Reciprocal Regulation between the Circadian Clock and Hypoxia Signaling at the Genome Level in Mammals

Graphical Abstract

Highlights

- The hypoxic response is gated by the circadian clock in vivo
- HIF1A is the regulatory node that connects hypoxia signaling with the circadian clock
- ChIP-seq analyses demonstrated that HIF1A and BMAL1 engage in synergistic crosstalk
- A heart attack model emphasizes the pathological significance of this crosstalk

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In Brief

Wu et al. reveal that the hypoxic response is gated by the circadian clock, and ChIP-seq analyses of HIF1A and BMAL1 show that this hypoxia-clock reciprocal regulation occurs at the genomic level. In a mouse model of heart attack, the circadian clock protects the heart from hypoxia-induced cell death.

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Reciprocal Regulation between the Circadian Clock and Hypoxia Signaling at the Genome Level in Mammals

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SUMMARY

Circadian regulation is critically important in maintaining metabolic and physiological homeostasis. However, little is known about the possible influence of the clock on physiological abnormalities occurring under pathological conditions. Here, we report the discovery that hypoxia, a condition that causes catastrophic bodily damage, is gated by the circadian clock in vivo. Hypoxia signals conversely regulate the clock by slowing the circadian cycle and dampening the amplitude of oscillations in a dose-dependent manner. ChIP-seq analyses of hypoxia-inducible factor HIF1A and the core clock component BMAL1 revealed crosstalk between hypoxia and the clock at the genome level. Further, severe consequences caused by acute hypoxia, such as those that occur with heart attacks, were correlated with defects in circadian rhythms. We propose that the clock plays functions in fine-tuning hypoxic responses under pathophysiological conditions. We argue that the clock can, and likely should, be exploited therapeutically to reduce the severity of fatal hypoxia-related diseases.

INTRODUCTION

The mammalian clock consists of a “core” transcriptional feedback loop with CLOCK and BMAL1 as positive factors and PERIOD and CRYPTOCHROME (or PER and CRY) as negative regulators. These components work in concert to generate a rhythm of roughly 24 hr duration. It has also been established that multiple “stabilizing” feedback loops are associated with the core loop; these function in structuring a hierarchical, multi-layered regulatory network (Zhang and Kay, 2010). Our previous genome-wide small interfering RNA (siRNA) screen established that many other cellular pathways, including cell-cycle signaling, both impinge on and are affected by the circadian clock (Zhang et al., 2009). Thus, the mammalian clock is intertwined with various signaling networks and profoundly influences multiple aspects of animal physiology.

In addition to normal physiological processes, circadian-related networks may also be associated with pathological events (Green et al., 2008; Takahashi et al., 2008). Hypoxia signaling is known to regulate both physiology and pathology in mammals. Therefore, hypoxia is viewed as a suitable experimental model system for testing hypotheses about the putative roles of circadian networks in hypoxia pathophysiology. The physiological responses that occur during hypoxia include angiogenesis, metastasis, and erythropoietin (EPO) production. Apoptosis and necrosis are pathological responses known to occur during hypoxia (Gilkes et al., 2014; Semenza, 2014). The key hypoxia signaling molecule HIF1A and its binding partner ARNT are both PER-ARNT-SIM (PAS)-domain-containing transcription factors that are structurally similar, respectively, to the circadian components CLOCK and BMAL1 (Table S1 for the detailed characteristics of the genes and drugs mentioned in this study) (Hogenesch et al., 1998; Weidemann and Johnson, 2008). This structural similarity suggests that these two pathways engage in crosstalk with each other.

Our previous screen identified HIF1AN, an inhibitory molecule of HIF1A, as a period-lengthening candidate (Zhang et al., 2009). The CircaDB online database predicts that various hypoxia-responsive genes are under daily cycling in mouse tissues (Hughes et al., 2009). These observations suggest that hypoxia signaling and the clock are possibly intertwined. Here, we uncovered a reciprocal regulation interaction between the clock and hypoxia signaling occurring at the whole-genome level. We subsequently developed a cardiac disease model to demonstrate that this regulation is likely of biomedical relevance.

RESULTS

The Hypoxic Response Is Controlled by the Circadian Clock In Vivo

To investigate the possible impact of the clock on hypoxia-responsive signaling, we examined the induction of known hypoxia-regulated genes in response to a fixed stimulation during a time course (i.e., the circadian gating assay; Millar and Kay,
Figure 1. Circadian Regulation of Hypoxic Responses In Vivo
(A) WT mice were injected with DMOG (300 mg/kg) or control at different times of the day (shown as ZT). Livers were harvested 3 hr after the injection. Data are shown in a time-dependent manner (n ≥ 3 for each time point per group, *p < 0.05 and **p < 0.01 for the induction).
(B) The qPCR data shown above were further analyzed by JTK_Cycle analysis to determine the rhythmicity and the phases of the peaks (time course threads of induction or non-induction, with both p and q values less than 0.05, were considered as rhythmic).

(legend continued on next page)
Intraperitoneal (i.p.) injection of dimethyloxalylglycine (DMOG), a low-toxicity compound commonly used to mimic hypoxia at both the cellular and tissue levels (Jaakkola et al., 2001; Lando et al., 2002a, 2002b), resulted in an acute and robust induction of the expression of hypoxia-responsive genes in various mouse tissues (Figure 1A; Figure S1). JTK_Cycle analysis (Hughes et al., 2010) showed that this induction was clearly under the control of the circadian clock. The expression of well-known Hif1α-target genes, including Glut1, Vegfa, Epo, and Egln1-3, was highly induced at zeitgeber time 6–9 (ZT6–ZT9) in the livers of DMOG-injected mice; the induction of their expression was much weaker or hardly induced at all at ZT18–ZT21 (Figures 1A and 1B). It should be noted that the strongest inductions of gene expression, which occurred during ZT6–ZT12, were coincident with the peaking of the expression of E-box genes, suggesting that this circadian gating phenomenon is likely governed by an E-box-containing gene. The strongest genes, suggesting that this circadian gating phenomenon is clock-regulated in vivo.

Similar to DMOG, the clinical trial III drug FG-4592 is also an EGLNs inhibitor and is able to stabilize Hif1α (Figure S2A). This drug is being used in trials (NCT: NCT02021318) to boost the production of EPO, a physiological target of HIFs, in patients with chronic kidney failure (Jain et al., 2016). Using WT mice as a model, we observed that the efficacy of this drug was gated by the circadian clock. This result has very important medical implications (Figure 1D). The induction of EPO production resulting from daytime stimulation was at least 1-fold greater than the induction from nighttime stimulation (p < 0.05). A recent study suggested that up to 50% of the genes in the mammalian genome undergo daily cycles in at least one tissue in vivo. Therefore, the timing of the administration of certain drugs is critically important for maximizing their efficacies (Zhang et al., 2014). Our results clearly support these notions and provide insight into the appropriate administration times for FG4592 in particular and perhaps for other hypoxia-related drugs generally.

The Presence of an E-box in the HIF1α Promoter Suggests Its Direct Regulation by the Clock

We were interested in identifying which, if any, E-box-containing gene may function as the master regulator for circadian gating of hypoxia signals. It is known that HIF1α plays a major role in mediating this signaling (Gilkes et al., 2014; Semenza, 2014). We therefore examined the expression pattern of the Hif1α gene in mouse liver (Figure 2A). We found that Hif1α expression is clearly under circadian control. Further, we found that its expression peaked in a manner consistent with the predicted E-box gene phase (ZT6–ZT12). A subsequent comparative genomics analysis identified a highly conserved canonical E-box (CACGTG) in the promoter region of the HIF1α gene (Figure 2B). Using a chromatin immunoprecipitation coupled with deep sequencing approach (ChIP-seq), we confirmed the binding enrichment of BMAL1 at this E-box site in human osteosarcoma U2OS cells (Figure 2C). Interestingly, re-analysis of previously published datasets of time course ChIP-seq using antibodies against clock proteins (Koike et al., 2012) showed that the presumptive E-box site that we identified also associates with core clock proteins, including Bmal1, Clock, and Cry1, in the mouse liver (Figure S2B). Moreover, its functionality was verified as transcription of the luciferase reporter driven by HIF1α promoter was activated by Clock/Bmal1 (Figure 2D, upper panel). Results from an RNA sequencing (RNA-seq) expression analysis of several wild-type (WT) and clock-deficient mouse fibroblasts also supported the assertion that Hif1α is an E-box-regulated gene: Hif1α expression was downregulated in cells lacking the circadian activator Bmal1 but was upregulated in the absence of the Cry1/Cry2 clock repressors (Figure 2D, lower panel). Thus, the HIF1α gene is directly regulated by the clock at the cellular transcriptional level. Consistently, DMOG treatment also led to dramatically stronger induction in cells absent of clock repressors: cells of the Per1−/−;Per2−/− genotype got the strongest boost from DMOG, presumably because these cells had the highest levels of Hif1α expression (Figure 2E; Figures S2C and S2D). Interestingly, the expression of the Hif1α-target genes Bnip3 and Noxa1, which are known to be pro-apoptotic (Diwan et al., 2007; Shimizu et al., 1996), was strongly induced in both Per1−/−;Per2−/− and Cry1−/−;Cry2−/− cells, suggesting that these cells are more vulnerable to cell death programs when they have been exposed to hypoxia signals. In fact, the much higher induction of the hypoxia-responsive genes in Per1−/−;Per2−/− cells may be partially attributable to the function of Per2, which has been reported to physically interact and stabilize Hif1α protein (Eckle et al., 2012). Interestingly, the E-box regulation of Hif1α expression may offer a major, but not a complete, explanation for the gating phenomenon, as Hif1α mRNA peaks around ZT10.5 (Figure 2A), while the maximal effect of Hif1α protein peaks around ZT6–ZT12 (Figures 1A and 1B). If Hif1α is the only player, we would expect to see some degree of phase delay in the gating phenomenon. Therefore, we suspect that some other unknown, potentially minor protein(s) may be acting synergistically alongside Hif1α to regulate the “gating.”

HIF1α Is the Regulatory Node that Connects Hypoxia Signaling with the Circadian Clock

Given that some hypoxia-related genes, such as HIF1AN, were identified as clock modifiers, we reasoned that hypoxia signals

(C) Clock mutants (left) and Bmal1+/– (right), together with their littermates, were injected with DMOG or control ZT9. The expression in kidney of the Egln3 gene is shown (n ≥ 3 for each, *p < 0.05 and **p < 0.01).

(D) Compound FG-4592 was injected into WT mice at ZT8 and ZT20. Each dot represents a serum EPO sample measured from an individual mouse (n ≥ 10 per group, **p = 0.02). All data are presented as the mean ± SEM from at least three independent experiments.
may be reciprocally transduced back to the clock (Zhang et al., 2009). To investigate the influence of hypoxia on the mammalian clock, we used two well-accepted anoxia-mimicking reagents, Ni²⁺ and Co²⁺, to treat ex vivo cultures of the master-clock tissue (mouse suprachiasmatic nuclei [SCN]) that harbored a luciferase reporter fused with the Per2 protein (Liu et al., 2007; Yoo et al., 2004). We observed a significant and reversible period-lengthening and amplitude-reducing phenotype with both the Ni²⁺ and the Co²⁺ treatments, suggesting that the hypoxia signals are able to prolong and suppress the intensity of circadian cycling (Figure 3A). Similarly, in U2OS cells, treatment with Ni²⁺, Co²⁺, or DMOG led to a period-lengthening and

![Figure 2. Expression of the HIF1A Gene Is Regulated by the Clock, Both In Vivo and In Vitro](image)

(A) The expression of the Hif1a gene is rhythmic in the mouse liver.
(B) A conserved canonical E-box (CACGTG) and its flanking sequence exists in the promoter region of the HIF1A genes of all 12 mammals examined.
(C) ChIP-seq profiling revealing that BMAL1 physically interacts with the promoter region of the HIF1A gene.
(D) Hif1a is functionally regulated by the clock in vitro. Top: a luciferase reporter assay showed that the mouse Hif1a promoter can be activated by Clock/Bmal1. A reporter with tandem E-boxes derived from the Dbp gene served here as a positive control. Bottom: RNA-seq profiling showed that the expression of Hif1a was stronger in Cry1⁻/⁻;Cry2⁻/⁻ fibroblast cells, but weaker in Bmal1⁻/⁻ cells, relative to WT controls (WT-T: Per2 trough phase in WT; WT-P: Per3 peak phase).
(E) Different genotypes of mouse fibroblast cells were treated with DMOG (1.25 mM), and the expression of hypoxia-related genes was measured by qPCR. To generate similar scales for the figure, we made the hypoxia genes Hif1a, Vegfa, Egln1, and Noxa1 at the left reference the y axis, where the mRNA expression of these genes in WT cells with a DMSO control treatment were set to 1; Bnip3 should be read as ten times greater (WT_DMSO read as 0.1) and Egln3 should be read as a further ten times greater (WT_DMSO read as 0.01). All data are presented as the mean ± SEM from at least three independent experiments.

![Graphs and diagrams showing expression levels and genotypes](figures)
amplitude-damping phenotype in a dose-dependent manner (Figures 3B and 3C). Taken together, these results enable us to conclude that pharmacologically induced hypoxia signals influence the period and the amplitude of the circadian clock.

We next used a short hairpin RNA (shRNA) lentivirus system to knock down the expression of HIF1A or HIF2A in U2OS cells in an attempt to determine which of these is responsible for the observed influence on the clock. The knockdown efficiencies of HIF1A and HIF2A were 89.2% and 73.2%, respectively (Figure S3A). Following a modest treatment dose of Co2⁺, the expression of hypoxia targets, including EGLN1, EGLN3, and VEGFA, was not induced in shHIF1A cells but was induced in scramble control and shHIF2A cells (Figure S3C), indicating that HIF1A is the main modulator of hypoxia signals in U2OS cells. This result is in line with a previous study, which reported that HIF1A is widely expressed and functions in a more general role, while HIF2A is expressed with a greater degree of spatial specificity and presumably performs relatively more specialized role(s) (Su et al., 2004). We therefore examined whether or not the loss of function of HIF1A in U2OS cells would interfere with normal clock function. Knockdown of HIF1A expression by up to 90% led only to subtle (if any) clock phenotypes (Figure 3D). However, these knockdown cells were much more resilient to pharmacological perturbations of the clock than were scramble shRNA control cells. The clock of HIF1A knockdown cells treated with Co2⁺ did not have larger changes in period or in the damping of the amplitude than did untreated HIF1A knockdown cells, while Co2⁺ treatment of WT control cells with the same dose both lengthened the period for up to 4 hr and crippled oscillation for at least three cycles in a week-long culture. Thus, HIF1A is required to transduce hyperactive HIF signals to the clock. Reminiscent of this idea, the clock period in U2OS cells was also lengthened by knockdown of the EGLN genes, which encode enzymes that induce the destabilization of HIF alpha proteins by hydroxylation of two key proline residues (P402/P564 of human HIF1A) (Masson et al., 2001) (Figure 3E; Figure S3B). Our previous siRNA screen identified HIF1AN as a long period hit (Zhang et al., 2009). HIF1AN hydroxylates a conserved Asparagine residue in HIF alpha proteins (N803 of human HIF1A) and reduces their activity by abolishing their interaction with the p300/CBP transcription co-activators (Lando et al., 2002a). Consistently, with our results with the knockdown of HIF1AN in U2OS cells confirm that stronger HIF signals lead to a longer period for the clock (Figure 3E; Figure S3D).

In order to eliminate the possibility that side effects from the treatment ions, small molecules, or off-target siRNAs caused the aforementioned clock phenotype (period lengthening and amplitude reducing), we used the tetR system to overexpress HIF1A and examine its role in regulating the clock. A constitutively active mutant of HIF1A was generated via mutations of P402A/P564A (HIF1A 2M) and subcloned into U2OS cells using the tetR inducible system. As expected, expression of the stabilized HIF1A protein in the tet-ON condition lengthened the period and severely dampened the clock (Figure 3F; Figure S3F). Thus, gain of function with HIF1A alone can cause period-lengthening and amplitude-reducing changes in the clock. Taken together, these results demonstrate that HIF1A is both required and is itself sufficient to mediate signal transduction from hypoxia to the clock.

HIF1A Binds Directly to the PER2 Promoter and Alters PER2 Expression

To identify which recipient site(s) of the clockwork may be the direct effectors of the activated HIF1A, we examined the expression of nine core clock genes in the inducible HIF1A 2M U2OS cells. The expression of all but one (PER1) of the five E-box-containing genes (PER2, DBP, NIR1D, and DEC1) was induced by at least 2-fold. The expression of the four non-E-box governing clock genes that we examined (BMAL1, CLOCK, CRY1, and CRY2) did not change significantly (Figure 3G). We reasoned that PER1 was less inducible by hypoxia because the tandem E-box elements in its promoter region made it more favorable to CLOCK/BMAL1 activation versus HIF1A/ARNT induction, a contrast to PER2 and other genes (Figure 3H). As controls for HIF1A activation, the expression of known HIF1A-targeted genes, including VEGFA, EGLN1/3, and GLUT1, was also induced significantly (Semenza, 2014). Similarly, in mouse fibroblast cells, the expression of the E-box-containing genes Nr1d1 and Per1/2 was induced by Co2⁺ or DMOG and was activated to a greater extent in Per1⁻/⁻/Per2⁻/⁻ or Cry1⁻/⁻/Cry2⁻/⁻ than in WT cells (Figure S3E).

Given that the core sequence of a hypoxia-responsive element (HRE) is “[A/G]CGTG” (Semenza, 2014), and considering that this sequence includes most of the BMAL1 binding E-box “CACCGT” (Hogenesch et al., 1998), we hypothesized that activated HIF1A may also bind to the promoters of some E-box-containing genes. To test this idea, we cloned the canonical E-box of DBP and the canonical HRE of EPO into luciferase reporter constructs and transfected these into 293T cells (Figure S3G). We found that the E-box was activated by both CLOCK/BMAL1 and HIF1A/ARNT, while EPO HRE was only activated by HIF1A/ARNT.

To establish which clock gene is the primary factor that transduces the HIF1A signal to the oscillator, we conducted a series of ChIP-coupled qPCR experiments (ChIP-qPCR) using the 2M cell line. We found that HIF1A is enriched in the known non-canonical E-box region of the PER2 promoter (Figure 3I). Thus, once it is stabilized either pharmacologically or genetically, HIF1A can directly occupy the PER2 promoter and increase PER2 expression. Changes in the abundance of PER2 in U2OS cells results in arrhythmicity of the cellular clock (Chen et al., 2009). Mathematical modeling provided the insight that PER2 is one of the most important factors for setting the period of the clock (Wilkins et al., 2007). Therefore, we conclude that PER2 is the direct coordinator linking the hypoxia signal with the clock network.

ChIP-Seq Analysis Reveals Crosstalk between HIF1A and BMAL1 at the Genomic Level

Belonging to members of the PAS-domain-containing transcription factor family, HIF1A and BMAL1 are able to dimerize and be co-immunoprecipitated (Figure S4A). To determine whether or not there is crosstalk between the HIF1A and BMAL1 proteins at the chromatin level, we conducted additional ChIP-seq analysis (Figure 4; Figures S4B–S4D; Table S2). We found substantial (30%–50%) overlap among the BMAL1 and HIF1A targets. As shown in a dual heatmap plotting 6,213 HIF1A-bound loci with both HIF1A and BMAL1 binding (Rahel et al., 2010), more than a third of HIF1A targets are co-occupied with BMAL1 (Figure 4A). In a seeming echo of the occupation of HIF1A on the PER2 locus, which is a well-known BMAL1 target (Figure 3I), both HIF1A and BMAL1 co-localize on the E-box site (Figures 4A and 4B). The
co-occupancy of HIF1A and BMAL1 is not necessarily completely overlapping. For instance, another clock gene, DBP, is bound by both, while the TEF and VEGFA promoters interact specifically (Figure 4C). Nevertheless, even when the most stringent criteria were applied (limiting the binding to within 1 kb of the promoter regions), there remained substantial (20%–30%) co-occupation for these two transcription factors (Figure 4D). Consistent with the previous hypothesis that HIF1A binds to some, but not all, E-box motifs, both the BMAL1 and HIF1A binding sites were enriched in E-box motifs; the BMAL1-specific targets sites were relatively more enriched in E-box motifs (Figure 4E). Motif analysis of three different categories (BMAL1/HIF1A co-occupant targets, BMAL1-specific targets, and HIF1A-specific targets) of the genes in Figure 4D suggested that there was strong enrichment of the CACGTG motif in the genes only occupied by BMAL1 and strong enrichment of the (T)ACGTG motif (HRE) in genes only occupied by HIF1A (Figure 4G). The promoters of genes co-occupied by these two transcription factors were strongly enriched in both the HRE and E-box elements. The frequency of E-box motifs at BMAL1 and HIF1A binding sites in the co-occupied genes is much higher than that of (T)ACGTG motifs (Figure 4F).

To distinguish which pathways are regulated by both hypoxia signaling and the circadian clock from the pathways that are regulated by only one of these, we performed KEGG analysis of the BMAL1 and HIF1A targets using the tools of DAVID bioinformatics at the NIH website (Figure S5) (Dennis et al., 2003; Huang et al., 2009). Interestingly, the circadian pathway is significantly overrepresented among genes co-occupied by both BMAL1 and HIF1A (p value is 6.70E−5). This enrichment was not evident for genes occupied only by BMAL1, further supporting our assertion that HIF1A can integrate circadian signals with hypoxia signals by directly regulating the E-box clock genes (Figure S5A). Additionally, other known circadian-regulated pathways, such as p53 signaling (Zhang et al., 2009), show more than 2-fold enrichment, indicating that they are co-regulated by hypoxia and the clock.

Synergistic Activation of HIF1A and BMAL1 at the Genomic Level

To determine how the HIF1A and BMAL1 proteins crosstalk at the chromatin level, competing or synergistic, we were set to examine their associations with the chromatin under either normoxia or hypoxia conditions. In addition, as we know that the hypoxia stimulations have highest effect when E-box genes peak in vivo (Figure 1), we wonder whether this is also true in vitro and, if so, whether it could be reflected in the transcription factor’s binding on the chromatin. To comprehensively investigate these issues, we conducted a series of ChIP-seq. We synchronized the U2OS cells by dexamethasone, stimulated them by DMOG, and simultaneously measured the genomic associations of BMAL1 and HIF1A at the peak (ZT30, or 30 hr post-synchronization) or trough (ZT42) of Per2-dLuc reporter (Figure 5A). Interestingly, we found that the ChIP signals of BMAL1 were drastically increased in most of BMAL1/HIF1A co-occupant loci, especially when Per2-dLuc was at peak (Figures 5B and 5C). Meanwhile, the signals at BMAL1-only binding sites were largely unchanged. These data strongly indicate that HIF1A can synergistically enhance the BMAL1 association to the co-occupant loci. In echo to previously demonstrated gating phenomenon in vivo, the BMAL1 association to the chromatin is much higher at ZT30 than at ZT42. Thus, the circadian gating phenomenon is also seen in terms of BMAL1 binding to the chromatin. Similar phenotype was also observed at HIF1A ChIP-seq (Figure 5D).

A Cardiac Disease Model Reveals the Significance of Co-regulation of Hypoxia and the Clock

Heart attack is a fatal, hypoxia-related disease that results from clots in the coronary artery that lead to an insufficient oxygen supply to the heart. It has been known for a long time that heart attacks happen most frequently in the early morning, presumably due to cardiovascular malfunction that occurs in response to the sharp increase of blood pressure during this period (Muller et al., 1985; Takahashi et al., 2008). Moreover, there have been clinical reports suggesting that morning heart attacks are the most deadly (Reiter et al., 2012; Suarez-Barrientos et al., 2011).

To evaluate this phenomenon in mice, we conducted coronary artery ligation surgery, mimicking heart attack in human patients (Virag and Lust, 2011). In agreement with our hypotheses, we observed significantly different ratios of myocardial infarction (MI) to the area at risk (AAR), i.e., the severity of tissue death, in mice treated with daytime versus nighttime surgery. More importantly, Per1+/−;Per2−/− mice showed more severe MI

Figure 3. Impacts of Hypoxia Signaling on the Circadian Clock

(A) The anoxic reagents Ni2+ (500 μM) and Co2+ (200 μM) reversibly prolong and cripple the clock in SCN explants.

(B) Treatment with the anoxic reagent DMOG (1.25 mM) results in period lengthening and amplitude damping in U2OS cells, as monitored by both Per2-dLuc and Bmal1-dLuc reporters.

(C) Dose-dependent effects of Co2+ on circadian parameters in Per2-dLuc cells.

(D) Cellular genetic experiments demonstrate the involvement of HIF1A in the clock modification in U2OS cells. Top: knockdown of HIF1A results in resistance against Co2+ (100 μM) effects in Bmal1-dLuc cells. Bottom: quantitative period alterations of the same cells by Co2+ or DMOG treatments.

(E) Upstream regulatory genes of HIF1A modify the clock. siRNA knockdown of HIF1AN (green) or simultaneous knockdown of all three EGLN genes (red) results in a long period and quick damping of the clock in Per2-dLuc cells. Bottom right: bars show the quantitative period changes.

(F) A time-inducible system was used to introduce the stabilized form (P402A/P564A or 2M) of HIF1A and evaluate its influence on the clock. Duplicates of 2M induction are shown after administration of doxycycline for 48 hr. Bottom right: bars show quantitative period change values.

(G) The expression of many clock genes, including PER2, was elevated in these 2M-induced cells, as was the expression of well-known hypoxia-responsive genes, such as EGLN1s, VEGFA, and GLUT1. To obtain similar scales, we scaled the expression of the ten clock genes to the values represented by the left y axis, while we scaled the values for four typical hypoxic genes to the values on the right y axis.

(H) A transient transfection luciferase assay was conducted in 293T cells to show the difference in the extent of hypoxia induction for Per1 or Per2. In the 2M-induced cells, stabilized HIF1A resides on the promoter region of the PER2 locus. The single arrow represents the transcription start site. ChIP-qPCR assays used IgG as a negative control. Data are presented as the mean ± SEM from at least three independent experiments.
Figure 4. ChIP-Seq Analysis Revealing the Molecular Basis of the Reciprocal Regulation of Clock-Hypoxia Signaling

(A) A dual heatmap illustrating the co-occupancy of BMAL1 and HIF1A in U2OS cells. Left: each site (6,213 sites in total) was rank ordered based on the amount of HIF1A bound at that locus, from the highest amount of HIF1A to the lowest amount, and flanking with ±2.5 kb sequence. Right: the exact location corresponding to that of BMAL1-bound site. Blue indicates enrichment, while white indicates no enrichment.

(B) ChIP-seq binding plots revealing the co-occupancy of both BMAL1 and HIF1A at the same promoter region of the PER2 locus.

(C) Representative gene loci for single or dual binding of BMAL1 and HIF1A. The DBP gene is bound by both transcription factors, while TEF is specifically bound by BMAL1, but not HIF1A, and VEGFA is bound by HIF1A only.

(D) Even under the most stringent criteria (BMAL1 or HIF1A enriched within 1 kb of promoter regions), the predicted BMAL1 and HIF1A target genes retain significant overlap: 425 out of the 1,461 HIF1A targets are also bound by BMAL1.

(E) Frequency of E-box motifs in proximity to BMAL1 binding sites in the 1,576 BMAL1-only targets and in the 425 targets of both BMAL1 and HIF1A and the frequency of E-box motifs in the proximity of HIF1A binding sites in the 1,036 HIF1A-only targets.

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damage than did the WT controls, suggesting a positive circadian role for protection from this deadly impairment (Figures 6A and 6B). Our results were consistent with previous study that Per2−/− mice showed more severe MI damage than the WT controls, and Per2 can mediate light-induced cardiac protection (Eckle et al., 2012). Then we examined the expression levels of hypoxic targets in the ligated heart tissues and found that Glut1 is induced to a greater extent in Per1−/−;Per2−/− mice ligated at night than in WT mice (Figure 6C). Interestingly, Per1−/−;Per2−/− mice exhibit elevated induction of the pro-apoptotic gene Bnip3 than WT mice (Figure 6C), which provides a plausible explanation for the relatively more severe MI damage observed in the in Per1−/−;Per2−/− mice.

**DISCUSSION**

It has long been suspected that the clock may engage in cross-talk with hypoxia signaling, as the key clock component BMAL1 (then named as MOP3) is known to interact with and form a complex with the HIF1A protein in vitro (Hogenesch et al., 1998). However, investigations at both the cellular level and in vivo have, for more than a decade, failed to establish a direct connection between these pathways. This likely results from the fact that, with the cells that are typically used in these studies, the drug-dose window is too narrow to enable researchers to distinguish phenotypes resulting from hypoxia reagent-induced clock effects from cell death phenotypes. For instance, when treated with anoxic reagents, such as Ni2+ or Co2+, mouse fibroblasts harboring a Per2::luciferase knockin reporter quickly dampen their clock oscillation, complicating data interpretation with respect to cell death phenotypes. Just as overexpression of EPO, VEGFA, and GLUT1, which help to supply more oxygen at the organism, tissue, and cell levels; the other group consists of pro-apoptotic genes, including Bnip3, which help to reduce oxygen consumption (Figure 6D). The former group of genes protect the tissue from hypoxia-induced damage. However, if the lack of oxygen continues or gets worse, the cell-death-prone genes are activated, and the tissue damage becomes exaggerated. Thus, MI-induced heart damage seems to result from some interplay between the expression of the protective genes and pro-apoptotic genes. It appears that the circadian clock plays a dual role in the regulation of the expression of both groups of genes (a situation consistent with Hif1a's role), and the pro-apoptotic one takes the lead at the end. We therefore conclude that MI-induced heart damage is a combination of inducing protective genes, such as Glut1, and pro-apoptotic genes, such as Bnip3. In the Per1−/−;Per2−/− mice, the damage-prone side appears to win. The confound, circadian clock-related, MI-induced heart damage may also be involved in myocardial metabolism, in which metabolites, such as NAD+, contribute to bridging these two parts together (Chatham and Young, 2013; Peek et al., 2016). Interestingly, it has also been reported that some events related to inflammatory cell infiltration, which are known to be triggered by MI or hypoxia signaling and known to be regulated by the circadian clock (Virag and Lust, 2014). This may help explain the heart damage phenotype described here. In conclusion, an intact circadian clock plays a role in protecting the heart from hypoxia-induced cell death. Shift workers, whose internal clocks may be significantly crippled by

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(F) Frequency of E-box motifs (CACGTG) and HREs ([T]ACGTG) in the proximity of BMAL1 and HIF1A binding sites in the 425 targets of both BMAL1/HIF1A targets and frequency of CTATAG motifs in the proximity of BMAL1 binding sites as a negative control.

(G) Table listing the binding motifs enriched within 1 kb of the promoter regions for the three groups of target gene loci described in (D) and the associated p values.
Figure 5. ChIP-Seq Profiling of the BMAL1 and HIF1A Co-occupancies in Hypoxic and Normoxic Conditions
(A) The time points indicate the administration of DMOG or control.
(B) Individual genes’ (PER1 and PER2) profiling of BMAL1 ChIP-seq.
(C) Comparison of heatmaps of BMAL1 ChIP-seq.
(D) Comparison of heatmaps of HIF1A ChIP-seq. Arrows indicate the cutoffs for the binding sites. The call peak numbers are indicated as below: for BMAL1-HIF1A co-binding (5,187), for BMAL1 only (6,292), and for HIF1A only (4,158).
The frequent day-night transitions of their work schedule, should be extremely cautious about heart attacks, which may be relatively more lethal in this population. Our results also immediately suggest the hypothesis that therapeutic modification of the clock should help reduce damage caused by heart attacks and other hypoxia-related diseases, like sleep apnea (Vgontzas et al., 2005). Circadian negative regulators, such as PERs and CRYs, can also repress hypoxic signals that may cause lethal damage in heart attacks, likely via the regulation of the expression of HIF1A. Recent studies that focused on chrono-therapies for type II diabetes have identified small-molecule compounds that boost the activity of CRYs and suppress the CLOCK/BMAL1 activity (Hirota et al., 2012). Our results suggest that these compounds might have an additional, potentially positive side effect for reducing the lethality of heart attacks. Obviously, clinical studies will be needed to evaluate the veracity and the clinical relevance of this hypothesis, but our results indicate a potential way, through regulating the circadian clock, to reduce the morbidity and mortality of hypoxia-related diseases in human patients.

**EXPERIMENTAL PROCEDURES**

**Animals**
Mice were housed in a specific-pathogen-free (SPF) environment under a 12 hr light/dark photoperiod with food/water ad libitum. All experiments with mice were performed following the guidelines of the Institutional Animal Care and Use Committee (IACUC) at NIBS.

**Chemical Reagents, Antibodies, Plasmid DNA, and Lentiviral Constructs**
Unless specifically noted, all chemical reagents were purchased from Sigma-Aldrich. ChIP grade α-HIF1A antibody was purchased from Active Motif (#39665); α-BMAL1 was from Abcam (#ab3350). Other antibodies, including anti-BNIP3 (Cat#: ab10433), anti-mouse Per2 (Cat#: PM083, MBL International), were purchased separately. Transient expression vectors were generated by inserting cDNA into the multi-cloning site of pC modelBuilder and hygro. Stable expression vectors were obtained by subcloning cDNA into pLenti-CMV/TO using LR clonase.

**Cell and Tissue Explant Culture**
U2OS cells, HEK293T cells, and mouse fibroblasts were cultured at 37°C with a 5% CO2 atmosphere in DMEM supplemented with 10% fetal bovine serum. SCN slices were prepared from the early postnatal stage (3-6 days old), as

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**Figure 6. Circadian Gating of Heart Failure in an Animal Model**
(A) Mice underwent coronary artery ligation surgery and recovered for 24 hr. Hearts were removed from the chest and stained with Evans Blue prior to being sectioned transversely. Triphenyltetrazolium chloride (TTC) staining was performed to measure the area of myocardial infarction (MI), and the ratio of MI to the area at risk (AAR) was used as the key parameter for the evaluation of damage. The data shown are representative images from WT and Per1+/−; Per2−/− mice that underwent surgery at ZT21. (B) Each dot represents the damage from an individual mouse. (n ≥ 6 per group, *p < 0.05 and **p < 0.01). Times indicated in ZT were the surgery starting time, with roughly ±2 hr of variation, owing to the time required to complete the surgeries. (C) Relative expression of hypoxic targets in the ligated heart tissues (n = 3, *p < 0.05 and **p < 0.01). (D) Proposed model of reciprocal regulation between the circadian clock and hypoxic response signals. The core oscillator of the circadian clock is driven by a negative feedback loop in which CLOCK/BMAL1 activates transcription of E-box-containing genes, whereas PER/CRY acts to repress the expression of these genes (the upper half). The hypoxic response is regulated by HIF1A/ARNT, which activate the transcription of genes containing HRE elements, including HIF attenuating factors (EGLNs), hypoxic protective genes, such as EPO/VEGFA/GLUT1, and hypoxia-induced, pro-apoptotic BNIP3/NOXA1 genes (the lower half). In normoxic conditions, HIF1A is hydroxylated by EGLNs and HIF1AN, leading to rapid protein degradation or the loss of its transcriptional activity. When hypoxia occurs, or when a pharmacological inhibitor (e.g., DMOG or FG-4592) of EGLNs is applied, stabilized HIF1A dimerizes with ARNT and can activate genes with promoters containing HREs. HIF1A can be recruited to the PER2 promoter and activate its transcription, resulting in the disruption of the circadian system. In turn, HIF1A transcription is regulated by CLOCK/BMAL1, thus gating hypoxic responses. All data are presented as the mean ± SEM from at least three independent experiments.
The cycling program was 95°C time PCR with a KAPA SYBR Fast qPCR Kit (#KP-KK4601, Kapa Biosystems). Kit (#RR036A, Takara). Gene expression was analyzed via quantitative real-time qRT-PCR generated from RNA using a PrimeScript RT Master Mix Real-time RT-PCR to the manufacturer’s instructions (#15596, Life Technologies). cDNA was RNA extracted from cells and mouse tissues with Trizol reagent according to previously described procedures (Virag and Lust, 2011).

Luminometry and Luciferase Assays
Cells harboring Per2-dLuc or Bmal1-dLuc were grown to confluence in 3.5 cm dishes and were then placed in experiment medium and sealed. Data were collected in a LumiCycle luminometer at 36°C for 5–7 days; data were analyzed with LumiCycle Analysis software (Actimetrica). Note that data from the first 24 hr cycle was not analyzed (Liu et al., 2007). Luciferase reporter assays were performed in Corning 96-well white bioassay plates with HEK293T cells as described previously (Sato et al., 2008).

qRT-PCR
RNA was extracted from cells and mouse tissues with Trizol reagent according to the manufacturer’s instructions (#15596, Life Technologies). cDNA was generated from RNA using a PrimeScript RT Master Mix Real-time RT-PCR Kit (RR036A, Takara). Gene expression was analyzed via quantitative real-time PCR with a KAPA SYBR Fast qPCR Kit (#KP-KK4601, Kapa Biosystems). The cycling program was 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Primer information is detailed in Table S3.

Chromatin Immunoprecipitation and High-Throughput Sequencing
Confluent U2OS cells treated with saline or doxycycline (2 μg/mL) were prepared and analyzed by ChIP using a ChIP-IT High Sensitivity Kit (#53040, Active Motif). The assays were performed following the protocol included with the kit. RNA-seq and ChIP-seq were conducted using the Illumina Rapid-Run model of the HiSeq2500 system. The data from these analyses were deposited in GEO under the accession numbers GSE69100 and GSE85096.

Epo Measurements
7- to 12-week-old mice were intraperitoneally injected with saline, DMOG (300 mg/kg), or FG4592 (25 mg/kg; #HY-13426, MedChem Express) for 3 hr; arterial blood samples were then collected from eyes for Epo measurement. Serum Epo was measured using a Mouse Erythropoietin Quantikine ELISA Kit (#MEP00B, R&D Systems), according to the protocol for the kit.

Myocardial Ischemia Surgery and Damage Evaluation
Myocardial ischemia was induced by ligation of the left anterior descending branch (LAD) of the coronary artery, and its effects were evaluated according to previously described procedures (Virag and Lust, 2011).

Statistical Analysis
In all experiments, unless noted, error bars represent SEM. Statistical significance was determined using two-sided Student’s t tests when only two groups were analyzed. One-way ANOVA was used when more than two groups were being analyzed. *p < 0.05, **p < 0.01.

ACCESSION NUMBERS
The accession numbers for the data from RNA-seq and ChIP-seq analyses reported in this paper are GEO: GSE69100 and GSE85096.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.09.009.

AUTHOR CONTRIBUTIONS
Y.W. and E.E.Z. conceived the study and designed the experiments. Y.W. performed the cellular experiments with help from N.L., D.T. and Y.W. conducted the animal experiments with help from H.Z. Y.L. dissected the SCN slides. Y.W. and D.T. analyzed the experimental data. Z.M. conducted the JTK_Cycle analysis. W.X. and H.H. analyzed the sequencing data. P.C. and X.Q. provided chemical reagents. E.E.Z. supervised the project and wrote the manuscript. All authors discussed the results and commented on the manuscript.

REFERENCES


