

Nal<sup>R</sup> variants of haplotype H58 and its derivative haplotypes (H34, H57, and H60 to H65) were isolated in Vietnam, India, and Pakistan, and other countries in southern Asia (Table 2). These Nal<sup>R</sup> variants represent at least five distinct *gyrA* mutations (K, L, M, N, and O), which arose during or before the mid-1990s (Fig. 2C). The frequency of *gyrA*<sup>+</sup> and mutation L has remained fairly constant since the mid-1990s, but H58 isolates with mutation K seem to have become more common in recent years, particularly in Vietnam (Table 2).

These results show that selection for antibiotic resistance has probably led to clonal expansion of H58 and its Nal<sup>R</sup> derivatives in southern Asia. These strains have now also reached Africa, given that one MDR H58 strain (isolated in Morocco in 2003) was included among nine rare, recent MDR isolates from Africa and that the sole MDR Nal<sup>R</sup> isolate from Africa that was tested (mutation K, isolated in central Africa in 2004) also belonged to H58 (Table 2). Thus, H58 is probably not ethnically restricted to southern Asians, and nalidixic acid-resistant typhoid fever may soon present an additional public health problem in Africa.

Despite the selection for resistance to nalidixic acid in southern Asia, the data do not show complete clonal replacement, which would be expected from periodic selection; about 20% of Typhi isolated in recent years in northern Vietnam and 5% of Typhi from southern Vietnam remain susceptible to nalidixic acid, as are many other recent H58 isolates (Fig. 2C). Furthermore, recent isolates from southern Asia also belong to other haplotypes, where mutations in *gyrA* are rare (Fig. 2B). Thus, the population structure indicative of neutral evolution has not been disrupted by strong selection for resistance to nalidixic acid during the past 15 years, except for the clonal expansion of H58. Possibly *gyrA* mutations in many haplotypes reduce fitness (23) or some cases of typhoid fever have not been treated with fluoroquinolones. But still another alternative is that the population structure of Typhi reflects two distinct epidemiological dynamics associated with different time scales: first, the human carrier state permitting slow neutral evolution (millennia), and second, infectious transmission facilitating a rapid response to selection in real time. Outbreaks of infections, similar to the recent expansion of H58 in southeast Asia, may be responsible for independent chains of intercontinental transmission. These, in turn, create a global distribution of carriers for multiple haplotypes. According to this interpretation, it is exactly because the environment selects that everything is everywhere in Typhi, thus inverting a hotly disputed (24) tenet of microbial ecology that was proposed by L. G. M. Baas Becking in 1934 (25).

The results presented here open multiple avenues for future research. Long-term epidemiology with larger strain collections is now possible on the basis of neutral SNPs (fig. S2), whereas classical microbiological methods do not seem to provide reliable markers for such purposes (table S3). Surveillance of haplotypes is particularly appropriate to provide early warning of the

continued spread of Nal<sup>R</sup> H58. Our overview of the current global population diversity in Typhi will allow comparisons of genomic sequences from representative strains without the risk of phylogenetic discovery bias (26). Finally, we suggest that the human carrier state may be of much greater importance for neutral evolution and genetic buffering than had been previously appreciated, an interpretation that would demand major changes in public health campaigns to reduce the incidence of typhoid.

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#### Supporting Online Material

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## Dissecting the Functions of the Mammalian Clock Protein BMAL1 by Tissue-Specific Rescue in Mice

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The basic helix-loop-helix (bHLH)–Per-Arnt-Sim (PAS) domain transcription factor BMAL1 is an essential component of the mammalian circadian pacemaker. *Bmal1*<sup>-/-</sup> mice lose circadian rhythmicity but also display tendon calcification and decreased activity, body weight, and longevity. To investigate whether these diverse functions of BMAL1 are tissue-specific, we produced transgenic mice that constitutively express *Bmal1* in brain or muscle and examined the effects of rescued gene expression in *Bmal1*<sup>-/-</sup> mice. Circadian rhythms of wheel-running activity were restored in brain-rescued *Bmal1*<sup>-/-</sup> mice in a conditional manner; however, activity levels and body weight were lower than those of wild-type mice. In contrast, muscle-rescued *Bmal1*<sup>-/-</sup> mice exhibited normal activity levels and body weight yet remained behaviorally arrhythmic. Thus, *Bmal1* has distinct tissue-specific functions that regulate integrative physiology.

Circadian rhythms control many aspects of mammalian physiology and behavior. The suprachiasmatic nuclei (SCN) act as pacemakers required for the generation of circadian behavioral rhythms as well as syn-

chronizers of autonomous peripheral tissue clocks (*1*). Molecular circadian regulation engages a transcription-translation feedback loop comprising the activating proteins CLOCK and BMAL1, which induce expression of the negative feed-

back elements *Per* and *Cry* (1). BMAL1 (also known as MOP3) was originally characterized by its high expression in brain and muscle (2, 3) and was identified as a heterodimeric binding partner of CLOCK (4, 5). *Bmal1*<sup>-/-</sup> mice not only lose behavioral circadian rhythmicity but also exhibit a variety of other phenotypes including decreased activity levels and body weight, progressive joint disease, and shortened life span (6–12). Therefore, in addition to circadian rhythm regulation, BMAL1 appears to play a role in a variety of functions that are potentially dependent on the tissue type in which it is expressed. To determine whether BMAL1 has unique tissue-specific functions, we generated transgenic mice that express *Bmal1* ubiquitously or in distinct tissue types. We then crossed these lines onto a *Bmal1* null background and determined which phenotypes could be rescued by exogenous, tissue-specific *Bmal1* expression.

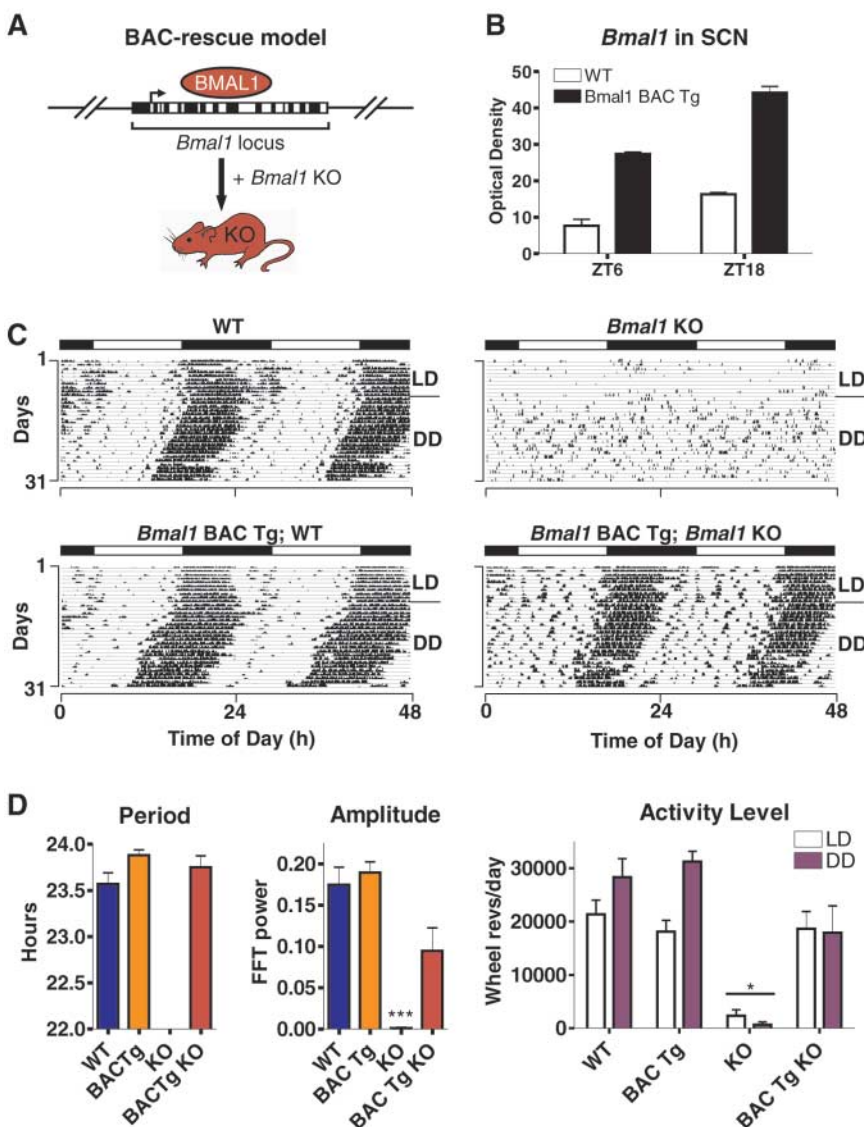
We first examined the effects of rescuing *Bmal1* ubiquitously by using a transgenic mouse line produced with *Bmal1*-containing bacterial artificial chromosome (BAC) clones (Fig. 1A) (13). Because the BAC clones contain the genomic coding and promoter sequence of *Bmal1*, expression of the transgene should occur in all tissues that normally express *Bmal1*. We measured increased *Bmal1* expression in the SCN of BAC transgenic mice at normal peak and trough times of *Bmal1* mRNA [ZT (zeitgeber time) 18 and 6, respectively], and also observed increased *Bmal1* expression during peak times in the liver (ZT 18 to ZT 2) (fig. S1) (13). BAC transgenic mice were then sequentially crossed with *Bmal1*<sup>+/-</sup> mice to produce BAC-rescued *Bmal1*<sup>-/-</sup> mice (13). Circadian rhythms of locomotor activity were then analyzed in a 12 hour:12 hour light:dark (LD) cycle followed by constant darkness (DD) conditions (Fig. 1C) (13).

Whereas *Bmal1*<sup>-/-</sup> mice exhibited no circadian rhythm of activity in DD and showed reduced activity levels, BAC-rescued *Bmal1*<sup>-/-</sup> mice displayed normal circadian rhythm characteristics (free-running period and amplitude of circadian rhythm) and activity levels in LD and DD that were similar to those of wild-type mice (Fig. 1D). In addition, 100% of BAC-rescued *Bmal1*<sup>-/-</sup> mice survived until the end of experimental analysis ( $\geq 10$  months old) compared to 29% of *Bmal1*<sup>-/-</sup>

mice. Therefore, long-term survival was restored in the BAC-rescued *Bmal1*<sup>-/-</sup> mice, and no gross abnormalities such as low body weight or joint calcification were observed in the BAC-rescued mice. Thus, *Bmal1* BAC transgenes completely rescued the phenotypes observed in *Bmal1*<sup>-/-</sup> mice.

We next determined whether expression of *Bmal1* in brain tissue could restore behavioral rhythms in *Bmal1*<sup>-/-</sup> mice as well as alleviate other phenotypes. To produce the brain-rescued line, we used the tetracycline trans-

activator (tTA) system, which requires two transgenes for expression of the target gene *Bmal1* (Fig. 2A) (14, 15). We used the promoter sequence of *Scg2*, which is expressed in brain and enriched in the SCN (16), to drive expression of the tetracycline trans-activator (tTA) (13). The tTA protein binds to the tetracycline operator (tetO) sequence and drives expression of downstream hemagglutinin (HA)-tagged *Bmal1* (*Bmal1*-HA) cDNA. Doxycycline (Dox) inhibits tTA binding to the tetO promoter, which halts expression of



**Fig. 1.** *Bmal1*-containing BAC transgenes rescue *Bmal1*<sup>-/-</sup> phenotypes. (A) *Bmal1* BAC clones were used to create transgenic (Tg) mice, which were consecutively crossed with *Bmal1*<sup>+/-</sup> mice to create BAC-rescued *Bmal1*<sup>-/-</sup> mice. (B) *Bmal1* mRNA levels in SCN were examined by in situ hybridization in wild-type (WT) and *Bmal1* BAC Tg mice, killed at ZT 6 and ZT 18 [shown are means  $\pm$  SEM; significant effect of genotype, generalized linear model analysis of variance (GLM ANOVA)]. (C) Representative wheel-running activity records from WT, *Bmal1* BAC Tg, *Bmal1*<sup>-/-</sup> (*Bmal1* KO), or BAC-rescued (*Bmal1* BAC Tg; *Bmal1* KO) mice. Mice were housed in LD and then released into DD for 3 weeks. (D) Bar graphs of means  $\pm$  SEM show that BAC-rescued mice ( $n = 6$ ) exhibit free-running period, amplitude of circadian rhythm, and activity levels that are not significantly different from those of WT. Amplitude is graphed as the peak amplitude of the proportion of the total variance in the time series in the circadian (~24 hours) range (\*\*\* $P < 0.001$ , one-way ANOVA; \*significant effect of genotype, GLM ANOVA).

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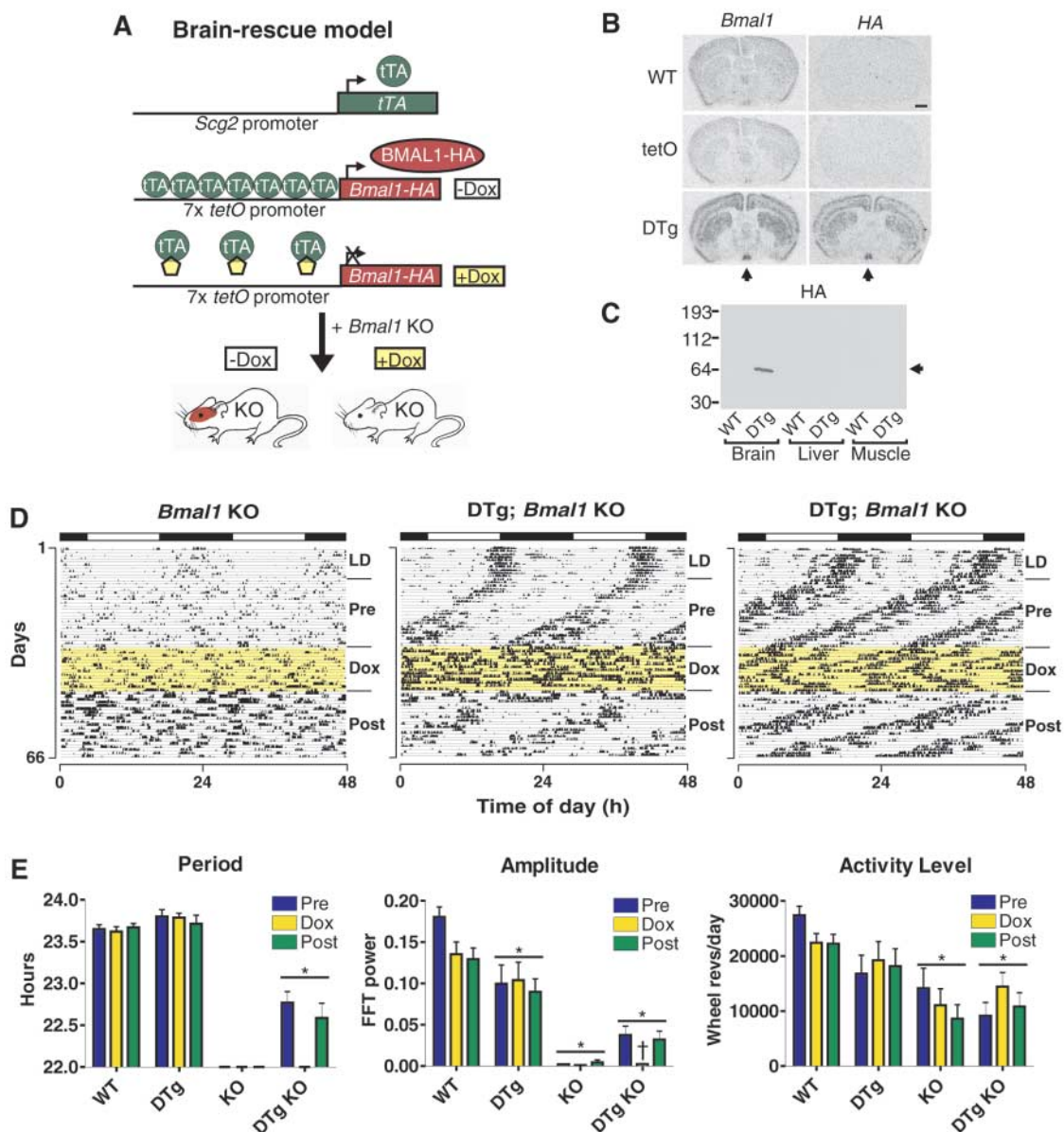
**Bmal1-HA.** In situ hybridization showed strong, specific expression of *Bmal1-HA* in *Scg2::tTA × tetO::Bmal1-HA* double transgenic mice only (Fig. 2B) (13). The pattern of expression observed is consistent with high *Scg2* expression in the SCN. HA-tagged protein at the correct molecular weight for BMAL1 (~69 kD) was produced specifically in double transgenic mouse brain extracts (Fig. 2C), and HA-tagged BMAL1 was shown to be functional by *Per1::luciferase* reporter gene assays (fig. S2) (13). The double transgenic mice were crossed onto a *Bmal1*<sup>-/-</sup> background to create brain-rescued *Bmal1*<sup>-/-</sup> mice, and wheel-running experiments were performed as described above (Fig. 2D and figs. S3 to S5).

Adult (≥8 weeks old) brain-rescued *Bmal1*<sup>-/-</sup> mice exhibited a consistent circadian rhythm of behavior in the initial (pre-Dox) DD period, which was completely abolished after 1 to 2 days of Dox administration and then regained during Dox withdrawal. However, the free-running period of brain-rescued mice was about 1 hour shorter than that of wild-type mice (Fig. 2E). This was likely due to the constitutive bioavailability of BMAL1 protein and/or the lack of peripheral tissue feedback to the SCN (fig. S6). In support of this idea, *Rev-Erba*<sup>-/-</sup> mice express *Bmal1* in the SCN at consistently high levels and exhibit shortened period length (17). In contrast to the restoration of circadian rhythmicity in brain-rescued mice, both amplitude and activity levels were

significantly lower than that seen in wild-type mice (Fig. 2E). Thus, brain-rescued mice exhibit restored circadian rhythms of behavior, but their locomotor activity is still impaired.

Because *Bmal1* is highly expressed in muscle, we investigated whether muscle-specific rescue might restore activity levels in *Bmal1*<sup>-/-</sup> mice. We produced muscle-specific *Bmal1* transgenic mice with the use of a DNA construct consisting of human  $\alpha$ -actin-1 (*Acta1*) promoter sequence positioned upstream of *Bmal1-HA* (Fig. 3A) (13). HA-tagged protein was specifically detected in transgenic muscle extracts (Fig. 3B) (13). Adult muscle-rescued *Bmal1*<sup>-/-</sup> mice did not express circadian rhythmicity of activity (Fig. 3, C and D); however, their level of locomotor

**Fig. 2.** Reversible restoration of circadian rhythms but not activity levels in brain-rescued *Bmal1*<sup>-/-</sup> mice. (A) Mice were created to express *Bmal1-HA* conditionally in brain tissue with the use of the tTA system. (B) In situ hybridization was performed with HA tag or *Bmal1* probes on brains from WT, *tetO::Bmal1-HA* (*tetO*), or *Scg2::tTA × tetO::Bmal1-HA* double transgenic (DTg) mice killed at ZT 6 (arrow indicates SCN; scale bar, 1 mm). (C) Western blot showing HA staining in brain, liver, and skeletal muscle protein extracts from WT or DTg mice killed at ZT 12 (arrow indicates correct size of BMAL1). (D) Representative wheel-running activity records from one *Bmal1*<sup>-/-</sup> mouse and two brain-rescued *Bmal1*<sup>-/-</sup> (DTg; *Bmal1* KO) mice. After 3 weeks in DD (Pre), mice were administered Dox for 2 weeks (Dox, highlighted yellow) and then spent an additional 3 weeks without Dox (Post). (E) Brain-rescued mice (*n* = 10) display a free-running period of 22.8 hours (Pre) and 22.6 hours (Post) when *Bmal1* is expressed; these values are significantly different from those of WT and DTg groups (\*significant effect of genotype, GLM ANOVA). Activity levels of KO and DTg KO mice were significantly reduced relative to WT. Amplitude of circadian rhythm was significantly different in all genotypes relative to WT, and a simultaneous loss of rhythm and decrease in amplitude were observed in DTg KO mice during Dox treatment (†significant effect of time interval). Graphs represent means ± SEM.

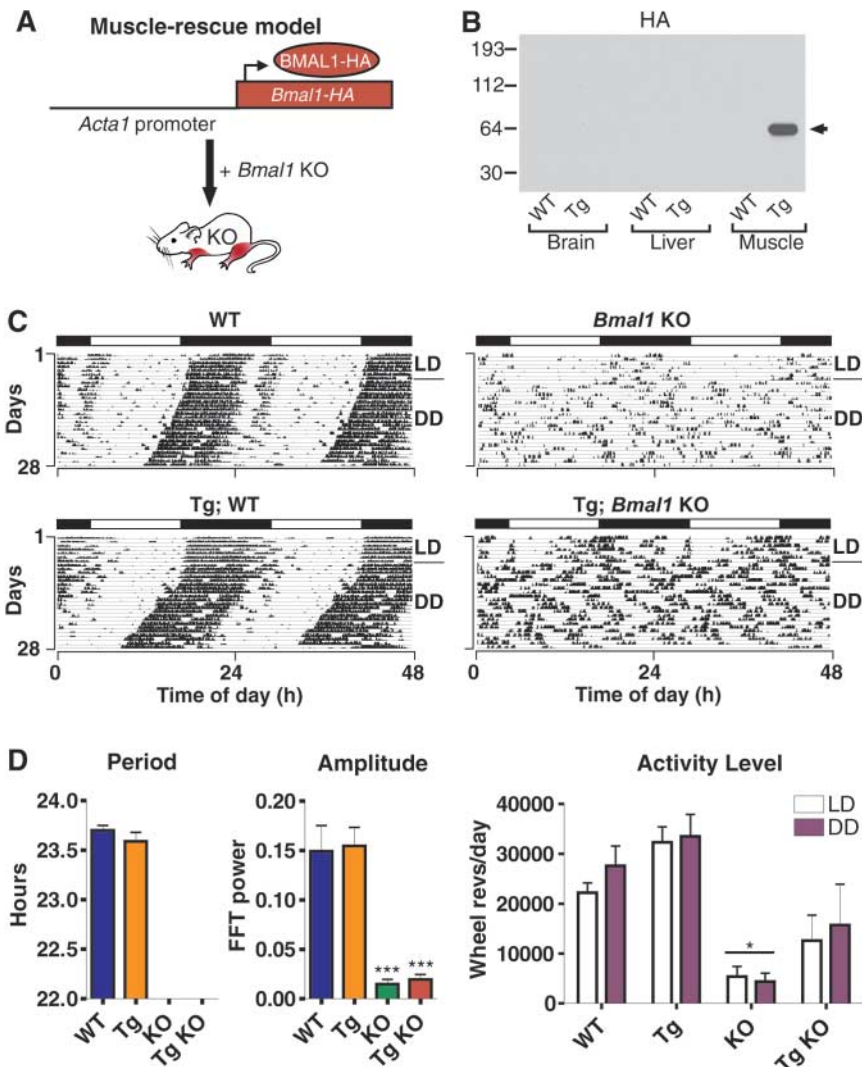


activity was not significantly different from that of wild-type mice (Fig. 3D).

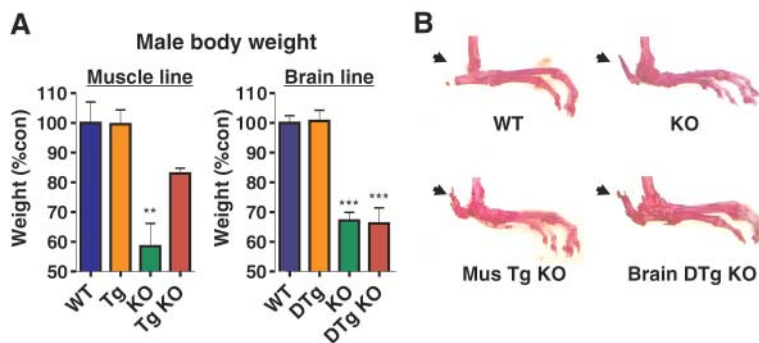
We also found that at 4 to 6 months of age, the *Bmal1*<sup>-/-</sup> and brain-rescued mice weighed significantly less than did wild-type mice. In contrast, the body weight of muscle-rescued mice was restored to a level not significantly different from that of wild-type mice (Fig. 4A) (13). Only 75% of brain-rescued mice survived to the end of the experiment, whereas 100% of muscle-rescued mice survived. These results suggest that BMAL1 function in muscle is important for activity as well as for body weight maintenance and longevity. In addition, bone phenotypes were examined by Alizarin Red stain; both brain- and muscle-rescued mice showed significant tendon calcification similar to that seen in *Bmal1*<sup>-/-</sup> mice (Fig. 4B). This suggests that *Bmal1*-HA was not expressed in bone in either line and that the calcification observed in *Bmal1*<sup>-/-</sup> mice was not improved by restoring BMAL1 expression in muscle or brain. Thus, three distinct patterns of rescue could be observed in these mice, relating to (i) circadian activity rhythms, (ii) activity level and body weight, and (iii) tendon calcification.

Unlike the BAC transgenic line, the brain and muscle transgenic lines were designed to constitutively express BMAL1-HA. To verify this, we measured similar levels of *Bmal1* mRNA and BMAL1-HA protein at normal peak and trough times in the brain and muscle transgenic lines (figs. S6 and S7) (13). We then examined mRNA levels of the key BMAL1 target genes *Per1* and *Per2* (6). Relative to wild-type and brain double transgenic mice, *Bmal1*<sup>-/-</sup> mice exhibited consistently low expression levels of *Per1* in the SCN (fig. S7). In contrast, the brain-rescued mice had increased amplitude of *Per1* expression, although peak levels remained significantly lower than those of wild-type mice (fig. S7). *Per2* expression was measured in both muscle and liver from the brain and muscle transgenic lines at normal peak time for *Per2* (ZT 12) (fig. S8) (13). *Per2* in muscle of *Bmal1*<sup>-/-</sup> mice was significantly reduced to below 50% of wild-type levels at ZT 12. This decreased expression was restored to wild-type levels in the muscle-rescued mice but not in the brain-rescued mice. These data suggest that the presence of BMAL1 is important for proper expression of *Per1* genes in brain and muscle tissue (but not liver; see fig. S8). Both *Rev-Erba* and *Dbp* exhibited substantial down-regulation in liver and muscle of *Bmal1*<sup>-/-</sup> mice at ZT 12 and showed increased expression only in muscle of muscle-rescued mice (fig. S8).

We have shown that all phenotypes of *Bmal1*<sup>-/-</sup> mice are alleviated only when *Bmal1* is rescued ubiquitously, whereas different parameters of behavioral activity (circadian rhythm and activity level), body weight, and



**Fig. 3.** Muscle-rescued mice exhibit restored activity level but not circadian rhythms. (A) Muscle-specific *Bmal1* Tg mice were created by fusing the *Acta1* promoter sequence to *Bmal1*-HA. (B) Western blot shows HA staining in brain, liver, and skeletal muscle protein extracts from WT or Tg mice killed at ZT 12 (arrow indicates correct size of BMAL1). (C) Representative wheel-running activity records are shown from WT, Tg, *Bmal1* KO, and muscle-rescued (Tg; *Bmal1* KO) mice. (D) Muscle-rescued mice ( $n = 6$ ) are arrhythmic in DD with significantly reduced amplitude of rhythm (\*\* $P < 0.01$ , one-way ANOVA) but display activity levels that are not significantly different from those of WT mice. Graphs show means  $\pm$  SEM (\*significant effect of genotype, GLM ANOVA).



**Fig. 4.** Effects of tissue-specific *Bmal1* expression on body weight and tendon calcification. (A) Brain-rescued mice and KO mice in both lines have significantly reduced body weight, whereas muscle-rescued mice exhibit body weight similar to that of WT mice (graphs represent means  $\pm$  SEM; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , one-way ANOVA). (B) Photographs of Alizarin Red-stained hindlimbs from WT, KO, muscle-rescued, and brain-rescued KO mice. Arrows indicate calcaneal tendon calcification in all but WT mice.

gene expression can be rescued separately by distinct spatial expression patterns of *Bmal1*. Genome-wide profiling experiments suggest that ~10% of the transcriptome is under circadian regulation; however, the majority of these cycling transcripts are tissue-specific (18–22). Our results are consistent with this tissue-specific diversity of circadian expression and further suggest that core circadian clock components may play distinct roles in different tissues, perhaps in addition to their function in regulating circadian rhythms. The restoration of circadian activity rhythms in brain-rescued *Bmal1*<sup>-/-</sup> mice is consistent with previous SCN transplant studies in rodents (23, 24). However, the transgenic approach used here has the advantages of preserving the anatomical integrity of the brain as well as allowing the conditional manipulation of the rescue via Dox treatment. The use of tissue-specific and conditional regulation of circadian clock gene expression should be a valuable method for understanding the molecular-,

cellular-, and systems-level regulation of circadian rhythms in mammals.

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#### Supporting Online Material

[www.sciencemag.org/cgi/content/full/314/5803/1304/DC1](http://www.sciencemag.org/cgi/content/full/314/5803/1304/DC1)

Materials and Methods

SOM Text

Figs. S1 to S8

References

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## A Bacterial Protein Enhances the Release and Efficacy of Liposomal Cancer Drugs

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*Clostridium novyi-NT* is an anaerobic bacterium that can infect hypoxic regions within experimental tumors. Because *C. novyi-NT* lyses red blood cells, we hypothesized that its membrane-disrupting properties could be exploited to enhance the release of liposome-encapsulated drugs within tumors. Here, we show that treatment of mice bearing large, established tumors with *C. novyi-NT* plus a single dose of liposomal doxorubicin often led to eradication of the tumors. The bacterial factor responsible for the enhanced drug release was identified as a previously unrecognized protein termed liposomase. This protein could potentially be incorporated into diverse experimental approaches for the specific delivery of chemotherapeutic agents to tumors.

There is no dearth of drugs that can kill cancer cells. The challenge is killing the cancer cells selectively while sparing the normal cells. Three basic strategies are currently used to achieve this specificity. The first (selective toxicity) uses drugs that have more potent growth-inhibitory effects on tumor cells than on normal cells (1, 2). This strategy underlies the success of conventional chemotherapeutic agents as well as those of newer targeted therapies such as imatinib (Gleevec). The second strategy (delivery) uses agents such as antibodies that specifically react with molecules

that are predominantly expressed in tumor cells (3, 4). The third strategy (angiogenic) exploits abnormal aspects of the tumor vasculature with agents such as bevacizumab (Avastin) (5, 6) or drugs incorporated into liposomes (7–9). Liposomes are relatively large particles that can penetrate through the fenestrated endothelium present in tumors and a limited number of other organs (8, 9). Once they gain access to tumors, they persist and eventually release their contents and raise local drug concentrations through the enhanced permeabilization and retention effect (10). Although each of these strategies has merit, the specificity achieved with any one of them is imperfect, limiting the amount of drug that can be safely administered without causing systemic toxicity.

Here, we describe our efforts to combine all three strategies. We investigated *C. novyi-NT*,

an attenuated strain of the obligate anaerobe *C. novyi*. Similar to other bacteriolytic therapies, *C. novyi-NT* can selectively infect and partially destroy experimental cancers because of the hypoxic nature of the tumor environment (11, 12). *C. novyi-NT* is also hemolytic (lyses erythrocytes). Because enzymes that rupture erythrocytes can disrupt lipid bilayers (13), we hypothesized that the bacterium's hemolytic properties could be exploited to enhance the release of liposome-encapsulated drugs within tumors. This approach would theoretically increase specificity by combining the selective tumor toxicity of chemotherapeutic agents, the selective delivery of *C. novyi-NT* to tumors, and the selective uptake of liposomes mediated by the abnormal tumor vasculature.

To test this hypothesis, we first treated syngeneic CT26 colorectal tumors in BALB/c mice. *C. novyi-NT* spores were injected intravenously, and once germination had begun in the tumors (~16 hours after injection), we administered a single intravenous dose of liposomal doxorubicin (Doxil). Doxil is a liposomal formulation that encapsulates doxorubicin, a widely used DNA-damaging chemotherapeutic agent. Liposome-encapsulated doxorubicin has been shown to result in improved outcomes compared with unencapsulated doxorubicin (14). As previously documented (15, 16), treatment with *C. novyi-NT* spores alone resulted in germination and necrosis within the centrally hypoxic region of tumors but left a well-oxygenated viable rim that eventually regrew (Fig. 1A). Neither doxorubicin nor Doxil alone resulted in prolonged therapeutic effects in these mice. The combination of Doxil and *C. novyi-NT* spores, however, resulted in complete regression of tumors in 100% of mice (Fig. 1A), and 65% of the mice were still alive at 90 days (Fig. 1B).

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