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The small molecule Nobiletin targets the molecular oscillator to enhance circadian rhythms and protect against metabolic syndrome

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SUMMARY

Dysregulation of circadian rhythms is associated with metabolic dysfunction, yet it is unclear whether enhancing clock function can ameliorate metabolic disorders. In an unbiased chemical screen using fibroblasts expressing *PER2::Luc*, we identified Nobiletin (NOB), a natural polymethoxylated flavone, as a Clock amplitude-Enhancing small Molecule (CEM). When administered to diet-induced obese (DIO) mice, NOB strongly counteracted metabolic syndrome and augmented energy expenditure and locomotor activity in a *Clock* gene-dependent manner. In *db/db* mutant mice, the clock is also required for the mitigating effects of NOB on metabolic

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SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures and Supplemental References, six Supplemental Figures, two Supplemental Tables and two Excel data files.

AUTHOR CONTRIBUTIONS

Z.C. conceived the project and wrote the manuscript; Z.C., S.-H.Y. and J.S.T. supervised research; B.H., K.N., N.P., Y.-S.P., B.G., Z.Z., S.-H.Y. and Z.C. conducted research; all authors contributed to data analysis and manuscript preparation.

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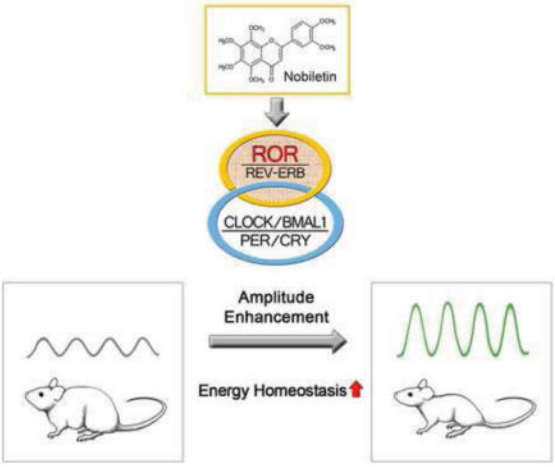
disorders. In DIO mouse liver, NOB enhanced clock protein levels and elicited pronounced gene expression remodeling. We identified retinoid acid receptor-related orphan receptors (RORs) as direct targets of NOB, revealing a pharmacological intervention that enhances circadian rhythms to combat metabolic disease via the circadian gene network.

Graphical Abstract



Keywords

Circadian clock; Nobiletin; natural flavonoid; retinoid acid receptor-related orphan receptors (RORs); metabolic syndrome; clock amplitude-enhancing small molecule

INTRODUCTION

Virtually all living organisms on Earth have evolved an intrinsic timing system, the circadian clock, to anticipate and exploit daily environmental changes. In mammals, the clock system is hierarchically organized, with the central pacemaker in the hypothalamic suprachiasmatic nuclei (SCN) coordinating peripheral tissue clocks to perform physiological functions (Takahashi et al., 2008). The cell-autonomous molecular oscillator is the basic component of the clock system, composed of interlocked feedback loops (Dibner et al., 2010). The core loop, consisting of positive (transcriptional activators CLOCK/BMAL1 or NPAS2/BMAL1) and negative (PER1/2 and CRY1/2) arms, is responsible for generating molecular rhythms, whereas competing nuclear receptors REV-ERBs and RORs regulate *Bmal1* expression to confer rhythm stability and robustness (Zhang and Kay, 2010). The molecular oscillators drive tissue-specific gene expression throughout the circadian cycle via both transcriptional and posttranscriptional mechanisms (Koike et al., 2012; Zhang et al., 2014).

A fundamental process tightly regulated by the clock system is metabolism (Asher and Schibler, 2011; Bass and Takahashi, 2010; Gerhart-Hines and Lazar, 2015; Green et al., 2008; Rutter et al., 2002), as both metabolites (Eckel-Mahan et al., 2012) and metabolic gene expression (Yang et al., 2006; Zhang et al., 2014) broadly exhibit circadian oscillation. In humans, circadian misalignment has been shown to cause metabolic disturbances such as

glucose intolerance and hyperlipidemia (Roenneberg et al., 2012; Scheer et al., 2009). Genetic studies have also revealed overlapping metabolic deficiencies in clock-disrupted mice (Green et al., 2008). For example, the circadian mouse mutant *Clock*$^{\Delta 19/\Delta 19}$ harboring a dominant negative allele has been found to exhibit a spectrum of metabolic disorders including obesity, hyperlipidemia, hepatic steatosis, hyperglycemia, hypoinsulinemia, and respiratory uncoupling (Marcheva et al., 2010; Shi et al., 2013; Turek et al., 2005).

Recent studies have explored the strategy of directly manipulating circadian rhythms to ameliorate the metabolic syndrome (Antoch and Kondratov, 2013; Chen et al., 2013; Farrow et al., 2012; Schroeder and Colwell, 2013). For example, time-restricted intake of high-fat diet (HFD) was shown to protect mice against metabolic disease (Hatori et al., 2012). Oscillatory amplitude of clock and metabolic gene expression was significantly enhanced in a nighttime-specific HFD regime, suggesting that the body can best expend the incoming nutrients by a concerted action of clock-associated pathways during the active period. To circumvent compliance issues inherent in behavioral interventions, a pharmacological approach involving clock-modulating small molecules was also examined (Chang et al., 2015; Chen et al., 2012; Chen et al., 2013; Hirota et al., 2012; Isojima et al., 2009; Meng et al., 2010; Solt et al., 2012; Wallach and Kramer, 2015). For example, small molecule agonists acting on the REV-ERB nuclear receptors showed beneficial metabolic effects (Solt et al., 2012), suggesting modulatory compounds can improve metabolism via clock components or clock-associated mechanisms.

We previously identified several synthetic Clock amplitude-Enhancing small Molecules (CEMs) in a high throughput chemical screen using reporter cells with highly robust rhythms (Chen et al., 2012; Chen et al., 2013). When applied to cultured heterozygous *Clock*$^{\Delta 19/+}$ *PER2::Luc* reporter cells where reporter rhythms oscillate with a weaker amplitude (approximately 1/3) relative to wild-type (WT) *Clock*$^{+/+}$ cells, these CEMs were able to restore the reporter rhythm amplitude to near normal levels. Here we report identification of a natural compound, Nobiletin, that enhances the clock and concordantly renders protection against metabolic syndrome in a *Clock* gene-dependent manner. We further identified RORs, nuclear receptors functioning in the stabilization loop of the molecular oscillator, as the direct targets of Nobiletin.

RESULTS

Identification of Nobiletin (NOB) as a clock modulator

To identify additional CEMs, we screened an in-house compound collection with 5,300 small molecules using the heterozygous *Clock*$^{\Delta 19/+}$ *PER2::Luc* reporter cells which exhibit sustained reporter rhythms at a dampened amplitude relative to WT cells (see Experimental Procedures and Supplementary Information). Interestingly, a naturally-occurring polymethoxylated flavonoid enriched in citrus peels, Nobiletin (NOB), was found independently from two sub-libraries to enhance the reporter rhythm of the *Clock*$^{\Delta 19/+}$ cells (Figures 1A and 1B). Tangeretin, a close analog of NOB, similarly enhanced the *PER2::Luc* reporter rhythm in *Clock*$^{\Delta 19/+}$ cells (Figures S1A and S1B). NOB robustly enhanced the amplitude of *Per2::LucSV* reporter rhythm (Chen et al., 2012) and also lengthened the period in a dose-dependent manner, with an estimated EC50 of <5.0 μM (Figure 1C).

Similar to previously reported CEMs (Chen et al., 2012; Chen et al., 2013), NOB was ineffective in restoring the rhythm in clock-disrupted homozygous *Clock*$^{\Delta 19/\Delta 19}$ reporter cells (Figure 1D). Importantly, NOB enhanced *PER2::Luc* reporter rhythms in peripheral tissue explants from both *Clock*$^{\Delta 19/+}$ and WT reporter knock-in mice (Figures 1E and S1C), but not in the SCN which is resistant to external perturbation due to robust inter-neuronal coupling (Figure S1D) (Buhr et al., 2010; Chen et al., 2012; Liu et al., 2007). In accordance with the resistance of the SCN to NOB manipulation, we also observed normal wheel-running activity and periodicity in WT C57BL/6J mice treated with NOB (Figure 1F).

NOB has shown a wide variety of beneficial effects (Ben-Aziz, 1967; Cui et al., 2010; Mulvihill et al., 2011; Nagase et al., 2005; Walle, 2007). However, its role as a modulator of the circadian clock was previously unknown. Whereas *Per2* transcript levels were moderately altered and reduced at CT20 by NOB in *PER2::LucSV* cells (Figure S1E), PER2 proteins were found to accumulate to greater levels (Figures S1F and S1G), consistent with the elevated bioluminescence and suggesting a post-transcriptional mechanism for PER2 enrichment. Expression of other core clock genes was also altered by NOB (Figures S1E – S1G). In particular, CRY1, the heterodimeric partner of PERs in the negative arm of the oscillator, showed a slight trend of greater protein abundance despite markedly reduced transcript level (Figures S1E and S1F). The concordant enrichment of PER2 and CRY1 is consistent with the idea that PER2 and CRY1 proteins stabilize each other (Yagita et al., 2002), and that an improved stoichiometric ratio of the negative arm can lead to enhanced overall circadian amplitude in mouse fibroblast cells (Lee et al., 2011).

Robust *Clock*-dependent metabolic protection by NOB in diet-induced obese mice

Recent studies suggested a protective role of NOB against metabolic syndrome (Kurowska and Manthey, 2004; Lee et al., 2013; Lee et al., 2010; Mulvihill et al., 2011; Roza et al., 2007). In accordance, pharmacokinetic studies revealed significant brain and systemic exposure of NOB (Figure S2A). To address whether metabolic protection by NOB depends on clock function, we first employed a diet-induced obesity (DIO) mouse model using both WT and clock-disrupted *Clock*$^{\Delta 19/\Delta 19}$ mice. In WT C57BL/6J mice fed with high-fat diet (HFD), 10-week NOB treatment significantly attenuated body weight gain relative to the vehicle control (Figures 2A and S2B). Food intake was not significantly altered by NOB relative to the vehicle control (Figures 2B and S2C). Body mass composition analysis revealed that the body weight loss was primarily attributable to reduction in fat mass (Figure 2C) and white adipose cell size (Figures 2D and S2D). Remarkably, NOB treatment only led to very modest reduction in body weight gain in HFD-fed *Clock*$^{\Delta 19/\Delta 19}$ mutant mice (Figures 2A and S2B), with the fat mass and adipocyte cell size essentially unchanged by NOB treatment (Figures 2C, 2D and S2D). While mutant mice consumed more food during the light phase than WT mice (Turek et al., 2005), NOB did not change food intake in the mutant mice (Figures 2B and S2C). Indicating elevated energy expenditure, NOB-treated WT mice exhibited greatly elevated oxygen consumption compared to the controls throughout the circadian cycle with the largest increase found in early dark phase (Figures 2E and S2E). Respiratory quotient was also increased in WT mice treated with NOB (Figure S2F), consistent with a switch from lipid-biased metabolism to a more balanced contribution from all major macronutrients. In contrast, *Clock*$^{\Delta 19/\Delta 19}$ mice showed no increase in energy

expenditure or respiratory quotient after NOB treatment (Figures 2E and S2F). Consistent with its effect on body weight and energy expenditure, NOB greatly increased wheel running activity levels in HFD-fed WT mice relative to control treatment (Figure 2F); in contrast, no significant difference in activity levels was detected between the treatments for *Clock*$^{\Delta 19/\Delta 19}$ mice (Figure 2G).

NOB also improved glucose and lipid homeostasis in WT but not *Clock*$^{\Delta 19/\Delta 19}$ mice. NOB lowered fasting glucose levels in WT mice (Figure 3A), and significantly improved glucose tolerance and insulin sensitivity (Figures 3B and 3C). Interestingly, blood insulin levels were strongly reduced in NOB-treated WT mice relative to vehicle controls (Figure 3D). Total triglyceride (TG) and cholesterol (TC) levels in WT serum and liver were also significantly diminished by NOB (Figures 3E and 3F). Hematoxylin and eosin (H&E) and Oil Red O staining revealed that NOB strongly improved liver steatosis and essentially abolished lipid droplet formation in DIO WT liver (Figures 3G, S2H – S2J). In contrast, NOB did not significantly improve glucose and lipid homeostasis in *Clock*$^{\Delta 19/\Delta 19}$ mice (Figure 3), and its beneficial effect on *Clock*$^{\Delta 19/\Delta 19}$ liver steatosis was markedly attenuated compared with that in WT (Figures 3G, S2H – S2J). In contrast to HFD, regular chow (RC) feeding did not lead to metabolic disorders and NOB treatment did not show significantly beneficial effects on metabolic homeostasis (Figure S3). Together, these results demonstrate a *Clock*-dependent efficacy of NOB against metabolic syndrome.

Non-methoxylated flavanones such as Naringin (NAR) and its aglycone derivative Naringenin are also naturally occurring flavonoids (Figure S4A) (Assini et al., 2013), yet failed to enhance cellular circadian rhythms in our primary screen (Figures S4B – S4F). Consistent with previous studies (Mulvihill et al., 2009; Mulvihill et al., 2011), compared with NOB, NAR showed significantly attenuated effects on body weight gain and lipid/glucose homeostasis (Figure S5). Importantly, the modest effects from NAR treatment were largely indistinguishable between WT or *Clock*$^{\Delta 19/\Delta 19}$ C57BL/6J mice.

Clock-dependent metabolic protection by NOB in *db/db* diabetic mice

Given the improved glucose homeostasis in DIO mice treated with NOB, we next investigated effects of NOB on *db/db* mice, an established genetic mouse model for obesity and diabetes that lacks functional leptin receptors, and the role of the clock. NOB treatment strongly blunted body weight gain in *db/db* mice (Figure 4A), lowered fasting glucose levels (Figure 4B), improved glucose tolerance and insulin sensitivity (Figures 4C and 4D), and reduced serum TG and TC levels in *db/db* mice (Figures 4E and 4F). In contrast, *db/db Clock*$^{\Delta 19/\Delta 19}$ double mutant mice exhibited a markedly attenuated response to NOB (Figure 4). Under Veh treatment, *db/db Clock*$^{\Delta 19/\Delta 19}$ mutant mice showed lower insulin levels than *db/db*, consistent with β-cell deficits and hypoinsulinemia previously reported in *Clock*$^{\Delta 19/\Delta 19}$ mice (Marcheva et al., 2010). NOB treatment strongly reduced circulating insulin levels in both *db/db* and *db/db Clock*$^{\Delta 19/\Delta 19}$ mutant mice, with the former exhibiting a more pronounced reduction (Figure 4G). These results are consistent with lower insulin sensitivity in *db/db Clock*$^{\Delta 19/\Delta 19}$ relative to *db/db*. Given the age-dependent deficits in pancreatic β-cell proliferation and insulin secretion in *Clock*$^{\Delta 19/\Delta 19}$ (Marcheva et al., 2010),

future studies will investigate the mechanistic relationship between the effects of NOB on insulin levels and β-cell function and/or insulin sensitivity.

Identification of hepatic NOB-responsive genes by microarray

To investigate the molecular basis of NOB action in HFD-fed WT mice, we focused on a major metabolic organ, the liver, where we observed a strong protection by NOB. Consistent with previous studies (Kohsaka et al., 2007), the oscillatory amplitude of clock gene expression was generally lower in the liver of HF.Veh mice relative to lean RC.Veh mice (Figures 5A and 5B). NOB improved circadian clock transcript oscillations and largely restored clock protein rhythms (Figures 5A and 5B) (HF.NOB vs. HF.Veh), concordant with its robust physiological efficacy. To investigate metabolic output gene expression, we conducted microarray analysis. Comparative analysis was conducted to analyze gene expression changes in pair-wise comparison, namely HF.Veh vs. RC.Veh (the comparison is denoted as HF/RC) and HF.NOB vs. HF.Veh (denoted as NOB/HF), at ZT2 and ZT14 time points, which revealed altered expression of 544, 229, 915 and 243 genes, respectively (Data S1). Importantly, a total of 251 and 56 genes were identified showing altered gene expression patterns in response to HFD (HF/RC) that were reversed by NOB (NOB/HF) at one or both time points (ZT2 and/or ZT14), respectively (Figure 5C, Figure S6A and Data S2). Functional classification (Gene Ontology, GO) of these NOB-responsive genes highlighted a prominent role in metabolic regulation (Figure 5D, Data S2 and Tables S1). Real-time qPCR analysis further illustrated a broad modulatory function of NOB in metabolic output gene expression (Figures 5E). For example, transcript expression of *Cidec/Fsp27*, known to function in lipid droplet formation (Eckel-Mahan et al., 2013; Matsusue et al., 2008; Puri et al., 2007), was induced by >10 fold in HFD.Veh mouse liver relative to RC.Veh and reverted back to baseline levels by NOB, consistent with the NOB efficacy in mitigating hepatic steatosis (Figure 3G). NOB also altered the expression of genes involved in gluconeogenesis and glycolysis (e.g., *Pdk4 and Pkm2).*

RORs as direct protein targets for NOB

Cross-examination of previous circadian ChIP-seq studies (Cho et al., 2012; Koike et al., 2012) revealed that 63% of the NOB-responsive genes showed promoter occupancy of core clock proteins (Figure S6B), particularly REV-ERBs. REV-ERBs and RORs function respectively as negative and positive transcription factors competing for binding to RORE promoter elements, playing important roles in circadian rhythms, metabolism and inflammation (Gerhart-Hines et al., 2013; Jetten et al., 2013; Kojetin and Burris, 2014). In a previous screen for inhibitors of RORγt, NOB was among a number of primary screen hits that instead activated RORγt and consequently were not validated further (Huh et al., 2011). ROR family receptors consist of α, β and γ isoforms. RORα and RORγ are more similar in tissue distribution and the ligand binding domain structure, whereas RORβ is more divergent (Kallen et al., 2002; Solt et al., 2010; Stehlin et al., 2001). We therefore focused on RORα and RORγ.

To investigate a possible direct interaction between NOB and ROR proteins, we employed a competitive radioligand binding assay for RORs using 25-[3H]-hydroxycholesterol (25-[3H]-OHC) (Kumar et al., 2010; Wang et al., 2010b). Saturation curves and Scatchard plots

validated the assay, with similar Kd values to that previously reported (Figures 6A and 6B) (Kumar et al., 2010; Wang et al., 2010b). Importantly, NOB showed robust competitive binding to the ligand binding domains (LBDs) of RORα and RORγ, with higher affinity for RORγ (Figure 6C; see Ki comparison). In contrast, NAR or its aglycone derivative Naringenin showed markedly diminished binding in the same concentration range (Figures 6C and 6D). Consistent with these binding assay results, we observed robust activities of NOB and the known ROR ligand SR1001 (Solt et al., 2011) for GAL4-RORα and GAL4-RORγ chimeric receptors in mammalian one-hybrid reporter assays, and NAR showed no activities (Figures 6E and 6F). Of note, regardless of the nature of the ligand (agonist or inverse agonist), ligand interaction with these chimeric receptors has been shown to reduce transcriptional activity of these chimeric receptors (Wang et al., 2010a; Wang et al., 2010b). These results together indicate direct binding of NOB to RORα and RORγ.

Next we conducted functional assays to determine the effect of NOB on RORα/γ transcriptional activity. NOB was found to dose-dependently increase *Bmal1* promoter-driven luciferase reporter activity with wild-type, but not mutant, RORE elements (Preitner et al., 2002) in the presence of RORα or RORγ in Hepa1-6 cells (Figure 6G). Conversely, knockdown of the *Rora/c* genes encoding RORα/γ by siRNAs abrogated the NOB-mediated induction of *Bmal1* promoter-driven luciferase reporter activity in both Hepa1-6 and U2OS cells (Figures 6H and S6C). Several ROR target genes (e.g., *Cyp7b1, IkBa* and *Gck*) were induced in NOB-treated DIO mouse liver relative to control treatment (Figure 6I), and Ingenuity pathway analysis (IPA) also showed an important role of RORs in the genome-wide NOB response (Figure S6D). Together, these results indicate that NOB directly binds to and activates RORs and that RORα/γ are necessary for the enhancing activity of NOB on *Bmal1* transcription.

DISCUSSION

In summary, our unbiased chemical screen identified clock-enhancing polymethoxylated flavones, particularly Nobiletin. Compelling evidence from both genetic and pharmacological studies demonstrates a *Clock* gene-dependent efficacy of NOB in preventing metabolic syndrome in mice, providing proof in mammals that strengthening circadian amplitude is a pharmacological intervention strategy for metabolic disease and other clock-related pathologies such as age-related decline (Chen et al., 2013). The beneficial outcome of enhanced circadian amplitude could include enhanced efficiency in physiological performance, greater stimuli range and sensitized response (Hatori et al., 2012; Hogenesch and Herzog, 2011; Ikeda et al., 2013; van Ooijen and Millar, 2012). Consistent with the notion that augmented circadian amplitude enhances energy metabolism, time-restricted feeding, and thus energy expenditure, plays a dominant role determining extent of obesity from high-fat diet feeding (Hatori et al., 2012).

Polymethoxylated flavones elicit diverse benefits in mice and humans, including mitigating effects against cancer, inflammation, atherosclerosis, and more recently metabolic disorders and neurodegenerative diseases (Cui et al., 2010; Evans et al., 2012; Kurowska and Manthey, 2004; Lee et al., 2013; Mulvihill et al., 2009; Nohara et al., 2015a). Polymethoxylated flavones generally show a favorable pharmacokinetic profile (Evans et al., 2012; Saigusa et

al., 2011), and no discernible toxicity was observed in chronic treatment of mice in this and previous studies (Lee et al., 2013; Mulvihill et al., 2011). Our recent studies showed a role of NOB in ammonia disposal via urea cycle regulation, and transcriptional induction of the rate-limiting *Cps1* gene by NOB was impaired in $Clock^{\Delta 19/\Delta 19}$ mutant mice (Nohara et al., 2015a). Providing a mechanistic basis for these functional results, the current study illustrates a direct role of NOB in the enhancement of circadian clocks and particularly the activation of ROR receptors. These findings collectively suggest a unifying circadian mechanism governing the diverse physiological effects of polymethoxylated flavones.

We identified the ROR nuclear receptors as the molecular target of NOB, which makes a direct link to the circadian gene network via the REV-ERB/ROR pathway that stabilizes the core CLOCK:BMAL1 transcriptional feedback loop (Sato et al., 2004). This function of NOB on RORs explains why there are relatively “subtle” changes in circadian gene expression, because the RORs primarily enhance *Bmal1* transcription in the core loop, which in turn leads to enhancement of both activation and feedback repression (Hogenesch and Herzog, 2011). This auto-regulatory feedback limits the dynamic range of transcriptional readouts as a consequence of this homeostatic, closed-loop system, also exemplified by the finding that transgenic *Clock* overexpression in mice led to greater stability, but only modest amplitude increase, in *Per* and *Cry* oscillation (Antoch et al., 1997).

Genetic and pharmacological studies illustrate a complex duality of ROR functions in metabolism and immunity. On the one hand, multiple ROR-deficient mouse mutants showed metabolic benefits including resistance to obesity and improved lipid homeostasis (Lau et al., 2008), suggesting a negative role of RORs in metabolic regulation. Likewise, pharmacological studies have mainly focused on inverse agonists for RORs (Jetten et al., 2013; Solt et al., 2010), due to their therapeutic potential in Th17-dependent autoimmunity (Huh et al., 2011; Solt et al., 2011) and metabolic syndrome (Chang et al., 2015; Jetten et al., 2013). On the other hand, RORs also play important positive roles, and agonists or activating compounds of RORs can effect physiological improvements. First, genetic studies support positive metabolic and immune functions of RORs (Garidou et al., 2015; Raichur et al., 2010). For example, attenuated RORα signaling by skeletal muscle-specific expression of RORα1 ΔDE (lacking the ligand-binding domain) led to various metabolic abnormalities in mice, including hyperglycemia and glucose intolerance (Raichur et al., 2010). RORα plays an anti-inflammatory role by interfering with NF-κB signaling (Delerive et al., 2001), and homozygous *Staggerer* mice exhibit an enhanced susceptibility to lung inflammation (Stapleton et al., 2005). Recent studies also showed that RORγ deficiency compromises immunity to pathogens and parasites (Ohnmacht et al., 2015; Okada et al., 2015). Second, cholesterol biosynthetic intermediates (CBI) have been identified as endogenous RORγt ligand(s) that stabilize the RORγ ligand-binding domain and induced coactivator recruitment in normal physiology (Santori et al., 2015). Finally, metabolic effects in ROR-deficient mice should be considered in the broader context of overall physiological health. For example, the *Staggerer* mice deficient in RORα suffer from severe developmental, physiological and behavioral deficits and require special husbandry care; notably, despite reduced adiposity and apparent resistance to high-fat diet, these mice also exhibit severe atherosclerosis (Jetten, 2009; Mamontova et al., 1998). Therefore, RORs play complex roles in both

immunity and metabolism, and it is possible that both agonists and antagonists can fine-tune ROR activities and exert beneficial roles. It is important to examine molecular details of NOB-ROR interaction, especially given that different inverse agonists of RORγt exhibit divergent effects on target gene expression via distinct molecular mechanisms (Xiao et al., 2014). Genetic models, such as double conditional KO and/or overexpression of functionally overlapping RORα and RORγ, will help elucidate the function of ROR ligands including NOB (Knight and Shokat, 2007).

In conclusion, our work reveals NOB as a clock-enhancing natural compound that activates RORs and protects against metabolic syndrome in a clock-dependent manner. This proof-of-principle study will spur future application of NOB and additional clock-enhancing compounds in other diseases (e.g., mood and sleep disorders) and aging known to suffer dampened circadian amplitude (Nohara et al., 2015b; Schroeder and Colwell, 2013).

EXPERIMENTAL PROCEDURES

Animals and cell lines

Animal husbandry for all the studies except tissue explant experiments was carried out under IACUC guidelines and the procedures were conducted as described in an animal protocol approved by the University of Texas Health Science Center at Houston (UTHSC-H). PER2::Luc reporter knock-in mice used for tissue explant experiments were maintained according to guidelines from IACUC at the University of Texas Southwestern Medical Center (UTSW). Adult mouse ear fibroblast and mouse embryonic fibroblast (MEF) cells were previously described (Chen et al., 2012).

High-throughput chemical screen and validation

The chemical screen for circadian clock modulators was conducted at the Chemical Genomics Core facility at the UTHSC-H. The in-house chemical library we screened consists of compounds from NIH Clinical Collection, NCI collection and Microsource Spectrum Collection. The screening was conducted largely based on the protocol previously described (Chen et al., 2012). Briefly, immortalized fibroblast cells from *Clock*$^{\Delta 19/+}$ heterozygous mice expressing the *PER2::Luc* bioluminescence reporter were plated into 96-well plates. Upon confluency, cells were incubated with 5 μM forskolin for 1–2 h followed by the addition of chemical compounds to the plates with robotic arms (Beckman), and then subjected to continuous monitoring over several days in a temperature-controlled EnVision microplate reader (Perkin Elmer). Data analysis was carried out by using the MultiCycle software (Actimetrics) for measurement of period, phase, and amplitude.

Mouse treatment and metabolic analyses

For diet-induced obesity, male mice at 6 weeks of age were fed with HFD (D12492, Research Diets) until the end of the experimental protocol. The mice were treated with either vehicle (DMSO) or NOB (200mg/kg body weight) via oral gavage every other day, in the time window of ZT8-10, throughout the experimental period. We chose the every-other-day dosing regimen at the indicated level based on several reasons as described in Supplemental Information. Metabolic assays and energy expenditure analyses were conducted as

previously described (Daniels et al., 2010; Garcia et al., 2013; He et al., 2015; Jeong et al., 2015). Please see Supplemental Information for details.

Microarray analysis

Total RNAs prepared from liver tissues from RC-fed, Vehicle-treated and HFD-fed, Vehicle or NOB-treated WT mice for 10 weeks were reverse transcribed into cDNAs, which were then biotin-UTP labeled and hybridized to the Illumina mouse WG-6v2.0 Expression BeadChip. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE78848 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78848).

Molecular and biochemical analyses

Real-time qPCR and Western blotting analyses of circadian gene expression were conducted as previously described (Yoo et al., 2013). Primers used are listed in Table S2. For mammalian one-hybrid assays, regardless of the nature of the ligand (agonist or inverse agonist), ligand interaction with these chimeric receptors has been shown to reduce transcriptional activity of these chimeric receptors (Wang et al., 2010a; Wang et al., 2010b). For radio-ligand receptor binding assays, we adopted previously described protocols with minor modifications (Kumar et al., 2010; Wang et al., 2010b).

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined by one-way or two-way ANOVA with Turkey's or Dunnett' tests for multiple group comparisons. $P<0.05$ was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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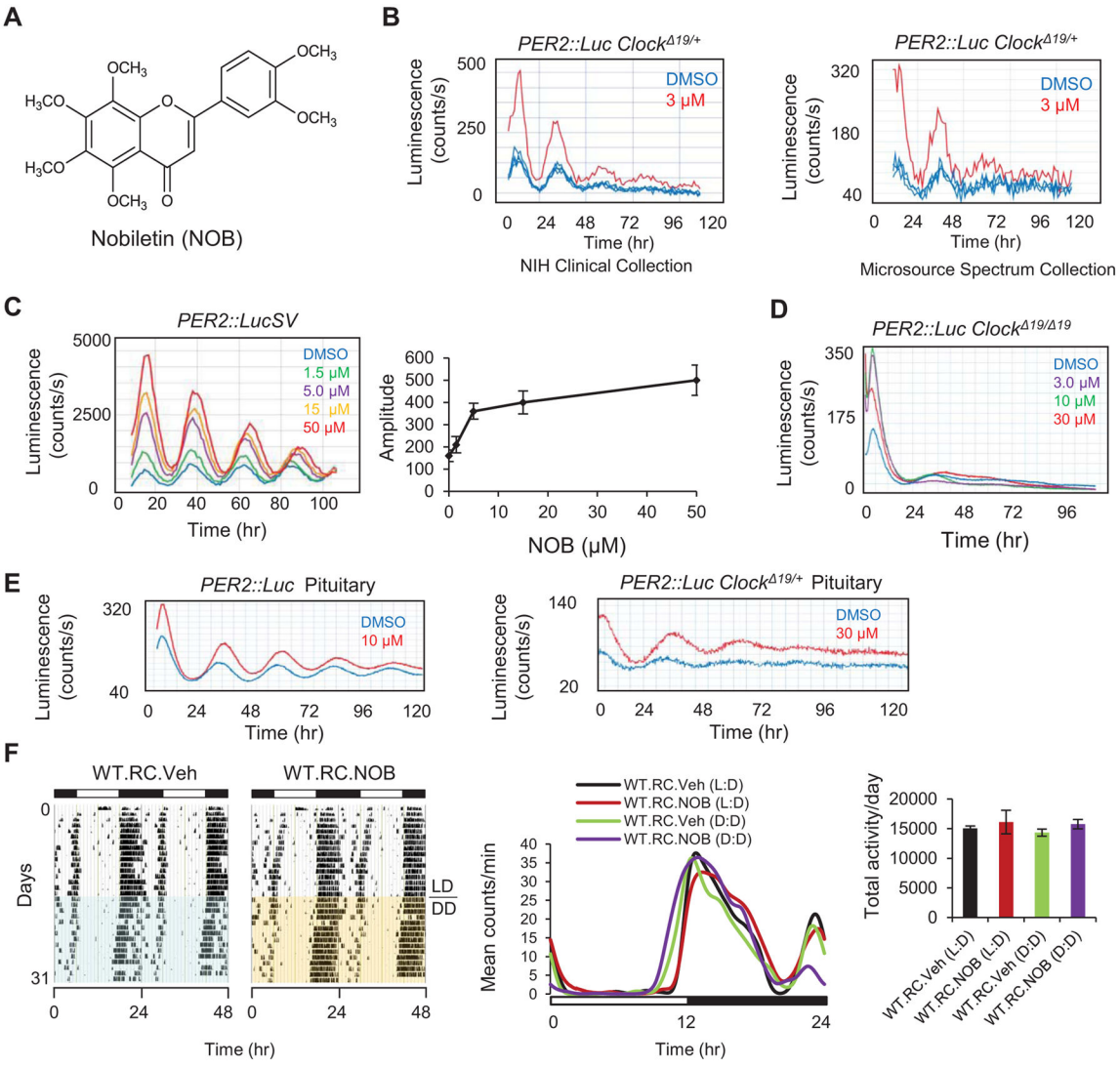

Figure 1. Nobiletin (NOB) enhances amplitude of circadian rhythms

(A) Nobiletin (NOB) chemical structure. (B) Primary screening of the NIH Clinical Collection (left) and Microsource Spectrum Collection (right) showing enhancement of the *PER2::Luc Clock*$^{\Delta 19/+}$ reporter rhythm by NOB. (C) Left: Dose-dependent effects of NOB on reporter rhythms from *PER2::LucSV* cells. Right: Quantification of amplitude response to NOB doses. (D) NOB was not able to rescue reporter rhythms in PER2::Luc Clock$^{\Delta 19/\Delta 19}$ fibroblast cells. (E) Clock-enhancing effects of NOB in pituitary explants from *PER2::Luc* WT (left) and *PER2::Luc Clock*$^{\Delta 19/+}$ (right) reporter mice. (F) Left: Actograms illustrating the effect of vehicle or NOB on circadian behavior in RC-fed WT mice (n=5). Middle: Average wave plots summarizing wheel-running activity during 12–14 days of LD (12hr light, 12hr dark) or DD (Constant darkness) for indicated genotypes (n=5). Right: Daily total wheel-running activity during L:D or D:D conditions for the indicated genotypes (n=5). Data are represented as mean ± SEM.

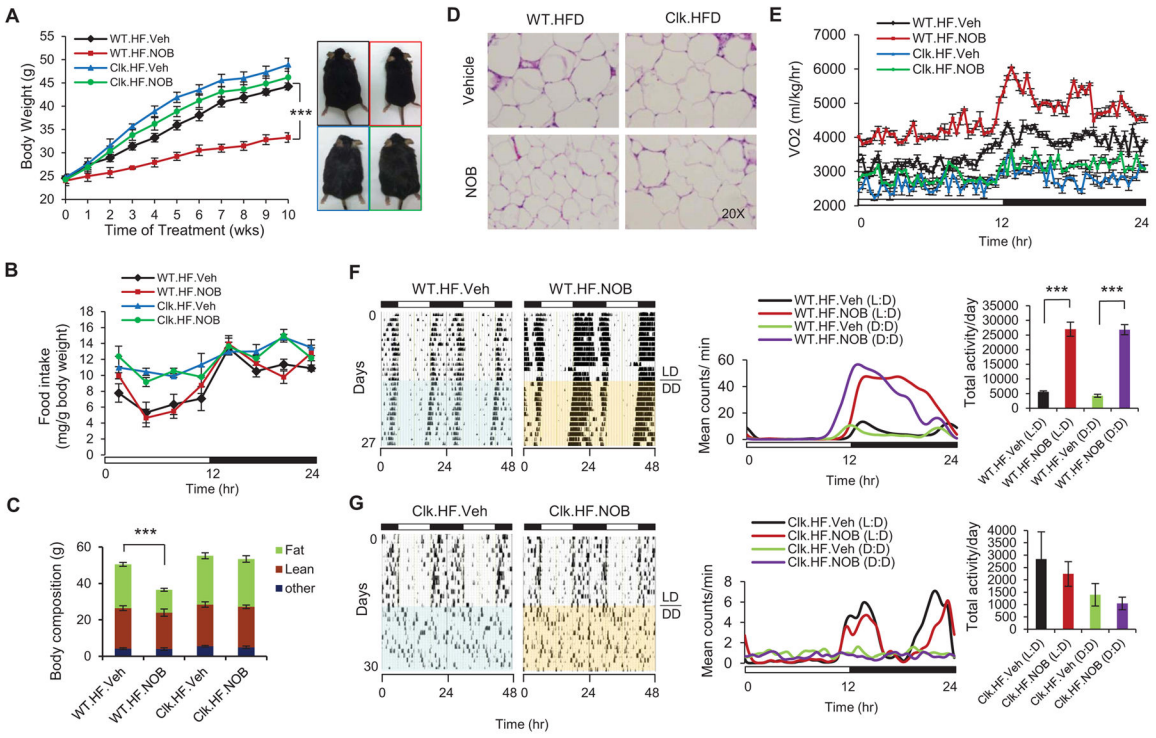

Figure 2. NOB modulated energy homeostasis and behavior in diet-induced obesity (DIO) mice in a clock-dependent manner

(A) Average body weight of WT or *Clock*$^{\Delta 19/\Delta 19}$ mutant mice fed with high-fat diet and treated with either vehicle or NOB (WT.HF.Veh, WT.HF.NOB, Clk.HF.Veh and Clk.HF.NOB) for 10 weeks (n=8–15). (B) Daily food intake for the above 4 groups of mice (n=8–15). (C) Body mass composition as analyzed by NMR (n=3). (D) Histological analysis of white adipose fat (WAT) after 10-week treatment. WAT was subjected to H&E staining. (E) The diurnal rhythms of VO_2 (volume of oxygen consumed) in the 4 groups of mice (n=8). (F) Left: Actograms illustrating the effect of vehicle or NOB on circadian behavior in HFD-fed WT mice (n=7). Middle: Average wave plots summarizing wheel-running activity during 12–14 days of LD (12hr light, 12hr dark) or DD (Constant darkness) for indicated genotypes (n=7). Right: Daily total wheel-running activity during L:D or D:D conditions for the indicated genotypes (n=7). (G) Left: Actograms illustrating the effect of vehicle or NOB on circadian behavior in HFD-fed *Clock*$^{\Delta 19/\Delta 19}$ mutant mice (n=3). Middle: Average wave plots summarizing wheel-running activity during 10–12 days of LD (12hr light, 12hr dark) or DD (Constant darkness) for indicated genotypes (n=3). Right: Daily total wheel-running activity during L:D or D:D conditions for the indicated genotypes (n=3). Data are represented as mean ± SEM. ***$p < 0.001$. WT.HF.NOB vs. WT.HF.Veh.

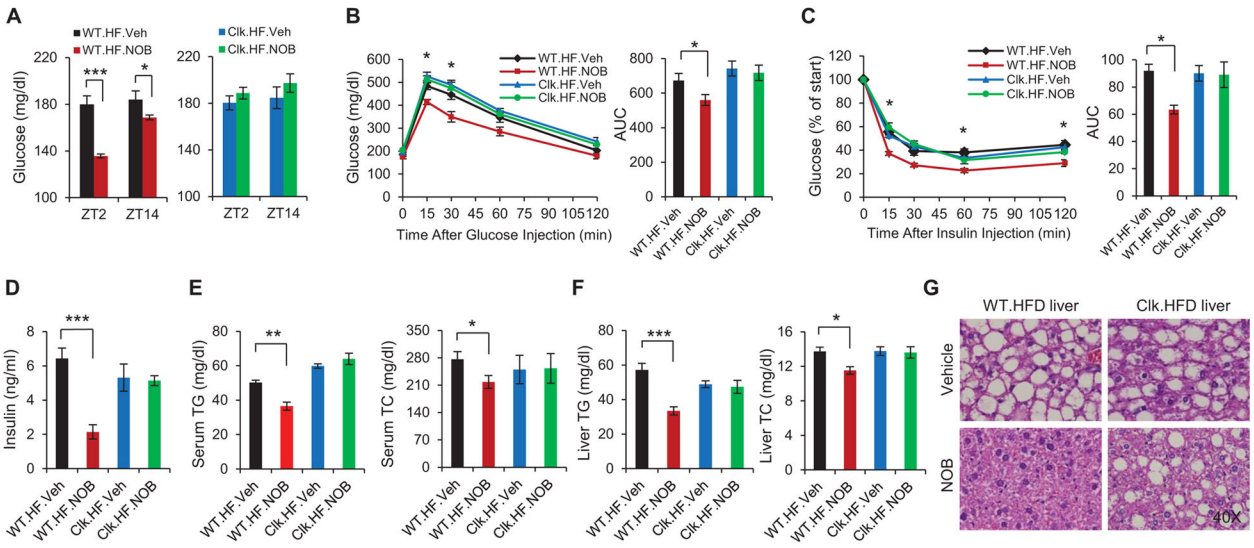

Figure 3. NOB improved glucose and lipid homeostasis in diet-induced obesity (DIO) mice in a clock-dependent manner

(A) Fasting blood glucose levels in HFD-fed WT (left) and *Clock*$^{\Delta 19/\Delta 19}$ mutant mice (right) with Vehicle or NOB treatment at two opposite time points (n=8–15). (B) Effect of NOB on glucose tolerance in HFD-fed WT and *Clock*$^{\Delta 19/\Delta 19}$ mutant mice as measured by glucose tolerance test (GTT) (n=8–15). Right panel: Area under curve (AUC). (C) Effect of NOB on insulin tolerance in HFD-fed WT and *Clock*$^{\Delta 19/\Delta 19}$ mutant mice as measured by insulin tolerance test (ITT) (n=8–15). Right panel: Area under curve (AUC). (D) Blood insulin levels in HFD-fed WT mice and *Clock*$^{\Delta 19/\Delta 19}$ mutant mice with Vehicle or NOB treatment (n=8–15). (E) Total triglyceride (TG) levels and cholesterol (TC) levels in blood after 10-week treatment (n=8–15). (F) Total triglyceride (TG) levels and cholesterol (TC) levels in liver after 10-week treatment (n=8–15). (G) H&E staining of whole livers from HFD-fed WT and *Clock*$^{\Delta 19/\Delta 19}$ mutant mice after 10-week treatment. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. WT.HF.NOB vs. WT.HF.Veh.

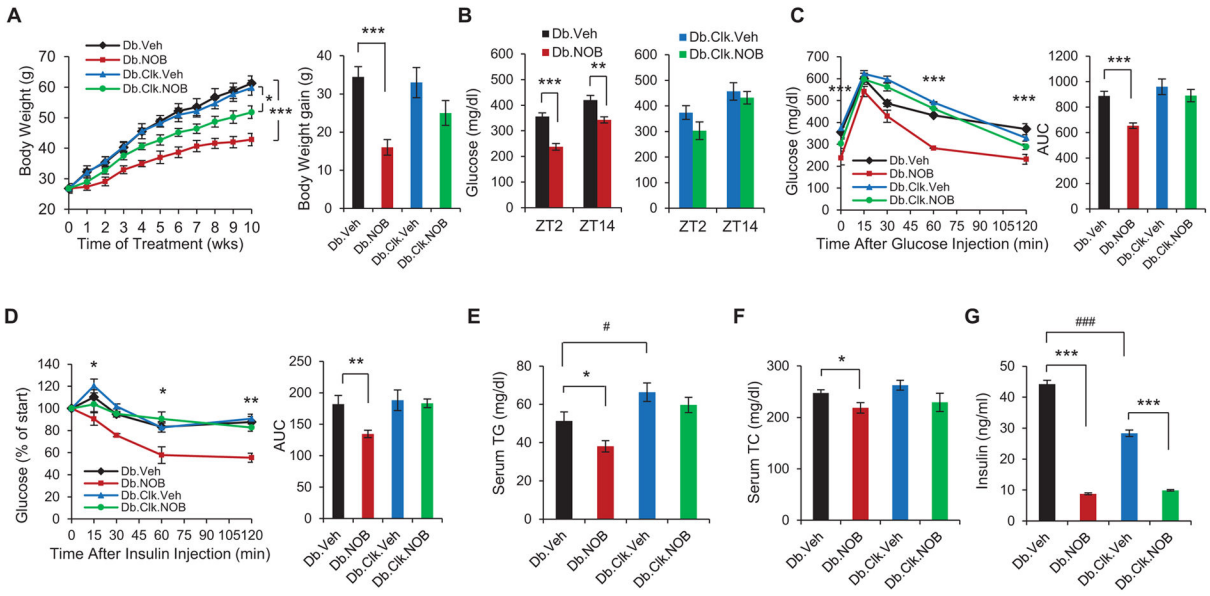

Figure 4. NOB efficacy against metabolic syndrome in Type 2 diabetic *db/db* mice requires a functional circadian clock

(A) Left panel: Average body weight of *db/db* or *db/db Clock*$^{\Delta 19/\Delta 19}$ double mutant mice fed with regular chow and treated with either vehicle or NOB (Db.Veh, Db.NOB, Db.Clk.Veh and Db.Clk.NOB) for 10 weeks (n=6–8). The mice were 6–8 weeks old at the beginning of the treatment. Right panel: Average body weight gain for these 4 groups of mice. (B) Fasting blood glucose levels in *db/db* (left) and *db/db Clock*$^{\Delta 19/\Delta 19}$ double mutant mice (right) at two opposite time points (n=6–8). (C) Effect of NOB on glucose tolerance in *db/db* and *db/db Clock*$^{\Delta 19/\Delta 19}$ double mutant mice as measured by glucose tolerance test (GTT) (n=6–8). Right panel: Area under curve (AUC). (D) Effect of NOB on insulin tolerance in *db/db* and *db/db Clock*$^{\Delta 19/\Delta 19}$ double mutant mice as measured by insulin tolerance test (ITT) (n=6–8). Right panel: Area under curve (AUC). (E and F) Blood total triglyceride (TG) levels and cholesterol (TC) levels in *db/db* and *db/db Clock*$^{\Delta 19/\Delta 19}$ mice (n=6–8). (G) Effects of NOB on circulating insulin levels in both *db/db* and *db/db Clock*$^{\Delta 19/\Delta 19}$ mice (n=6–8). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NOB vs. Veh. #$p < 0.05$ and ###$p < 0.001$. Db.Veh vs. Db.Clk.Veh.

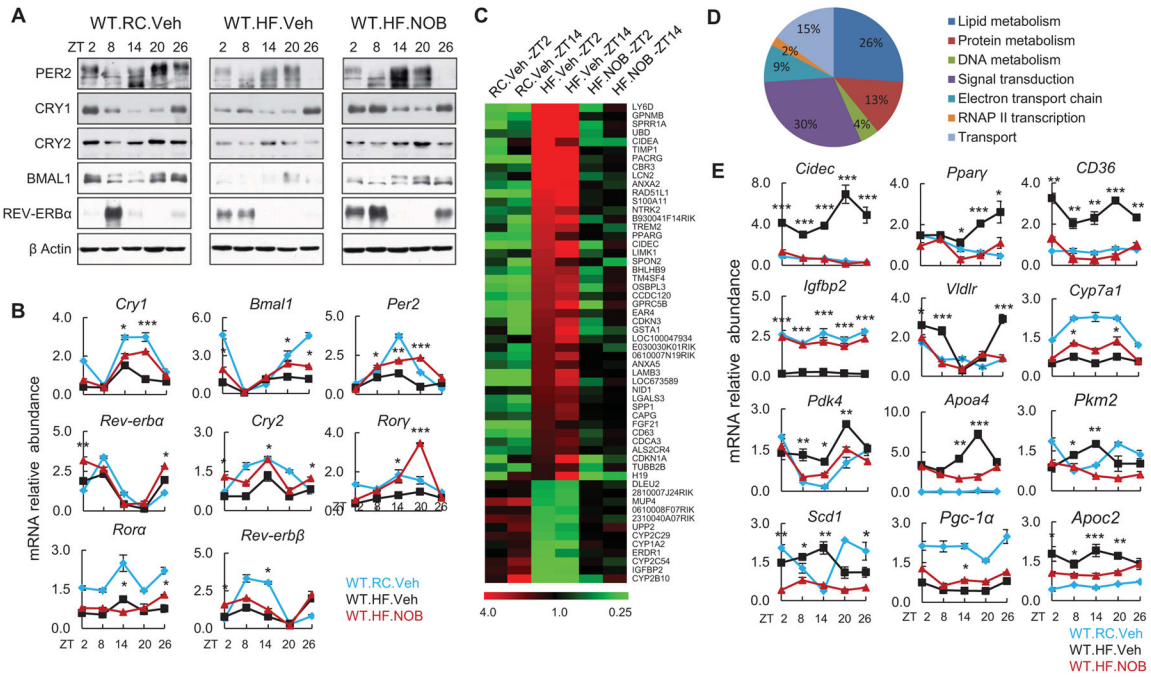

Figure 5. NOB restored circadian oscillation of clock and metabolic output genes in the liver of HFD-fed WT mice

(A and B) Liver samples were collected from HFD-fed WT mice with vehicle or NOB treatment (WT.HF.Veh and WT.HF.NOB). WT mice fed with regular chow (WT.RC.Veh) were used as controls for comparison (N=3–4). Western blotting (A) and Real-time qPCR (B) analyses were performed to determine protein and mRNA expression of clock genes. (C) Heat map of microarray gene expression data indicating that the expression patterns of 56 genes were altered by HFD, and NOB reversed, to varying degrees, their expression to approximate RC levels in WT mouse liver at both ZT2 and ZT14 time points. Color scale indicates median normalized signal intensity in relative values. (D) Functional classification of 56 genes in (C) by the Gene Ontology (GO) program. Percentages of genes sharing GO biological processes are shown. (E) Real-time qPCR analysis of mRNA expression of clock-controlled metabolic output genes in the livers from treated mice as above. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001. WT.HF.NOB vs. WT.HF.Veh.

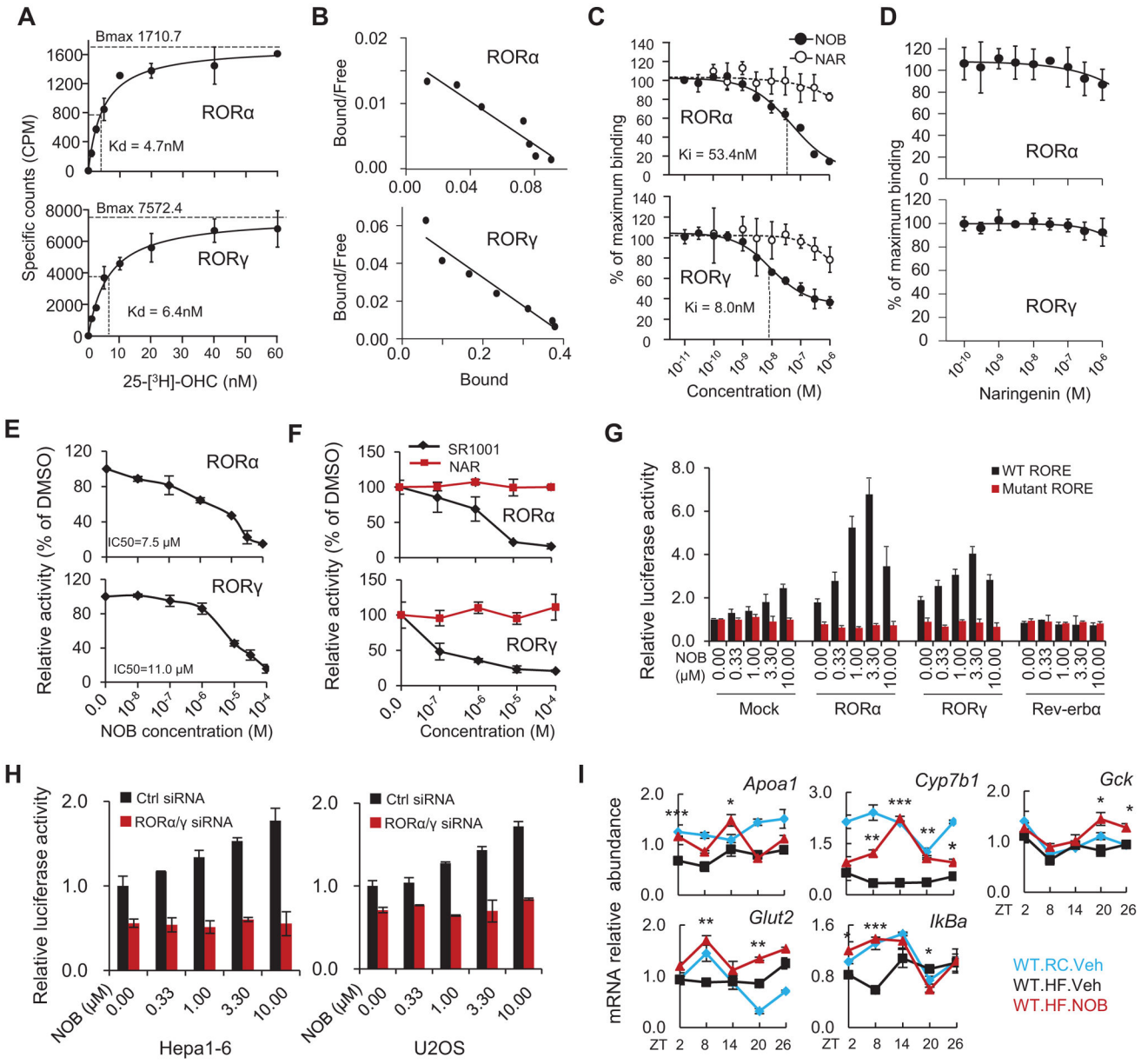

Figure 6. NOB enhanced RORα/γ transcriptional activity via direct binding to RORα/γ

(A and B) Saturation curves and Scatchard plots from filter binding assays for RORα-LBD and RORγ-LBD. (A) Saturation curves for 25-[3H]-OHC binding were generated with 100ng of RORα-LBD (top) and 200ng of RORγ-LBD (bottom) (n=3). Dissociation constant values are shown. (B) Saturation curve results in Fig. 5E were subjected to Scatchard analysis and Scatchard plots for 25-[3H]-OHC are shown for RORα-LBD (top) and RORγ-LBD (bottom) corresponding to (n=3). This analysis gave a dissociation constant (Kd) of 6.10 nM and a total number of binding sites (Bmax) of 100 fmol/mg of protein for RORα, and 6.67 nM and 410 fmol/mg of protein for RORγ. (C and D) *In vitro* competitive radio-ligand binding assay indicating the direct binding of NOB (C), but not NAR (C) or Naringenin (D), to RORα-LBD and RORγ-LBD within the indicated dose range. Inhibitory

constant values are shown. (E and F) Mammalian one-hybrid assays showing ligand interaction with ROR-LBD. HEK293T cells were cotransfected with a GAL4 reporter construct with expression vectors for GAL4 DBD-RORα LBD or GAL4 DBD-RORγ LBD. Cells were treated with varying concentrations of NOB (E) and its non-methoxylated analog NAR (F). SR1001 served as a positive control in (F). (G) NOB dose-dependently increased *Bmal1* promoter-driven luciferase reporter expression with wild-type, but not mutant, RORE in the presence of RORα or RORγ in Hepa1-6 cells. (H) Knockdown of RORα/γ expression by siRNAs abrogated NOB induction of *Bmal1* promoter-driven luciferase reporter expression in both Hepa1-6 and U2OS cells. (I) Real-time qPCR analysis of RORα/γ target genes from the same mouse liver samples as in Figure 5A. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. WT.HF.NOB vs. WT.HF.Veh.