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Histone Lysine Demethylase JARID1a Activates CLOCK-BMAL1 and Influences the Circadian Clock

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In animals, circadian oscillators are based on a transcription-translation circuit that revolves around the transcription factors CLOCK and BMAL1. We found that the Junomijc (JmjC) and ARID domain–containing histone lysine demethylase 1a (JARID1a) formed a complex with CLOCK-BMAL1, which was recruited to the Per2 promoter. JARID1a increased histone acetylation by inhibiting histone deacetylase 1 function and enhanced transcription by CLOCK-BMAL1 in a demethylase-independent manner. Depletion of JARID1a in mammalian cells reduced Per promoter histone acetylation, dampened expression of canonical circadian genes, and shortened the period of circadian rhythms. Drosophila lines with reduced expression of the Jarid1a homolog, lid, had lowered Per expression and similarly altered circadian rhythms. JARID1a thus has a nonredundant role in circadian oscillator function.

To gain insight into the dynamics of chromatin modifications and the function of CLOCK-BMAL1 transcription factors in the circadian clock, we measured the state of two histone modifications that correlate with active transcription, acetylation of histone 3 (H3) lysine 9 (H3K9Ac), and trimethylation of H3 lysine 4 (H3K4me3) at the Per2 promoter (1). In mouse liver, both modifications synchronously oscillated at the Per2 gene promoter CLOCK-BMAL1 E2−binding site (“E-box”) (2), with lowest amounts at circadian time (CT, the endogenous, free-running time) 3 hours after the onset of activity (CT3) and peak amounts at CT12 (Fig. 1A). The peaks of histone modification were followed by those of Per2 mRNA abundance. We also found BMAL1 abundance rhythms at the E-box, which reached a maximum at CT9 (Fig. 1B). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) generate rhythms in histone acetylation and have important roles in circadian rhythms (3, 4). H3K4me3 modification at promoter regions correlates with transcriptional potential, which suggests that this mark helps maintain a transcriptionally poised state (5). Recently, the H3K4 methyltransferase MLL1 was shown to have a necessary role in CLOCK-BMAL1–dependent transcription (6).

We focused on a JumonjiC (JmjC) domain–containing H3K4me3 demethylase family with four mammalian and one insect gene members (fig. S1). In murine liver chromatin, JARID1A was enriched at the Per2 E-box, and its profile at this site coincided with that of BMAL1 (Fig. 1B). Jarid1a expression in liver did not show robust oscillations (fig. S2), which suggested that recruitment of JARID1A to the Per2 promoter might be mediated by the circadian machinery. Indeed, JARID1A recruitment to the Per2 promoter E-box but not at a non–CLOCK-BMAL1 JARID1A target is reduced in Bmal1−/− cells (Fig. 1C and fig. S1A). Consistently, immunoprecipitation of endogenous CLOCK or BMAL1 from nuclear extracts copurified with endogenous Jarid1a (Fig. 1D). Similarly, CLOCK and BMAL1 associated with immunoprecipitated Jarid1a (fig. S3). Overexpression of Jarid1a enhanced CLOCK-BMAL1–mediated transcription from Per2 (Fig. 1E) and Per1 (fig. S4A) promoters in a dose-dependent manner but failed to coactivate expression from an unrelated (E74-Luc) reporter (fig. 5D). Furthermore, coactivation of CLOCK-BMAL1 by Jarid1a did not require its histone demethylase activity, as Jarid1a mutants that carry a loss-of-function mutation (H483A, in which Ala replaces His383 (7) or that lack the JmjC domain enhanced CLOCK-BMAL1 activity, reversed
HDAC1 repression, and increased acetylation amounts at the Per2 E-box (Fig. 1F and figs. S4B, S4C, and S5). In contrast, JARID1b and JARID1c repressed CLOCK-BMAL1–mediated activation of Per2 transcription (figs. S6, A and B). Accordingly, overexpression of JARID1b and JARID1c, but not JARID1a, reduced H3K4me3 modification at the Per2 promoter (fig. S6C). Overall, these results suggest that JARID1a enhances CLOCK-BMAL1 activation of the Per2 promoter, whereas JARID1b and JARID1c might function to reverse its H3K4me3 levels during the repression phase of Per transcription.

To test whether JARID1a has a nonredundant role in the cell-autonomous oscillator, we stably transfected a Per2–Luciferase (Per2:Luc) reporter into fibroblasts derived from either Jarid1a+/+ (wild type; WT) or Jarid1a−/− (knockout, KO) mice (7) and monitored circadian rhythms in Per2:Luc luminescence. In cell populations (Fig. 2A and fig. S7A) and single cells (fig. S8), Per2 transcriptional rhythms in a Jarid1a−/− genetic background showed a significantly shorter period than those of WT controls [WT = 23.7 ± 0.2 hours, KO = 22.4 ± 0.3 hours, P = 6.68 × 10−8, n = 10 each genotype, one-way analysis of variance (ANOVA)].

**Fig. 1.** JARID1a coactivates CLOCK-BMAL1–mediated transcription of Per genes. (A) H3K4 trimethylation and H3K9 acetylation oscillations at the Per2 promoter E-box in mouse liver shown alongside Per2 mRNA expression (secondary y axis). Normalized average (+SEM, n = 3) of chromatin immunoprecipitations (ChIPs) performed with antibodies against H3K4me3 or H3K9Ac followed by qPCR analysis. Chromatin modifications shown as a percentage of total chromatin used (input) normalized to the corresponding lowest value (H3K4me3 = 0.27%, H3K9Ac = 0.25%) for ease of comparison. (B) Abundance of BMAL1 and JARID1a in mouse liver at the Per2 promoter E-box and flanking regions. (C) Absence of JARID1a from the Per2 promoter E-box in Bmal1−/− cells. (D) Association of endogenous JARID1a and CLOCK-BMAL1. Immunoprecipitates (IPs) obtained with the indicated antibodies from asynchronous U2OS (a human osteosarcoma cell line) nuclear extracts were immunoblotted (IB) with antibodies against JARID1a, CLOCK, BMAL1, or βACTIN. (E) Effect of JARID1a on CLOCK-BMAL1–dependent transcription from a Per2:Luc reporter. Luciferase activity (light counts) from HEK293T cells transiently expressing full-length JARID1a cDNA and Per2:Luc reporter construct are shown. (F) Wild-type (WT) or demethylase-mutant JARID1a H483A (MUT) rescues CLOCK-BMAL1 activation of a Per2 reporter construct in Jarid1a−/− cells.

**Fig. 2.** JARID1a is required for normal circadian function. (A) Real-time bioluminescence from Jarid1a+/+ (black) and Jarid1a−/− (red) mouse fibroblasts stably expressing a Per2:Luc construct. (Middle and right) Average period lengths (+SEM, n = 10) and amplitude estimates (**P < 0.001, two-tailed Student’s t test). (B) Per2, Cry1, Dbp, and SDF1 mRNA abundance in Jarid1a+/+ cells (black) and Jarid1a−/− cells (red). Forty-eight hours after circadian synchronization, cells were collected at three-hour intervals over the course of 24 hours, and the mRNA abundance was analyzed by reverse transcription (RT)–qPCR and normalized to that of Gapdh. Average normalized values (+SEM, n = 3) are plotted at various times after cell synchronization. (C) Representative real-time luminescence from U2OS cells stably expressing Bmal1:Luc and transfected with scrambled (S, black) or Jarid1a siRNA (red). Effects of different Jarid1a siRNA concentrations (10, 20, and 40 nM) on the endogenous Jarid1a mRNA abundance (average + SEM, n = 3) and period length (average + SEM, n = 5) relative to those of cells transfected with scrambled siRNA (40 nM). Representative results from at least three independent repetitions are shown.
(ANOVA) (Fig. 2A). Amounts of endogenous Per2 transcript were also reduced and oscillated with a shorter period in Jarid1a−/− cells (Fig. 2B). Similarly, other CLOCK-BMAL1 targets were significantly reduced in Jarid1a−/− cells, and their rhythms were either absent or dampened (Fig. 2B and fig. S9). In contrast, abundance of SDF1 mRNA, a repressive target of the histone demethylase (KDM) activity of Jarid1a (7), was increased in Jarid1a−/− fibroblasts. Acute small interfering RNA (siRNA)–mediated depletion of Jarid1a mRNA also shortened circadian period length in a dose-dependent manner (Fig. 2C and fig. S10). These lid10424/CyO flies showed normal activity distribution under a 12 hours of light:12 hours of dark (LD) cycle (Fig. 3B), and their total activity (1030 ± 44, average beam breaks for 3 days in LD, SEM, n = 75) was equivalent (P > 0.13, one-way ANOVA) to that of WT y;ry flies (831 ± 85, SEM, n = 16) or +/CyO (938 ± 60 SEM, n = 45) flies, which implied no deficit in locomotion or light:dark entrainment of the circadian clock.

However, under constant darkness (DD), the lid10424/CyO flies exhibited disrupted circadian activity rhythms. The majority of lid10424/CyO flies showed no or weak circadian activity rhythms (Fig. 3, A and C). Those lid10424/CyO flies with detectable rhythms showed significant period shortening (Fig. 3C) (lid10424/CyO = 22.91 ± 0.04 hours, y;ry = 23.61 ± 0.06 hours; average ± SEM, n = 16 and 75; P < 0.001). Expression of a genomic copy of lid in lid10424/lid10424 homozygous flies (lid10424/lid10424;gLid) restored Lid protein expression to near-WT amounts and also restored circadian activity rhythms (Fig. 3C and fig. S11). Furthermore, a demethylase-deficient Lid allele (8) partially rescued the circadian phenotype (lid10424/lid10424;gLmjC*). Under LD and DD, the lid10424 flies exhibited attenuated day:night differences in activity. Specifically, the lid10424 flies exhibited increased activity during midday and reduced dark anticipation (Fig. 3D). Comparable circadian rhythm disruption and reduced expression of CLOCK-BMAL1 (or CYC) targets in both Drosophila and mammalian cells supports a conserved role of lid/Jarid1 in the insect and mammalian circadian oscillator.

In Jarid1a−/− mouse fibroblasts, the Per2 promoter region showed an increased abundance of H3K4me3 (fig. S12A), but a significantly reduced amount of acetylated H3K9 relative to those of WT controls (Fig. 4A). Conversely, overexpression of JARID1a in human embryonic

Fig. 3. Circadian rhythm disruption in lid10424 flies. (A) RT-QPCR of lid, per, cry, and tim mRNA in the heads of y;ry (black) and lid10424/CyO (red) flies collected every 4 hours after 72 hours in constant darkness (±SEM, n = 3). (B) Representative circadian rhythms of wild-type lid10424 (y;ry), lid10424/CyO, lid10424/lid10424;gLid, and lid10424/lid10424;gLmjC* flies under LD or DD conditions. Arrowheads indicate transition from LD to DD. (C) (Left) Normalized average activity of y;ry (black) and lid10424/CyO flies (red) (n = 33) under LD and DD conditions. (Right) Summary of the circadian activity rhythms in various Drosophila strains tested. Histogram shows the percentage of flies with normal (R, rhythmic), weak (W) or no detectable (A, arrhythmic) circadian activity rhythms (±SEM). (D) Normalized average activity (±SEM; n = 16 to 75) in 30-min bins during the light-dark period show two major phases of activities in y;ry flies at morning and evening.
kidney−293 cells (HEK293T cells) increased accumulation of H3K9Ac at the Per2 promoter (Fig. 4B), but not at a control promoter (Fig. S12C). This suggests that coordinated increases in both H3K4me3 and histone acetylation are required for sufficient Per induction. Recruitment of Jarid1a to Per promoters might promote H3K9 acetylation levels during Per transcription.

Lid inhibits histone deacetylase Rpd3 to activate transcription at specific loci (9). Such HDAC inhibition by Lid, which is independent of its KDM activity, is consistent with the observed HDAC inhibition by Lid, which is independent of its KDM activity, as JARID1a associates with HDAC1 in mammalian cells, but dampened Per2:Luc oscillations (Fig. S15). Affinity-purified JARID1a impaired the ability of HDAC1 to deacetylate acetylated histone H3 in vitro, whereas another JmjC domain-containing protein, JMJD6, could not (Fig. 4E and fig. S16).

These results suggest a model in which Jarid1a mediates transition from repression to robust activation of Per transcription. During the activation phase, both H3K4me3 and acetylated histones at CLOCK-BMAL1 target sites accumulate in parallel with Per transcription. Jarid1a appears to be recruited to the Per promoter in complex with CLOCK-BMAL1, where it inhibits HDAC1 activity to enhance histone acetylation and transcriptional activation. Although purified Jarid1a or Lid inhibits HDAC activity in vitro, this mode of regulation is likely gene- and context-dependent in vivo. Jarid1a−/− cells have increased amounts of H3K4me3 at the Per2 promoter (Fig. S12), and overexpression of the JmjC domain of Jarid1a slightly repressed Per transcription (Fig. S5), which suggest that, in vivo, Jarid1a may mediate demethylation or that its presence in the transcriptional complex of target genes is required for proper recruitment of other proteins that carry out this function. However, whereas the lysine demethylase activity of Jarid1a is dispensable for normal circadian rhythms in mammalian cells, its HDAC-inhibiting function appears to be necessary for proper oscillator function.

References and Notes
Superfast Muscles Set Maximum Call Rate in Echolocating Bats

Coen P. H. Elemans,† Andrew F. Mead,‡ Lasse Jakobsen,¹ John M. Ratcliffe‡*  

As an echolocating bat closes in on a flying insect, it increases call emission to rates beyond 160 calls per second. This high call rate phase, dubbed the terminal buzz, has proven enigmatic because it is unknown how bats are able to produce calls so quickly. We found that previously unknown and highly specialized superfast muscles power rapid call rates in the terminal buzz. Additionally, we show that laryngeal motor performance, not overlap between call production and the arrival of echoes at the bat’s ears, limits maximum call rate. Superfast muscles are rare in vertebrates and always associated with extraordinary motor demands on acoustic communication. We propose that the advantages of rapid auditory updates on prey movement selected for superfast laryngeal muscle in echolocating bats.

Laryngeal echolocation and insectivory characterize about 70% of present-day bat species (1–3). Over the course of an attack on a flying insect, bats increase their echolocation call emission rates as they progress from prey detection, through approach, to the terminal buzz (1, 2, 4) (Fig. 1A). Increasing call emission rates means more information updates per unit time from returning echoes on the relative position of the target. All aerial hawking bats studied to date produce the buzz, which is sometimes subdivided into “buzz I” and “buzz II” phase calls, the former occurring at rates of ~100 to 160 calls/s, and the latter ≥160 calls/s (1, 5) (Fig. 1B). Bats do not call at rates exceeding those reached during this final stage of aerial hawking attack (2, 4), and we hypothesize that call production, echo processing, or both limit maximum echolocation call rate. Laryngeal nerve-cut experiments reveal that each call a bat emits is under active neuromuscular control (6, 7). Consequently, muscle performance might place an upper limit on the rate at which bats produce calls. Alternatively, if prey echoes overlap with or return after the next call is emitted, accuracy in target ranging may suffer as a result of ambiguity in matching echoes to calls (I, 8). While hunting, most species avoid potential ambiguity by not producing the next call until target echoes reach the bat’s ears (I), potentially limiting maximum call rates during the buzz. To investigate these hypotheses, we first measured sound production during aerial attack sequences in free-flying Daubenton’s bats (Myotis daubentonii, Vespertilionidae) using a 12-microphone array (Fig. 1C) (9) and determined when the start of buzz II calls, echo matching. In fact, for buzz II calls, the repetition rate could theoretically exceed 400 calls/s without any such ambiguity (Fig. 2C and fig. S1), a rate twice as high as the 190 calls/s observed in our study (Fig. 1). Our results also demonstrate that, because call duration decreases during the buzz, there is no overlap between a call and its echo until the bat is less than 5 cm from its target (Fig. 2B), corroborating previous estimates for

Fig. 1. Echolocation and flight kinematics during aerial attack sequences in Myotis daubentonii. (A) Spectrogram and oscillogram of emitted echolocation calls as recorded on the center microphone of the array. (B) Instantaneous call repetition rates from sequence in (A). The start of buzz I is defined as call repetition rate >100 calls/s (cyan circles, light gray zone). In buzz II, the repetition rate is >160 calls/s (red circles, medium gray zone); here, peak fundamental frequency of the calls drops from 45 to 25 kHz. (C) Four reconstructed flight paths from a single bat using a 12-microphone array (10). Red arrows indicate flight direction.