. 1L), suggesting that translated uORFs may contribute as an
 135 NMD-activating feature to endogenous mRNA upregulation in *Smg6* mutants. Stronger
 136 correlations were observed with the lengths of the CDS (Fig. 1M) and 3' UTRs (Fig. 1N). The
 137 latter association is consistent with the ability of long 3' UTRs to function as NMD-activating
 138 features. Altogether these associations match those observed for other NMD loss-of-function
 139 models, e.g. in Hela cells subjected to *Upf1* knockdown²². Taken together, we concluded that
 140 our genetic model based on mutant *Smg6* was suitable to analyze endogenous targets and
 141 functions of the NMD pathway.



143 **Figure 1. A novel conditional NMD loss-of-function allele based on PIN nuclease domain mutant *Smg6*.** **A.** Schematic of the genetic
144 model. *Smg6*^{flx} expresses wild-type SMG6 protein encoded by the blue exons; after Cre-mediated recombination to *Smg6*^{mut}, point-mutated
145 exons 18 and 19 (yellow) lead to expression of mutant SMG6 (D1352A, D1391A). **B.** The mutated aspartic acid residues are within the
146 catalytic triade of the PIN nuclease domain, shown in the structure of the human protein (PDB accession 2HWW; ¹⁶). **C.** For cellular studies,
147 tail fibroblasts from adult male mice (*Smg6*^{flx} and wild-type littermates) were cultured until spontaneous immortalization, and tamoxifen-
148 activatable CreERT2 expression was achieved by a retrovirus. Upon 4-hydroxytamoxifen (4-OHT) treatment, NMD mutants (yellow) were
149 compared to different control cells (shades of blue). **D.** PCR-based genotyping of genomic DNA extracted from cells depicted in C. indicates
150 efficient recombination upon 4-OHT treatment. **E.** A luciferase reporter containing an intron in the 3' UTR is upregulated in 4-OHT-treated
151 *Smg6*^{flx/flx} cells, as expected under NMD-inactive conditions. N=2-6 plates/group, adjusted p=0.0038; multiple Student's t-test. **F.** Density
152 plot showing transcriptome-wide mRNA/pre-mRNA ratio distributions calculated from RNA-seq, in NMD-inactive (yellow) vs. control cells.
153 **G.** The difference in mRNA/pre-mRNA ratios between NMD-inactivated (*Smg6*^{flx/flx} + 4-OHT) and control cells (*Smg6*^{flx/flx} + vehicle) is
154 consistent with higher stability of annotated NMD substrates (purple, N=2681) and transcripts with retained introns (green, N=3369).
155 Moreover, the broad distribution and shift to positive values for non-NMD-annotated protein coding transcripts (beige, N=3232) is indicative
156 of transcriptome-wide NMD regulation. **H.** Read coverage on the *Hnmp1* and **I.** *Srsf11* loci indicates the specific upregulation of transcript
157 isoforms that are NMD-annotated (purple) and that can be identified via specific exons (marked by arrows in insets). **J.** Differential expression
158 analysis at the exon level, comparing *Smg6*^{flx/flx} + 4-OHT vs. *Smg6*^{flx/flx} + vehicle conditions, reveals significant upregulation of NMD-
159 annotated exons (purple; N=492; 169 genes), retained introns (green; N=521; 122 genes), and a sizeable number of constitutive exons
160 (beige; N=1787, 382 genes), suggestive of NMD regulating many protein coding genes. **K.** Correlation analysis between mRNA/pre-mRNA
161 ratio change upon NMD activation and expression levels in wild-type cells shows significant anticorrelation. The lengths of **L.** the 5' UTR,
162 **M.** the CDS and **N.** the 3' UTR are all positively correlated with mRNA/pre-mRNA ratio change upon NMD inactivation. Pearson correlation
163 coefficient (r), slope and p-values were calculated by a linear model.

164 **NMD inactivation lengthens free-running circadian periods in fibroblasts and in liver**

165 We next investigated how mutant *Smg6* affected the circadian clock. First, we stably
166 transfected the above fibroblasts with a circadian reporter gene, *Dbp-Luciferase* ²³, and
167 recorded their free-running circadian rhythms upon NMD inactivation with 4-OHT. Briefly, we
168 synchronized the cellular oscillators using temperature cycles ²⁴, released them at 37°C, and
169 continued real-time bioluminescence recordings for an additional 5 days under constant
170 conditions (**Fig. 2A**). These experiments revealed a lengthening of the free-running circadian
171 period in NMD-deficient cells by ca. 1.5 hours (**Fig. 2B**). We next wished to corroborate a
172 potential period phenotype using an alternative peripheral clock model that was more relevant
173 for circadian physiology and functions *in vivo*. We thus crossed into the *Smg6*^{flx} mouse line a
174 hepatocyte-specific *CreERT2* (driven from the *Albumin* locus ²⁵) and a circadian reporter,
175 *mPer2::Luc* ²⁶. After intraperitoneal tamoxifen injections into young adult mice, animals were
176 sacrificed 4 weeks later, a time at which highly efficient recombination to *Smg6*^{mut} had taken
177 place (**Fig. 2E**). We then prepared organotypic slices (tissue explants) for real-time recording
178 of luciferase rhythms *ex vivo* (**Fig. 2C**). In these experiments we observed a strong and
179 specific period lengthening by almost 3 hours in liver explants from animals with inactivated
180 NMD (tamoxifen-treated *Smg6*^{flx/flx} mice) as compared to livers from identically treated
181 littermate animals of the control genotype (**Fig. 2D**). As an additional specificity control, we
182 recorded kidney explant rhythms from the same animals. Free-running periods were generally
183 longer in this organ, as reported previously ²⁶, yet we did not observe any differences between
184 genotypes (**Fig. 2D**), in line with the hepatocyte-specificity of *CreERT2* expression in our
185 genetic model.



186

187 **Figure 2. NMD inactivation through *Smg6* mutation lengthens free-running circadian periods.** **A.** Real-time recording of
188 bioluminescence rhythms in immortalized fibroblasts of genotypes *Smg6^{flox/flox}* and *Smg6^{+/+}* (both transduced with *CreERT2* retrovirus and
189 circadian reporter *Dbp::Luc*); after temperature-entrainment (24-h periodic square wave; 35°C-37°C), cells were released into free-running
190 conditions (37°C) from day 4. Representative traces show longer free-running period in *Smg6^{mut}* cells (yellow) as compared to control cells
191 (blue). **B.** Quantification of several experiments as in A. *Smg6* mutants (yellow) showed significantly longer periods in comparison to controls
192 (*Smg6^{flox/flox}* treated with vehicle in blue, or *Smg6^{+/+}* with/without 4-OHT in grey). N=5 for *Smg6^{flox/flox}* cells; N=3 for *Smg6^{+/+}* cells; period
193 difference between 4-OHT and vehicle-treated *Smg6^{flox/flox}* cells is 1.6 hours; Bonferroni's multiple comparisons test adjusted $p = 0.0228$. **C.**
194 Adult *Smg6^{flox/flox}* male mice and their *Smg6^{+/+}* littermates (all carrying tamoxifen-activatable *AlbCreERT2* and the circadian reporter
195 *Per2::Luc*) were sacrificed 4-5 weeks after tamoxifen injections. Liver and kidney explants were used for real-time recording of luciferase
196 rhythms. Representative traces from livers show strong free-running period lengthening in the *Smg6^{mut}* (yellow) vs. control (blue). **D.**
197 Quantification of several experiments as in C. Robustly increased periods, by almost 3 hours, were observed in NMD-deficient liver explants
198 (yellow; mean = 25.36 ± 2.23 h) in comparison to control livers (blue; mean = 22.0 ± 0.90 h). No period length difference was observed for
199 kidney explants from the same animals (grey; *flox/flox* mean \pm SD = 25.2 ± 1.19 h, control mean \pm SD = 24.4 ± 1.83 h). Liver tissue: N=16
200 slices *Smg6^{flox/flox}*; N=17 slices for controls; Mann-Whitney test $p < 0.0001$. Kidney tissue: N=16 for *Smg6^{flox/flox}*; N=20 for controls, Mann-
201 Whitney test $p = 0.0771$. 1-4 tissue slices were used per mouse; analyses performed blindly. **E.** Efficient recombination was validated by
202 genotyping of livers (PCR analysis of genomic DNA). **F.** Cartoon depicting the *in vivo* recording setup (RT-Biolumicorder); *Smg6^{flox/flox}* or
203 *Smg6^{+/+}* mice carrying *AlbCreERT2* and *mPer2::Luc* alleles were implanted with a luciferin-loaded mini osmotic pump 4 weeks after
204 tamoxifen injections before placing in the recording device. **G.** Left panel: After 2 days under LD12:12, bioluminescence rhythms (photons)
205 and activity (infrared signal) were recorded for 5 days under photoskeleton photoperiod conditions (indicated by yellow vertical lines at ZT12
206 and before ZT0). The plot shows mean signal (solid trace) and SEM (shaded) over the whole course of the experiment in the left panel, and
207 compiled data, averaged from all days and mice, in the right panel. **H.** Quantification of *PER2::LUC* bioluminescence signal showed no
208 difference in phase between tamoxifen-injected *Smg6^{flox/flox}* (yellow) and *Smg6^{+/+}* (blue) control animals. N=6 per group; Mann-Whitney test
209 $p = 0.7251$.

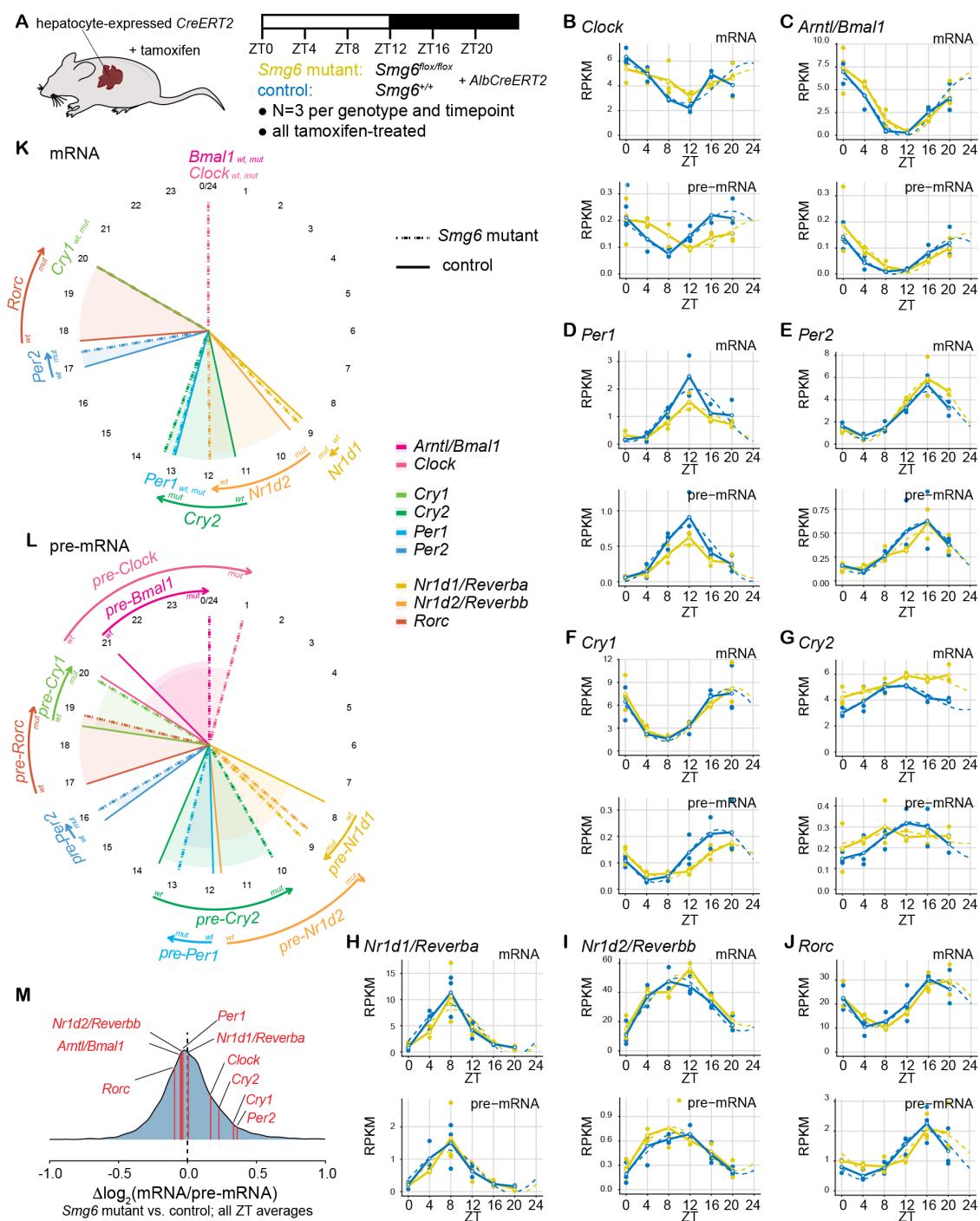
210 *In vivo*, and according to oscillator theory^{27,28}, a difference in period lengths between the
211 entraining clock (here: wild-type period SCN) and the entrained clock (here: long period *Smg6*
212 mutant hepatocytes) will typically translate to a phase shift of the entrained oscillator. Thus,
213 we expected that the long period mPER2-Luc rhythms seen in liver explants *ex vivo* would
214 lead to a change in phase *in vivo*. To evaluate this prediction, we used a method for the real-
215 time recording of daily liver gene expression in freely moving mice^{29,30} that relies on luciferase
216 reporters, luciferin delivery via an osmotic minipump, and highly sensitive bioluminescence
217 photon counting (**Fig. 2F**). Using the same *mPer2::Luc* reporter knock-in animals (NMD-
218 deficient vs. controls) as for the above tissue explant experiments, real-time recording was
219 carried out under conditions that ensured light-entrainment of the SCN clock to an external
220 24-hour light-dark cycle by means of a skeleton photoperiod, i.e. two 30 min light pulses
221 applied at times corresponding to the beginning and to the end of the light phase in a 12h-
222 light-12h-dark (LD12:12) cycle. We observed high-amplitude rhythmic bioluminescence
223 rhythms in both genotypes (**Fig. 2G**) with phases that were, however, indistinguishable (**Fig.**
224 **2H**). Next, we also investigated the effect of the *Smg6* mutation on the central clock in the
225 SCN. We stereotactically injected an adeno-associated virus (AAV) expressing Cre::eGFP to
226 induce recombination (**Fig. S1A, B**) and scored circadian clock parameters by two different
227 assays: *in vivo*, we measured behavioral locomotor rhythms under constant conditions (free-
228 running clock) by running wheel assays (**Fig. S1C, D**) and *ex vivo*, we recorded mPER2::LUC
229 rhythms from SCN explants (**Fig. S1E, F**). Neither assay revealed an effect of the *Smg6*
230 mutation on free-running periods for the SCN clock; yet, as a caveat, we also noted overall
231 less efficient recombination as compared to our liver experiments (**Fig. S1G**). We concluded
232 that loss of NMD triggered by the *Smg6* mutant allele had a strong period lengthening effect,
233 notably for peripheral clocks and in particular in liver explants.

234 **NMD inactivation differentially affects the phases of core clock gene expression in the
235 entrained liver**

236 We next analyzed the apparent discrepancy between the long periods of liver rhythms *ex vivo*
237 (**Fig. 2C, D**) and the lack of a phase phenotype *in vivo* (**Fig. 2G, H**). Briefly, other tissues than
238 liver (e.g. kidney³¹) may have contributed to the overall bioluminescence signal detected in
239 the *in vivo* recording experiments, thereby masking a hepatic phase phenotype. Moreover,
240 systemic cues that are dependent on the SCN, yet do not require a functional hepatocyte
241 clock, can drive rhythmic PER2 accumulation in liver^{32,33}; therefore, mPER2::LUC signal may
242 not be representative of the intrinsic liver clock phase. In order to evaluate in a comprehensive
243 fashion how rhythmic gene expression was altered *in vivo*, we collected livers at 4-hour
244 intervals around-the-clock from LD12:12-entrained *Smg6* mutant and control mice, with
245 timepoints ZT0 (*Zeitgeber* Time 0, corresponding to time of “lights-on”), ZT4, ZT8, ZT12

246 (“lights-off”), ZT16 and ZT20 (**Fig. 3A**). We carried out RNA-seq on all individual mouse liver
247 samples (triplicates per genotype and timepoint) and assembled the data into two time series
248 representing the diurnal liver transcriptome under conditions of an inactive vs. active NMD
249 pathway. As a means of quality control, we first validated that known NMD targets were
250 upregulated in *Smg6* mutant livers. Indeed, as in the fibroblasts (**Fig. 1H, I**), NMD-annotated
251 isoform exons were increased in abundance (**Fig. S2A**). Other transcripts diagnostic for an
252 inactive NMD pathway showed the expected post-transcriptional upregulation as well. For
253 example, mRNAs encoding components of the NMD machinery itself were post-
254 transcriptionally upregulated (**Fig. S2B**), as reported previously from cell lines ⁷. This
255 phenomenon has been proposed to represent an autoregulatory mechanism that involves as
256 NMD-activating features the long 3' UTRs that these mRNAs carry. Similarly, the uORF-
257 regulated *Atf4* and *Atf5* transcripts, which are documented NMD substrates ^{5,34} and encode
258 key transcription factors in the integrated stress response (ISR) ³⁵, showed the expected
259 upregulation (**Fig. S2C**). Of note, higher ATF5 protein accumulation (**Fig. S2D, E**) occurred in
260 the absence of general ISR activation (as judged by eIF2 α phosphorylation levels that were
261 only weakly affected; **Fig. S2D**), pinpointing the lack of direct NMD regulation rather than
262 proteotoxic stress as the likely trigger.

263 We then analyzed the daily dynamics of core clock gene expression at the mRNA and pre-
264 mRNA levels (**Fig. 3B-J**). Consistent with the *in vivo* recording of *mPer2::Luc* animals, *Per2*
265 mRNA and pre-mRNA rhythms were highly similar between the two genotypes (**Fig. 3E**). By
266 contrast, several other core clock genes - notably those encoding the main transcriptional
267 activators, *Clock* and *Arntl/Bmal1* (**Fig. 3B, C**), as well as *Cry1* (**Fig. 3F**) and *Rorc* (**Fig. 3J**) -
268 showed phase-delayed pre-mRNAs indicative of transcription occurring several hours later.
269 The complete analysis of core clock mRNA (**Fig. 3K**) and pre-mRNA (**Fig. 3L**) rhythms
270 revealed that the considerable phase differences seen for many core clock genes at the
271 transcriptional (pre-mRNA) level (**Fig. 3L**), only partially propagated to the mRNA level (**Fig.**
272 **3K**). Of the core loop constituents, *Cry2* mRNA showed a substantial delay by ca. 2 hours
273 (**Fig. 3G, K**). The later phase did not have its origins in the timing of transcription, which rather
274 appeared to be advanced (**Fig. 3G, L**). Other delays in mRNA accumulation that we observed
275 affected the two nuclear receptors and components of the stabilizing loop, *Nr1d2/Rev-erbb*
276 and *Rorc* (**Fig. 3H, J, K**).

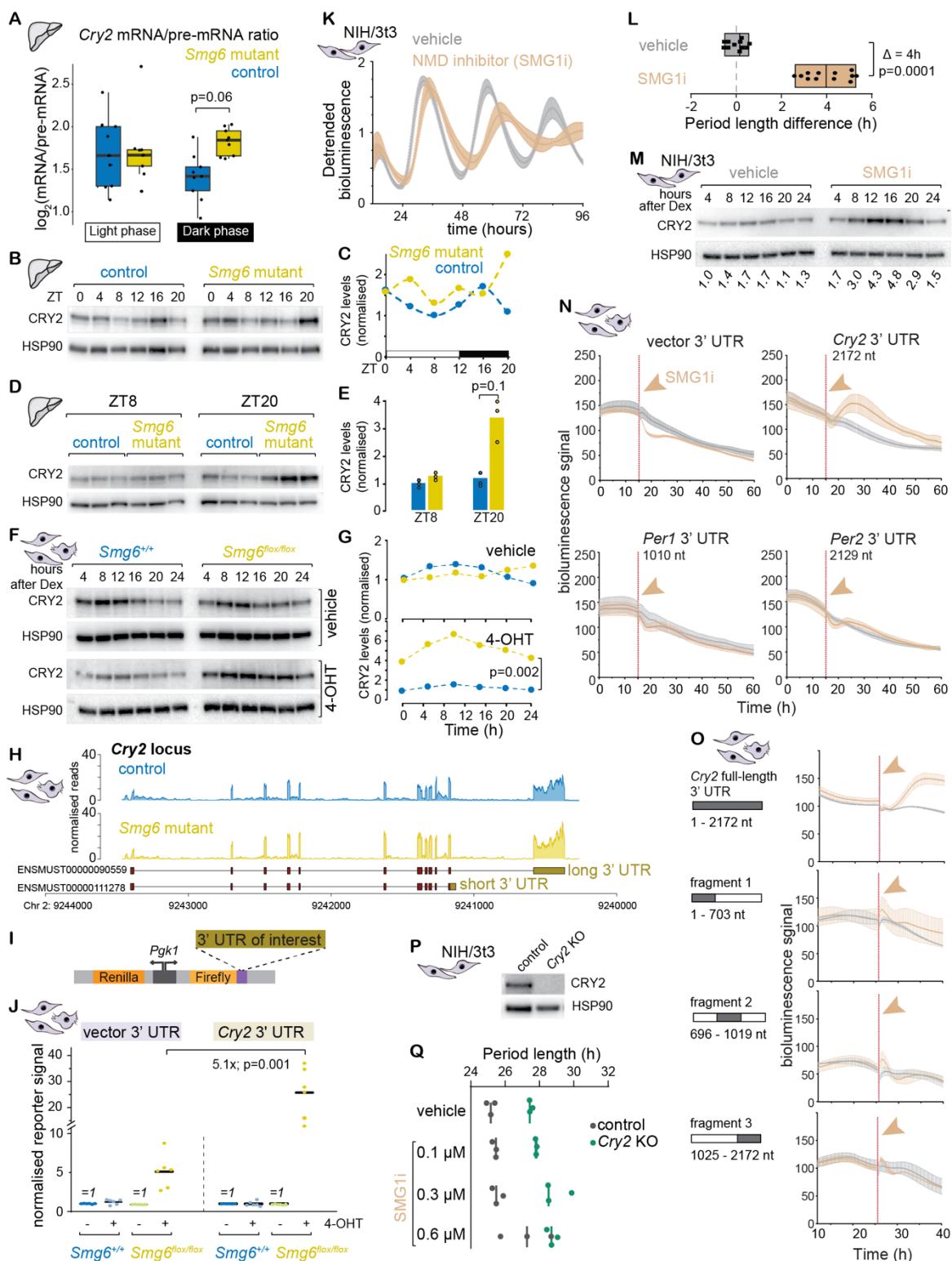


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278 **Figure 3. *Smg6* mutation differentially affects hepatic core clock pre-mRNA and mRNA rhythms. A.** Schematic of the around-the-
279 clock RNA-seq experiment, which was carried out on a time series of liver samples collected from LD12:12 entrained male *Smg6* mutant
280 (*Smg6*^{fl/fl}; AlbCreERT2; tamoxifen-treated) and control (*Smg6*^{+/+}; AlbCreERT2; tamoxifen-treated) mice. **B.-J.** RNA-seq data is plotted
281 for indicated core clock genes for mRNA (upper panels; exonic reads) and pre-mRNA (lower panels; intronic reads) for *Smg6* mutants
282 (yellow) and controls (blue). RPKM values (Reads Per Kilobase of transcript, per Million mapped reads) of individual mice are shown as
283 dots with solid lines connecting the means for each timepoint. The dashed lines represent the rhythmic data fit using the parameters from
284 Metacycle³⁶. **K.** Circular plot representing the phases of peak mRNA abundances according to the Metacycle fits for *Smg6* mutants (dashed)
285 and controls (solid) for indicated core clock genes. *Cry2*, *Nr1d2* and *Rorc* accumulated several hours later in *Smg6* mutants, whereas minor
286 effects were seen for the other genes. **L.** As in K. but for pre-mRNA rhythms. Several core clock pre-mRNAs showed later phases, indicative
287 of transcriptional shifts; notable exceptions being *Per2* (almost invariable) and *Cry2* and *Nr1d2*, which both showed a phase advance. **M.**
288 Similar to Fig. 1F, mRNA/pre-mRNA ratios were calculated for the liver RNA-seq data; briefly, average mRNA counts were first averaged
289 over all samples per genotype, before dividing by average pre-mRNA counts. Three components of the negative limb, *Cry2*, *Cry1* and *Per2*,
290 show higher mRNA/pre-mRNA ratios in *Smg6* mutants.

291 **NMD regulation of *Cry2* mRNA occurs through its 3' UTR and limits CRY2 protein
292 accumulation in the dark phase**

293 Among the core clock genes, the observed change in the daily *Cry2* expression profile (i.e. a
294 peak in *Cry2* mRNA levels at ZT8-12 with subsequent decrease in control animals; yet *Cry2*
295 mRNA abundances persisting on a high plateau until ZT20 in *Smg6* mutants; **Fig. 3G**) was
296 consistent with the hypothesis that the *Cry2* transcript became stabilized in the absence of
297 NMD. Indeed, the analysis of *Cry2* mRNA/pre-mRNA ratios across all liver samples suggested
298 elevated stability during the dark phase of the cycle (ZT12-20) (**Fig. 4A**). Western blot analysis
299 of total liver proteins revealed that the prolonged mRNA abundance under NMD-inactive
300 conditions led to corresponding changes in the levels of CRY2 protein, whose peak
301 accumulation was delayed by 4 hours in *Smg6* mutant animals (peak at ZT20) compared to
302 controls (peak at ZT16) (**Fig. 4B, C**). Moreover, the analysis of individual livers showed that
303 CRY2 reproducibly accumulated to >2x higher levels in *Smg6* mutant livers towards the end
304 of the dark phase, at ZT20 (**Fig. 4D, E**). Furthermore, increased CRY2 levels were also
305 apparent in *Smg6* mutant fibroblasts (**Fig. 4F, G**). These observations were consistent with a
306 direct regulation of *Cry2* mRNA stability through NMD. To explore this hypothesis, we
307 analyzed whether the *Cry2* mRNA contained any specific NMD-activating features. First, we
308 inspected RNA-seq coverage on the *Cry2* locus in our fibroblast data, which revealed the
309 expression of a single *Cry2* transcript isoform carrying a long 3' UTR of ~2.2 kb (**Fig. 4H**;
310 identical observations were made in the liver RNA-seq data; data not shown), i.e. well beyond
311 the ~1 kb cut-off that has been used as a benchmark for the definition of potential endogenous
312 NMD substrates^{2,6,7}. There was no evidence that a second annotated mRNA isoform with a
313 shorter 3' UTR (~0.4 kb; **Fig. 4H**) or any other, additional transcript variants were generated
314 from the locus. Finally, with a 5' UTR that is particularly short (20 nt) and no evidence for
315 translating ribosomes upstream of the annotated start codon according to previous ribosome
316 profiling data from liver²¹ or murine fibroblasts³⁷ (data not shown), we excluded the possibility
317 that the transcript contained NMD-activating uORFs. We thus assessed whether the ~2.2 kb
318 *Cry2* 3' UTR would confer NMD regulation to a luciferase reporter gene (**Fig. 4I**). Dual
319 luciferase assays revealed that inactivating NMD in fibroblasts led to a >5-fold activity increase
320 for the *Cry2* 3' UTR-carrying reporter as compared to the control reporter (**Fig. 4J**), providing
321 evidence that the *Cry2* 3' UTR acts to elicit NMD.



322

323 **Figure 4. NMD regulation of Cry2 mRNA via its 3' UTR.** **A.** mRNA/pre-mRNA ratios across individual liver samples – grouped into light
324 (ZT0, 4, 8) and dark phase (ZT12,16, 20) samples – indicate Cry2 mRNA stability increase in Smg6 mutants, which is visible in particular in
325 the dark phase; p-value=0.06; ANOVA. **B.** Western blot analysis of total liver proteins, for CRY2 and HSP90 (loading control). Each sample
326 is a pool of 3 individual mice. **C.** Quantification of Western blot shown in B; CRY2 intensity was normalized to the loading control, HSP90.
327 **D.** Western blot as in panel B, but from individual animals at ZT8 and ZT20, indicating that CRY2 is reproducibly more abundant at ZT20 in
328 Smg6 mutants. **E.** Quantification of Western blot in D; p=0.1; Mann-Whitney non-parametric test. **F.** Western blot analysis of total protein
329 extract from fibroblasts (cells as shown in Fig. 1C) reveals CRY2 upregulation specifically in 4-OHT-treated Smg6^{flox/flox} cells. **G.**
330 Quantification of Western blot shown in F; p=0.002; Mann-Whitney non-parametric test. **H.** RNA-seq read coverage on the Cry2 locus

331 (fibroblasts). Only one transcript isoform - carrying the 2.2 kb 3' UTR - is expressed; there is no evidence for expression of the short UTR
332 isoform. **I.** Schematic of the lentiviral dual luciferase system used in assays to test 3' UTRs of interest for NMD regulation. **J.** Dual-luciferase
333 assays reveal that the *Cry2* 3' UTR confers NMD regulation. Non-treated cells for each genotype/reporter were internally set to 1. Vector
334 UTR alone shows ca. 5-fold upregulation under *Smg6*^{fl/fl} + 4-OHT conditions (an effect coming from both Firefly luciferase up- and Renilla
335 luciferase downregulation). Against this background of the assay, the *Cry2* 3' UTR confers an additional >5-fold increase. Cells of each
336 genotype/reporter condition without 4-OHT treatment were internally set to 1, and the signal of 4-OHT-treated cells relative to these untreated
337 cells is reported; N=5 from 3 different experiments; p=0.001; Mann-Whitney non-parametric test. **K.** Bioluminescence traces of NIH/3t3 cells
338 carrying the DBP-Luciferase reporter, with (orange) and without (grey) 0.6 μ M SMG1i treatment. Traces show average (mean) signal and
339 standard deviation from 3 independent experiments. **L.** Quantification of experiments shown in K, showing reproducible period lengthening
340 by ca. 4h in the presence of 0.6 μ M SMG1i (N=11-12; p<0.001; Mann-Whitney test). **M.** Western blot analysis of total protein extract from
341 NIH/3t3 cells treated with vehicle or 0.6 μ M SMG1i; values of CRY2 abundance normalized to HSP90 (loading control) below the lanes. **N.**
342 Primary fibroblast (genotype *Smg6*^{+/+}, no 4-OHT) were stably transduced with luciferase reporters carrying different 3' UTRs (as in panel I).
343 Real-time recording of Firefly luciferase signal was carried until the signal reached stable state, before addition of 1 μ M SMG1i (orange) or
344 vehicle (grey). The reporter carrying the *Cry2* 3' UTR was specifically upregulated, as compared to *Per1*, *Per2* or vector 3' UTRs. Traces
345 show average (mean) signal and standard deviation; N=3. **O.** In assays as in N., only full-length *Cry2* 3' UTR showed upregulation upon
346 SMG1i treatment, but not individual fragments (N=2). **P.** Western blot showing absence of CRY2 in Crispr/Cas9-generated *Cry2* knockout
347 NIH/3t3 cells. **Q.** Period length of *Dbp*-luciferase reporter traces in NIH/3t3 cells - controls (grey) or *Cry2* knockouts (green) - treated with
348 0.1 μ M, 0.3 μ M or 0.6 μ M of SMG1i or with vehicle (DMSO) corresponding to the volume used in highest SMG1i treatment.

349 We wished to further validate NMD regulation of the *Cry2* 3' UTR by an approach that would
350 allow more rapid and direct readout of reporter activity after NMD inhibition, rather than having
351 to rely on prolonged 4-OHT treatment of reporter-expressing cells to induce the *Smg6*
352 mutation. To this end, we used a pharmacological inhibitor of the kinase SMG1, hSMG-1
353 inhibitor 11e (SMG1i in the following)³⁸. Briefly, for this compound an IC50 in the sub-
354 nanomolar range had originally been reported³⁸, yet subsequent studies *in vitro*³⁹ and in cells
355 (e.g.⁴⁰) have applied SMG1i at considerable higher concentrations (0.2-1 μ M) to inhibit NMD;
356 additional effects on other kinases (e.g. mTOR³⁸) cannot be excluded under these conditions.
357 Indeed, we observed a strong effect of 0.6 μ M SMG1i on circadian period in two commonly
358 used circadian model cell lines, murine NIH/3t3 fibroblasts (**Fig. 4K, L**) and human U2OS
359 osteosarcoma cells (**Fig. S3**). Of note, the period lengthening phenotype caused by the
360 compound (~4 hours; **Fig. 4L**) was considerably stronger than that seen in the genetic *Smg6*
361 fibroblast model (~1.5 hours; **Fig. 2B**), in line with possibly broader activity of SMG1i.
362 Moreover, cellular toxicity was observable after prolonged SMG1i treatment for several days
363 (data not shown). We thus concluded that this compound would be most appropriate for short-
364 term NMD inhibition up to 24 hours, which is also the timeframe in which it increased
365 endogenous CRY2 protein abundance (**Fig. 4M**). We then assessed how acute SMG1i
366 treatment affected the activity of lentivirally delivered luciferase reporters carrying various core
367 clock gene 3' UTRs, using real-time bioluminescence recording in mouse fibroblasts. Upon
368 addition of SMG1i, output from a reporter carrying the *Cry2* 3' UTR increased rapidly within a
369 few hours (**Fig. 4N**). By contrast, neither the vector 3' UTR, nor the 3' UTRs of other core clock
370 genes that were similar in length to the *Cry2* 3' UTR, namely that of *Per1* (~1 kb) and *Per2*
371 (~2.1 kb), showed increased reporter output. Based on this outcome, we concluded that the
372 *Cry2* 3' UTR was a specific target of the NMD pathway. We next reasoned that the *Cry2* 3'

373 UTR may be NMD-activating due to its length or, alternatively, that it could contain specific
374 *cis*-acting elements important for NMD activity, e.g. specific binding sites for RNA binding
375 proteins (RBPs). To distinguish between these two scenarios, we tested individual,
376 overlapping fragments of the full-length *Cry2* 3' UTR in the reporter assay. In contrast to full-
377 length *Cry2* 3' UTR, none of the fragments was associated with reporter upregulation upon
378 SMG1i treatment (**Fig. 4O**). We concluded that most likely the considerable length of the *Cry2*
379 3' UTR was responsible for downregulation via NMD.

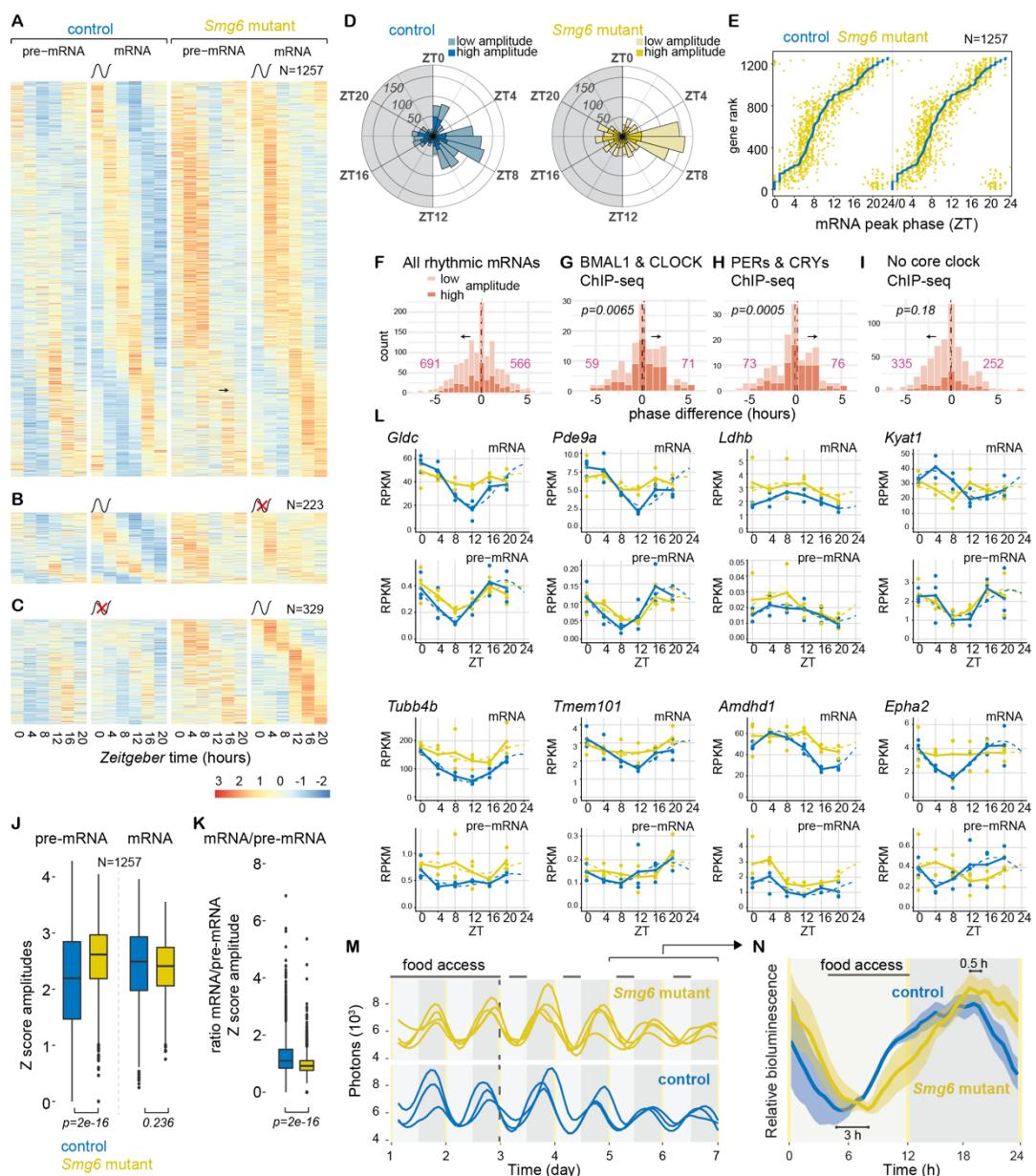
380 With NMD downregulation leading, on the one hand, to longer periods and, on the other hand,
381 to altered abundance and accumulation dynamics of CRY2, we next attempted to investigate
382 whether there was a causal link between both effects. To this end, we produced *Cry2*-deficient
383 NIH/3t3 cells (**Fig. 4P**). We treated these cells with SMG1i, based on the reasoning that NMD
384 inhibition may have a less severe phenotype in the absence of a functional *Cry2* gene.
385 However, in this setup we did not uncover an evident modulation of SMG1i-mediated period
386 lengthening by the absence of *Cry2* (**Fig. 4Q**). A similar outcome was obtained in *Cry2*-
387 deficient U2OS cells (**Fig. S3**). We concluded that the SMG1i-provoked period phenotype was
388 not dependent on *Cry2*. However, given the questions surrounding the specificity of SMG1i
389 detailed above, an interaction of the phenotype with *Cry2* may have been masked by other,
390 stronger effects of the compound. Dedicated experiments using *Smg6*^{mut} cells/livers will thus
391 be required in the future to evaluate to what extent NMD-mediated regulation of *Cry2*
392 contributes to period lengthening.

393 **Transcriptome-wide analyses uncover the extent of rhythmic gene expression 394 reprogramming in the entrained liver**

395 We next analyzed how, beyond the core clock genes (**Fig. 3**), the global rhythmic
396 transcriptome was affected in *Smg6* mutant livers *in vivo*. Our expectation was that we would
397 find a complex overlay of direct and indirect effects, due to (i) NMD directly controlling the
398 mRNA stability for some clock-controlled output genes, which would post-transcriptionally
399 impact on their amplitudes and phases; (ii) the altered phase of *Cry2* and other core clock
400 components (**Fig. 3K**) impacting on the transcriptional timing and dynamics at clock-controlled
401 loci; and (iii) additional secondary consequences that could be both transcriptional and post-
402 transcriptional in nature, as a result of the above effects. We first investigated whether there
403 were global changes in the populations of rhythmic transcripts between the two genotypes,
404 analyzing the RNA-seq datasets from the above cohort (**Fig. 3A**). Using established
405 rhythmicity detection algorithms (MetaCycle R package ³⁶), we found that the majority of
406 mRNAs classified as rhythmic in controls were also rhythmic in the *Smg6* mutant livers
407 (N=1257; **Fig. 5A**) and visual inspection of the pre-mRNA heatmaps further suggested that
408 most of these rhythms were of transcriptional origin. A lower number of transcripts passed the

409 rhythmicity criteria in only one of the genotypes, indicating possible loss (N=223; **Fig. 5B**) or
410 gain (N=323; **Fig. 5C**) of oscillations in the *Smg6* mutants. Inspection of the heatmaps,
411 however, indicated that in many cases, the alleged lack of rhythmicity in one or the other
412 genotype was probably the result of effects such as lower/noisier expression levels rather than
413 clear-cut loss of daily oscillations (a well-known phenomenon when comparing rhythmic gene
414 expression datasets, see ^{41,42}) . We thus first focused our analyses on the common mRNA
415 rhythmic genes. Their peak phase distributions globally resembled each other in the two
416 genotypes (**Fig. 5D**). A large group of mRNAs showed maximal abundance around ZT6-12
417 (an interval that overlaps with the expected peak mRNA phase of direct BMAL1:CLOCK
418 targets containing E-box enhancers ⁴³), and this cluster appeared phase-advanced in *Smg6*
419 mutants. Moreover, several phases were underrepresented in mutants as compared to
420 controls, such as the distinct group of transcripts with maximal abundance at the beginning of
421 the light phase (ZT0-2) in controls that was absent in *Smg6* mutant livers (**Fig. 5D**). For a more
422 quantitative analysis of these effects, we calculated transcript-specific phase differences,
423 which indicated that mRNA phases in *Smg6* mutants globally followed those in controls, with
424 advances and delays spread out across the day (**Fig. 5E**). Overall, more transcripts were
425 phase advanced than delayed in *Smg6* mutant livers (**Fig. 5F**). This outcome was unexpected
426 given that the expression profiles for core clock transcripts (**Fig. 3B-J**), and specifically the
427 findings on *Cry2* (**Fig. 4**), had rather pointed towards a delay of the entrained liver clock in
428 *Smg6* mutants. To investigate these observations further, we overlaid our rhythmic transcript
429 set with data from a large circadian mouse liver ChIP-seq study ²⁰. Our analyses revealed that
430 mRNAs arising from loci with binding sites for BMAL1 and CLOCK (**Fig. 5G**) or PER and CRY
431 proteins (**Fig. 5H**) were indeed significantly skewed towards phase delays, in contrast to
432 rhythmic genes that were not direct targets of these core clock proteins (**Fig. 5I**). We
433 concluded that multiple factors engendered phase changes at the rhythmic transcriptome level
434 in *Smg6* mutants, manifesting in delays for many direct BMAL1:CLOCK targets, and overall
435 advanced phases for many other rhythmically expressed mRNAs. Next, we compared peak-
436 to-trough amplitudes between the genotypes, given that for rhythmic mRNAs that are direct
437 targets of NMD, increased transcript stability in *Smg6* mutants should lead to amplitude
438 reduction. To explore this possibility, we used the Z-scores (**Fig. 5A**) for the common rhythmic
439 transcripts to calculate the amplitudes (maximum-to-minimum fold-changes) for mRNAs and
440 for pre-mRNAs, which we compared between the two genotypes. In *Smg6* mutants, median
441 mRNA amplitudes were lower than in controls, but pre-mRNA amplitudes were higher (**Fig.**
442 **5J**); when normalizing mRNA amplitudes for pre-mRNA fold-changes – as a means to control
443 for differences in transcriptional rhythmicity at the locus – the decrease in rhythmic transcript
444 amplitudes in *Smg6* mutants became highly significant (**Fig. 5K**). This outcome indicated that
445 higher stability of rhythmic mRNAs in *Smg6* mutants was detectable at the global level. In the

446 extreme scenario, an mRNA that is rhythmic in control animals would lose its amplitude to the
 447 extent that it would not anymore be considered as rhythmic at all; it would then group within
 448 the N=223 genes shown in **Fig. 5B**. We inspected their individual gene expression profiles,
 449 which led to the identification of a sizeable number of transcripts that displayed severely
 450 blunted mRNA amplitudes in *Smg6* mutants, despite similar rhythmic pre-mRNAs (i.e.
 451 oscillations in transcription) (**Fig. 5L**). For several of the cases, we can speculate about
 452 possible NMD-eliciting features. For example, according to our previous mouse liver ribosome
 453 profiling data²¹, *Glycine decarboxylase* (*Gldc*) contains translated uORFs (data not shown);
 454 in the case of *Lactate dehydrogenase B* (*Ldhb*), a regulatory mechanism entailing stop codon
 455 readthrough has been demonstrated [31] and could potentially link *Ldhb* translation to NMD
 456 regulation. For the other transcripts shown in **Fig. 5L** (*Pde9a*, *Kyat1*, *Tubb4*, *Tmem101*,
 457 *Amdhd1*, *Epha2*), no obvious candidate NMD-eliciting features were found.



458

459 **Figure 5. Rhythmic RNA expression is altered in *Smg6* mutant livers under entrainment.** **A.** Heatmap of transcripts with significant
460 rhythms at the mRNA level in both genotypes. Expression levels are represented as Z-scores for mRNA or pre-mRNA with color code for
461 low (blue) to high (red) expression. Z-scores were calculated separately for mRNA and pre-mRNA data, but on a common scale for both
462 genotypes. Transcripts are phase-order for the control genotype. **B.** Heatmap as in A., but for transcripts with significant rhythms only in
463 control animals (N=223) and not in mutants. **C.** Heatmap as in A., but for transcripts with significant rhythms only in mutant animals (N=329)
464 and not in controls. **D.** Radial diagrams showing peak phase of rhythmic mRNAs in control (blue) and *Smg6* mutant (yellow) liver for the
465 common transcripts shown in A. Dark shaded: high amplitude rhythmic transcripts; light shaded: low amplitude rhythmic transcripts; high/low
466 cut-off on \log_2 peak-trough amplitude of 1. **E.** Peak phase of mRNA in *Smg6* mutants (orange) relative to control phase (blue), ranked
467 according to the phase in the control (*Smg6* wt) for the transcripts shown in A (N=1257). **F.** Peak phase difference between mutant and
468 control mice for all common rhythmic mRNAs (N=1257). **G.** Peak phase difference of commonly rhythmic mRNAs as in F., but restricted to
469 loci with ChIP-seq binding sites for BMAL1 and CLOCK (N=130), according to ²⁰; p=0.0065; permutation test, calculated by 1000x
470 subsampling of N=130 transcripts from the “all rhythmic transcripts” (N=1257) of panel F, then comparing the means of these subsampling
471 groups with the observed mean (t-test). **H.** Peak phase difference of commonly rhythmic mRNAs as in F., but restricted to loci with ChIP-
472 seq binding sites for the ensemble of proteins PER1, PER2, CRY1 and CRY2 according to ²⁰; p=0.0005; permutation test as in panel G. **I.**
473 Peak phase difference of commonly rhythmic mRNAs as in F., but restricted to loci with no ChIP-seq binding sites for any of the proteins
474 BMAL1, CLOCK, PER1, PER2, CRY1 or CRY2 according to ²⁰; p=0.18; permutation test as in panel G. **J.** Z-score amplitudes – defined as
475 the difference between the maximum and minimum Z-score values, calculated independently for mRNAs and pre-mRNAs of commonly
476 rhythmic transcripts (N=1257) - show lower mean mRNA (p=0.236) and higher mean pre-mRNA amplitudes in mutants (p=2e-16);
477 significance calculations from a linear model (equivalent to t-test). **K.** Transcript mRNA/pre-mRNA Z-score amplitude ratios (from the N=1257
478 common rhythmic transcripts) stratified by genotype show decrease in mutants; p=2e-16; Student’s t-test. **L.** RNA-seq data is plotted for
479 indicated genes for mRNA (upper panels; exonic reads) and pre-mRNA (lower panels; intronic reads) for *Smg6* mutants (yellow) and controls
480 (blue). Rhythmicity of mRNA levels observed in control (blue) is damped or lost in *Smg6* mutant liver (yellow). RPKM values of individual
481 mice are shown as dots with solid lines connecting the means for each timepoint. The dashed lines represent the rhythmic data fit using the
482 parameters from Metacycle ³⁶. **M.** RT-Biolumicorder traces of individual mice in food shifting experiment. After 2 days under *ad libitum*
483 feeding, bioluminescence rhythms (photons) and activity (infrared signal) were recorded for 4 additional days under light-phase-restricted
484 feeding conditions (ZT10-20; horizontal black bar at top); skeleton photoperiod entrainment indicated by yellow vertical lines at ZT12 and
485 ZT0. Each line represents the signal from a control (blue) or a liver-specific *Smg6* mutant (yellow) animal. **N.** Compiled data, averaged over
486 the last two days of the experiment. Mean signal (solid trace) and SEM (shaded). Indicated phase differences calculated from rhythmic fits
487 to the data.

488 Collectively, these analyses demonstrated that the stably entrained liver clock, under *ad*
489 *libitum* feeding and LD12:12 conditions, was subject to phase and amplitude alterations at the
490 level of clock-controlled gene expression. Our *in vivo* recording experiments (**Fig. 2F-H**) had
491 been insensitive to picking up such differences in liver rhythms due to the use of the
492 *mPer2::Luc* reporter allele, whose phase was unaffected by *Smg6* mutation under stable
493 entrainment conditions. We reasoned that under conditions where the stable entrainment was
494 challenged, a phenotype may be unmasked also for *mPer2::Luc*. To this end, we carried out
495 food shifting experiments i.e., switching from *ad libitum* to daytime feeding. Under these
496 conditions, the liver clock receives conflicting timing cues from the SCN and from
497 feeding/fasting cycles, which are not anymore aligned and will eventually lead to an inversion
498 of hepatic oscillator phase due to the dominance of feeding signals for peripheral oscillators
499 ⁴⁴. The kinetics and endpoint of phase adaptation can also be understood as a paradigm of
500 clock flexibility and can be recorded using the RT-Biolumicorder setup ³⁰. Our experiments
501 showed that in *Smg6* mutant animals, after 3 days of feeding during the light phase, daily
502 cycles in bioluminescence had readjusted to a new phase that substantially differed between
503 control and *Smg6* mutant animals (3 hours difference at trough/0.5 hours at peak; **Fig. 5M**,
504 **N**). We concluded that NMD contributes to the adaptation of circadian gene expression to food

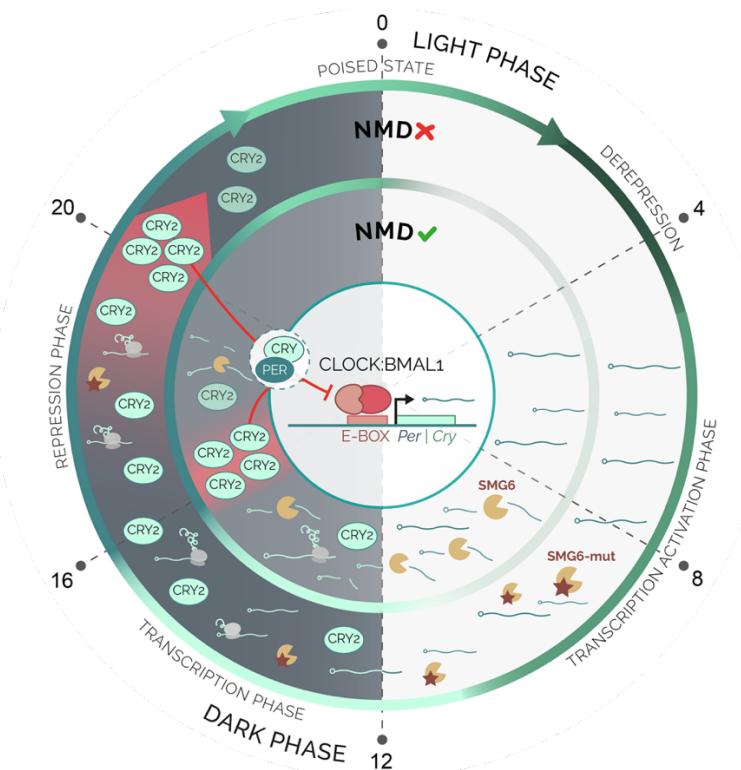
505 entrainment in mouse liver. More generally, the data point to notable differences between
506 *Smg6* mutant and control animals with regard to how different timing cues are integrated within
507 the core clock circuitry.

508 **Discussion**

509 Our novel conditional *Smg6* endonuclease-mutant allele provides unique possibilities to
510 explore *in vivo* activities of the NMD pathway and has allowed us to uncover an unexpected
511 role within the mammalian circadian system, which is a conserved, key mechanism for the
512 organization of daily rhythms in behavior, physiology and metabolism. We find that NMD loss-
513 of-function has a striking impact on free-running circadian periods in two peripheral clock
514 models, primary fibroblasts and liver. Moreover, we determine a specific core clock
515 component, *Cry2*, as NMD-regulated and attribute the NMD-eliciting activity to its long 3' UTR.
516 Although it is widely accepted that efficient mRNA decay is critical for the establishment of
517 gene expression oscillations, which specific pathways mediate the decay of transcripts
518 encoding core clock components has remained largely unknown. That NMD has been co-
519 opted for this purpose, as we find to be the case for *Cry2*, is surprising at first sight – yet it
520 may simply reflect that nature and evolution are opportunistic and employ the available
521 molecular pathways in the most efficient fashion. In line with this idea is the finding that a
522 sizeable number of other rhythmic transcripts appears to rely on NMD to ensure efficient
523 mRNA turnover as well (**Fig. 5L**). Our observations may change the way we should perceive
524 the evolutionary drives relating to NMD: for example, it has been speculated why many
525 mammalian mRNAs contain long 3' UTRs but evade NMD, and a model has been put forward
526 suggesting that such mRNAs have evolved to recruit NMD-inhibiting RBPs in spatial proximity
527 of the termination codon ⁶. However, an opposite drive to attract and retain NMD regulation
528 would be plausible as well – acting on endogenous transcripts, such as *Cry2*, whose intrinsic
529 instability is physiologically important. This idea is in line with findings that in the circadian
530 systems of *Neurospora* ¹⁵, *Arabidopsis* ¹³ and *Drosophila* ¹⁴, roles for NMD have emerged as
531 well.

532 In the absence of NMD, CRY2 protein in liver accumulates to higher levels and for an extended
533 time (**Figure 6**). Based on the experiments presented in our study, we are not yet fully in the
534 position to evaluate to what extent these effects are involved in the period lengthening
535 phenotype. Still, it would be plausible that the phase delay of CRY2 seen in the *Smg6* mutants
536 could be particularly critical. According to around-the-clock ChIP-Seq data from wild-type
537 mouse liver, CRY2 binds and represses its target genes at circadian time CT15-16 ²⁰, thus
538 closely matching the timing of maximal CRY2 abundance in our control mice (ZT16). The
539 ChIP-seq data from wild-type livers further indicates that by CT20, CRY2 is cleared and

540 replaced by CRY1, which binds to chromatin with a peak at around CT0 and is associated
541 with a transcriptionally repressed, but poised state of BMAL1:CLOCK activity. Period
542 lengthening through the prolonged availability of CRY2 may thus involve an extended CRY2-
543 mediated repressive phase and/or CRY2 denying its homolog CRY1 access to its targets,
544 causing a delay in the handover to CRY1. Of note, the period lengthening we observe is
545 phenotypically comparable to that reported for a chemical, selective stabilizer of CRY2 protein,
546 which also prolongs period in reporter assays across several cell types and species ⁴⁵.
547 Moreover, period lengthening has also been reported upon CRY2 stabilisation (in a *Cry1*-
548 deficient background) induced by genetic inactivation of the CRY-specific ubiquitin ligase
549 *Fbxl3* ⁴⁶. For these reasons – and reminiscent of findings on CRY1 accumulation ⁴⁷ – the
550 changed timing of CRY2 accumulation, rather than its generally higher levels, may be a critical
551 feature for the period phenotype and for the phase effects seen in the entrained liver. We thus
552 propose that limiting temporal *Cry2* mRNA availability, mediated through NMD, is an important
553 mechanism within the core loop of the clock by which CRY2 protein biosynthesis is restricted
554 to the beginning of the dark phase when it acts in sync with PER1 and PER2 to repress
555 CLOCK:BMAL1-mediated transcription. Only after duly removal of this repressive complex
556 can CRY1 join and advance the cycle through the late repressive and poised states, eventually
557 leading to the next transcriptional cycle at CLOCK:BMAL1-bound E-box enhancers.



559 **Figure 6. Model of how the daily dynamics of CRY2 accumulation are regulated by NMD.** In the entrained liver clock, *Cry2* mRNA is
560 translated and the protein accumulates with a peak in the dark phase (ZT16 in wild-type). In the absence of a functional NMD pathway, *Cry2*
561 mRNA is stabilized, reaches higher levels, and its translation leads to increased CRY2 at later times (ZT20). The specific phases and states
562 noted at the periphery of the circle (poised, derepression etc.) refer to the findings from Koike *et al.* ²⁰ on E-box binding of clock proteins.

563 Intriguingly, our findings suggest specificity of the phenotype for peripheral clocks. Thus, we
564 were unable to detect an impact on circadian period of the master clock in the SCN. Different
565 explanations may underlie this observation. First, we cannot exclude lack of phenotype due
566 to technical reasons, in particular the lower efficiency of Cre-mediated recombination in SCN
567 neurons, or slow replacement kinetics of wild-type SMG6 by its mutant version due to high
568 protein stability in neurons. For possible biological explanations, the decay of NMD substrates
569 may be less reliant on SMG6 in neuronal cells, or the strong intercellular coupling in the SCN
570 ⁴⁸ renders the clocks resilient against the genetic NMD perturbation and the resulting changes
571 in the critical NMD-regulated transcript. Finally, if the phenotype actually does involve CRY2,
572 it is interesting that it has been reported that the relative importance of the two homologs,
573 CRY1 and CRY2, in the negative feedback loop can be rather tissue-specific, with CRY1 being
574 the main transcriptional repressor in the SCN ⁴⁶, leading to another potential explanation for
575 the observed cell type-specificity. Future experiments will be required to distinguish between
576 these possibilities.

577 In summary, the unexpected role of NMD that we uncover within the circadian system
578 illustrates the ongoing shift in perception of NMD from surveillance to housekeeping functions.
579 We anticipate that our mouse model will provide valuable insights into so-far unidentified NMD
580 targets and functions in mammals *in vivo*, including in the context of pathologies such as
581 neurological diseases ⁴⁹ and cancer ^{50,51}, and in situations where NMD has been identified as
582 a promising therapeutic target ^{52,53}.

583 **Methods**

584 **Animals**

585 All animal experiments were performed according to the cantonal guidelines of the Canton of
586 Vaud, Switzerland, license VD3611. Healthy adult male mice of age 12 – 24 months were
587 used. All mouse lines were maintained on a C57BL/6J background. The alleles *AlbCre-ERT2^{ki}*
588²⁵ and *mPer2::Luc^{ki}*²⁶ have been previously described. The novel *Smg6^{flox}* allele was
589 generated in collaboration with Taconic (official nomenclature of line:
590 *Smg6^{tm5498(D1352A,D1391A)Tac}*).

591 **Primary fibroblasts and immortalization**

592 Adult male *Smg6^{flox/flox}* and *Smg6^{+/+}* control littermate mice were euthanized and approximately
593 1 cm of tail tip was recovered and further sliced into thin pieces under sterile conditions. Tissue
594 fragments were overnight digested with 1 mg/ml collagenase type 1A (Sigma Aldrich) in
595 culture medium at 37°C. The culture medium consists of 15% of fetal calf serum (FCS), 1%
596 Penicillin-Streptomycin-Glutamine (Thermo Fisher Scientific, 10378016), 1% non-essential
597 amino acids (Thermo Fisher Scientific, 11140050), 1 mM sodium pyruvate (Thermo Fisher
598 Scientific, 11360070), 87 mM β-mercaptoethanol, 18 mM HEPES pH 7.0 (Thermo Fisher
599 Scientific, 15630080), 2.5 µg/ml Amphotericin B (Thermo Fisher Scientific, 15290018) and 2.5
600 µg/ml Plasmocin (InvivoGen).

601 Isolated fibroblasts became spontaneously immortal upon continuous culture, creating
602 *Smg6^{flox/flox}* or *Smg6^{+/+}* cell lines. Immortalized fibroblasts were transduced with a retrovirus
603 carrying a tamoxifen-inducible Cre and puromycin resistance (MSCV *CreERT2* puro, Addgene
604 plasmid #22776)⁵⁴. Retrovirus production was performed using the pCL-eco (Addgene,
605 12371)⁵⁵ and pCMV-VSV-G (Addgene, 8454)⁵⁶ plasmids in 293FT HEK cells using the
606 CalPhos™ Mammalian Transfection Kit (Takara bio, 631312). Following 2 µM tamoxifen
607 treatment, renewed every 24h for 4 consecutive days, the cells were utilized for experiments
608 after 7-10 days from the treatment initiation.

609 **DNA genotyping**

610 DNA from cell cultures, liver or kidney tissue was extracted using the DNeasy® Blood & Tissue
611 Kit (Qiagen, 69504) according to the manufacturer's protocol. Genotyping PCR reaction was
612 performed using HotStar Taq DNA polymerase (Qiagen, 203207), 0.4 uM primers
613 (Microsynth), 0.2 uM dNTP mix (PROMEGA, U1511) and approximately 200-700 ng of DNA
614 template. The primer sequences are as follows (5'-3'): Forward: gaa ata cca ggg ccc ttg c ,
615 Reverse1: cat cac tac cca gct cag gaa c, Reverse2: gga ttg gct cct ctt tgc tg. The PCR program
616 is as follows : 15 sec at 95°C, 35 cycles : 1 min at 94°C, 1 min at 61°C, 1 min at 72°C and

617 final elongation at 72°C for 10 min. DNA extraction from dissected SCN tissue was done by
618 Arcturus® PicoPure® DNA Extraction Kit (Thermo Fisher Scientific, KIT0103). PCR reaction
619 was set up as above. The primer sequences are as follows (5'-3'): Forward gaa ata cca ggg
620 ccc ttg c, Reverse2: tct agc tcc ttt ctg cct ctt c. The PCR program is as follows : 15 sec at
621 95°C, 40 cycles : 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and final elongation at 72°C for
622 10 min.

623 **Luciferase reporters and lentiviral production**

624 *CreERT2 Smg6^{flx/flx}* and *Smg6^{+/+}* immortalized fibroblasts were transduced with a lentivirus
625 carrying a dual luciferase (Firefly/Renilla) NMD reporter or a control vector. For the generation
626 of dual luciferase reporter plasmids, the prLV1 dual luciferase reporter plasmid ¹¹ was used,
627 with or without the introduction of an intron downstream of the *Firefly* stop codon. For the latter,
628 the chimeric intron of the pCI-neo vector (Promega, E1841) was cloned into the 3' UTR of the
629 prLV1 vector. The following primers were used for PCR amplification: forward: aaagcggccGCTCGTTAGTGAACCGTC (introducing a NotI restriction site) and reverse:
630 tTTCTCGAGCTGTAATTGAACTGGGAG (introducing a Xhol restriction site). *Dbp-Luciferase*
631 ²³ and the 3' UTR luciferase reporters ¹¹ have been described previously. Lentiviral particles
632 were produced in 293T cells using the envelope vector pMD2.G and the packaging plasmid
633 psPAX2 as previously described ⁵⁷. Filtered viral supernatant was spun 2h at 24,000 rpm, 4°C
634 using Optima L-90K Ultracentrifuge (SW32Tirotor; Beckman, Brea, CA), then viral particles
635 were resuspended with normal growth medium and used for cell transduction.

637 **Circadian bioluminescence recording of cell cultures**

638 Fibroblasts cultured in 35 mm culture dishes (Falcon, 353001) were synchronized either with
639 serum shock (50% horse serum for 3h) or with temperature entrainment (cycles of 16h at 35°
640 C and 8h at 37°C for 5 days). During recording cells were cultured in phenol-free DMEM
641 (Gibco, Thermo Fisher Scientific, 11880028) containing 10% FBS, 1% PSG and 0.1 mM of
642 luciferin, sealed with parafilm to avoid evaporation, in the LumiCycler setup (Actimetrics) at
643 37°C and 5% CO₂. NIH/3T3 murine fibroblasts were cultured under the same conditions as
644 the immortalized fibroblasts but synchronized with 100 nM Dexamethasone treatment for 15
645 min. SMG1 inhibitor (hSMG-1 inhibitor 11e; Probechem Cat. No. PC-35788) ³⁸ was used as
646 10 mM stock (dissolved in DMSO) and, if not indicated otherwise, used at a concentration of
647 0.6 µM (NIH/3T3 experiments) to 1µM (*Smg6^{flx}* fibroblasts).

648 **Dual Luciferase assay**

649 After lentiviral transduction cells were collected using 5x Passive Lysis Buffer (Promega) and
650 luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega,

651 E1910) according to the manufacturer's protocol. *Firefly*-Luciferase signal was normalized to
652 *Renilla*-Luciferase, and for each construct (3' UTR or NMD reporter) this signal was then
653 normalized to that of lentivector-control plasmid (only containing generic vector 3' UTR)
654 treated with vehicle (for each experiment).

655 **RNA sequencing and analysis**

656 Reads were mapped on the mouse genome GRCm38 (Ensembl version 91) using STAR ⁵⁸
657 (v. 2.7.0f; options: --outFilterType BySJout --outFilterMultimapNmax 20 --
658 outMultimapOrder Random --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --
659 outFilterMismatchNmax 999 --alignIntronMin 20 --alignIntronMax 1000000 --
660 alignMatesGapMax 1000000). Read counts in genes loci were evaluated with htseq-count ⁵⁹
661 (v. 0.13.5) for transcript mapped reads (i.e. exons; options: --stranded=reverse --order=name
662 --type=exon --idattr=gene_id --mode=intersection-strict) and for whole locus mapped reads
663 (i.e. exons plus introns; options: --stranded=reverse --order=name --type=gene --
664 idattr=gene_id --mode=union). Read counting for exon analysis was not possible with htseq-
665 count (most reads spanned multiple exons and would have been discarded) so a new python
666 script was developed for this task. To avoid counting reads spanning different exons multiple
667 times, the script calculated average read depth for each exon. Read pileups for gene loci were
668 calculated using samtools depth ⁶⁰ (v. 1.9) and plotted using R (v 4.1.1). Differential expression
669 analysis was done in R using DESqe2 package ⁶¹. RNA stability analysis was performed using
670 RPKM normalised reads counts. Phase analysis was performed using RPKM normalised
671 reads counts and the MetaCycle R package ³⁶.

672 **Induction of liver-specific *Smg6* mutation**

673 8-12 week old male *Smg6*^{fl/fl} mice, carrying the liver-specific Albumin-driven CreERT2
674 (allele *Alb*^{tm1(cre/ERT2)Mtz} ²⁵), and their control littermates (*Smg6*^{+/+}) received 4 intraperitoneal
675 injections of 20 mg/ml tamoxifen (Sigma-Aldrich) in corn oil at a dosage of 75 mg tamoxifen/kg
676 of body weight. The mice were admitted for experiments 4 weeks later.

677 **Liver and kidney explants**

678 Male *Smg6*^{fl/fl} mice and their control littermates *Smg6*^{+/+} were euthanized following deep
679 anesthesia by isoflurane inhalation. Liver and kidney tissue were excised and put immediately
680 in ice-cold Hank's buffer (Thermo Fisher Scientific). The outermost edges of the tissues were
681 carefully excised in a sterile cabinet, and immediately placed on a 0.4 micron Millicell cell
682 culture inserts (PICMORG50) in a 35 mm dish with phenol-free DMEM (Thermo Fisher
683 Scientific, 11880028) containing 5% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml

684 streptomycin and 0.1 mM luciferin. The parafilm-sealed plates were placed for recording in the
685 LumiCycler (Actimetrics) at 37°C and 5% CO₂.

686 **RT-Biolumicorder experiments**

687 Adult male mice, 12-20 weeks of age, carrying the genetically encoded circadian reporter
688 allele *mPer2::Luc*²⁶ were used for the RT-Biolumicorder experiments. The experimental
689 procedure followed our recently published protocol²⁹. Briefly, Alzet mini-osmotic pumps
690 (model 100D5 or 2001) were filled with 90 mg/ml with D-Luciferin sodium salt, dissolved in
691 Phosphate Buffered Saline (PBS, pH 7.4) under sterile conditions. The pumps were closed
692 with blue-colored flow moderators (ALZET) and activated at 37°C according to the
693 manufacturer's instructions, followed by the subcutaneous, dorsal implantation. As analgesics
694 Carprofen (Rimadyl, 5 mg/kg subcutaneous), and paracetamol (2 mg/ml, via drinking water)
695 were administered. Prior implantation the dorsal area of the mouse at the site where the liver
696 is positioned was shaved using an electric razor. The RT-Biolumicorder (Lesa-Technology)
697 consists of a cylindrical cage for a single mouse with photon-reflecting walls, equipped with a
698 photomultiplier tube (PMT), water and food containers and a built-in infrared sensor that
699 records locomotor activity^(29,30). The RT-Biolumicorder records photon and activity levels in 1
700 min intervals. The data, which also contain light and food access information, were saved as
701 text files and later analyzed using the MatLab-based "Osiris" software according to²⁹ or a
702 custom-made R script.

703 **Running wheel experiments**

704 12-16 week old male mice were single-housed in cages equipped with a running wheel and
705 were placed in a light-tight cabinet. After approximately 10 days of habituation in 12h light-12h
706 dark the mice were released in constant darkness for approximately 14 days. For the running
707 wheel experiments with SCN-specific *Smg6* mutant recombination, the same protocol was
708 used, followed by 14 days of post-injection recovery under 12h-light-12h-dark conditions and
709 a second period of constant darkness for 14 days (adapted from⁶²).

710 **SCN-specific *Smg6* mutant mice**

711 Male adult *Smg6*^{fl/fl} mice and their control littermates (*Smg6*^{+/+}) received bilateral
712 stereotactic injections of CMV.HI-Cre::eGFP AAV5 particles (AddGene, 105545) into the SCN
713 (400 nl per site). Stereotactic coordinates: AP= - 0.34 ML= +/- 0.4, V=5.5. Ketamine/Xylazine
714 (80/12.5 mg/kg) by intraperitoneal injection was used as anesthetic and 5 mg/kg carprofen
715 was administered subcutaneously for analgesia. Additionally, paracetamol (2 mg/ml) was
716 administered via drinking water prior and 3 days following the procedure. Animal recovery was
717 monitored for ten days. Mice carrying *mPer2::Luc*²⁶ in addition to *Smg6*^{fl/fl} (experimental)

718 or *Smg6*^{+/+} (control) were used for the bioluminescence recording of SCN slices. For
719 evaluation of viral targeting, mice were transcardially perfused with phosphate-buffered saline
720 (PBS) followed by 4% paraformaldehyde (PFA). Brains were post fixed overnight in 4% PFA
721 at 4°C and then cryopreserved in 30% sucrose solution in PBS for at least 24 hours at 4°C
722 (until completely sunk to the bottom of the container). Cryopreserved brains were frozen and
723 sliced in 25 µm thick sections. Sections were mounted using DAPI-fluoromount. Fluorescent
724 images were acquired on a ZEISS Axio Imager.M2 microscope, equipped with ApoTome.2
725 and a Camera Axiocam 702 mono. Specific filter sets were used for the visualization of green
726 (Filter set 38 HE eGFP shift free [E] EX BP 470/40, BS FT 495, EM BP 525/50) and blue (Filter
727 set 49 DAPI shift free [E] EX G 365, BS FT 395, EM BP 445/50) fluorescence. For genomic
728 DNA extraction, fresh brain tissue was collected in RNAlater solution and kept at 4°C for 2
729 weeks. Then 250 µm thick sections containing the SCN were sliced using a microtome and
730 the SCN region was microdissected under a fluorescent equipped stereomicroscope (Nikon
731 SMZ-25).

732 **SCN slices and bioluminescence recording**

733 Approximately 14 days later, following bilateral stereotactic injections, the mice were sacrificed
734 and the SCN was dissected. Slices of 350 µm around the area of SCN were prepared with a
735 tissue chopper between ZT4.8 and ZT6.3; 2 slices per animal were used. Slicing and recovery
736 buffer contained of NMDG aCSF (85 mM NMDG, 9 mM MgSO₄, 2.3mM KCl, 1.1 mM
737 NaH₂PO₄, 0.5 mM CaCl₂, 23 mM D-Glucose, 28 mM NaHCO₃, 18 mM Hepes, 3 mM Na-
738 pyruvate, 5 mM Na-ascorbate and 2 mM thiourea; pH 7.3-7.4; 300-310 mOsm/Kg according
739 ⁶³. Each slice was cultured in a single well of a 24-well plate in 300 µl of culture medium (0.7
740 x MEM Eagle medium with 1.7 mM MgSO₄, 0.8 mM CaCl₂, 11 mM D-Glucose, 17 mM
741 NaHCO₃, 25 mM Hepes, 0.4 mM GlutaMAX, 17% Horse serum, 0.8 mg/L Insulin, 0.8495 mM
742 Ascorbic acid, 1% penicillin/streptomycin and 100 µM Luciferin; pH 7.3-7.4; 300-310
743 mOsm/Kg according ⁶³). Viral infection and accurate injection localization of the SCN was
744 evaluated with fluorescent imaging with THUNDER Imaging Systems widefield microscope
745 (Leica) on the 8th day in culture. Circadian bioluminescence was monitored by using
746 photomultiplier tubes (PMTs) for approximately one week at 34.5 °C with 5% CO₂ (in-house
747 built device).

748 **Protein extraction and Western Blot**

749 Total proteins from mouse liver were extracted in principle according to the NUN procedure
750 ⁶⁴. Freshly harvested liver extracts were homogenized in 2 tissue volume of Nuclear Lysis
751 Buffer (10 mM Hepes pH 7.6, 100 mM KCl, 0.1 mM EDTA, 10% Glycerol, 0.15 mM spermine,
752 0.5 mM spermidine) for 20 seconds using a Teflon homogenizer. 4 tissue volumes of 2x NUN

753 Buffer (2M Urea, 2% NP40, 0.6 M NaCl, 50 mM Hepes pH 7.6, 2 mM DTT, 0.1 mM PMSF and
754 supplemented with complete protease inhibitor tablets, Roche) were added dropwise and on
755 a vortex with constant low speed to ensure immediate mixing. The lysates were incubated on
756 ice for 30 min and then cleared through centrifugation at 10000 rpm, 4°C, for 20 min.
757 Supernatants were stored at -80°C. Aliquots of the lysates (20-30 µg of protein loaded per
758 lane, either from a pool from 3 mice or from individual mice, as indicated) were separated by
759 SDS-PAGE and transferred to PVDF membrane by dry transfer using an iBlot 2 gel transfer
760 device. After blocking (5% milk in TBST; for 1 hour at room temperature), the membrane was
761 incubated overnight at 4°C with appropriate dilutions of primary antibodies, including anti-
762 CRY2 (kind gift from Ueli Schibler, Geneva), anti-ATF5 (Abcam-ab184923), and anti-HSP90
763 (Cell signaling-4874), p-eif2alpha (Cell signaling-9721), eif2alpha (Cell signaling-9722).
764 Following TBST washing (3 x 5 minutes), the membranes were incubated with the appropriate
765 secondary antibody conjugated with HRP for 60 minutes at room temperature, followed by
766 washing as above. Chemiluminescence signal was detected with Supersignal West Femto
767 Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095), as described by the
768 manufacturer. The quantification of bands was performed using ImageJ software.

769 **Data and script availability**

770 Data has been deposited at GEO (reviewer token). Computational scripts are accessible at:

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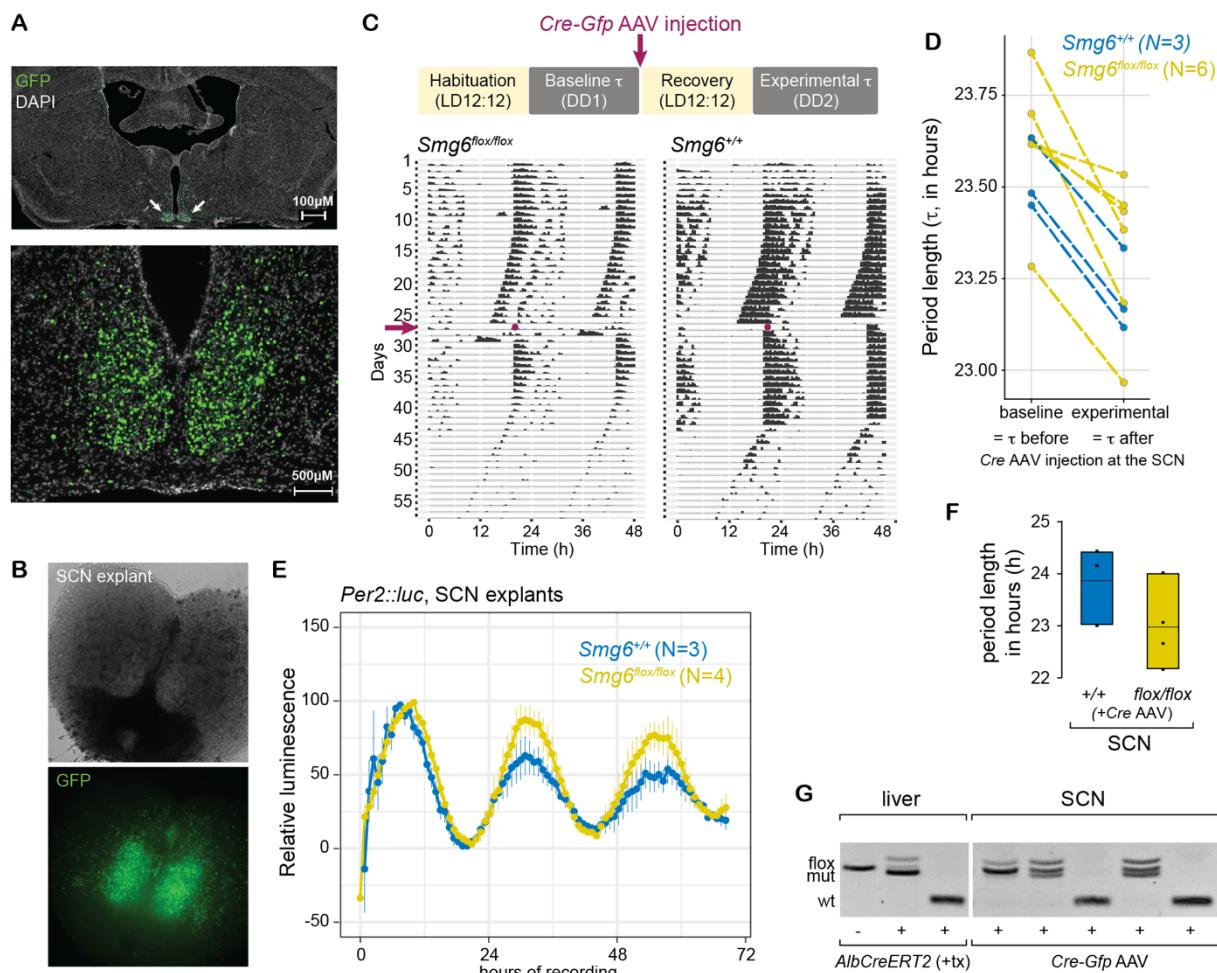
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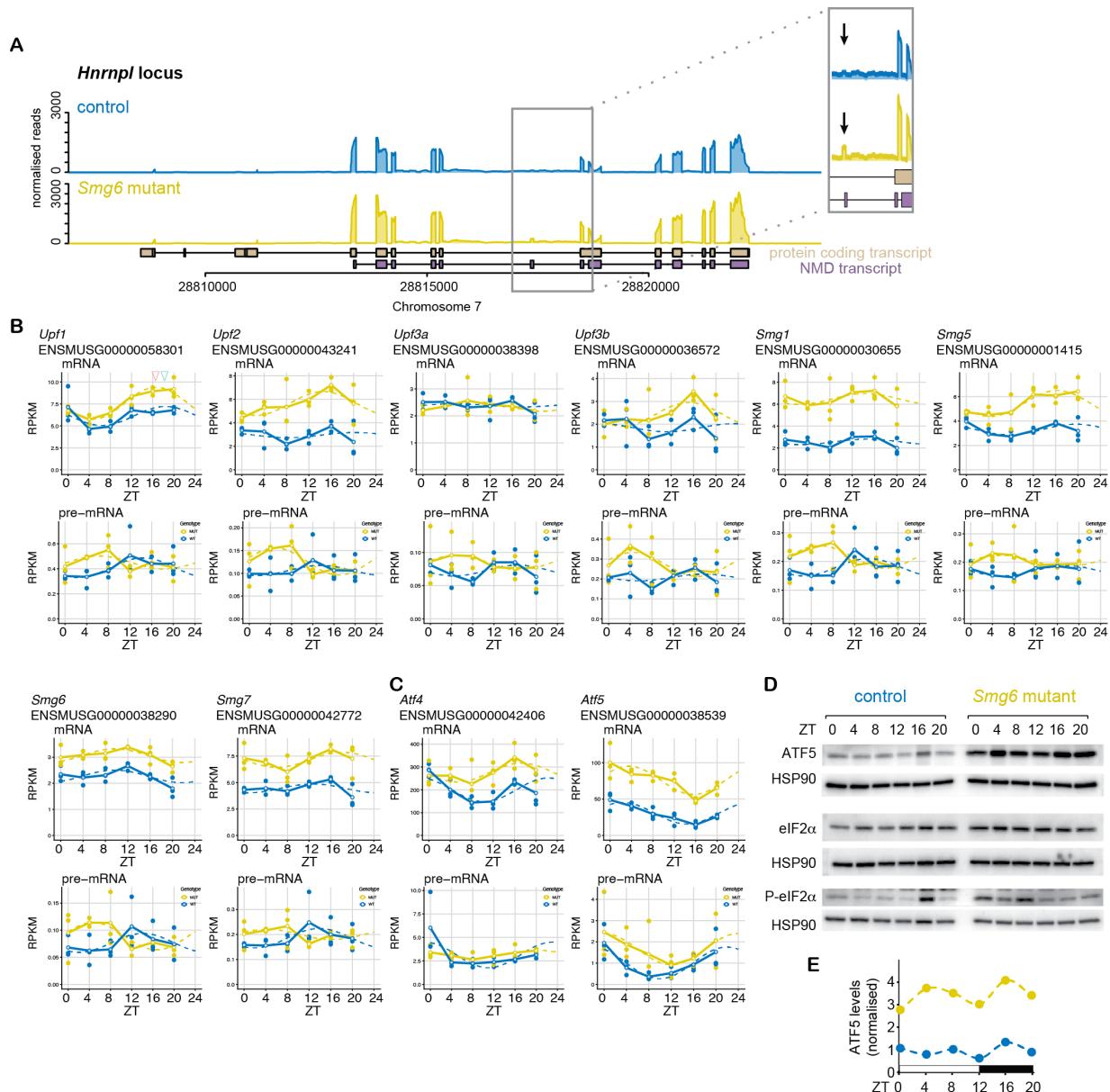
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921

922 **Supplementary material**

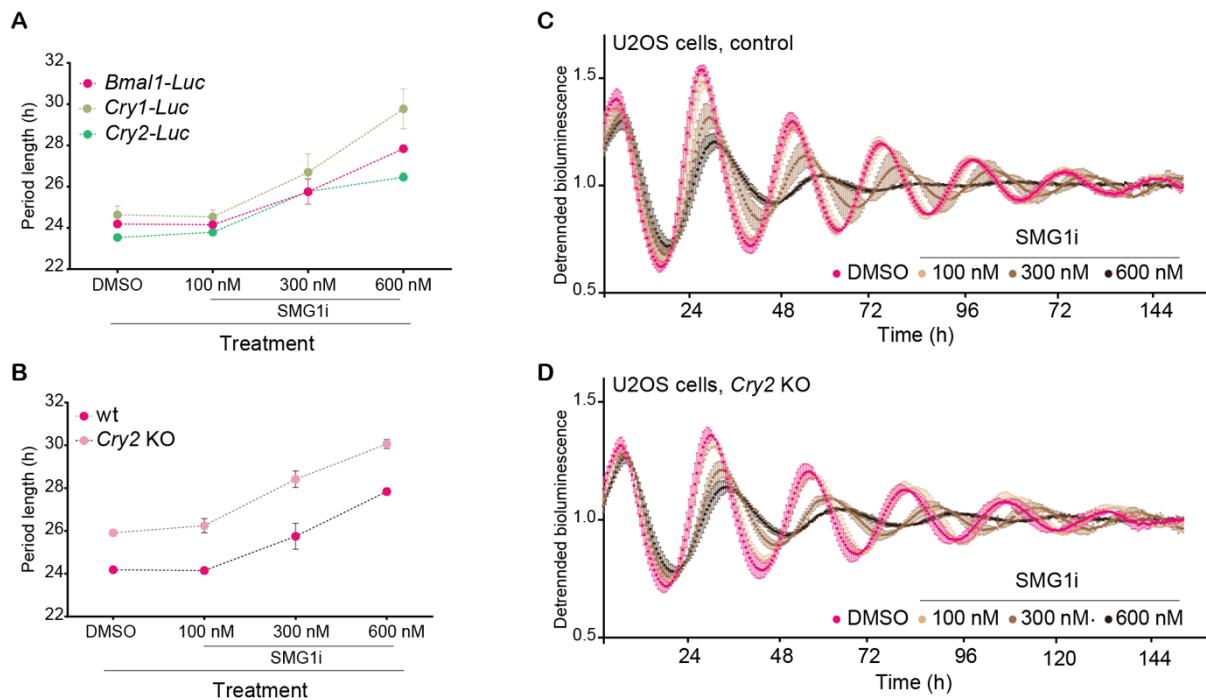


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924 **Supplementary Figure S1.** **A.** Representative microphotographs of SCN sections to assess effective targeting of
925 the SCN. Viral expression can be estimated from GFP signal, encoded with Cre on the same virus. **B.** Same as A,
926 but image taken during bioluminescence recording of SCN slices. **C.** Upper diagram: Rhythms of voluntary
927 locomotor activity were recorded prior to and after the SCN injection of the Cre- and GFP-expressing AAV. Lower:
928 Representative actograms of a *Smg6^{flox/flox}* and *Smg6^{+/+}* mouse. The day of Cre::eGFP AAV injection is marked by
929 an arrow and a dot. **D.** Period lengths of circadian locomotor activity rhythms of *Smg6^{flox/flox}* (in yellow) and *Smg6^{+/+}*
930 (in blue) mice before (DD1) and after (DD2) stereotaxic surgery. **E.** Averaged traces of *mPer::Luc* rhythms of AAV-
931 injected *Smg6^{flox/flox}* (yellow) and *Smg6^{+/+}* (blue) SCN explants. **F.** Period lengths of *mPer::Luc* expression in AAV-
932 injected *Smg6^{flox/flox}* (yellow) and *Smg6^{+/+}* (blue) SCN explants. **G.** Recombination efficiency following Cre induction
933 was evaluated by genotyping of genomic DNA extracted from SCN slices (liver-specific mutants served as controls
934 for the genotyping).



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Supplementary Figure S2: A. Read coverage on the *Hnrnp1* locus indicates the specific upregulation of transcript isoforms that are NMD-annotated and that can be identified by specific exons (see arrows in insets) in liver tissue. **B.** RNA-seq data is plotted for indicated genes – that all encode components of the NMD machinery itself – for mRNA (upper panels; exonic reads) and pre-mRNA (lower panels; intronic reads) for *Smg6* mutants (yellow) and controls (blue). RPKM values of individual animals are shown as dots with solid lines connecting the means for each timepoint. The dashed lines represent the rhythmic data fit using the parameters from Metacycle. **C.** RNA-seq data is plotted for *Atf4* and *Atf5* for mRNA (upper panels; exonic reads) and pre-mRNA (lower panels; intronic reads) for *Smg6* mutants (yellow) and controls (blue). RPKM values of individual animals are shown as dots with solid lines connecting the means for each timepoint. The dashed lines represent the rhythmic data fit using the parameters from Metacycle. **D.** Western blot analysis of liver tissue (as in Fig. 4D) for ATF5, eIF2 α and phospho-eIF2 α in *Smg6* mutant and control liver samples; HSP90 served as loading control. **E.** Quantification of ATF5 signal, normalized to HSP90 as loading control, from Western blot shown in D.



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949 **Supplementary Figure S3: Pharmacological NMD inhibition prolongs circadian period in human**
950 **osteosarcoma U2OS cells. A.** Period length of the circadian reporters *Bmal1-Luc* (fuchsia), *Cry1-Luc* (khaki) or
951 *Cry2-Luc* (green) in the presence of increasing concentrations of SMG1i or vehicle (DMSO, equal volume as for
952 the highest SMG1i dose). **B.** Period length of the circadian reporter *Bmal1-Luc* in wt (pink) or *Cry2* KO (fuchsia)
953 U2OS cells **C.** Traces of *Bmal1-Luc* detrended bioluminescence signal in wild-type U2OS cells treated with
954 increasing dosage of SMG1i or vehicle. Solid circles represent mean, error bars represent standard deviation. **D.**
955 Average traces of *Bmal1-Luc* detrended bioluminescence signal in *Cry2* KO U2OS cells treated with increasing
956 concentrations of SMG1i or vehicle. Solid circles represent mean, error bars represent standard deviation.