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Core circadian clock genes regulate leukemia stem cells in AML

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SUMMARY

Leukemia stem cells (LSCs) have the capacity to self-renew and propagate disease upon serial transplantation in animal models, and elimination of this cell population is required for curative therapies. Here, we describe a series of pooled, *in vivo* RNA interference (RNAi) screens to identify essential transcription factors (TFs) in a murine model of acute myeloid leukemia (AML) with genetically- and phenotypically-defined LSCs. These screens reveal the heterodimeric, circadian rhythm TFs *Clock* and *Bmal1* as genes required for the growth of AML cells *in vitro* and *in vivo*. Disruption of canonical circadian pathway components produces anti-leukemic effects, including impaired proliferation, enhanced myeloid differentiation, and depletion of LSCs. We

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AUTHOR CONTRIBUTIONS

R.V.P., O.R.R., F.A., A.R., and B.L.E. designed and supervised the research. R.V.P., M.S.K., C.G.D., R.K.S., P.G.M., M.M., Z.T., H.T., D.H., M.J., M.C.C., and H.L. performed the research. A.T. provided experimental assistance for the continuous bioluminescence studies. R.V.P., M.S.K., C.G.D., R.K.S., M.M., G.S.C., F.A., and B.L.E. analyzed the data. R.V.P. and B.L.E. wrote the manuscript.

find that both normal and malignant hematopoietic cells harbor an intact clock with robust circadian oscillations, and genetic knockout models reveal a leukemia-specific dependence on the pathway. Our findings establish a role for the core circadian clock genes in AML.

INTRODUCTION

Acute myeloid leukemia (AML) is a clonal disorder of hematopoietic stem and progenitor cells (HSPCs) characterized by enhanced proliferation and impaired differentiation. Recurrent mutations in transcription factors (TFs) and epigenetic regulators identified in AML (Dohner et al., 2015; Ley TJ, 2013) suggest that aberrant transcriptional circuits are a common feature of leukemogenesis. Collectively, these circuits drive oncogenic gene expression programs that inhibit differentiation and activate self-renewal, generating leukemia stem cells (LSCs) responsible for the initiation and propagation of disease (Chao et al., 2008; Reya et al., 2001; Somervaille and Cleary, 2006).

In leukemias with rearrangements of the *Mixed Lineage Leukemia* (*MLL*) gene, activation of a self-renewal circuit involving the *Hox* gene cluster is a key aspect in the transformation of committed myeloid progenitor cells (Krivtsov et al., 2009). Studies have identified multiple transcription factor dependencies within this circuitry, including *HoxA9*, *Meis1*, *β-Catenin*, *Myc*, and *Myb*, demonstrating that re-wired transcriptional circuits in LSCs may be targeted by blocking the activity of single TFs (Faber et al., 2009; Wang et al., 2010; Wong et al., 2007; Zuber et al., 2011a). Although both LSCs and normal hematopoietic stem cells (HSCs) share the hallmark feature of self-renewal, differences in their transcriptional wiring and the degree of functional redundancy within their stemness circuits may offer selective therapeutic opportunities (Huntly and Gilliland, 2005; Novershtern et al., 2011).

Recent studies demonstrate that leukemia-specific targets may be identified using short-hairpin RNA (shRNA) screens to interrogate multiple genes in primary AML cells *in vivo* (Jaras et al., 2014; Miller et al., 2013; Zuber et al., 2011b). These screens employ disease models that reflect the phenotypic and functional cellular heterogeneity observed in human AML (Lapidot et al., 1994) and aim to study leukemia cells in the context of the niche (Lane et al., 2009; Schepers et al., 2015). *In vivo* screening using primary cell models enables the identification of both cell autonomous and cell non-autonomous mechanisms relevant to disease biology. We therefore used pooled, *in vivo* shRNA screening to identify transcription factor dependencies in leukemia stem cells.

RESULTS

Pooled In Vivo RNA Interference Screening in LSCs

To identify essential TFs in leukemia and to highlight transcriptional mechanisms of self-renewal in LSCs, we performed an *in vivo* RNA interference (RNAi) screen using a serial transplantation model of *MLL-AF9*-driven, myelomonocytic AML derived from dsRed^{+} mice (Figure S1A). LSCs in this model are aggressive, immunophenotypically-defined, and may be enriched by prospectively sorting dsRed-labeled cells with high expression of the c-Kit cell surface marker (Hartwell et al., 2013) (Figure S1B). The short-latency, high

penetrance quaternary transplant leukemias are highly enriched for LSCs and are suitable for *in vivo* screening (Miller et al., 2013).

Given the limited number of genes that can be effectively examined in such an *in vivo* screen, we first identified candidate TFs on the basis of gene expression (Bullinger et al., 2004; Chen et al., 2008; Krivtsov et al., 2009; Metzeler et al., 2008; Novershtern et al., 2011; Somervaille et al., 2009; Valk et al., 2004; Wang et al., 2010). We selected DNA-binding proteins that have higher expression in stem cells (LSCs or HSCs) compared to myeloid progenitor cells and are co-expressed with the canonical LSC regulators *HoxA9* and *Meis1*. The top 152 genes, based on this analysis, were selected for the screen (Table S1A).

To test the functional importance of these candidate genes, we generated a custom pool of 770 lentiviral shRNAs (approximately 5 shRNAs per gene) and 7 control shRNAs that lack complementarity with any known murine genes. Freshly sorted quaternary transplant leukemia cells were transduced with 6 subpools of lentiviral shRNAs. An aliquot of these cells was banked 24 hours later, and the remainder was transplanted into sublethally irradiated mice (5 replicate mice per subpool). Two weeks after transplantation, dsRed^{+} leukemia cells were banked from the bone marrow of moribund recipient mice (Figure S1C). Genomic DNA was harvested from both banks of cells and shRNAs were PCR amplified and sequenced on the Illumina platform (Moffat et al., 2006).

We compared the quantitative representation of shRNAs in the input and leukemic bone marrow samples, and observed strong concordance between biological replicates (Figures 1A and S1D). We identified 35 transcriptional regulators targeted by at least 3 shRNAs depleted greater than 20-fold (Table S1B), indicating that these genes are essential for AML cells *in vivo*. Top hits from the screen are established regulators of LSC function, including *HoxA9*, *Meis1*, *β-Catenin*, *Pbx1*, and *Myc* (Figure 1B). Other genes that scored are implicated in myeloid specification (*PU.1*), Myc activity (*Mlxip*), PML bodies (*Pml*, *Mzf1*, *Trim27*, *Rere*), Mef2c signaling (*Mef2c*, *Foxj3*), and the circadian rhythm (*Clock* and *Bmal1*). For key hits, we validated that top scoring shRNAs effectively decrease expression of their target gene (Figure S1E).

Clock and Bmal1 are Required for Leukemia Cell Growth

Two hits from our *in vivo* screen were *Clock* and *Bmal1*, encoding bHLH domain-containing TFs that regulate transcription as a heterodimer (Huang et al., 2012). Multiple shRNAs targeting *Clock* or *Bmal1* were depleted greater than 20-fold *in vivo*, with several shRNAs depleted up to 1000-fold (Figure 1B). *Clock* and *Bmal1* are core components of an autoregulatory loop that drives robust oscillations in gene expression to regulate circadian physiology (Partch et al., 2014; Wager-Smith and Kay, 2000). In the hematopoietic system, the circadian clock regulates HSC egress from the bone marrow microenvironment (Mendez-Ferrer et al., 2008), hematopoietic engraftment (D'Hondt et al., 2004; Rolls et al., 2015), and bone marrow mitotic activity (Clark and Korst, 1969; Smaaland et al., 1991). Cell autonomous and cell non-autonomous functions of the circadian rhythm in leukemia have not been rigorously established.

We validated a role for *Clock* and *Bmal1* in leukemia *in vivo* with shRNAs co-expressed with GFP from single lentiviral vectors. We transduced murine *MLL-AF9* leukemia cells with individual shRNAs, transplanted the cells into sublethally irradiated recipient mice, and compared the percentage of GFP^+ leukemia cells before transplant and after 2 weeks *in vivo*. GFP^+ cells expressing *Clock* or *Bmal1* shRNAs were strongly depleted *in vivo,* while cells expressing the *luciferase* (*Luc*) control shRNA were stable over time (Figure 1C). To evaluate whether this effect is independent of the bone marrow microenvironment or cell engraftment, we performed an equivalent experiment *in vitro* with cytokine supplementation (IL3, IL6, and SCF). Similar to the phenotype *in vivo*, GFP^+ cells were depleted over 12 days in culture following transduction with *Clock* or *Bmal1* shRNAs but not with the *Luc* control (Figure 1D).

To account for possible off-target effects of the shRNAs, we performed a cDNA rescue experiment. We generated a retroviral vector that co-expresses GFP and a mutant *Bmal1* cDNA with silent point mutations in the shRNA binding sites. We sequentially transduced murine leukemia cells with the rescue vector and *Bmal1* shRNAs, followed by puromycin selection of shRNA-expressing cells. In this experiment, an increase in the percentage of GFP^+ cells reflects selection for the shRNA-resistant *Bmal1* cDNA and confirms on-target effects. Indeed, we observed a significant increase in the GFP^+ fraction *in vitro* for two *Bmal1* shRNAs (A1 and A6) normalized to the *Luc* control (Figure 1E).

As an added confirmation of specificity, we used CRISPR-Cas9-based genome editing to introduce loss-of-function mutations in the *Bmal1* gene in primary murine leukemia cells. We transduced quaternary transplant *MLL-AF9* cells with lentiviral SFFV-driven *Cas9* followed by puromycin selection of cells with stable *Cas9* expression (Heckl et al., 2014). Leukemia cells were then transduced with a separate lentiviral vector that co-expresses RFP657 and a single guide RNA (sgRNA) targeting *luciferase*, *Bmal1*, or *HoxA9* as a positive control. Consistent with the shRNA results, a sgRNA targeting *Bmal1* was progressively depleted (Figures 1F–G and S1F).

These data demonstrate that *Bmal1* and its heterodimeric partner *Clock* are functionally required for LSCs. Accordingly, we found that *Bmal1* expression is induced by *MLL-AF9* (Figure S1G) and is correlated with c-Kit, a marker of LSCs in our serial transplantation model of AML (Figures 2A and S2A–B; Table S2).

Circadian Disruption Induces Myeloid Differentiation and Impaired Cell Cycle Progression

We next examined the consequences of acute disruption of the circadian rhythm in AML using a small molecule. SR9011 is a potent and specific agonist of the nuclear hormone receptors *RevErb α* and *Rev-Erb β,* which inhibit *Bmal1* transcription via a feedback loop (Burris et al., 2013; Solt et al., 2012). Treatment of quaternary transplant *MLL-AF9* cells with SR9011 for 3 days *in vitro* resulted in a dose-dependent reduction in cell viability (EC_{50} = 1.8 μM) (Figure 2B). In agreement with known roles of *Rev-Erb α* and *Rev-Erb β* within the circadian pathway*, s*hort duration treatment of leukemia cells with the drug resulted in decreased expression of *Bmal1* and its target gene *Per*2 (Figure 2C). In addition, we observed dose-dependent suppression of key regulators LSC self-renewal (*HoxA9,*

Meis1, Ctnnb1), indicating that the circadian rhythm circuitry influences functions related to stemness (Figure 2D).

Consistent with these observations, we found that *Clock* or *Bmal1* shRNAs in leukemia cells induce morphologic changes of monocytic differentiation, including decreased nuclear to cytoplasmic ratio and enhanced granularity (Figure 2E). Using flow cytometry, we observed reduced cell surface expression of stem cell marker, c-Kit, and increased expression of myeloid differentiation markers, CD11b and Gr1 (Figures 2F–G and S2C). Additionally, leukemia differentiation following circadian gene knockdown is associated with a reduction in the percentage of actively dividing cells (S/G2/M) and an increase in the fraction of cells in G0/G1 (Figures 2H and S2D).

Murine Leukemia Cells Harbor a Functional Circadian Clock

Our finding that AML cells are dependent on *Clock* and *Bmal1* expression implies that leukemia cells, and perhaps their normal hematopoietic counterparts, harbor an intact circadian clock that regulates rhythmic gene expression. To test this possibility in primary murine leukemia cells, we used reporter mice with an in-frame knock-in of *luciferase* at the 3′ end of the *Per2* gene (Yoo et al., 2004). We generated primary *MLL-AF9* leukemias in the *Per2::Luc* genetic background, transplanted these leukemias into wild type (WT) recipients entrained to a 12 hour light - 12 hour dark (LD12:12) schedule, and followed the mice for 4 to 6 weeks for the development of disease (Figures S2E–F). Continuous bioluminescence recordings of leukemic spleen explants from moribund mice revealed oscillations with a period of approximately 24 hours (Figure 3A). Thus, murine leukemia cells have the capacity for circadian-dependent gene expression independent of the suprachiasmatic nucleus (SCN), the dominant circadian pacemaker in the central nervous system. Additionally, continuous bioluminescence measurements of purified *Per2::Luc MLL-AF9* bone marrow cells suspended in liquid culture with cytokines confirmed that these rhythms are not dependent on an intact stromal microenvironment (Figure S2G).

Canonical Circadian Machinery Operates in Human AML Cells

We next examined whether the circadian circuitry is intact in human AML, as it is in murine leukemia. We found that *MLL*-WT (SKM-1) and *MLL*-rearranged (THP-1) leukemia cell lines are highly sensitive to *CLOCK* and *BMAL1* knockdown using multiple shRNAs (Figures 3B and S2H–I). In THP-1 cells, reciprocal co-immunoprecipitation assays demonstrated a physical association between CLOCK and BMAL1, as would be expected for cells with an intact circadian mechanism (Figure 3C).

To define a set of CLOCK/BMAL1 targets in human AML, we performed chromatin immunoprecipitation sequencing (ChIP-Seq) for both TFs. We called peaks individually for CLOCK (269 peaks) and BMAL1 (9584 peaks) in THP-1 cells and defined a high-confidence set of 140 regions associated with 229 genes bound by both factors (Figure S2J; Table S3A). Pathway analysis revealed that CLOCK/BMAL1 targets are significantly enriched for genes involved in the circadian rhythm (“Circadian Rhythm – Mammal”; MSigDB; Hyper FDR 2.09E-8), including *BHLHE40/41*, *CRY1/2*, *NR1D1*, *PER1/2*, and *DBP* (Figure 3D; Table S3B). Gene ontology (GO) analysis demonstrated that high-

confidence loci are involved in diverse cellular processes, including metabolism, differentiation, immune function, cell cycle regulation, and proliferation, as reported in other tissue types (Miller et al., 2007; Richards and Gumz, 2012) (Figure 3E). In addition, we found CLOCK/BMAL1 binding to Wnt/β-Catenin pathway genes (*WNT11*, *FZD3*, *WISP1, CSNK2A3*) and several genes associated with cancer (*HRAS*, *CEBPA*, *ZEB1*, *EGLN2*). E-Box sites are among the most enriched motifs in the high-confidence binding regions, consistent with the known specificity of these TFs (Weirauch et al., 2014) (Figure 3F; Table S3C).

Identification of Additional Circadian Regulators in Leukemia Cells

Given evidence for binding of CLOCK/BMAL1 to multiple conserved circadian genes in human AML cells, we hypothesized that additional components of the pathway are required for LSC function. We evaluated this possibility by performing an *in vivo* minipool screen in murine *MLL-AF9* cells with shRNAs targeting 22 genes in the canonical circadian pathway, including *Clock* and *Bmal1* and several key regulators. This screen confirmed a role for Clock/Bmal1 and highlighted additional pathway genes (*Per1/3*, *Cry1*, and *Csnk1e/2a1/2b*), raising the possibility that both positive and negative elements of the circadian transcriptional-translational feedback loop impact LSC function (Figure 3G; Table S4). Interestingly, a subset of the hits from this screen (*Per1*, *Cry1*) are directly bound by the Clock/Bmal1 complex and may represent essential mediators of *Clock* and *Bmal1* activity in AML (Figure 3G).

Role of the Circadian Rhythm Pathway in Normal Hematopoiesis

Having established a functional requirement for *Clock* and *Bmal1* in leukemia, we next examined their roles in normal hematopoiesis. Continuous bioluminescence studies with spleen explants from healthy *Per2::Luc* transgenic mice entrained to LD12:12 revealed persistent oscillations with a period of approximately 24 hours (Figure 4A). The circadian activity observed in these mice could reflect either rhythms in hematopoietic cells or oscillations in extra-hematopoietic cells within the spleen. We thus performed bone marrow transplantation experiments to study the rhythm specifically in HSPCs and their progeny. We harvested bone marrow from healthy *Per2::Luc* mice and transplanted c-Kit enriched cells to lethally irradiated WT mice. Continuous bioluminescence recordings of spleen explants from these mice revealed robust rhythms with a period of approximately 24 hours (Figure 4B).

The presence of an intact clock in normal hematopoietic cells, in conjunction with evidence that *Clock* and *Bmal1* are more highly expressed in HSPCs compared to differentiated cells (Figures 4C–D), raises the possibility that the circadian rhythm impacts HSC function. We sought to determine the effect of circadian gene disruption on normal hematopoiesis using germline *Bmal1* knockout mice that lack a functional circadian pacemaker in all tissues (Bunger et al., 2000). These mice display premature death, infertility, organ shrinkage, age-related pathologies such as cataracts, and increased reactive oxygen species, indicating that *Bmal1* may be required for normal tissue stem cell homeostasis (Alvarez et al., 2008; Kondratov et al., 2006; Kondratov et al., 2009).

We used multi-parameter flow cytometry (Figures S3A–D) to assess *Bmal1*$^{-/-}$ mice (Figure S4A) for defects in hematopoietic function and/or differentiation. We first gated lineage^{-}, Scal^{+}, c-Kit^{+} (LSK) cells, with further separation based on the SLAM markers CD48 and CD150, to examine the stem cell compartment in these mice (Kiel et al., 2005). We identified a significant reduction in the percentage of both LSK cells and long-term HSCs (LT-HSCs) in *Bmal1*$^{-/-}$ animals compared to age-matched *Bmal1*$^{+/-}$ controls (Figures 4E–G; Table S5). However, we did not observe a corresponding reduction in the frequency of mature myeloid or lymphoid cells in the bone marrow or spleens of *Bmal1*$^{-/-}$ mice, possibly due to compensatory mechanisms (Figure 4H and S4B). Similarly, an evaluation of myeloid progenitor cells did not reveal significant abnormalities in common myeloid progenitor (CMP), granulocyte monocyte progenitor (GMP), or megakaryocyte erythroid progenitor (MEP) populations (Figure 4I; Table S5).

Because germline *Bmal1*$^{-/-}$ mice have disrupted central pacemaker function, globally altered molecular and behavioral rhythms, and diverse tissue phenotypes, the stem cell finding in these mice may be related to either cell autonomous or cell non-autonomous effects. Consequently, we sought to highlight cell intrinsic effects by inactivating *Bmal1* in adult hematopoiesis with preservation of the circadian rhythm in the SCN and non-hematopoietic peripheral tissues.

We examined the role of *Bmal1* in hematopoietic cells in competitive repopulation assays using conditional knockout mice in which LoxP sites flank the critical bHLH domain of *Bmal1* (Storch et al., 2007). We mixed WT cells expressing CD45.1 with equal numbers of either *Bmal1*$^{f/f}$; *MxCre*$^{+}$ cells or *Bmal1*$^{+/+}$; *MxCre*$^{+}$ cells marked by CD45.2, and transplanted the mix to lethally irradiated CD45.1^{+} recipient mice. Following engraftment, we treated the full cohort of mice with polyinosinic-polycytidylic acid (poly(I:C)), inducing Cre expression and *Bmal1* inactivation in *Bmal1*$^{f/f}$; *MxCre*$^{+}$ hematopoietic cells. We serially measured the peripheral blood CD45.2 chimerism by flow cytometry over 24 weeks *in vivo*, and did not identify significant abnormalities in repopulating ability or multi-lineage differentiation of *Bmal1* knockout HSPCs compared to control HSPCs (Figures 4J and S4C–F). Importantly, we did not detect differences in the frequencies of CD45.2-derived LSK cells or LT-HSCs between the 2 groups, and no significant hematopoietic phenotypes were observed with secondary transplantation (Figures 4K–L and S4G–H).

Consistent with these data, we found that normal murine LSK cells are resistant to *Bmal1* knockdown with multiple shRNAs (Figure S4I). In addition, analyses of non-competitive *Bmal*$^{f/f}$*; MxCre*$^{+}$ mice and germline *Per2* mutant (*Per2*$^{m/m}$) mice (Zheng et al., 1999) did not reveal gross hematopoietic deficits (Figures S5A–I). Collectively, these studies demonstrate that loss of *Bmal1* (or *Per2*) in adult hematopoiesis is well tolerated.

Genetic Knockout Confirms a Leukemia-Specific Dependence on Bmal1

We used the *Bmal1* conditional knockout model to evaluate the phenotypic consequences of complete loss of *Bmal1* activity on leukemia maintenance. We generated murine *MLL-AF9* leukemias in the *Bmal1*$^{f/f}$ background and retrovirally transduced the cells with *PIG-Cre*, resulting in efficient excision (Figure 5A). Consistent with our shRNA and CRISPR-Cas9

results, homozygous *Bmal1* loss resulted in an *in vitro* leukemia growth defect compared to the $Bmal1^{+/+}$ control (Figure 5A).

We next evaluated the effect of *Bmal1* inactivation on the initiation and propagation of *MLL-AF9* leukemia. We treated $Bmal1^{f/f}$; $MxCre^{+}$ or $Bmal1^{+/+}$; $MxCre^{+}$ animals with poly(I:C), harvested bone marrow from these mice, and confirmed excision (Figure S5J). C-Kit enriched cells from both groups of mice were retrovirally transduced with *MIG-MLL-AF9* and transplanted into lethally irradiated WT recipients. Although we did not observe differences in leukemia latency or overall survival between the primary transplant groups, secondary recipient mice receiving $Bmal1^{-/-}$ *MLL-AF9* cells lived significantly longer than mice receiving $Bmal1^{+/+}$ *MLL-AF9* cells (Figure 5B). Thus, *Bmal1* is required for disease propagation.

Given these findings, in conjunction with the HSC knockout studies, we reasoned that AML cells may be differentially sensitive to circadian disruption with the *Rev-Erb α/β* agonist SR9011. To test this hypothesis, we measured the viability of quaternary transplant *MLL-AF9* cells and normal murine LSK cells following treatment with SR9011 for 3 days *in vitro*. We found that murine leukemia cells are more sensitive than HSPCs to various concentrations of drug (Figure 5C). Similarly, SR9011 had greater activity in human AML cell lines (NOMO-1 and MOLM-13) compared to CD34+ cord blood cells purified from healthy donors (Figure 5D). These data indicate that myeloid leukemia cells, in contrast to normal hematopoietic cells, are highly sensitive to perturbation of the core circadian rhythm genes.

Finally, we interrogated genomic data from The Cancer Genome Atlas (TCGA) to determine if central circadian components (*Clock*, *Bmal1*, and *Per2*) are somatically disrupted in human AML (Ley TJ, 2013). In the 191 cases in the TCGA AML dataset, we did not identify mutations or homozygous deletions in *Clock*, *Bmal1*, or *Per2* (Figure S5K), indicating that key components of the circadian circuitry are intact in AML.

DISCUSSION

Cell non-autonomous roles for the circadian rhythm in hematopoiesis are well established (Casanova-Acebes et al., 2013; Lucas et al., 2008; Mendez-Ferrer et al., 2010; Mendez-Ferrer et al., 2008; Nguyen et al., 2013). Our findings demonstrate a previously unrecognized role for the core circadian clock genes in AML. We found that disruption of the circadian circuitry, mediated by shRNA knockdown, targeted mutation, or genetic deletion of key TFs, impairs the growth of murine leukemia cells *in vivo* and human cell lines *in vitro*.

We evaluated the cellular mechanisms of leukemia depletion and found that circadian perturbation induces myeloid differentiation, including a reduction in cell surface expression of c-Kit, a marker of LSCs. Using the Rev-Erb α/β small molecule agonist SR9011, we identified a dose-dependent transcriptional down-regulation of *HoxA9*, *Meis1*, and *Ctnnb1*; together, these self-renewal TFs cooperate to induce myeloid leukemia in mice and are broadly required in multiple AML models (Heidel et al., 2012; Wang et al., 2010). Similarly,

in the skin, the circadian clock regulates the expression of stem cell self-renewal genes, including those in the Wnt/β-catenin pathway, and loss of *Bmal1* results in stem cell arrhythmia in squamous cell tumors (Janich et al., 2011). Thus, links between the circadian rhythm and self-renewal pathways may be conserved in multiple tissue contexts.

We also found that *Clock* and *Bmal1* regulate cellular proliferation and cell cycle progression in AML cells, consistent with phenotypes reported in other systems (Grechez-Cassiau et al., 2008; Matsuo et al., 2003; Miller et al., 2007). Although some studies indicate that specific clock genes may act as tumor suppressors (Fu et al., 2002), other studies suggest a more complex relationship between the circadian rhythm and cancer (Antoch et al., 2013). Our finding that AML cells require *Clock* and *Bmal1* expression reinforces recent work demonstrating tumor-maintaining roles for the core circadian rhythm genes in a variety of cancers (Antoch et al., 2008; Cui et al., 2015; Elshazley et al., 2012; Janich et al., 2011; Li et al., 2013; Ozturk et al., 2009; Xiao et al., 2014).

Furthermore, targets and binding partners of CLOCK and BMAL1 are relevant to the biology and survival of AML cells. A previous study demonstrated that CLOCK and BMAL1 form a complex with wild type MLL1, a histone methyltransferase required for *MLL-AF9* leukemia (Katada and Sassone-Corsi, 2010; Thiel et al., 2010). This association results in cyclical H3K4 trimethylation, establishing a permissive chromatin state for genome-scale circadian transcription (Koike et al., 2012). If the critical interaction domain of MLL1 is preserved in MLL fusions, CLOCK and BMAL1 may physically bind the MLL-AF9 complex, which includes the H3K79 methyltransferase DOT1L, and target it to genomic loci required for leukemia survival (Bernt et al., 2011).

A critical feature of the circadian rhythm is oscillating gene expression in both the SCN as well as peripheral tissues. Peripheral circadian rhythms are cell-autonomous and occur in the absence of external input (Ko and Takahashi, 2006). Using *Per2::Luc* knock-in mice, we confirmed that *MLL-AF9* leukemia cells have the capacity to cycle. Continuous bioluminescence recordings of leukemic tissue explants cultured in isolation demonstrated persistent oscillations with a period of approximately 24 hours, indicating the presence of an intact clock in primary AML cells. Rhythms were maintained with serial transplantation, and remarkably, were present in moribund mice with advanced hematologic disease.

Consistent with cyclical gene expression patterns, canonical circadian regulatory mechanisms are preserved in leukemia. ChIP-Seq studies demonstrated that the CLOCK/BMAL1 complex binds to multiple known circadian pathway genes, and binding occurred with specificity to E-box regulatory elements (Yu et al., 2006). The MYC/MAX complex also has specificity for E-box motifs, indicating that MYC targets may display circadian-dependent regulation and contribute to cancer phenotypes. In addition, a subset of Clock and Bmal1 targets, including *Per1* and *Cry1*, were highlighted in a circadian pathway minipool screen as genes required for *MLL-AF9* leukemia. Given diverse outputs of the peripheral clock in most tissues, it is likely that a set of targets, rather than a single gene, modulates LSC function.

Although rhythmic, circadian-dependent gene expression was present in both normal cells and in leukemia, murine genetic knockout studies indicate that *Bmal1* is not absolutely required for normal hematopoietic function. Interestingly, in the setting of germline loss of *Bmal1*, we observed a reduction in the frequency of LSK cells and LT-HSCs. Despite altered stem cell numbers, mature myeloid and lymphoid cells in both the bone marrow and spleen were grossly normal and recent work indicates that HSCs from these mice have preserved function (Ieyasu et al., 2014). Additionally, we found that selective loss of *Bmal1* in adult hematopoiesis does not significantly alter HSPC differentiation or multilineage repopulating activity in competitive transplantation assays.

In contrast to normal cells, leukemia cells in the *MLL-AF9* model depend on *Bmal1* expression. Genetic deletion of *Bmal1* in established leukemias results in a competitive disadvantage. Leukemia initiation experiments demonstrated that primary leukemias may be derived from *Bmal1*-deficient HSPCs transformed with *MLL-AF9*. However, serial transplantation revealed that loss of *Bmal1* increases leukemia latency and impairs disease propagation. Consistent with the knockout studies, we found that AML cells, as compared to normal HSPCs, are differentially sensitive to circadian perturbation with SR9011.

It remains to be determined if the rhythms of HSCs and LSCs are similar *in vivo*. Differences in these rhythms could be exploited for chronotherapy, in which chemoradiation is dosed at a time of day when leukemia cells are selectively sensitive due to cell cycle status and/or expression of specific clock output genes. Additionally, drugs that alter the circadian rhythm may sensitize leukemia cells to traditional anti-cancer therapies and facilitate targeting of cancer stem cells to eliminate disease more effectively.

EXPERIMENTAL PROCEDURES

Mouse Maintenance, Transplants, and Genotyping

All mouse experiments were performed with an IUCAC-approved animal protocol at our facility. Specific strains used in this study include *C57BL/6J* (Jackson Labs), *B6.SJL* (Taconic), *Actin-dsRed* (006051, Jackson Labs), *B6.129S4(Cg)-Arntl*^{tm1Weit} (007668, Jackson Labs), *B6.129-Arntl*^{tm1Bra} (009100, Jackson Labs), *B6.129S6-Per2*^{tm1Jt} (006852, Jackson Labs), and *B6.Cg-Per2*^{tm1Brd}*; Tyr*$^{c-Brd}$*/J* (003819, Jackson Labs). CD45.1^{+} cells isolated from *B6.SJL* mice (Taconic) were used for the competitive transplantation studies. For all other mouse experiments, *C57BL/6J* mice (Jackson Labs) were used as controls. Prior to tail vein or retro-orbital transplantation, mice were sublethally (1 × 6 Gy [600 rads]) or lethally (1 × 9.5 Gy [950 rads]) irradiated as specified. A multiplexed PCR reaction was used to genotype *Bmal1*$^{f/f}$ mice and to assess the efficiency of excision, as described previously (Storch et al., 2007). *Per2::Luc* knock-in mice were genotyped using primers oIMR6588, oIMR6589, and oIMR6590 according to Jackson Labs protocols.

All mice used in this study were maintained on a standard LD12:12 schedule. Bone marrow and spleen harvests for murine knockout and leukemia studies were generally performed between 1 and 5 hours after the initiation of light (Zeitgeber time, ZT1 to ZT5). Control and experimental cohorts were always harvested at the same time of day.

Quaternary Transplant MLL-AF9 Leukemia Model

GMPs were isolated from the bone marrow of *Actin-dsRed* mice, as described previously (Krivtsov et al., 2006). GMPs were spinfected twice (2500 rpm, 90 min, 37° C) with *MSCV-MLL-AF9-Neo* retrovirus in SFEM (Stem Cell Technologies) supplemented with murine IL3 (mIL3, 10 ng/ml, Peprotech), murine IL6 (mIL6, 10 ng/ml, Peprotech), and murine SCF (mSCF, 10 ng/ml, Peprotech), followed by transplantation to lethally irradiated WT recipients. After disease onset, leukemic spleen cells were harvested and subsequently transplanted to sublethally irradiated secondary recipients. Two additional rounds of transplantation resulted in the generation quaternary transplant leukemias with high penetrance and short disease latency (Hartwell et al., 2013). Flow cytometry was performed using antibodies for the lineage markers CD3, CD4, CD8, B220, CD19, IL7R, and Ter119 (Caltag), c-Kit (17-1172-83, eBioscience), Sca-1 (11-5981-82, eBioscience), CD34 (11-0341-85, eBioscience), and CD16/32 (25-0161-82, eBioscience).

Isolation of Leukemia Stem Cells

The long bones and hips of moribund quaternary transplant leukemic mice were harvested, cleaned, crushed, and sequentially passed through 100 μM and 70 μM filters (Falcon). Following red blood cells lysis (Qiagen), leukemic bone marrow cells were stained with APC-conjugated anti-c-Kit (clone 2B8; eBioscience) and Hoechst 33258 (H21491, Invitrogen) in PBS with 2% FBS (Omega). LSC-enriched populations were isolated by sorting Hoechst^{-}, dsRed^{+}, c-Kit^{high} (top 10–20 percent) cells (FACS Aria II, Becton Dickinson). In the quaternary transplant *MLL-AF9* model, the c-Kit^{high} fraction is sufficiently enriched for leukemia-initiating cells, as described previously (Hartwell et al., 2013; Miller et al., 2013). Quaternary transplant leukemia cells were used for experiments presented in Figures 1A–G, 2B–H, and 5C.

ChIP-Seq

ChIP-Seq was performed as previously described (Kowalczyk et al., 2012) with minor modifications. Specifically, THP-1 cells were fixed in 2 mM EGS (Thermo Scientific) for 30 or 60 minutes followed by 1% formaldehyde for 30 or 15 minutes at RT, respectively. Chromatin was sonicated to a size less than 500 bp and then incubated overnight with either anti-Clock IgG (ab3517, Abcam) or anti-Bmal1 IgG (ab3350, Abcam). ChIP-Seq libraries were prepared through the process of DNA end-repair (Epicentre), A-base addition, and adaptor ligation using indexed Illumina adaptors followed by enrichment PCR. All enzymatic steps were carried out using enzymes from New England Biolabs. Final libraries were pooled, size selected, and sequenced on HiSeq2500 with 25 paired-end reads.

ChIP-Seq Data Analysis

Paired-end reads for both ChIP and input samples were mapped to the hg19 version of the human genome using Bowtie 2 (Langmead and Salzberg, 2012), only keeping read pairs that map uniquely to the genome. Peaks were called using findPeaks from the Homer suite (v4.7.2) (Heinz et al., 2010) with “-style factor -fdr 0.01 -LP 0.01 -P 0.1” parameters and using input data as background. The intersection of peaks for CLOCK and BMAL1 was used to define a high-confidence set of binding sites. Identification of target genes associated

with these binding sites and functional enrichment analysis were completed using GREAT (v2.0.2) (McLean et al., 2010). H3K4me3 peaks (SRA accession ERS148524) were set as background to control for activity/inactivity of genes. We identified 269 peaks associated with 406 genes for CLOCK and 9584 peaks associated with 8027 genes for BMAL1. Focusing on the intersection of CLOCK and BMAL1 peaks, we identified 140 binding sites associated with 229 genes. Motif analysis of high-confidence sites was performed using RSAT peak-motifs (Thomas-Chollier et al., 2012). Gene ontology analysis (Ashburner et al., 2000) was completed using the GO Slim web portal (http://go.princeton.edu). ChIP-Seq data presented in this study may be accessed on Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with GEO ID GSE70686.

Statistics

Kaplan Meyer analysis (Mantel-Cox test) and EC_{50} calculations were completed using GraphPad Prism 6. Statistical analysis was completed using R (www.r-project.org) or Excel (Microsoft) software. Mean values are shown (unless otherwise specified) and error bars represent standard error of the means. Flow cytometry gating and MFI analysis were completed using FlowJo 7.6.1 software. Statistical significance was determined using a two-sided Student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Detailed descriptions of additional experimental and analytical methods are included in the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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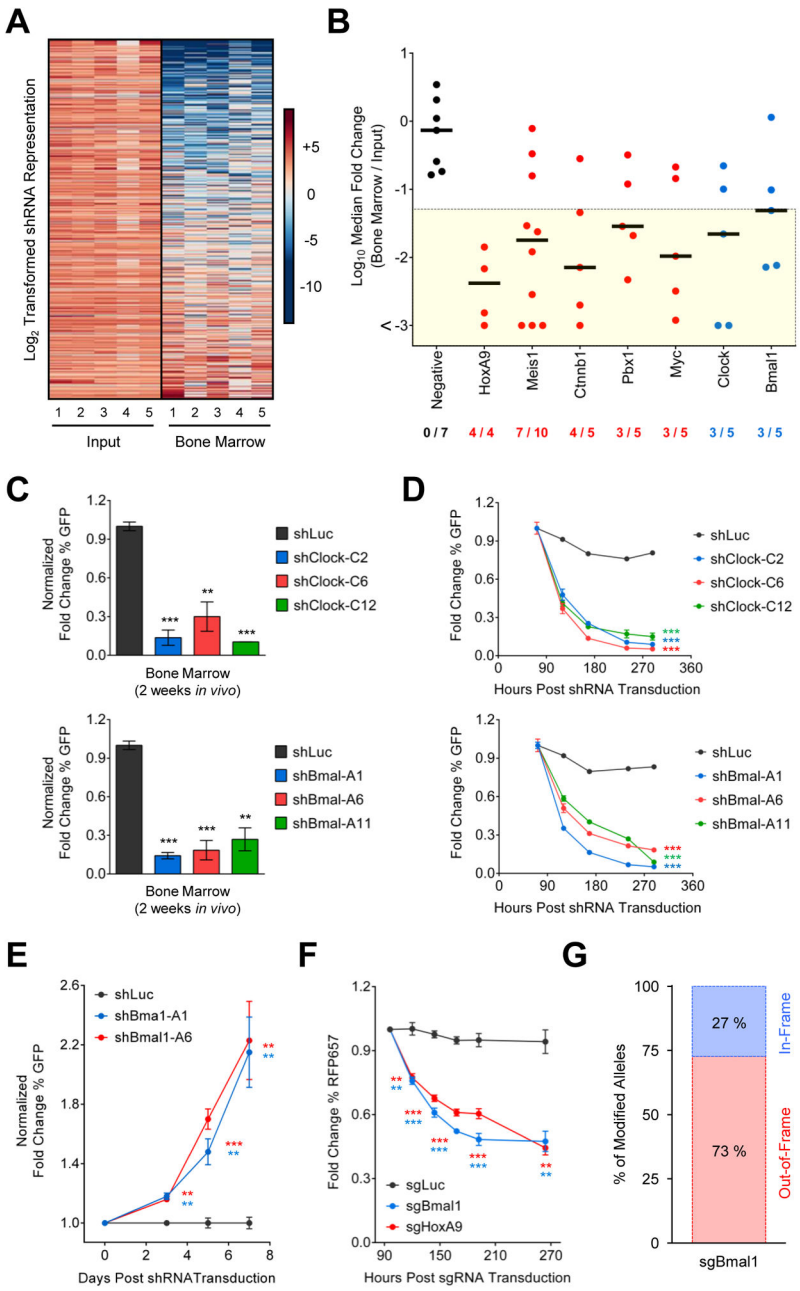

Figure 1. *Clock* and *Bmal1* are required for leukemia cell growth

(A) Pooled RNAi screen identified shRNAs depleting murine *MLL-AF9* leukemia cells *in vivo*. Heat map depicting the $\log_2$ transformed representation of individual shRNAs (rows) in the input replicates (n=5, left columns) and leukemic bone marrow replicates (n=5, right columns). Depleted shRNAs are shown in blue. **(B)** Performance of shRNAs targeting negative control genes (black), positive control genes (red), and circadian rhythm genes (blue). Plots depict the $\log_{10}$ median fold change for each shRNA. Fraction of shRNAs with fold depletion > 20 is shown. **(C–D)** Fold change in the percentage of GFP^+ murine *MLL-AF9* leukemia cells transduced with single *Clock* (C2, C6, C12) (n=3), *Bmal1* (A1, A6,

A11) (n=3), or *Luc* (n=3) shRNAs co-expressed with GFP **(C)** *in vivo* or **(D)** *in vitro*. **(E)** Phenotypic rescue of *Bmal1* knockdown with an shRNA-resistant *Bmal1* cDNA in murine *MLL-AF9* leukemia cells. **(F)** *In vitro* tracking of *Cas9*-expressing murine *MLL-AF9* leukemia cells transduced with sgRNAs targeting *Bmal1* (sgBmal) (n=3), *HoxA9* (sgHoxA9) (n=3), or *Luc* (sgLuc) (n=3) co-expressed with RFP657. **(G)** Detection of in-frame and out-of-frame mutations at the *Bmal1* locus in cells expressing *Cas9* and sgBmal1 by Sanger sequencing of individual clones. *p<0.05, **p<0.01, ***p<0.001, determined by a two-sided Student's t-test. Mean values are shown unless otherwise specified, and error bars represent +/− S.E.M.

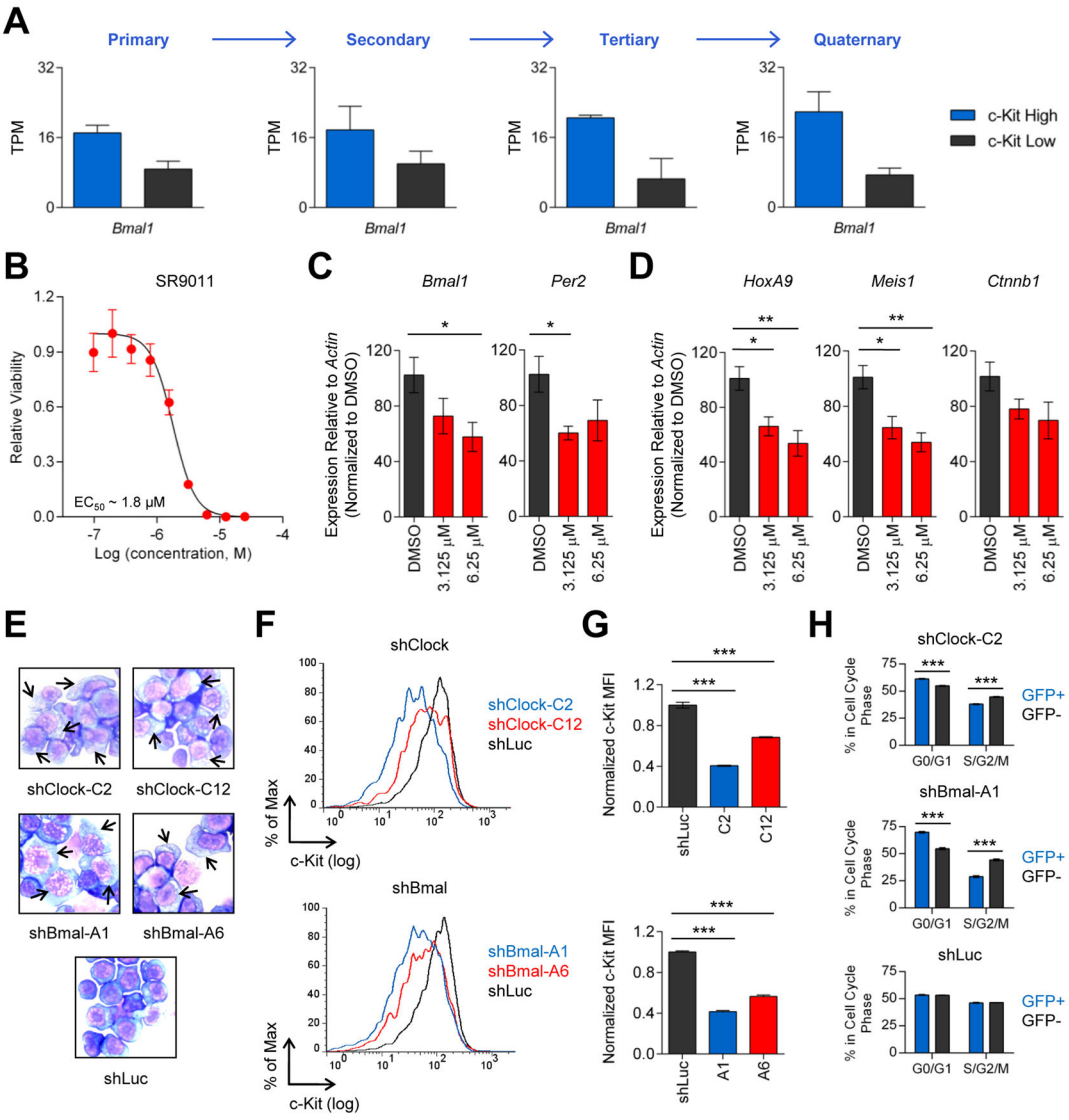

Figure 2. Disruption of the circadian rhythm machinery induces leukemia differentiation

(A) RNA-Seq analysis of *Bmal1* expression in c-Kit^{high} (top 10 percent, n=3) and c-Kit^{low} (bottom 10 percent, n=3) leukemic bone marrow cells from primary, secondary, tertiary, and quaternary transplant *MLL-AF9* mice. TPM, transcripts per million. **(B)** Dose response curve showing relative viability (y-axis) of murine *MLL-AF9* leukemia cells treated for 3 days with varying concentrations of SR9011 (x-axis, log scale) (n=3). **(C–D)** Expression of **(C)** circadian rhythm genes (*Bmal1*, *Per2*) or **(D)** regulators of LSC self-renewal (*HoxA9*, *Meis1*, *Ctnnb1*), measured by quantitative RT-PCR following treatment of murine *MLL-AF9* leukemia cells with 3.125 μM SR9011 (n=4), 6.25 μM SR9011 (n=4), or DMSO (n=4) for 24 hours. **(E)** Wright-Giemsa staining of murine *MLL-AF9* leukemia cells transduced with single *Clock* (C2, C12) or *Bmal1* (A1, A6) shRNAs revealed monocytic differentiation (black arrows). **(F)** Representative flow cytometry histograms of c-Kit cell surface expression (x-axis, log scale) in GFP^{+} murine *MLL-AF9* leukemia cells expressing *Clock* (C2, C12), *Bmal1* (A1, A6), or *Luc* shRNAs. **(G)** Quantification of c-Kit cell surface

expression by median fluorescence intensity (MFI) analysis. **(H)** Hoechst33342 cell cycle analysis of murine *MLL-AF9* leukemia cells transduced with *Clock* (C2, n=3), *Bmal1* (A1, n=3), or *Luc* (n=3) shRNAs. Data for both transduced (GFP^+) and untransduced (GFP^-) cells within the same replicate are shown. *p<0.05, **p<0.01, ***p<0.001, determined by a two-sided Student's t-test. Mean values are shown unless otherwise specified, and error bars represent +/− S.E.M.

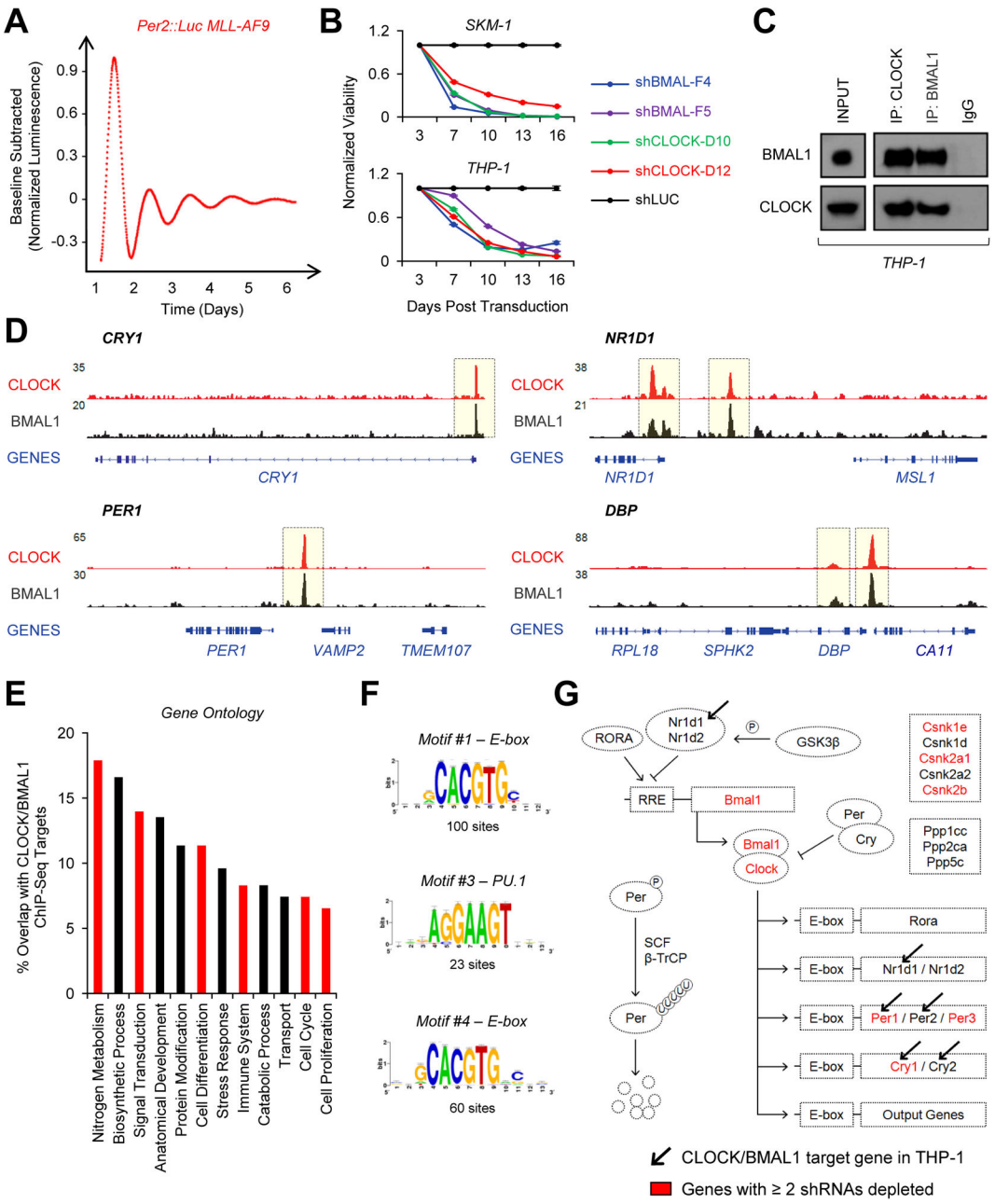

Figure 3. Canonical circadian regulatory mechanisms are intact in primary murine leukemia and human AML

(A) Continuous bioluminescence recording of a leukemic spleen explant from a WT secondary recipient mouse transplanted with *Per2::Luc MLL-AF9* leukemia cells. **(B)** Cell titer glo assay showing viability of SKM-1 or THP-1 cells following transduction with shRNAs targeting *CLOCK* (D10, D12) (n=5), *BMAL1* (F4, F5) (n=5), or *LUC* (n=5). Equal numbers of puromycin-selected cells were plated 3 days after transduction and followed for 2 weeks *in vitro*. Data is normalized to the shLUC control at each timepoint. **(C)** CLOCK and BMAL1 reciprocal co-immunoprecipitation in THP-1 cells. **(D)** UCSC genome browser tracks of CLOCK (red) and BMAL1 (black) occupancy at the *CRY1, NR1D1, PER1,* and

DBP loci in THP-1 cells based on normalized ChIP-Seq read coverage. Track heights are indicated. **(E)** Gene ontology analysis of pathways represented by high-confidence CLOCK/BMAL1 targets. Pathways of interest are highlighted in red. **(F)** RSEM motif enrichment analysis of high-confidence regions identified in CLOCK and BMAL1 ChIP-seq. **(G)** Minipool shRNA screen of 22 canonical circadian regulators in murine *MLL-AF9* leukemia cells. Hits from the screen are shown in red and high-confidence Clock/Bmal1 ChIP-Seq targets are marked with a black arrow. Mean values are shown unless otherwise specified, and error bars represent +/− S.E.M.

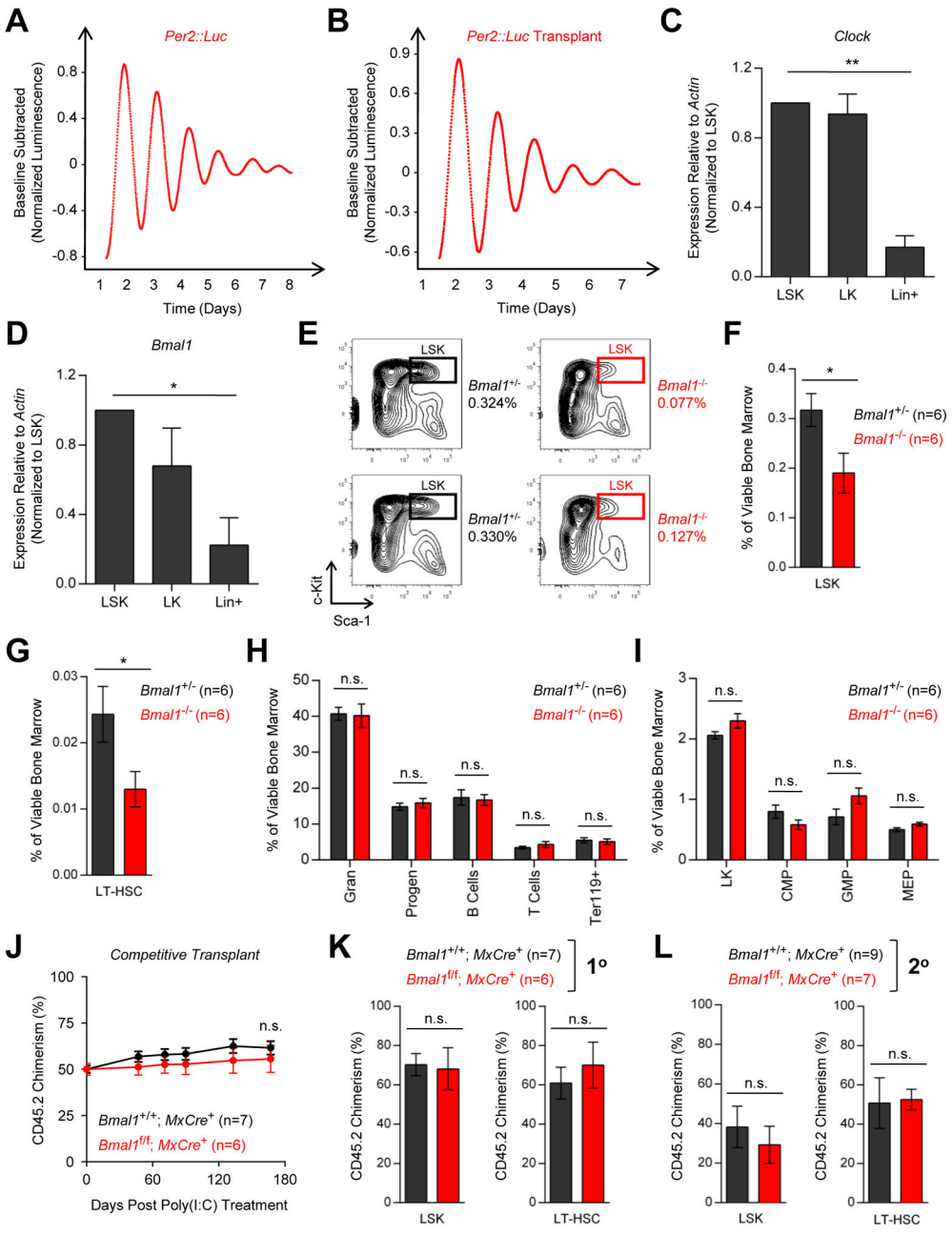

Figure 4. *Bmal1* is dispensable for normal HSC function

(A–B) Continuous bioluminescence recording of spleen explant from **(A)** homozygous *Per2::Luc* transgenic mouse or **(B)** lethally irradiated WT mouse transplanted with c-Kit enriched *Per2::Luc* bone marrow. **(C–D)** Expression of **(C)** *Clock* or **(D)** *Bmal1* in murine LSK cells (n=3), LK cells (n=3), and lineage+ cells (n=3) purified from wild type mice, as measured by qPCR. **(E)** Representative flow cytometry contour plots of LSK cells in the bone marrow of germline $Bmal1^{-/-}$ and $Bmal1^{+/-}$ mice. LSK frequency is presented as a percentage of viable whole bone marrow. **(F–I)** Quantification of **(F)** LSK cells, **(G)** LT-HSCs, **(H)** lineage^{+} cells, and **(I)** myeloid progenitor cells in the bone marrow of germline

Bmal1$^{-/-}$ (n=6) and *Bmal1*$^{+/-}$ (n=6) mice. Data is presented as a percentage of viable whole bone marrow. **(J)** Peripheral blood CD45.2 chimerism in *Bmal1*$^{+/+}$; *MxCre*$^{+}$ (n=7) and *Bmal1*$^{f/f}$; *MxCre*$^{+}$ (n=6) competitive transplant mice following poly(I:C) treatment. **(K–L)** CD45.2 chimerism in the bone marrow LSK and LT-HSC compartments of *Bmal1*$^{+/+}$; *MxCre*$^{+}$ and *Bmal1*$^{f/f}$; *MxCre*$^{+}$ **(K)** primary or **(L)** secondary competitive transplant mice. *p<0.05, **p<0.01, ***p<0.001, determined by a two-sided Student's t-test. Mean values are shown unless otherwise specified, and error bars represent +/− S.E.M.

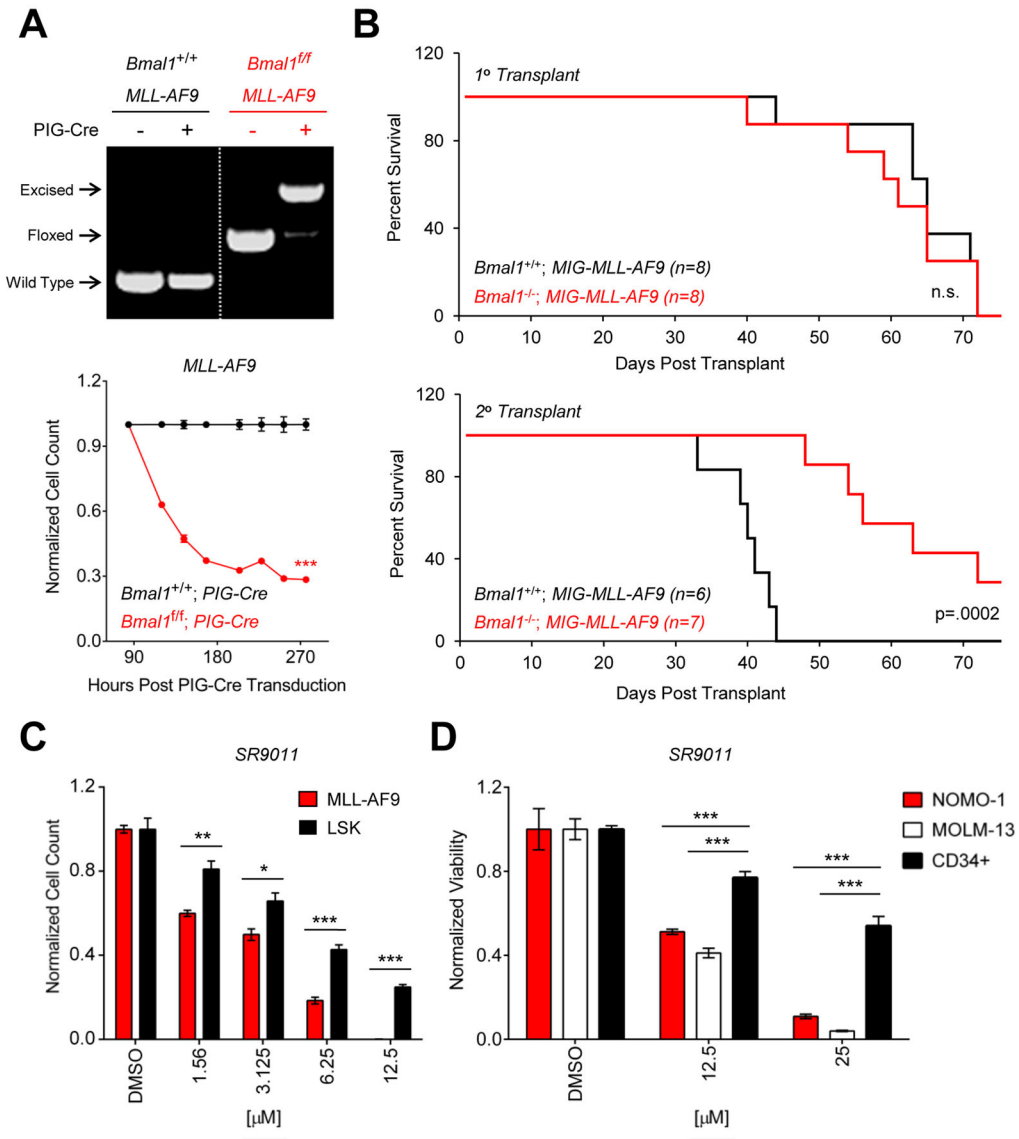

Figure 5. Genetic knockout models reveal a leukemia-specific dependence on *Bmal1*

(A) Top panel shows genotyping of *Bmal1*$^{f/f}$ and *Bmal1*$^{+/+}$ *MLL-AF9* leukemias before and after transduction with *PIG-Cre*. Dotted white line indicates spliced gel. Bottom panel shows normalized cell counts of *Bmal1*$^{f/f}$ (n=4) and *Bmal1*$^{+/+}$ (n=4) *MLL-AF9* leukemias transduced with *PIG-Cre in vitro*. Puromycin-selected cells were plated 84 hours after transduction and followed over time. **(B)** *Bmal1*$^{f/f}$; *MxCre*$^{+}$ mice and *Bmal1*$^{+/+}$; *MxCre*$^{+}$ controls were treated with poly(I:C) to induce hematopoietic-specific *Cre* expression. Bone marrow cells harvested from these mice were c-Kit enriched, transduced with *MIG-MLL-AF9*, and transplanted to lethally irradiated WT recipient mice. The resulting *Bmal1*$^{-/-}$ or *Bmal1*$^{+/+}$ *MLL-AF9* leukemias were transplanted to sublethally irradiated secondary recipients. Kaplan-Meier survival curves for the primary and secondary transplants are shown. **(C)** Normalized cell counts of quaternary transplant *MLL-AF9* cells (n=3) and murine LSK cells (n=3) following treatment with DMSO or SR9011 for 3 days *in vitro*. **(D)**

Cell titer glo assay showing normalized viability of NOMO-1 (n=3), MOLM-13 (n=3), and CD34+ cord blood cells (n=6) following treatment with DMSO or SR9011 for 4 days *in vitro*. *p<0.05, **p<0.01, ***p<0.001, determined by a two-sided Student's t-test. Mean values are shown unless otherwise specified, and error bars represent +/− S.E.M.