RESEARCH ARTICLE

A Novel Bmal1 Mutant Mouse Reveals Essential Roles of the C-Terminal Domain on Circadian Rhythms

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Abstract

The mammalian circadian clock is an endogenous biological timer comprised of transcriptional/translational feedback loops of clock genes. Bmal1 encodes an indispensable transcription factor for the generation of circadian rhythms. Here, we report a new circadian mutant mouse from gene-trapped embryonic stem cells harboring a C-terminus truncated Bmal1 (Bmal1GT ΔC) allele. The homozygous mutant (Bmal1GT ΔC/GT ΔC) mice immediately lost circadian behavioral rhythms under constant darkness. The heterozygous (Bmal1+/GT ΔC) mice displayed a gradual loss of rhythms, in contrast to Bmal1+/- mice where rhythms were sustained. Bmal1GT ΔC/GT ΔC mice also showed arrhythmic mRNA and protein expression in the SCN and liver. Lack of circadian reporter oscillation was also observed in cultured fibroblast cells, indicating that the arrhythmicity of Bmal1GT ΔC/GT ΔC mice resulted from impaired molecular clock machinery. Expression of clock genes exhibited distinct responses to the mutant allele in Bmal1+/-/GT ΔC and Bmal1GT ΔC/GT ΔC mice. Despite normal cellular localization and heterodimerization with CLOCK, overexpressed BMAL1GT ΔC was unable to activate transcription of Per1 promoter and BMAL1-dependent CLOCK degradation. These results indicate that the C-terminal region of Bmal1 has pivotal roles in the regulation of circadian rhythms and the Bmal1GT ΔC mice constitute a novel model system to evaluate circadian functional mechanism of BMAL1.

Introduction

Most living organisms harbor biological timers called circadian clocks to drive daily physiological and behavioral rhythms. In mammals, the molecular circadian clock is composed of interlocked feedback loops. The CLOCK:BMAL1 heterodimer activates the transcription of clock genes such as Periods (Per1/2), Cryptochromes (Cry1/2), Rors and Rev-erbs. The translated PER
and CRY proteins subsequently repress the activity of CLOCK:BMAL1. RORs and REV-ERB proteins contribute to rhythmicity via the competitive regulation of Bmal1 promoter activity [1, 2].

The clock is endowed with the intrinsic redundancy due to homologous genes encoding functionally overlapping components. For example, while Per1 and Per2 double knock-out mice exhibited an immediate loss of circadian rhythm in constant darkness, single gene knock-out mice retained largely normal rhythms. Interestingly, Bmal1 is unique among the core clock genes in that its disruption alone leads to arrhythmicity [3]. Bmal1−/− mice have also been shown to display various physiological deficits including defective glucose/lipid metabolism, progressive arthropathy, early aging and decreased longevity [4–6]. Therefore, Bmal1−/− mice have been a valuable animal model for evaluating the impact of circadian rhythms on physiology and behavior. However, several lines of evidence from tissue-specific rescue experiments indicated that certain phenotypes may result from the consequences of tissue-specific, as opposed to core clock, functions of Bmal1 and expression of its paralogous gene Bmal2 can rescue several phenotypes of Bmal1−/− mice including circadian rhythmicity [7–9]. Taken together, various phenotypes of Bmal1−/− mice, which are not related to the rhythm itself have limited further analysis of its circadian and physiological roles.

The C-terminal region of BMAL1 plays an important regulatory function for periodic oscillation. The deletion or site-directed mutagenesis in this region leads to loss of circadian rhythms [10, 11]. Several studies demonstrated that the binding sites for transactivation factors or CRYs reside in the C-terminal region [12–14]. In particular, the G and H domains of BMAL1 have been reported as important regions for generating circadian rhythms [11]. These results raise the possibility that BMAL1 C-terminal truncation or specific mutation is sufficient to abrogate rhythms while leaving other domains intact in vivo.

Here, we report a new animal model for evaluating the roles of Bmal1 in circadian rhythm and physiology. The mice carrying a C-terminus truncated Bmal1 allele (Bmal1GTAC) were generated from gene-trapped ES cells. Although the heterozygous (Bmal1+GTAC) and homozygous (Bmal1GTACGTAC) mutant mice also suffer loss of circadian rhythms, these mice displayed significantly distinct molecular and physiological phenotypes compared with the severely compromised Bmal1−/− mice, providing a novel animal model for understanding the roles of Bmal1 in mammalian clock functions.

Materials and Methods

Generation of Bmal1GTAC mice

To generate Bmal1GTAC mice, ES cells harboring C-terminus truncated Bmal1 gene were obtained from Sanger Institute Gene Trap Resource (SIGTR, Cambridge, UK) [15]. The ES cells were injected into blastocysts and chimeric mice were generated as described previously [16]. The genotypes were determined by using three primer polymerase chain reaction (PCR) method (WT-F, 5′-CCTCTCCAGGCGTCTCTGTTTCTG-3′, WT-R, 5′-TGACGCTGCCCTGATAG-3′ and Bmal1GTAC-R, 5′-GGCCAAGTTTGTTTCCCTGTA-3′). The mice were backcrossed for six generations on C57BL/6 mice purchased from the Orient Bio (Orient Bio Inc., Seongnam, Korea). For generating littermates, ten-weeks-old male mice were paired at a ratio of 1:2 with female mice.

Housing conditions and monitoring of wheel running activity

After genotype selections, male mice were transferred to a conventional animal facility and housed individually in Nalgene cages (Nalge Nunc, Rochester, NY). All mice were maintained in a specific pathogen free (SPF) animal facility and a sound-proof isolated room with a
constant temperature (22–23°C) and humidity (50 ± 10%). Lights were on at 08:00 AM and off at 20:00 PM (L:D = 12h:12h). Illumination was provided by 32 W cool white fluorescence bulbs adjusted to 350 lux at the bottom of cage and completely blocked during the scotophase (< 0.5 lux). Mice were housed with a wood-chip bedding [17] and fed ad libitum with NIH-31 rodent chow (Zeigler Brothers, Gardners, PA) and tap water. All animal experiments were made to minimize suffering and approved by Seoul National University Institutional Animal Care and Use Committee. For wheel running activity experiments, the mice were transferred to a cage supplemented with a wheel running assembly at 8 weeks of age (Mini-Mitter, Bend, OR). After 1 week of adaptation, the wheel running activity was recorded at 6 min intervals and the raw data files were analyzed as described previously [18]. The fast Fourier transform (FFT) and free running period (FRP) were analyzed by ClockLab data analysis program (Actimetrics, Wilmette, IL). For the circadian tissue sampling, mice were sacrificed every 6hr from 30hr to 48hr in a constant darkness condition.

Cell culture, transfection, and luciferase assay

NIH-3T3 and mouse embryonic fibroblasts (MEFs) were maintained in DMEM containing 10% FBS, 100 units penicillin and 100 g/ml streptomycin. For MEF preparations, 13.5 day old mouse embryos were collected and dissociated by mincing and Trypsin-EDTA treatment [19]. Transfections were performed by using Lipofectamine and Plus reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). For luciferase assays, 10 ng of pmPer1 6.8Kbp-luciferase construct was co-transfected with other effectors and total quantity of DNA was adjusted by pcDNA 3.0. Cells were harvested and luciferase activities were measured by dual luciferase assay system. The transfection efficiency was adjusted by renilla luciferase activity of pRL-TK (Promega, Madison, WI). All assays were performed in triplicate and repeated three times independently.

In situ hybridization

Mouse brains were immediately frozen in liquid nitrogen-chilled isopentane. Frozen sections (12mM) were cut coronally and fixed with 4% paraformaldehyde and dehydrated. Riboprobes were produced from the plasmids containing Per2 (GenBank accession number: AF036893, n.t. 186–555) and Bmal1 (GenBank accession number: AF022992, n.t. 1261–1614) cDNA. The probes for Bmal1 mRNA were designed to detect both Bmal1wt and Bmal1GTΔC. Antisense ribopores were prepared by in vitro transcription using α-[35S] UTP as manufacturer’s instruction (Promega, Madison, WI). Sections were hybridized with the riboprobes overnight at 52°C and washed with sodium-saline citrate. Then, the sections were treated with RNase A (20 mg/ml) for 30 min at 37°C, and rinsed. Radioactive signals were visualized by exposing the sections to β-max film for 7 days (Kodak, Rochester, NY).

mRNA analysis

Mouse liver samples were immediately frozen by liquid nitrogen at the indicated times and stored at -80°C until the analysis. For mRNA analysis, total RNAs were extracted from the liver by the single step acid guanidinium thiocyanate-phenol-chloroform method. The purified RNA was reverse-transcribed according to the manufacturer’s instructions (Promega). The mRNA levels were quantified by the real-time PCR using following primer sets and normalized by GAPDH levels. Per1-F, 5'-GTGTCTGGATTAATAAGTGAG-3', Per1-R, 5'-ACCACTCATGCTGTGGCC-3'; Per2-F, 5'-GGGGATGCTGTTGGAAATCTT-3', Per2-R, 5'-GCTCTTTCCAGGCTCCCTATC-3'; Bmal1-F, 5'-CTAATTCTCAGGCGCACAG-3', Bmal1-R, 5'-TCAGCTCGTGGCATCATTGAGT-3', Clock-F, 5'-TTGCTCCAGGGAATCT-3'.
Clock-R, 5′-GGAGGGAAAGTGCTCTGTTGTAG-3′, Rev-erbα-F, 5′-AAGACATGACGACCCTGGAC-3′, Rev-erbα-R, 5′-GAATGCAACTCCCT-3′, Cry1-R, 5′-AAAAATTCAGCCACAGGAG-3′, Dbp-F, 5′-CAAGAACATGAAAGCAAGCAGAGG-3′, Dbp-R, 5′-AGGGCAAGACAGGAACAATTAC-3′, GAPDH-F, 5′-CATGGCCTCCTTGTTCTCT-3′, GAPDH-R, 5′-CCTGCTTCACACCTCTCTTGA-3′. The probes for Bmal1 mRNA were designed to detect both Bmal1wt and Bmal1GTAC.

Immunoprecipitation (IP) and immunoblotting (IB)

For IB experiments, mouse liver tissues and cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 8.0], 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na3VO4, and 1X protease inhibitor cocktail (Sigma, St. Louis, MO). Proteins were resolved on 6% or 8% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membrane was blocked for 1 h at room temperature in 10% skim milk solution and incubated with several primary antibodies such as anti-BMAL1 [20], CLOCK (SantaCruz, Dallas, TX), β-GAL (Promega, Madison, WI), MYC (SantaCruz, Dallas, TX), ACTIN (SantaCruz, Dallas, TX). Immunoreactive bands were visualized with ECL reagents (Thermo Scientific, Waltham, MA) according to manufacturer’s instructions. For IP experiments, NIH3T3 cells were transfected with the indicated plasmids. At 36 hours posttransfection, the cells were lysed with RIPA buffer and centrifuged at maximum speed for 20 min at 4°C. Equal amounts of total protein were incubated with 2 μg of anti-Flag M2 (Sigma, St. Louis, MO) for 1.5 h at 4°C and then added protein A/G-Sepharose bead slurry. The final immune complexes were analyzed by immunoblotting with indicated antibodies [21].

Real-time monitoring of bioluminescence

To observe endogenous circadian rhythms of Bmal1WT and Bmal1GTAC/GTAC, MEFs were generated from embryos of mutant mice that were bred with PER2::LUC knock-in mice [22]. At 6th passage, those MEFs were incubated with 1μM dexamethasone (DEX) for 2hr, then, media were changed with a recording media containing 100μM D-luciferin (Promega). To analyze the effect of Bmal1GTAC on the circadian rhythm by the transient transfection, immortalized wild type (WT) MEFs were transfected with mutant clones and dual-color luciferases (Per2-SLR2 and Bmal1-Eluc). After 36hr of transfection, cells were synchronized by 1μM DEX treatment and bioluminescence was measured for 1min at 10min interval using KronosDio (ATTO Corporation, Tokyo, Japan) [21]. Each luciferase activity was calculated as previously reported [23]. The period length and amplitude were calculated as previous described [21].

Fluorescence and bimolecular fluorescence complementation (BiFC) assays

To analyze the effect of Bmal1 C-terminal region on the localization of BMAL1 and CLOCK simultaneously, VENUS protein was fused to BMAL1WT and BMAL1GTAC, and CERULEAN (CERUL) to CLOCK as indicated [24]. For BiFC assay, amino acid residues 1 to 172 (VN) and 173 to 238 (VC) of VENUS protein was fused to BMAL1 and CLOCK, respectively. NIH-3T3 cells were transfected with the indicated constructs, incubated at 37°C for 36hr and visualized by using Delta-Vision fluorescence microscope (Applied Precision, Isaquah, WA).
Statistical analysis
Data were analyzed by one-way or two-way analysis of variance (ANOVA) with Tukey post-tests using GraphPad PRISM (GraphPad Prism Software, La Jolla, CA). A p value less than 0.05 was considered as a significant difference.

Results

Generation of the Bmal1<sup>GTΔC</sup> mice
We obtained ES cells from SIGTR where the gene trap vector was inserted in the Bmal1 gene locus. To identify the precise insertion site, we sequenced the 18th intronic DNA region of Bmal1 based on SIGTR sequence tag information (#CE0167, Arntl<sup>Gr(CE0167)Wtsi</sup>) [15]. The gene trap vector containing Engrailed 2 (En2), self-cleaving sequence, β-Geo (β-Galactosidase + neomycin) and poly(A) signal was found to be inserted at 3704 bp away from the 18th exon (Fig 1A and S1A Fig). cDNA sequencing revealed that the 18th intron of Bmal1<sup>GTΔC</sup> was successfully spliced out and the 17th exon was connected to the En2 exon, self-cleaving sequence 2A and β-Geo to generate a functional mRNA (Fig 1A and S1A Fig). Domain structures of WT and mutant BMAL1 proteins are illustrated (Fig 1B and S1B Fig). Although the hypothetical size of the fusion protein is approximately 180 kDa, β-GALACTOSIDASE (β-GAL) antibody detected 110 kDa proteins, indicating a functional self-cleaving 2A peptide sequence (Fig 1D). The genotypes were further determined by three primers PCR and immunoblotting (Fig 1C and 1D). Collectively, these results indicated that the gene trap vector was inserted at the 18th intronic region of Bmal1 generating C-terminus truncated BMAL1 (residues 1–538) plus 52 amino acids from EN2 and the self-cleaving sequence. Using these heterozygous male and female mutant mice, we generated WT, heterozygous and homozygous mice for the experiments. The genotypes of littermates exhibited a 1:2:1 ratio, indicating no overt effect of Bmal1<sup>GTΔC</sup> allele on embryonic development (Table 1).

Altered circadian rhythms in Bmal1<sup>GTΔC</sup> mice
We next examined the circadian behavior of Bmal1<sup>GTΔC</sup> mice by monitoring wheel-running activities. To directly compare with Bmal1 null mutants, we also examined the behavior of Bmal1<sup>+/+</sup> and Bmal1<sup>−/−</sup> mice. Bmal1<sup>+</sup>/<sup>GTΔC</sup> mice showed unstable circadian periodicity for ~20 days under the constant darkness (DD) condition. Compared with WT and Bmal1<sup>−/−</sup> mice (Fig 2A), 90% (10/12) of the Bmal1<sup>+</sup>/<sup>GTΔC</sup> mice showed arrhythmic behaviors after ~20 days in DD. The remaining mice also lost their rhythms after showing extremely long FRP (S2A Fig). Initially, Bmal1<sup>+</sup>/<sup>GTΔC</sup> mice did not have significantly altered FRP during first 5 days in DD. However, their FRPs were lengthened continuously followed by rhythm loss. The FRP in the last 5 days before losing the rhythms was significantly increased (Fig 2B). However, the amplitude of Bmal1<sup>+</sup>/<sup>GTΔC</sup> mice in the last 5 days was not significantly changed (S2C Fig). These unusual circadian behaviors of Bmal1<sup>+</sup>/<sup>GTΔC</sup> mice were not found in WT and Bmal1<sup>−/−</sup> mice (Fig 2A). As expected, Bmal1<sup>GTΔC/GTΔC</sup> immediately became arrhythmic under DD. Furthermore, Bmal1<sup>−/−</sup>/<sup>GTΔC</sup> mice also lost behavioral rhythms (S2B Fig). Interestingly, the total locomotor activities of Bmal1<sup>GTΔC/GTΔC</sup> mice were comparable to those of WT mice, whereas Bmal1<sup>−/−</sup> mice manifested significantly decreased activities regardless of LD cycles as reported previously (Fig 1C and S2D Fig) [9]. These results strongly suggested that the truncation of BMAL1 C-terminal region is sufficient to disrupt circadian rhythm, which may involve a different mechanism compared to the null mutant mice.
Altered mRNA and protein expression of clock genes in \textit{Bmal1}\textsuperscript{GTAC} mice

To understand the arrhythmic circadian behavior of \textit{Bmal1}\textsuperscript{GTAC} mice, we examined the mRNA and protein expression profiles of clock genes. As shown in Fig 3A, \textit{Per2} mRNA expression of...
WT mice showed peak expression at CT12 in the SCN, while reaching nadir expression at CT24. These patterns were also observed in Bmal1\textsuperscript{+/GTΔC} mice. However, Per2 mRNA expression was arrhythmic in Bmal1\textsuperscript{GTΔC/GTΔC} mice and remained at low levels. Interestingly, although Bmal1 mRNA expression was rhythmic in both WT and Bmal1\textsuperscript{+/GTΔC} mice, greater levels were observed in Bmal1\textsuperscript{GTΔC/GTΔC} mice. The increase in mRNA levels was more pronounced in Bmal1\textsuperscript{GTΔC/GTΔC} mice that showed arrhythmic expression.

To further substantiate and quantify these patterns, we examined the mRNA expression profiles of several clock genes in the liver. The oscillation of Per1 and Per2 mRNA was abrogated in Bmal1\textsuperscript{GTΔC/GTΔC} mice, but there was no difference in their rhythmic expression between WT and Bmal1\textsuperscript{+/GTΔC} mice (Fig 3B). However, those of Rev-erbα and Dbp mRNA were significantly decreased in both heterozygous and homozygous mutant mice. Interestingly, the levels of Bmal1 and Cry1 mRNAs were increased in both Bmal1\textsuperscript{+/GTΔC} and Bmal1\textsuperscript{GTΔC/GTΔC} mice, whereas Clock mRNA expression showed no difference (two-way ANOVA with Tuckey’s post-tests, *p*<0.01). Furthermore, these observed increases were coupled with decreased Rev-erbα mRNA expression. In light of the reduced clock gene mRNA levels in Bmal1\textsuperscript{-/-} mice [3], these results implied that the rhythm disruption in Bmal1\textsuperscript{GTΔC} mice entails a different mechanism compared with the null mutants.

In addition to mRNA profiles, we also examined levels of clock proteins in the liver. As expected, β-GEO proteins were only found in Bmal1\textsuperscript{+/GTΔC} and Bmal1\textsuperscript{GTΔC/GTΔC} mice, and the size of BMAL1\textsuperscript{GTΔC} proteins was slightly reduced compared with BMAL1\textsuperscript{wt}. Despite robust circadian behavior during the first several days in DD, Bmal1\textsuperscript{+/GTΔC} mice did not exhibit robust oscillations of BMAL1\textsuperscript{wt} and BMAL1\textsuperscript{GTΔC} proteins compared with WT and Bmal1\textsuperscript{+/+} mice. In accord with behavioral and mRNA data, there was no circadian oscillation of BMAL1\textsuperscript{GTΔC} proteins in Bmal1\textsuperscript{GTΔC/GTΔC} mice, and no BMAL1 protein was detected in Bmal1\textsuperscript{+/-} mice. In contrast to the increased Bmal1 mRNAs levels of the liver of Bmal1\textsuperscript{+/GTΔC} and Bmal1\textsuperscript{GTΔC/GTΔC} mice, there were only subtle changes of BMAL1\textsuperscript{wt} and BMAL1\textsuperscript{GTΔC} protein levels (S3A Fig). Furthermore, no difference in CLOCK level was observed among the different mouse genotypes, consistent with its mRNA patterns (Figs 3B and 4 and S3A Fig).

### Disrupted circadian rhythms at molecular and cellular levels

The above data from locomotor activities, mRNA and protein expressions clearly demonstrated circadian arrhythmicity of Bmal1\textsuperscript{GTΔC/GTΔC} mice. To determine whether the arrhythmicity resulted from disrupted molecular clock or deficits at in vivo systemic level, we generated Per2::luc knock-in MEFs harboring WT, Bmal1\textsuperscript{+/GTΔC}, Bmal1\textsuperscript{GTΔC/GTΔC}, Bmal1\textsuperscript{+/-} and Bmal1\textsuperscript{-/-} alleles. Despite the fact that Bmal1\textsuperscript{+/-/GTΔC} mice gradually lost the circadian locomotor rhythm, the rhythms of PER2::LUC expression were maintained. The period length of Bmal1\textsuperscript{+/-/GTΔC} mice was significantly decreased, but there was no change in the amplitude, compared with those of WT and Bmal1\textsuperscript{+/-} MEFs (Fig 5B and S3B Fig). As expected, the circadian oscillation in Bmal1\textsuperscript{GTΔC/GTΔC} MEFs was disrupted to similar degrees as in Bmal1\textsuperscript{+/-} MEFs, indicating cellular defects in rhythm generation (Fig 5). To substantiate these results, we introduced a BMAL1\textsuperscript{GTΔC} expressing vector to WT MEFs, and examined circadian oscillation of Per2 and

### Table 1. The progeny genotypes of Bmal1 mutant and null mice.

|        | Bmal1\textsuperscript{+/-} X Bmal1\textsuperscript{+/-} | Bmal1\textsuperscript{+/-} X Bmal1\textsuperscript{+/-} |
|--------|--------------------------------------------------------|--------------------------------------------------------|
|        | +/+ (+/GTΔC)                                           | GTΔC/GTΔC                                              |
|        | 21                                                     | 60                                                     |
|        | +/+                                                   | 28                                                     |
|        | 27                                                     | 59                                                     |
|        | +/-                                                   | 30                                                     |

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Bmal1 promoter activity by using the dual-color luciferase technique [23]. Using this experimental system, we also tested N- and C-terminal truncated Bmal1 constructs (Fig 6A). The overexpression of Bmal1GTAC and Bmal1ΔC clearly disrupted circadian rhythms in WT MEFs, whereas no change was observed in WT and Bmal1ΔN transfected cells (Fig 6B). These results
Fig 3. Altered circadian gene expression of WT, Bmal1<sup>+/GTΔC</sup> and Bmal1<sup>GTΔC/GTΔC</sup> mice. (A) In situ hybridization results of Per2 and Bmal1 mRNA expression in the SCN of WT, Bmal1<sup>+/GTΔC</sup> and Bmal1<sup>GTΔC/GTΔC</sup> mice. (B) The mRNA expression in the liver. Data are represented as the mean ± S.E.M. (n = 3–6 per group). 

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indicated that the loss of rhythms in Bmal1\textsuperscript{GT\textDeltaC/GT\textDeltaC} mice resulted from the defective molecular clock machinery at the cellular and molecular level.

Impaired transcriptional activity and degradation of CLOCK: BMAL1\textsuperscript{GT\textDeltaC} heterodimer

Next, we examined the transcriptional activity of BMAL1\textsuperscript{GT\textDeltaC} on Per1 promoter. Overexpression of BMAL1\textsuperscript{GT\textDeltaC} with CLOCK failed to activate Per1 promoter activity. Interestingly, co-transfection of Bmal1\textsuperscript{wt} and Bmal1\textsuperscript{GT\textDeltaC} partially blocked WT CLOCK:BMAL1 activity, suggesting competition between intact and mutant BMAL1 as reported previously (Fig 7A) [10]. To determine whether the negative role of Bmal1\textsuperscript{GT\textDeltaC} allele resulted from protein cellular localization, we examined sub-cellular expression of BMAL1\textsuperscript{wt} and BMAL1\textsuperscript{GT\textDeltaC} in combination with CLOCK. Either alone or co-expressed with CLOCK, the fluorescent protein tagged BMAL1\textsuperscript{GT\textDeltaC} protein showed similar expression with BMAL1\textsuperscript{wt} (Fig 7B). We also did not observe any difference in the dimerization with CLOCK in BiFC assays (Fig 7C). To analyze these results quantitatively, we performed IP experiments. As shown in Fig 7D, CLOCK proteins in BMAL1\textsuperscript{GT\textDeltaC} -transfected cells showed significantly higher input and IP levels, perhaps
as a result of activity-dependent degradation of the CLOCK:BMAL1$^{\Delta C}$ dimer or uneven expression levels. To distinguish these possibilities, we conducted dose-dependency test [25].

The level of CLOCK was rapidly decreased with increased BMAL1$^{\text{wt}}$ amount, as reported previously (Fig 7E). However, increased BMAL1$^{\text{GTAC}}$ expression did not attenuate the level of CLOCK. These results are consistent with the compromised transcriptional activation as shown in Fig 7A and the previous report [26].
Discussion

In the present study, we developed and characterized C-terminal truncated Bmal1 mutant mice (Bmal1\textsuperscript{GTAC}). Molecular and behavioral studies indicated that the circadian rhythms of Bmal1\textsuperscript{GTAC} mice were disrupted in homozygous mutant mice. Interestingly, the heterozygous mutant mice also showed gradual loss of rhythmicity in DD, indicating a semi-dominant negative allele. These results reveal that C-terminal region of BMAL1 plays a key role in generating circadian rhythms \textit{in vivo}.

Fig 6. The effects of C-terminal region of Bmal1 on the molecular circadian rhythm. (A) The structures of BMAL1\textsuperscript{WT} and Bmal1 mutant constructs, Bmal1\textsuperscript{GTAC}, Bmal1\textsuperscript{AC} and Bmal1\textsuperscript{DN}. (B) The representative circadian oscillation profiles of the constructs in (A). The constructs were transfected to WT MEFs. The luciferase activities of Bmal1 and Per2 promoters were distinguished by the wavelength separation method (n = 3).

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Circadian mutant animal models have revealed important roles of the circadian clock in physiology, behavior and metabolism [27]. Interestingly, loss of rhythmicity in animal models invariably required homozygous mutations. For example, ClockΔ19, a classical mammalian circadian mutant allele, led to lengthened and arrhythmic circadian rhythm in homozygous (ClockΔ19/Δ19), but the heterozygous mutant (Clock+/-Δ19) mice maintain the rhythmicity with lengthened FRP (24.42±0.057, (BALB × B6) F2 progeny) [28]. Here, we report the first circadian "heterozygous" mutant mice showing disrupted rhythmicity in extended DD (Fig 2A).

McDearmon et al. (2006) demonstrated that the reduced locomotor activity of Bmal1-/- mice was rescued by muscle-specific recovery of Bmal1. Furthermore, constitutively expression of its paralog Bmal2 in Bmal1-/- mice rescued circadian behavior [7]. Interestingly, the total activities of Bmal1GTΔC/GTΔC mice were not decreased (Fig 2B). These results suggest that Bmal1GTΔC/GTΔC mice retain the partial roles of Bmal1 on tissue specific functions.

Fig 7. Underlying molecular mechanisms of Bmal1 GTΔC. (A) Effects of Bmal1GTΔC on Per1 promoter activity. (B) Bmal1wt, Bmal1GTΔC and CLOCK were tagged with fluorescence proteins and the localizations were examined. The heterodimerization and cellular localization of CLOCK:Bmal1wt and CLOCK:Bmal1GTΔC were examined by BiFC assays (C) and IP experiments (D). (E) The dose dependent degradation of CLOCK by Bmal1wt and Bmal1GTΔC (n = 3).

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Although CLOCK:BMAL1 is well known to transactivate clock controlled genes, a dual mode of activation and repression has also been suggested in light of the elevated Cry1 mRNA levels in Bmal1–/– mice [29]. In addition to CLOCK:BMAL1, Cry1 expression is also under regulation of REV-ERBs When REV-ERBα and β bind to the 5’ upstream region of Cry1 gene [32]. Consistently, Bmal1GTAC/GTAC mice showed decreased Rev-erba and increased Cry1 mRNA expressions, indicating that these mice also employ similar mechanisms of Bmal1–/– mice in regulating Cry1 expression. Surprisingly, we found significantly decreased mRNA levels of Dbp and Rev-erba in the heterozygous mutant mice. Considering no significant change in Per1 and Per2 mRNA expression between WT and Bmal1+/GTAC mice, these unexpected results indicate that the circadian transcriptional network was disrupted in Bmal1+/GTAC mice (Fig 3B). However, there are still a functional WT allele in Bmal1+/GTAC mice which can sustain rhythms for several days in DD. The abnormal circadian transcription including normal circadian Per1/2, decreased Rev-erba/Dbp and increased Cry1 expression, in Bmal1+/GTAC mice may exacerbate the molecular feedback loop over time and lead to gradual rhythm loss of Bmal1+/GTAC mice under DD. In these contexts, Bmal1GTAC mice represent an excellent model for evaluating the complex interactions of circadian transcriptional network [33].

The roles of the BMAL1 C-terminal region in the circadian clock have been characterized in vitro. Collectively, CRY1 and coactivators competitively bind to the transactivation domain (TAD) of BMAL1 encompassing residues 579–626. Deletions, site-directed mutation and replacement of the BMAL1 TAD demonstrated that the TAD is important for both activation and repression of circadian transcriptional activity and plays a crucial role in the generation of circadian rhythms [10, 11, 14]. The previous paper demonstrated that competitive binding of CRY1 and CBP (p300) to the TAD generates circadian cycling. As expected, our mutant mice express truncated BMAL1GTAC without half of the G domain and the entire H domain including the TAD [11]. Furthermore, our mutant mice express BMAL1GTAC protein which heterodimerize with CLOCK (Fig 7). Compared with the null mutant mice, BMAL1GTAC also enables investigation of the circadian interaction between the TAD and other circadian transcription factors. Therefore, our Bmal1GTAC mice provide important in vivo evidence for the rhythm-generating function of the TAD and an excellent system for further investigations of the circadian control of transcriptional activities.

However, the mechanism underlying the gradual loss of circadian behavior in Bmal1+/GTAC mice remains unknown. As shown in Fig 7E, CLOCK protein levels were not decreased by overexpressed BMAL1GTAC. Previous studies on the degradation dynamics of CLOCK and BMAL1 proteins suggested that CLOCK:BMAL1 activation and proteasomal degradation are tightly linked [25, 26, 34–36]. Stabilized CLOCK:BMAL1GTAC without transcriptional activity may perturb the balance of BMAL1wt and BMAL1GTAC expression level in Bmal1+/GTAC mice (Fig 7). However, we did not observe any significant difference in the CLOCK and BMAL1 expression levels among WT, Bmal1+/GTAC, Bmal1GTAC/GTAC and null mice (Fig 4 and S3A Fig). Complex regulatory mechanisms may impinge on mRNA and protein levels in the gradual loss of rhythmicity in Bmal1+/GTAC mice, e.g., decreased Rev-erba mRNA, poly(A) tail from the gene trap vector and stabilized BMAL1GTAC protein. Further investigations into the arrhythmic behavior transition and unusual mRNA expression of Bmal1+/GTAC mice should be conducted.

Although PER and CRY are known as repressors of the CLOCK:BMAL1 complex, recent ChIP-seq data showing distinct spatio-temporal occupancy of CRY1 related to PERs and CRY2 have revised the roles of CRY1 [37]. Furthermore, several recent studies have highlighted potential non-repressive roles of PERs and CRYs on the CLOCK:BMAL1 complexes [38, 39].
As previously reported, the C-terminal region of BMAL1 determines the balance between circadian transcriptional activation and suppression by competitive binding of CRY1 and CBP (p300) [10, 11]. In this context, Bmal1<sup>G Tac</sup> mice constitute an excellent animal model system for investigating the binding dynamics of PERs and CRYs on CLOCK:BMAL1.

Taken together, we have developed and characterized a novel circadian mutant mice, Bmal1<sup>G Tac</sup> that exhibited arrhythmicity but otherwise distinct molecular perturbation compared to Bmal1<sup>+/−</sup> mice. Further studies of this mutant will provide crucial insight into molecular mechanisms of the circadian clockwork.

Supporting Information

S1 Fig. The sequences of genomic DNA, mRNA and protein of Bmal1<sup>G Tac</sup> mice. (A) The structure and insertion site of the gene trap vector on the DNA region. Additional 7bps (underlined) and followed En2 intron (bold) were inserted on the 18<sup>th</sup> intronic region of Bmal1. (B) The mRNA and protein structures. The truncated Bmal1 mRNA was linked to En2 and the following coding sequences. The translated protein was cleaved by the self-cleaving sequence.

S2 Fig. Representative actograms of Bmal1<sup>+/G Tac</sup> and Bmal1<sup>+/−G Tac</sup> mice. (A) Long term monitoring of wheel running activity in Bmal1<sup>+/G Tac</sup> mouse that showed circadian rhythms for more than 40 days. (B) Wheel running activity of Bmal1<sup>+/−G Tac</sup> mouse. (C) FFTs of each genotype. The FRPs of WT and Bmal1<sup>+/−</sup> mice were calculated from the data of initial 10 days in DD. Those of Bmal1<sup>+/G Tac</sup> mice were divided as two different periods. “First” indicates the initial 5 days when the mice were in DD and “Last” indicates the interval of 5 days before losing their rhythms. (D) Effects of genotypes on total activity of wheel running activity in LD. Asterisks indicate significant differences (**p<0.001) compared with WT mice. Data are represented as the mean ± S.E.M. (n = 4–8 per group).

S3 Fig. Comparison of CLOCK and BMAL1 expression levels and the amplitude changes in WT, Bmal1<sup>+/G Tac</sup> and Bmal1<sup>+/−</sup> MEFs. Representative expression levels of CLOCK and BMAL1. The liver samples of indicated genotypes were collected at two circadian time points and analyzed in one blot. (B) Amplitudes of WT, Bmal1<sup>+/G Tac</sup> and Bmal1<sup>+/−</sup> cells. Data are represented as the mean ± S.E.M. (n = 3).

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Author Contributions

Conceived and designed the experiments: NP S Cheon KK. Performed the experiments: NP S Cheon HR JL. DH. Analyzed the data: NP S Cheon S Cho. Contributed reagents/materials/analysis tools: NP HK S Cheon HR JL. Wrote the paper: NP S Cheon KK.
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