NAD⁺-SIRT1 control of H3K4 trimethylation through circadian deacetylation of MLL1

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The circadian clock controls the transcription of hundreds of genes through specific chromatin-remodeling events. The histone methyltransferase mixed-lineage leukemia 1 (MLL1) coordinates recruitment of CLOCK–BMAL1 activator complexes to chromatin, an event associated with cyclic trimethylation of histone H3 lys4 (H3K4) at circadian promoters. Remarkably, in mouse liver circadian H3K4 trimethylation is modulated by SIRT1, an NAD⁺-dependent deacetylase involved in clock control. We show that mammalian MLL1 is acetylated at two conserved residues, K1130 and K1133. Notably, MLL1 acetylation is cyclic, controlled by the clock and by SIRT1, and it affects the histone deacetylase activity of MLL1. Moreover, H3K4 methylation at clock-controlled-gene promoters is influenced by pharmacological or genetic inactivation of SIRT1. Finally, levels of MLL1 acetylation and H3K4 trimethylation at circadian gene promoters depend on NAD⁺ circadian levels. These findings reveal a previously unappreciated regulatory pathway between energy metabolism and histone methylation.

A wide variety of biological processes are under circadian control, as illustrated by rhythms in mammalian behavior, physiology and metabolism. The core transcription factors circadian locomotor output cycles kaput (CLOCK) and BMAL1 dimerize to drive the expression of clock-controlled genes (CCGs), through a mechanism that relies on coordinated chromatin-remodeling events. Circadian transcription is associated with rhythmic changes in epigenetic marks at circadian promoters, such as H3K4 trimethylation and H3K9 and H3K14 acetylation. A key event in circadian transcriptional activation is the interaction of CLOCK–BMAL1 with proteins associated with the Set1 (COMPASS) complex component MLL1, whose enzymatic activity leads to the transcription-activating histone mark trimethyl H3K4 (H3K4me3; ref. 5). MLL1 contributes to the recruitment of CLOCK–BMAL1 to chromatin and thereby to the expression of CCGs.

Circadian metabolism and the epigenome intersect at various levels, and the circadian clock has been proposed to control part of this interplay through sirtuins, a class of NAD⁺-dependent deacetylases. In addition, the intracellular levels of many metabolites oscillate in a circadian manner. Specifically, circadian fluctuation of coenzyme NAD⁺ levels induce rhythmicity in SIRT1 enzymatic activity. Remarkably, NAD⁺ oscillation is dictated by CLOCK–BMAL1, which directly controls the gene Nampt, encoding the nicotinamide phosphoribosyltransferase (NAMPT) enzyme, which catalyzes the rate-limiting step in the NAD⁺ salvage pathway. Moreover, genetic ablation or pharmacological inhibition of SIRT1 activity alters circadian rhythmicity of CCGs. Also, as previously reported, SIRT1 appears to control circadian acetylation of H3K9 and H3K14 at a number of CCG promoters and to regulate the expression of a large, specific subset of circadian genes.

Although these studies have shown that circadian regulators operate in response to diverse cellular metabolic cues, the molecular mechanisms governing their cross-talk have remained largely unexplored. One of the unanswered questions is indeed related to whether cyclic chromatin modifications are controlled by the circadian clock. Hence, we sought to determine whether the function of H3K4 methyltransferase MLL1 on circadian gene promoters is influenced by metabolic regulation governed by the circadian clock. Here, we present evidence that the function of the metabolite NAD⁺ extends to modulation of the circadian epigenome through a molecular interplay between SIRT1 and MLL1. As a consequence, levels of the epigenetic mark H3K4me3 at circadian gene promoters are responsive to intracellular NAD⁺ levels. We found that MLL1 is an acetylated protein and that its enzymatic activity is controlled by SIRT1-dependent deacylation. These findings may provide new insights into the circadian alterations that occur during aging, in which NAD⁺ decay is paralleled by misregulation in the amplitude and phase of clock-controlled genes.

RESULTS

SIRT1 controls cyclic levels of H3K4me3

During our studies on the role of SIRT1 in circadian transcription, we performed chromatin immunoprecipitation (ChIP) assays at different circadian times in dexamethasone (DEX)-synchronized wild-type (WT) and SIRT1-deficient (Sirt1−/−) mouse embryonic fibroblasts (MEFs). As previously reported, H3K4me3 displayed robust circadian oscillation on the Dbp promoter and coding region, being high at circadian time (CT) 18 and low at CT 30 (Fig. 1a, Dbp E1 and Dbp I1). We obtained analogous results in several CCGs, including Per2.

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Received 12 November 2014; accepted 11 February 2015; published online 9 March 2015; doi:10.1038/nsmb.2990
Also, H3K4me3 levels in Sirt1−/− MEFs, exhibited markedly higher levels than in WT MEFs along the circadian cycle (Fig. 1a, Dbp E1 and Dbp I1), an intriguing observation because H3K4 trimethylation and H3K9 and H3K4 acetylation have been functionally associated.

To explore whether the increase of H3K4me3 is directly linked to SIRT1, we treated WT MEFs with the specific inhibitor EX527. This treatment resulted in an increase of H3K4me3 at the Dbp coding region (Fig. 1a, Dbp E1 and Dbp I1) but not at the 3′ untranslated region (UTR) used as control for specificity (Fig. 1a). We obtained analogous results in vivo by analyzing livers from Sirt1−/− mice, with a liver-specific deletion in the catalytic domain of the Sirt1 gene. Indeed, similarly to the results in MEFs, H3K4me3 levels at circadian-gene promoters showed higher amplitudes in Sirt1 mutants as compared to WT littermates (Fig. 1b), a difference associated with parallel changes in circadian gene expression (Supplementary Fig. 1a,b). Importantly, H3K4me3 and expression levels from the noncircadian housekeeping genes Tbp and Gapdh, and the MLL1-controlled genes Hoxa9 and Emilin1 (refs. 22–24), displayed no significant changes upon Sirt1 deletion (Fig. 1c and Supplementary Fig. 1c,d). Thus, SIRT1 appears to specifically control a subset of MLL1 targets, namely circadian genes. These results highlight the specificity of the control of the circadian epigenome at clock-controlled genes, which are governed by a dedicated molecular machinery to maintain the correct circadian output. Also, monomethylated H3K4 (H3K4me1) levels did not cycle, nor were they altered upon deletion or pharmacological inhibition of SIRT1 (Supplementary Fig. 2a). However, dimethylated H3K4 (H3K4me2) levels at the Dbp gene were increased in Sirt1−/− MEFs and in cells treated with EX527 (Supplementary Fig. 2a). Thus, SIRT1 appears to specifically affect H3K4 circadian methylation levels at CCGs.

NAD+--dependent cyclic H3K4 trimethylation

NAD+ metabolism is intimately linked to circadian rhythms. The NAD+ salvage pathway is clock controlled because the expression
of the rate-limiting enzyme NAMPT is driven by the clock, and NAD^+ levels are circadian, thus leading to cyclic SIRT1 enzymatic activity. Because our findings suggest a cross-talk between energy metabolism and the epigenetic modifier MLL1, we explored whether alterations in the levels of intracellular NAD^+ would influence MLL1-mediated circadian gene transcription (Fig. 1d). Increasing doses of NAD^+ progressively dampened MLL1-mediated activation of Dbp and Per1 expression (Fig. 1d and Supplementary Fig. 2b). Also, the NAD^+ precursors β-nicotinamide mononucleotide (β-NMN) and nicotinic acid (NA) elicited a similar effect (Fig. 1d and Supplementary Fig. 2b). The treatment with the by-product of NAD^+ consumption, nicotinamide (NAM), elicited a substantial and dose-dependent increase in MLL1-mediated activation of Dbp expression (Fig. 1d and Supplementary Fig. 2b). A similar trend was apparent in Sirt1^−/− MEFs only when cotransfected with SIRT1 but not in the catalytically inactive mutant SIRT1 H363Y (Supplementary Fig. 2c). Next, we investigated whether H3K4-methylation levels at circadian promoters would be modified by changing the NAD^+ concentration (Fig. 1e). ChIP experiments on NAD^+ - and β-NMN-treated cells showed that H3K4me3 levels became constitutively low, and oscillation was partially or totally lost, and these treatments had no effect in Sirt1^−/− MEFS (Supplementary Fig. 2d). Concomitantly, H3K4me3 and H3K4me2 levels were higher after treatment with FK866, a specific chemical inhibitor of NAMPT, showing greater amplitude in oscillation than in nontreated cells (Supplementary Fig. 2e). As expected, these changes were accompanied by decreased oscillation of H3 acetylation after FK866 treatment (Supplementary Fig. 2e).

**SIRT1 controls MLL1 methyltransferase activity**

From these findings, we reasoned that SIRT1 could directly modulate MLL1 function. Coexpression of Flag-MLL1-myc and a tagged version of SIRT1 and subsequent coimmunoprecipitation revealed that SIRT1 interacts with MLL1 (Fig. 2a and Supplementary Fig. 3a). The interaction is specific, as shown by the use of the related deacetylase SIRT2 (Fig. 2a). Endogenous SIRT1 and MLL1 proteins also interact, as demonstrated by coimmunoprecipitation assays (Fig. 2b and Supplementary Fig. 3b,c). We next followed the MLL1-SIRT1 interaction through the circadian cycle (Fig. 2c), preparing nuclear extracts at different circadian times from DEX-entrained MEFs. We confirmed circadian rhythmicity by monitoring the oscillation in BMAL1 phosphorylation (methodology in refs. 25,26; Fig. 2c). Coimmunoprecipitation assays revealed that MLL1 interacts with endogenous SIRT1 in a circadian time-specific manner. This interaction peaked at CT30–CT36, paralleling the Dbp circadian profile when H3K4 methylation is at its trough (Figs. 1 and 2c). We did not detect the MLL1-SIRT1 interaction in unsynchronized WT MEFS (Fig. 2c, CT0), results indicating that the clock machinery promotes this molecular interplay.

To identify the regions involved in the SIRT1-MLL1 interaction, we generated various truncated Flag-tagged versions of the MLL1 protein (Fig. 2d, F–M1 to F–M6). After coexpression in HEK293 cells, coimmunoprecipitations of SIRT1 with the various MLL1 truncations revealed that an N-terminal region of MLL1 containing its DNA-binding domain (amino acids 650–1327) interacts with SIRT1, thus revealing that an N-terminal region of MLL1 containing its DNA-binding domain (amino acids 650–1327) interacts with SIRT1, thus confirming specificity of the interaction. (Fig. 2e and Supplementary Fig. 3d,e). Importantly, this protein domain is involved in the interaction of MLL1 with different protein complexes, regulating MLL1 recruitment to specific promoters in the genome. Using a mammalian two-hybrid assay (Fig. 2f), we found that the same MLL1 region required for the interaction with SIRT1 mediates the interaction with CLOCK and that MLL1 interaction with CLOCKA19 was much weaker (Fig. 2f).

**MLL1 is acetylated**

The MLL1-SIRT1 interaction suggested that SIRT1 might control MLL1 through deacetylation, an event that could modulate MLL1...
function and activity. To address this question, we first determined whether MLL1 is indeed an acetylated protein by using an anti-pan-acetyllysine antibody (Fig. 3a and Supplementary Fig. 4a). The interactions between MLL1 and the histone acetyltransferases (HATs) CBP or p300 have been extensively characterized, including their cooperation in gene activation. Thus, we investigated whether SIRT1 deacetylates MLL1, a specific antibody detecting acetylated lysines K1130 and K1133 from MLL1. Using anti–pan-acetyllysine antibody, which preferentially detects C-terminal acetylation of MLL1, we found that both CBP and p300 markedly enhanced MLL1 acetylation at both its N- and C-terminal domains. Importantly, PCAF, a HAT related to CBP and p300, did not acetylate MLL1 (Supplementary Fig. 4b). We further confirmed specificity because other HATs known to interact with MLL1, including CLOCK and MOF as well as HAT1, do not acetylate MLL1 (Supplementary Fig. 4b).

Next we sought to determine the regulation of MLL1 deacetylation. To do so we coexpressed MLL1 and CBP and then treated cells with trichostatin A (TSA), an inhibitor of class I and class II histone deacetylases (HDACs), and/or NAM, an inhibitor of class III HDACs (Fig. 3c). We found that acetylation of MLL1 was increased by NAM treatment but not by TSA treatment (Fig. 3c), thus indicating that a sirtuin is responsible for MLL1 deacetylation. To establish whether SIRT1 deacetylates MLL1, we used a deacetylation assay (Supplementary Fig. 4c). Acetyl-MLL1 is readily deacetylated by SIRT1 in an NAD+–dependent manner (Fig. 3d). This activity is not caused by a possible contamination of the immunoprecipitated proteins with class I or class II HDACs, because addition of TSA had no effect. Also, previously described nuclear sirtuins SIRT3, SIRT6 and SIRT7 (refs. 32–34) were not able to deacetylate MLL1 (Supplementary Fig. 4d), thus demonstrating that MLL1 deacetylation has to be attributed to SIRT1.

SIRT1-mediated circadian deacetylation of MLL1

Although, to our knowledge, MLL1 acetylation has not been studied previously, MS profiling for the global protein acetylome identified four lysine residues as acetylated in MLL1: K636, K1130, K1133 and K1235 in the human isoform of MLL1 (ref. 35). Interestingly, K1130,
K1133 and K1235 are located within the MLL1 region that interacts with CLOCK and SIRT1 (Fig. 2d,e). Importantly, K1130 and K1133 are highly conserved (Supplementary Fig. 5a), thus indicating a likely role for acetylation at these two residues. Mutation of K1130 and K1133 into arginines resulted in a protein that is functionally unable to compete with the MLL1-mediated CLOCK–BMAL1 coactivation of a circadian gene promoter, whereas the WT form of the peptide was able to do so (Supplementary Fig. 5b). Thus, the K1130 and K1133 residues are functionally relevant for the activity of the complex (Supplementary Fig. 5b). We obtained similar results when coexpressing SRT1. We thereby raised a polyclonal antibody that specifically recognizes MLL1 acetylated at K1130 and K1133 (Fig. 3f). In contrast, a catalytically inactive SIRT1 with a single–amino acid substitution (H363Y) or another sirtuin such as SIRT2 did not influence acetylation at these residues (Fig. 3f). Moreover, pharmacological inhibition of endogenous SIRT1 with EX527 markedly increased acetylation of K1130 and K1133 (Fig. 3f), thus confirming that these are specific and direct targets of SIRT1.

Because SIRT1 deacetylase activity is circadian40, we reasoned that its MLL1 target K1130 and K1133 residues could be rhythmically deacetylated. To test this possibility, we entrained cells with DEX and prepared nuclear extracts at various CTs (Fig. 3g). MLL1 showed a robust circadian acetylation peaking at CT18. Remarkably, the MLL1 deacetylation profile paralleled its cyclic interaction with SRT1 (Fig. 2c and Fig. 3g). Although the SIRT1–MLL1 interaction peaked at CT30, we observed some deacetylation at CT24. This effect is the result of various factors. First, circadian levels of NAD+ are high at CT24, thus enhancing SIRT1 activity; although the interaction with MLL1 might be weak at this time, deacetylation still occurs stochastically. Second, the specificity of SIRT1 targets is dictated not only by enzymatic kinetics but also by other factors such as additional interacting proteins (i.e., DBC1) or the intranuclear availability of NAD+. Remarkably, the strongest deacetylation of MLL1 was at CT30, coincident with the most efficient interaction with SIRT1. We extended our analysis in vivo by preparing liver nuclear extracts at different zeitgeber times (ZT) and then monitored acetylation of K1130 and K1133. We found that MLL1 was acetylated rhythmically in the liver, with a peak at ZT9 (Supplementary Fig. 5c). Importantly, genetic ablation of SIRT1 disrupted circadian MLL1 acetylation (Supplementary Fig. 5c), and MLL1 protein levels were not regulated by SIRT1 and remained constant during the circadian cycle (Supplementary Fig. 5d). Remarkably, acetylation of MLL1 K1130 and K1133 was enhanced after treatment with FK866 and EX527 as well as in Sirt1−/− MEFs, but it was lower after treatment with NAD+ or β-NMN, thus demonstrating a molecular cross-talk between MLL1, SIRT1 and energy metabolism (Fig. 3h).

The SIRT1–MLL1 interplay controls circadian transcription

We have previously reported that H3K4 trimethylation at CGGs is mediated by MLL1 (ref. 5). To investigate whether SIRT1 influences MLL1-mediated function, we analyzed the effect of SIRT1 on the MLL1-mediated activation of the Dbp promoter (Supplementary Fig. 6a). As previously reported5, MLL1 increased CLOCK–BMAL1-dependent Dbp promoter activity in a dose-dependent manner (Supplementary Fig. 6a). This activity was dependent on MLL1 catalytic activity, because a mutated protein lacking the catalytic SET domains acted as a dominant-negative regulator (Supplementary Fig. 6a). A truncated version of MLL1 lacking a portion of the N-terminal region, which includes the two acetylated residues, also lost the transactivation potential, thus highlighting the relevance of this domain in CLOCK–BMAL1–dependent transcription (Supplementary Fig. 6a). Remarkably, SIRT1 strongly reduced CLOCK–BMAL1–mediated transcriptional activation (Supplementary Fig. 6b), an effect dependent on the enzymatic activity, because a catalytically inactive SIRT1 (H363Y) was not able to elicit this function (Supplementary Fig. 6b).

To gain insight into the functional implications of the MLL1–SIRT1 interplay, we coexpressed MLL1 with increasing amounts of SIRT1 (Fig. 4a and Supplementary Fig. 6c). Remarkably, SIRT1 decreased MLL1–mediated transcriptional activation of the Dbp promoter in a dose-dependent manner (Fig. 4a), an effect abolished when SIRT1 catalytic activity was impaired (Fig. 4a and Supplementary Fig. 6c, SIRT1 H363Y). We confirmed this result by pharmacological inhibition of endogenous SIRT1 in cells treated with increasing doses...
of EX527 (Fig. 4b). Therefore, SIRT1 modulates the MLL1-mediated activation of circadian gene expression. SIRT1-mediated deacetylation has been linked to the function of various transcriptional regulators. Thus, we questioned whether MLL1 circadian methyltransferase activity could be controlled by SIRT1. This was indeed the case, and SIRT1 deacetylase activity was required for this regulation (Fig. 4c).

DISCUSSION

The cyclic transcription of hundreds of clock-controlled genes represents a remarkable paradigm to study harmonic changes in chromatin remodeling. The spatial and temporal organization of the circadian epigenome appears to involve ‘nuclear hubs’ where coordinately regulated genes are associated in circadian interactomes. The molecular mechanisms that control these events involve chromatin reorganization. Accumulating evidence has implicated a variety of chromatin remodelers in circadian control, which lead to rhythmic acetylation, phosphorylation and methylation of specific residues on histone tails at circadian-gene promoters. Although a role for CLOCK–BMAL1 in directly influencing chromatin has been proposed, its recruitment to chromatin loci permissive for transcription is thought to be involved in activator complexes containing enzymatically active remodelers. Among these, MLL1 has been shown to play a major role in directing cyclic H3K4 trimethylation and critically contributing to CLOCK–BMAL1 recruitment to chromatin.

One unanswered question has concerned how chromatin remodelers may interact to ideally translate changes in cellular metabolism to the homeostatic oscillations in gene expression governed by the circadian clock. In this study, we reveal a previously unrecognized connection between NAD⁺ and the activity of MLL1, a histone methyltransferase. This link extends to the modulation of H3K4 methylation of CGGs, thereby affecting their transcriptional efficacy (Fig. 5). Our findings suggest a scenario in which oscillating levels of NAD⁺ control MLL1 acetylation and thereby CLOCK–BMAL1–dependent transcriptional activation. Specifically, when NAD⁺ levels are low, MLL1 is acetylated and cooperates with CLOCK–BMAL1 in circadian gene activation (Fig. 5a). When acetylated, MLL1 enzymatic activity is high (Fig. 4c), paralleling H3K4me3 levels (Fig. 1a,b) and the peak of circadian transcription (Supplementary Fig. 1a,b). NAD⁺ levels rise at ZT11–ZT15, thereby promoting SIRT1 deacetylase activity (Fig. 5b). SIRT1 in turn deacetylates MLL1, an event that reduces its enzymatic activity and leads to decreased H3K4me3 levels (Fig. 5c).

Our findings reveal a previously unexplored regulatory pathway in which clock-driven levels of NAD⁺ synthesis lead to cyclic changes in H3K4 methylation, thereby exerting control on clock genes through the interplay of two epigenetic regulators, SIRT1 and MLL1.

It is generally accepted that H3K4 methylation has a major role in transcription-coupled processes. Importantly, recent work has revealed that manipulation of H3K4me3 levels may affect transcriptional consistency, thus indicating a role in robustness of transcriptional outputs. Interestingly, stochastic variations in transcription, such as genome instability, increase during aging, and some of these effects are at least partially mediated by SIRT1 (ref. 47). Also, it is well established that robustness of circadian gene expression is associated with increased life span, and MLL1 is critical to this mechanism, at least in the central pacemaker, the suprachiasmatic nuclei. Here, we provide mechanistic insights into the control of cyclic H3K4me3 levels by SIRT1, indicating that the robust regulation of the circadian epigenome might have potential implications in sustaining correct rhythms on gene transcription.

Finally, the cofactor NAD⁺ is emerging as a key regulator of metabolism and circadian rhythms. We have extended this concept by demonstrating that modulation of intracellular NAD⁺ affects H3K4me3 levels at circadian gene promoters, notably through SIRT1 activation. Importantly, this regulatory pathway does not seem to influence noncircadian MLL1-controlled genes. Thereby, our findings provide an additional paradigm by which metabolism and chromatin remodeling act in concert to keep adequate homeostasis.

Understanding the mechanisms that enable cells to sense and respond to environmental and metabolic cues through the circadian clock is of critical importance. The physiological implications of this cross-talk are likely to provide yet-unexplored connections between circadian rhythms, cellular homeostasis and aging. Our findings reveal a previously unappreciated role of NAD⁺–SIRT1 in the control of histone methylation, possibly paving the way for the exploration of new strategies for therapeutic applications contributing to the modulation of various aging-related diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank all the members of Sassone-Corsi laboratory for helpful discussion and technical support. We also thank C.D. Allis (Rockefeller University), P. Ernst (Geisel School of Medicine at Dartmouth), J. Hess (Indiana University School of Medicine), E. Verdin (University of California, San Francisco), J. Hish (Memorial Sloan Kettering), P. Puigserver (Harvard Medical School), M. Oshimura (Tottori University), Y. Murakami (National Institute of Infectious Diseases Japan), J. Hirayama (Tokyo Dental and Medical University), N.J. Zeleznik-Le (Loyola University Chicago), J. Auwerx (École Polytechnique Fédérale de Lausanne) and K. Yagita (Kyoto Prefectural University of Medicine) for sharing reagents and technical support. We also thank C.D. Allis (Rockefeller University), P. Ernst (Geisel School of Medicine at Dartmouth), J. Hess (Indiana University School of Medicine), E. Verdin (University of California, San Francisco), J. Hish (Memorial Sloan Kettering), P. Puigserver (Harvard Medical School), M. Oshimura (Tottori University), Y. Murakami (National Institute of Infectious Diseases Japan), J. Hirayama (Tokyo Dental and Medical University), N.J. Zeleznik-Le (Loyola University Chicago), J. Auwerx (École Polytechnique Fédérale de Lausanne) and K. Yagita (Kyoto Prefectural University of Medicine) for sharing reagents and technical support.

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to R.O.-S.), INSERM (France), and the US National Institutes of Health (grants AG041504, GM082634 and DA036408 to P.-S.-C.).

AUTHOR CONTRIBUTIONS
L.A.-A., S.K. and P.-S.-C. conceived and designed the project. L.A.-A., S.K. and R.-O.-S. contributed newly generated reagents and materials. L.A.-A. designed and performed experiments. L.A.-A. and P.-S.-C. analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Plasmids. Flag-MLL1-pCXN2, MLL1 carrying the SET-domain deletion (Flag-MLL1ASET-pCXN2), Flag-MLL1-myc-pCXN2, myc-CLOCK-pCDNA3, myc-BMAL1-pCDNA3 and mDbp and mPer2 promoters fused to luciferase have been described previously. The plasmid carrying a deletion of exons 3–5 on the MLL1 protein (Flag-MLL1AN-pCDNA3) was a kind gift from Dr. Peter Ernst. The plasmid Flag-MLL1-myc-pCXN2 was used as a template to generate by PCR the MLL1 truncation fragments, which were subcloned into the NotI–Xhol sites of a Flag-pCDNA3 backbone plasmid. The following primers were used to generate the truncated MLL1 fragments (in Supplementary Table 1): Flag-M1, M1TAFw and M1Rv; Flag-M2, M2TAFw and M2Rv; Flag-M3, M3TAFw and M3Rv; Flag-M4, M4TAFw and M4Rv; Flag-M5, M5TAFw and M5Rv; and Flag-M6, M6TAFw and M6Rv. The plasmids VP16-M1 to VP16-M6 were obtained by digestion of the corresponding plasmids Flag-M1 to Flag-M6 with NotI and Xhol restriction enzymes and subsequent cloning into these sites in the pVP16AD vector (Clontech). pGAL4BD-CLOCK-pGM4polyII, pGAL4BD-CLOCKA19-pGM4polyII, Flag-SIRT1-pCDNA3 and Flag–SIRT1 H363Y-pCDNA3 are described in ref. 10. These were used as templates to obtain the plasmids GAL4BD-SIRT1-pGM4polyII and GAL4BD-SIRT1 H363Y-pGM4polyII. More precisely, the SIRT1 and SIRT1 H363Y-pCDNAs were amplified by PCR from the corresponding primers with the plasmids ClasSIRT1Fw and SactSIRT1Rv (Supplementary Table 1). The PCR fragments were digested with Clal and SacI restriction enzymes and cloned in a similarly digested pGM4polyII plasmid. The plasmids Flag–SIRT1 to Flag–SIRT7 were kind gifts from Dr. Ervin. The vector myc-SIRT2 was kindly provided by M. Osimiura. The plasmid Flag-PCAF-pCI was purchased from Addgene and is described elsewhere. The plasmids HA-p300-pCDNA3 and HA-CPB-pCDNA3 were a gift from S. Sahar. The myc-MOF-pCDNA3 plasmid was obtained by cloning the MOF cDNA obtained by PCR amplification from total mouse cDNA into the BamHI and NotI sites. The myc-HAT1-pCDNA plasmid is described in ref. 53. All the vectors were examined and verified by restriction analysis and sequencing.

Antibodies and reagents. The antibodies used in this study are as follows: anti-H3K4me3, Active Motif cat. no. 39159; anti-H3K4me2 and anti-H3K4me1, Abcam cat. nos. ab7766 and ab8895, respectively; anti-H3K9/14Ac, Diagenode cat. no. C15410200; anti-MLL1 N terminal, Active Motif cat. no. 39829 and Bethyl Laboratories cat. no. A300-087A; anti-MLL1 C terminal, Bethyl Laboratories cat. no. A300-374A; anti-SIRT1, Millipore cat. no. 09-844; anti-BMAL1, Abcam cat. no. ab93806; anti-p84, GenTex cat. no. GTX70220; anti-actin, Abcam cat. no. ab3280; anti-α-tubulin, Sigma cat. no. T5618; anti-acetylated lysine, Cell Signaling cat. no. 9411; anti–Flag M2, Sigma F7425; anti–Myc tag, Millipore cat. no. 05-419; anti-GAL4 (DBD), Santa Cruz cat. no. sc-510; anti–HA tag, Millipore cat. no. 05-904. The anti–Flag M2 Affinity Gel used for immunoprecipitation of Flag-tagged proteins was purchased from Sigma (cat. no. 2220). All purchased antibodies were validated for mammalian studies (as shown on the manufacturers’ websites). The rabbit polyclonal antibody to acetylysines K1130 and K1133 from human MLL1 was generated by immunization of rabbits with KHL conjugates of the peptide NH2-APPIK(ac)PIK(ac)PVTR (Millipore cat. no. ABE344). Specificity of the antibody was validated by performing the appropriate controls, as depicted across the text, and original images of blots presented in this study can be found in Supplementary Data Set 1. EX526 was purchased from Tocris Bioscience. FK866 was purchased from Axon Medchem. NAD+ (N8353), nicotinamide (N0636), β-nicotinamide mononucleotide (N3051) and nicotinic acid (N0765) were purchased from Sigma.

Cell culture. HEK293 cells were maintained in high-glucose DMEM (HyClone) supplemented with 10% (v/v) FBS ( Gibco) and antibiotics. MEFs from SIRT1- and MLL1-knockout mice were obtained as described previously10 and cultured in DMEM supplemented with 5% FBS, 5% newborn calf serum (NBBCs, Gibco) and antibiotics. The stably transfected MEF cell line Mll1−/−Flag-Mll1 was a kind gift from J. Hess and is described in refs. 22, 27. All cell lines tested negative for mycoplasma contamination.

SIRT1-inhibitor EX527 treatment. MEFs in growing phase (60–80% confluent) were synchronized with dexamethasone (DEX)41. DEX was washed out, and synchronized cells were treated during 18 h with 50 µM EX527 before being harvested at each time point. For C70, cells were treated with EX527 during 18 h before and during synchronization. For CT12, EX527 was added in the medium during the 2 h of DEX synchronization.

Luciferase reporter assay. HEK293 cells or MEFs were seeded as a monolayer in 24-well plates. Semiconfluent cells (70–80%) were transfected with 25 ng of luciferase reporter and LucZ plasmids, together with (or without) 50 ng of CLOCK and BMAL1 in the presence or absence of increasing amounts of MLL or SIRT1 plasmids, with BioT transfection reagent (Bioland) according to the manufacturer’s instructions. The total amount of applied DNA per well was adjusted by addition of pcDNA3 vector. The amount of each transfected cDNA was determined by consideration of their molecular sizes and the promoter strength of each vector. At 16 h after transfection, the culture medium was replaced with new medium. The luciferase activities were measured 36 h after transfection as described previously42. The luciferase activities were normalized to transfection efficiency by a colorimetric β-galactosidase assay. For all the transfections presented across the manuscript, control experiments were performed with or without CLOCK-BMAL1 transfections to ensure that, in these conditions, 100 ng of CLOCK-BMAL1 transfection led to ~6 times more transactivation on the Dbp-luc reporter than Dbp-luc alone, and 300 ng of MLL1 addition was equivalent to ~30 times more transactivation.

Treatments on transiently transfected cells. Each compound, at the indicated concentrations, was added to the cells in new culture medium 16 h after transfection. Cells were harvested for luciferase assays after 16 h.

Preparation of cell extracts and nuclear extracts. Harvested cells were washed twice with cold phosphate-buffered saline (PBS) and lysed for 15 min at 4 °C in RIPA buffer supplemented with HDAC inhibitors (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl2, 1% NP40, 1× protease-inhibitor cocktail (Roche), 1 mM DTT, 1 mM PMSF, 1 µM TSA, and 10 mM NAM). Lysates were cleared by centrifugation, and the supernatants were stored at −80 °C. For preparation of the nuclear extracts from cells, after the PBS washes, 3 × 107 cells were resuspended and washed twice in fresh hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, and 1× protease-inhibitor cocktail (Roche)) by collection with centrifugation during 10 min at 600g. The pellets were resuspended in 500 µl of buffer II (hypotonic buffer + 0.1% NP-40) and mixed at 4 °C during 10 min. After a centrifugation step during 10 min at 4 °C, the supernatant was recovered and stored at −80 °C as the cytoplasmic fraction. The pellet containing the nuclear fraction was washed twice with hypotonic buffer and resuspended in 700 µl of hypertonic buffer (20 mM HEPES, pH 7.9, 450 mM NaCl, 1.5 mM MgCl2, 0.5 mM DTT, and 1× protease-inhibitor cocktail (Roche)) by collection with centrifugation during 10 min at 600g. The pellets were resuspended and washed twice in fresh hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, and 1× protease-inhibitor cocktail (Roche)) and mixed at 4 °C during 10 min. After a 10-min centrifugation at 4 °C at maximum speed, the nuclear fraction was recovered from the supernatant. The nuclear extracts from mouse liver tissue was obtained as follows: one-quarter of frozen liver was minced and suspended in 5 ml of fresh, ice-cold buffer A (10 mM HEPES, pH 7.8, 25 mM KCl, 320 mM sucrose, 0.3% Triton X-100, 1 mM EGTA, 1 mM EDTA, 0.5 mM spermidine, 150 µM spermine, 0.5 mM PMSF, 1 mM DTT, 10 mM NaF, and 1× protease-inhibitor cocktail (Roche)). The liver tissue was then homogenized with a motorized tissue grinder. The samples were washed twice in buffer A, with centrifugation at 3,000 r.p.m. for 10 min at 4 °C after each wash. The pellets were resuspended in 1 ml of ice-cold low-salt buffer (10 mM HEPES, pH 7.8, 25 mM KCl, 20% glycerol, 1 mM EGTA, 1 mM EDTA, 0.5 mM spermidine, 150 µM spermine, 0.5 mM PMSF, 1 mM DTT, 10 mM NaF, and 1× protease-inhibitor cocktail (Roche)) and washed twice, with centrifugation at 2,000 r.p.m. for 10 min at 4 °C after each wash. The pellets were then resuspended in one volume of low-salt buffer, and two volumes of high-salt buffer.
ChIP assays. After DEX synchronization, cells were cross-linked at the time points of interest with 1% formaldehyde at room temperature for 15 min. The reaction was stopped by addition of glycine to a final concentration of 125 mM. The cells were then washed and resuspended in ice-cold PBS. After centrifugation (800 r.p.m., 10 min at 4 °C), the cells were washed with 1 ml of ChIP buffer I (10 mM HEPES, pH 6.5, 0.25% Triton X-100, 10 mM EDTA, and 0.5 mM EGTA) and agitated at 4 °C for 10 min. The pellets were collected by centrifugation (800 r.p.m., 10 min at 4 °C) and similarly washed with 1 ml of ChIP buffer II (10 mM HEPES, pH 6.5, 200 mM NaCl, 10 mM EDTA, and 0.5 mM EGTA). The pellet was then resuspended in lysis buffer (50 mM Tris-HCl, pH 8, 1% SDS, 10 mM EDTA, 1 mM PMSF, and 1× protease-inhibitor cocktail (Roche)) and stored at −80 °C until all the time points were processed. The samples were then sonicated on ice across the linked chromatin was sheared to an average DNA fragment length of 0.2–0.6 kb. After centrifugation (10 min, 12,000g), soluble cross-linked chromatin was diluted 1:10 in immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 8, 1,500 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, and 1× protease-inhibitor cocktail (Roche)). The chromatin preparations were precleared by incubation with 60 µl of protein G PLUS-Agarose solution (Santa Cruz Biotechnology sc-2002) for 2 h at 4 °C under rotation. The protein G was removed by centrifugation, and the precleared chromatin was immunoprecipitated by incubation with the antibody and 60 µl of protein G PLUS-Agarose solution overnight at 4 °C. The immunoprecipitates were washed in buffers TSE I (20 mM Tris-HCl, pH 8, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, and 2 mM EDTA) TSE II (TSE I with a final concentration of 500 mM NaCl), buffer III (10 mM Tris-HCl, pH 8, 1% NP-40, 1% deoxycholate, 0.25 M LiCl, and 1 mM EDTA) and three times with Tris-EDTA (TE) buffer. To reverse the cross-linking, the washed beads were resuspended in 200 ml of direct elution buffer (10 mM Tris-HCl, pH 8, 0.5% SDS, 300 mM NaCl, 5 mM EDTA, and 0.05 mg/ml proteinase K) and incubated overnight at 65 °C. A treatment with RNase A was performed at 37 °C during 30 min, and samples were extracted twice with phenol-chloroform. The DNA was precipitated with ethanol in the presence of 0.1 mg/ml of glycerol as a carrier. The DNA pellets were resuspended in MQ water and stored at −20 °C.

Quantitative real-time PCR. cDNA was obtained by retrotranscription of 1 µg of total mRNA with iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. Real-time RT-PCR was done with the real-time CFX96 detection system (Bio-Rad). The PCR primers are detailed in Supplementary Table 1. For a 20-µl PCR reaction, 50 ng of cDNA template was mixed with the primers to final concentrations of 200 nM and mixed with 10 µl of iQ SYBR Green Supermix (Bio-Rad). The reactions were done in triplicates with the following conditions: 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C and 40 s at 60 °C.

SIRT1-dependent deacetylation assay on MLL1. Flag-MLL1-myc and SIRT1-7 Flag-tagged plasmids were transfected in HEK293 cells with BioT reagent (Bioland). Cells were lysed 36 h after transfection with modified RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl2, 1% NP-40, 1× protease-inhibitor cocktail (Roche), 1 mM DTT, and 1 mM PMSF). Equal amounts of total proteins were immunoprecipitated with anti-Flag M2 agarose overnight at 4 °C. Immunoprecipitated material was washed twice with Tris-buffered saline (TBS) and one time with SIRT-deacetylation buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 4 mM MgCl2, and 1 mM DTT). The beads with Flag-SIRT1 were distributed in different tubes, according to the number of reactions, and the supernatant was removed. The Flag-MLL1-myc tagged protein was eluted from the agarose beads with Flag peptide (Sigma F3290) previously reconstituted in SIRT-deacetylation buffer, and equal amounts of this extract were added to the Flag-SIRT1-containing tubes. The reactions were set up in a final volume of 60 µl of SIRT-deacetylation buffer supplemented with the adequate compounds (NAM, 1 mM; NAD+, 1 mM; or TSA, 400 nM). The reactions were incubated during 2.5 h at 37 °C, stopped by the addition of Laemmli sample buffer, boiled, and, after a brief centrifugation, analyzed by western blotting.

MLL1 methyltransferase assay. Flag-MLL1-myc plasmid was transfected in HEK293 cells either alone or in the presence of GAL4BD-SIRT1 or GAL4BD–SIRT1 H363Y. After 12-h transfection, cells were treated either with the small-molecule inhibitor of SIRT1 EX527 (final concentration 50 µM) or with the vehicle (ethanol) during 24 h. Cells were harvested in modified RIPA buffer supplemented with deacetylase inhibitors. The expression of the proteins at similar levels was tested by western blot on total extracts. Similar amounts of extracts were subjected to immunoprecipitation with anti-Flag M2 agarose at 4 °C overnight. The immunoprecipitated Flag-MLL1-myc was used for the methyltransferase assay according to the protocol described in ref. 56, with some modifications. Briefly, reactions were set up in a final volume of 30 µl of KMT buffer (50 mM Tris-HCl, pH 8, 5 mM MgCl2, and 4 mM DTT) in the presence of 1.2 µl of S-adenosyl-l-[3H]-methionine (PerkinElmer NET155H001MC) and 1 µg of recombinant histone H3.3 (New England BioLabs M25075). These were incubated at 30 °C for 2 h. 10 µl of each reaction was spotted onto Whatman P-81 paper circles (Whatman 3698-325) and processed for liquid scintillation counting as in ref. 56. The rest of the reaction was stopped with SDS sample buffer and boiled. After a brief centrifugation, 5 µl was loaded in a 5.5% acrylamide gel for WB analysis with anti-myc antibody. The rest was loaded on a 15% acrylamide gel, run, stained with Coomassie brilliant blue, destained and photographed. The gel was then treated for 1 h with freshly made enhancer solution (1M sodium salicylate NS and 2% glycerol), dried and directly exposed to a Kodak Biomax MS film (Kodak 822 2648), with an intensifying screen, at −80 °C over 2–4 nights. Methylated H3.3 was visualized upon developing. Original images of gels, autoradiographs and blots can be found in Supplementary Data Set 1.

Statistics and image analyses. Comparisons between samples were performed with two-tailed t-tests, with the assumption that data followed a normal distribution, as assessed with graphical methods and K-S test. Statistics were performed with GraphPad Prism 6.0 software. Western blot analyses and image processing were performed with ImageJ software.

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