Report

Insulin-FOXO3 Signaling Modulates Circadian Rhythms via Regulation of *Clock* Transcription

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Summary

Circadian rhythms are responsive to external and internal cues, light and metabolism being among the most important. In mammals, the light signal is sensed by the retina and transmitted to the suprachiasmatic nucleus (SCN) master clock [1], where it is integrated into the molecular oscillator via regulation of clock gene transcription. The SCN synchronizes peripheral oscillators, an effect that can be overruled by incoming metabolic signals [2]. As a consequence, peripheral oscillators can be uncoupled from the master clock when light and metabolic signals are not in phase. The signaling pathways responsible for coupling metabolic cues to the molecular clock are being rapidly uncovered [3–5]. Here we show that insulin-phosphatidylinositol 3-kinase (PI3K)-Forkhead box class O3 (FOXO3) signaling is required for circadian rhythmicity in the liver via regulation of *Clock*. Knockdown of *FoxO3* dampens circadian amplitude, an effect that is rescued by overexpression of *Clock*. Subsequently, we show binding of FOXO3 to two Daf-binding elements (DBEs) located in the *Clock* promoter area, implicating *Clock* as a transcriptional target of FOXO3. Transcriptional oscillation of both core clock and output genes in the liver of FOXO3-deficient mice is affected, indicating a disrupted hepatic circadian rhythmicity. Finally, we show that insulin, a major regulator of FOXO activity [6–9], regulates *Clock* levels in a PI3K- and FOXO3-dependent manner. Our data point to a key role of the insulin-FOXO3-*Clock* signaling pathway in the modulation of circadian rhythms.

Results

In the past few years, it has become evident that Forkhead box class O (FOXO) transcription factors are of key importance in the underlying mechanisms of aging and the regulation of metabolism [5, 8, 9]. FOXO transcription factors are known to integrate signals from different pathways and form a transcriptional endpoint of the insulin-phosphatidylinositol

3-kinase (PI3K) signal transduction pathway. In *Drosophila*, FOXO has been shown to modulate the stress sensitivity of the circadian clock [10]. Furthermore, metabolites and hormones, such as glucagon and insulin, are thought to play important roles in the dynamic interplay between metabolism and circadian rhythms [11]. In line with these observations, we hypothesized that mammalian FOXOs play a key role in the regulation of mammalian circadian rhythms upon incoming metabolic cues.

To identify a possible interplay between FOXO transcription factors and the mammalian circadian clock, we first transiently overexpressed FOXO1, FOXO3, and FOXO6 [12] in NIH 3T3 cells and analyzed the effect on circadian rhythmicity (Figure 1; see also Figure S1 available online). Overexpression of FOXO1 or FOXO3 did not affect circadian oscillation, as both amplitude and period were unchanged (Figures 1A and 1C). In contrast, overexpression of FOXO6 resulted in a considerable reduction of the amplitude (to 24% ± 5%; Figures 1A and 1C). We next analyzed the effect of transiently suppressing the expression of *FoxO* in NIH 3T3 cells (Figures 1 and S1). Small interfering RNA (siRNA) directed against *FoxO3* reduced the amplitude, whereas *FoxO1* siRNA did not disrupt the oscillation (Figures 1B and 1C). It should be noted that the oscillation in the presence of FoxO3 siRNA is irregular and that the period is variable. Interestingly, downregulation of *FoxO6* expression did not affect the amplitude, but slightly increased the period of the oscillation (Figures 1B and 1C). These results point to a differential effect of FOXO proteins on circadian rhythm modulation.

In order to further investigate the mechanism underlying the effect of FOXO3 and FOXO6 on circadian rhythms, we performed a microarray study on synchronized NIH 3T3 cells upon transient knockdown of *FoxO3* (siO3) or overexpression of *FoxO6* (oeO6). Cells were harvested for RNA isolation 24, 30, 36, and 42 hr after synchronization (Figure S1G). Gene expression analysis was performed with the Agilent two-color microarray platform and, to allow a direct comparison, all samples were hybridized (n = 4 per condition) against a common pool of mRNA ("reference"). As gene expression is not constant throughout the circadian cycle, we focused on genes that showed a significantly changed expression at least at one time point. 115 genes were differentially regulated in both arrays (Figure 2D), but among these no members of the core clock machinery were present. Gene ontology (GO) analysis of this group of genes (Figure S1I; Table S1) showed cell-cycle-related genes to form the predominant group. We further focused on the unique genes that were differentially expressed in either the FoxO6 (255 genes) or FoxO3 (1,240 genes) arrays. Remarkably, while in the FOXO6 overexpression experiment no genes belonging to the core oscillator were identified for which the expression was significantly changed (Figure 2E, left), mRNA levels of five core clock genes were altered (familywise error rate [FWER], p < 0.05) at minimally one time point after *FoxO3* knockdown (Figure 2E, right). Among the core clock genes modulated by *FoxO3* knockdown, *Clock* expression was most clearly modified, showing a significant downregulation (p < 0.05) at three time points (Figure 2E, right). Interestingly, most core clock genes
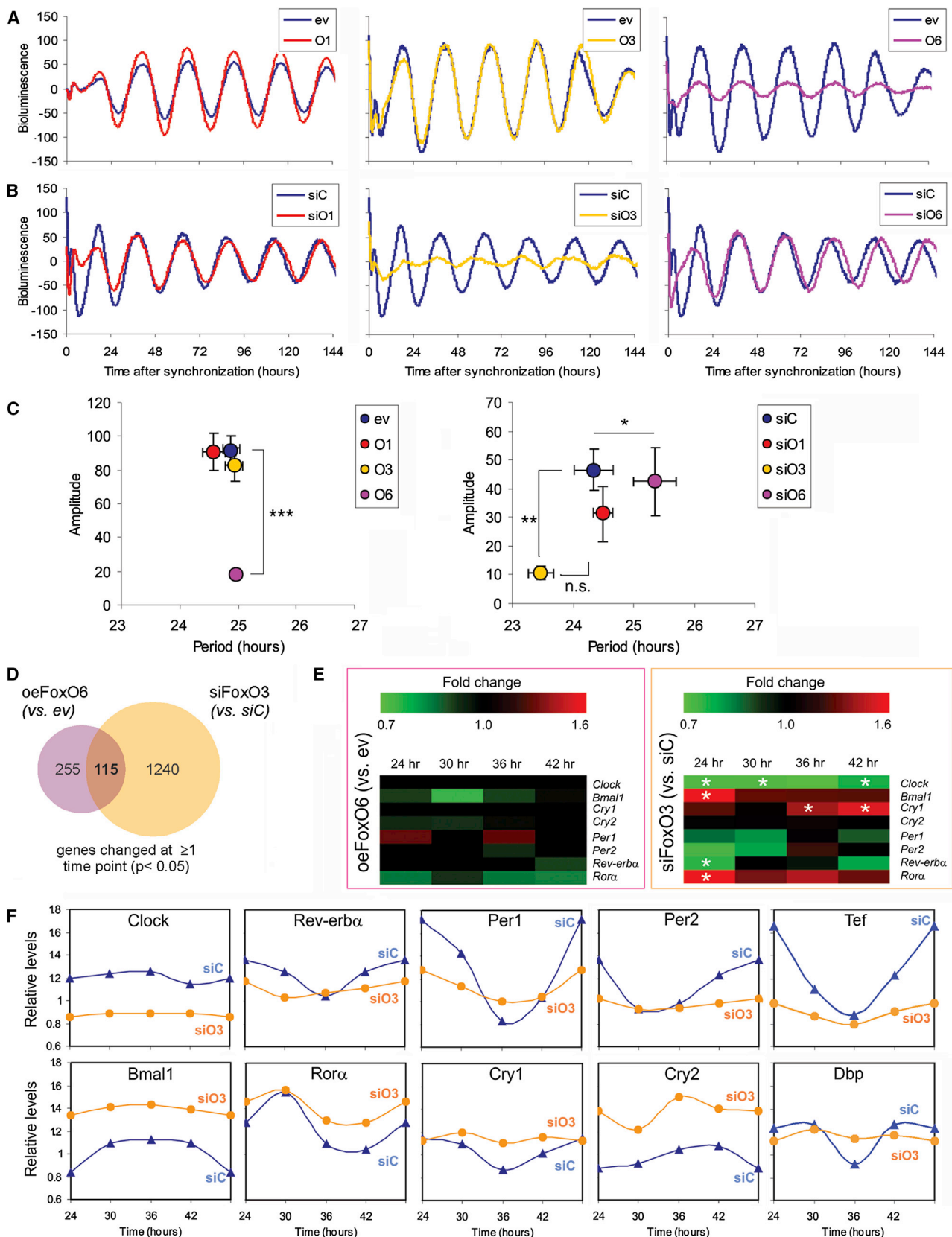
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Figure 1. Differential Effect of FOXO Transcription Factors on Circadian Oscillation of NIH 3T3 Cells

(A) Representative examples of bioluminescence rhythms in cells cotransfected with an mBmal1::luciferase reporter construct and either empty vector (blue line), FOXO1-EGFP (left, red line), FOXO3-EGFP (middle, yellow line), or FOXO6-EGFP (right, magenta line).

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show a decrease in circadian amplitude following *FoxO3* knockdown, indicating FOXO3 to be a modulator of circadian rhythmicity (Figure 2F).

The data presented clearly show a difference in FOXO3- and FOXO6-mediated regulation of circadian rhythms. Whereas FOXO3 modulates circadian oscillation via a core clock-related mechanism, FOXO6 affects rhythmicity through an alternative, as yet unknown, pathway. We further focused on the FOXO3-mediated effect on circadian rhythms. As shown, knockdown of *FoxO3* results in a strong decrease of circadian amplitude (Figure 1C), similar to the effect observed upon *Clock* knockdown in U2OS cells [13]. This prompted us to investigate the possibility that FOXO3 is modulating circadian oscillation via regulation of *Clock* transcription. We scanned the genomic context of the murine *Clock* gene for Daf-binding elements (DBEs), recognition sites of FOXO [14], and identified two perfect DBEs, the first of which was located at 2,070 nt upstream of the transcription initiation, and the second of which was situated in the second intron at 1,743 nt downstream of the transcription initiation (Figure 2A). To determine whether FOXO3 or FOXO6 is able to specifically bind these DBEs, we performed a chromatin immunoprecipitation (ChIP) assay in NIH 3T3 cells transfected with FoxO3-EGFP or FoxO6-EGFP. Crosslinked chromatin was pulled down with anti-GFP antibodies, and rabbit immunoglobulin G (IgG) was used as a negative control. The results show that only FOXO3 specifically binds to the identified DBEs within the *Clock* locus (Figures 2B and 2C), suggesting that FOXO3 can regulate *Clock* transcription. We next investigated whether the *FoxO3* knockdown phenotype could be rescued by *Clock* overexpression (Figures 2 and S2). As shown in Figures 2D and 2E, overexpression of *Clock* restores to a large extent the oscillation in cells transfected with siRNA directed against *FoxO3*, further indicating that FOXO3 regulates *Clock* transcription. This effect is specific for FOXO3, validating the idea that FOXO6 is acting via a different mechanism (Figures 2F and 2G).

Our data suggest that FOXO3 is essential for the correct regulation of circadian rhythmicity. Given the fact that FOXO3 is expressed in the suprachiasmatic nucleus (SCN), we analyzed the wheel-running behavior of *FoxO3* knockout mice [15]. The circadian behavior of *FoxO3*$^{-/-}$ is similar to that of the *FoxO3*$^{+/+}$ controls (Figures 3A and 3B), suggesting that the FOXO3 deficiency does not affect the SCN master clock. Nevertheless, FOXO3 could be involved in the regulation of circadian rhythms in peripheral clocks, such as the liver. In fact, FOXOs have been shown to be important in hepatic glucose and lipid metabolism [16]. To address this hypothesis, we followed real-time luminescence of *FoxO3*$^{-/-}$ *Per2::Luc* tissue explants [17] (Figure 3C). Circadian oscillation in *FoxO3*$^{-/-}$ and *FoxO3*$^{+/+}$ SCN slices does not differ, confirming the lack of a behavioral phenotype. Similarly, lung explants and primary fibroblasts (Figure S3A) from

FoxO3 knockout mice and control littermates show circadian oscillations with comparable amplitudes. In marked contrast, liver explants derived from FOXO3-deficient mice display a very weak (almost absent) rhythm, suggesting FOXO3 to be a modulator of hepatic circadian rhythms. We attribute the difference observed between NIH 3T3 cells and primary fibroblasts to the differences in cell type (NIH 3T3 are immortalized) and to the type of experiment (NIH 3T3 cells were transfected). The clear difference between the liver and the SCN (and other tissues) can be explained by differences in expression: FOXO3 is highly expressed in the liver, and *FoxO3* mRNA displays a robust oscillation in this organ [18, 19], whereas in the SCN FoxO3 mRNA is present at low, weakly oscillating levels [20]. To understand how the FOXO3 deficiency affects the liver circadian clock and its output in vivo, we performed gene expression analysis on liver tissue collected from *FoxO3*$^{-/-}$ mice and wild-type control littermates sacrificed at zeitgeber time (ZT) 2, 6, 10, 14, 18, and 22. Our results validate the oscillation of *FoxO3* mRNA in the liver [19] (Figure S3B) and show that the hepatic oscillator is affected in the absence of FOXO3 (Figures 3D and S3B). Oscillation of most clock genes is flattened and the oscillation of clock-controlled and output genes is altered in *FoxO3* knockout livers. Interestingly, a group of liver metabolic genes displays an oscillation with a period of 10–12 hr (statistical analysis represented in Tables S2 and S3). This suggests that the coupling with the circadian clock is lost and these genes only respond to feeding, which has been shown to display two daily peaks [21]. Although FoxO3 siRNA affects the expression and oscillation of the mBmal1::luciferase construct, expression of Bmal1 in the FoxO3-deficient liver is not largely affected. This is likely due to differences in transcriptional regulation between the overexpressed plasmid (carrying part of the promoter) and the endogenous locus. Circadian oscillation in expression of the *Clock* gene is among the most affected in the *FoxO3* knockout mouse, further confirming regulation of *Clock* transcription via FOXO3. Interestingly, circadian expression of the insulin and insulin growth factor (IGF) receptors is disrupted (Figures 3D and S3B). This result points to an incorrect insulin responsiveness in the *FoxO3* knockout livers.

Our data show that FOXO3 modulates circadian rhythms via transcriptional regulation of *Clock*, suggesting that hepatic circadian rhythms can be modulated by insulin signaling, which directly regulates activity of FOXO3 via the PI3K axis. In this scenario, insulin should have a negative effect on *Clock* transcription and decrease the level of *Clock* mRNA. To test this hypothesis, we analyzed the effect of insulin on *Clock* mRNA levels in a liver cell line (Hepa 1-6). As shown in Figure 4A, the levels of *Clock* mRNA were significantly lower in the presence of insulin as compared to mock-treated cells. Importantly, the effect of insulin on *Clock* expression could be reversed by Ly294002, an inhibitor of PI3K (Figure 4A).

(B) Representative examples of bioluminescence rhythms in cells cotransfected with a mBmal1::luciferase reporter construct and control siRNA (blue line), *FoxO1* siRNA (red line), *FoxO3* siRNA (yellow line), or *FoxO6* siRNA (magenta line).
(C) Graphical representation of the amplitude (y axis) and period length (x axis) under FOXO overexpression (left) or *FoxO* knockdown (right). Error bars represent SD. *p < 0.05, **p < 0.01, ***p < 0.001.
(D) Venn diagram showing the genes for which expression is significantly regulated (p < 0.05) at least at one time point in FOXO6 versus empty vector (magenta) and si*FoxO3* versus siControl (yellow). 115 genes overlap between the two arrays.
(E) Heatmap representation of core clock genes in the FoxO6 array (left) and FoxO3 array (right). White asterisks indicate significance (FWER, p < 0.05).
(F) Relative expression of clock-controlled genes in time (FoxO3 array). This is a different means of representing the data shown in (E). Blue line corresponds to siControl; yellow line corresponds to si*FoxO3*. The y axis represents fold change compared to the common reference. Raw bioluminescence data and expression data are shown in Figure S1.
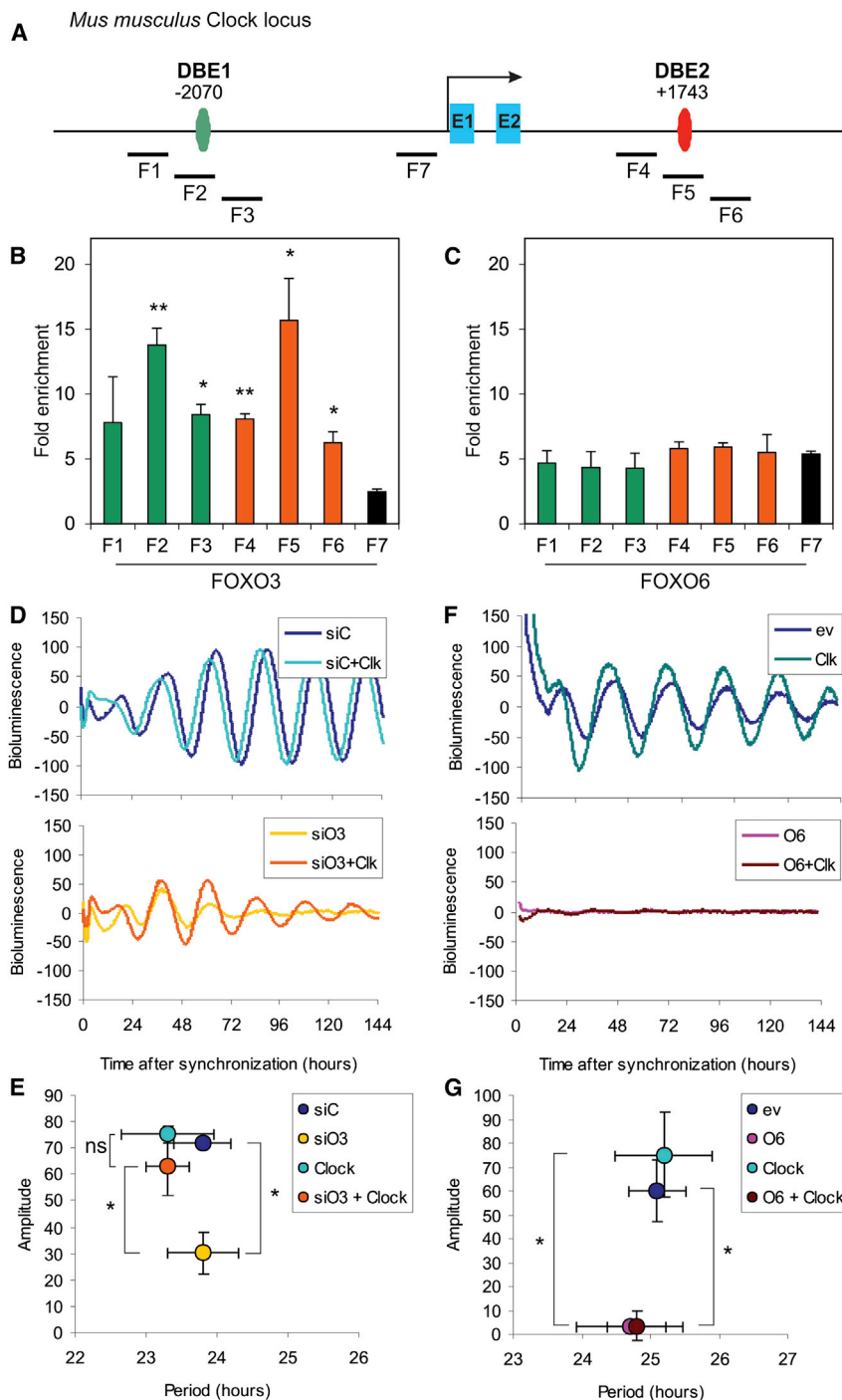
A *Mus musculus* Clock locus

Figure 2. *Clock* Can Rescue the FoxO3 siRNA Phenotype

(A) Schematic representation of part of the mouse *Clock* gene locus. The arrow represents transcription initiation. E1 and E2 are the first two exons. Ovals represent DBEs (DBE1, green; DBE2, orange). The numbers indicate the position relative to transcription initiation (base pairs).

(B and C) Graphic representation of the ChIP assay (FOXO3-EGFP in B, FOXO6-EGFP in C). Green bars, fragments around DBE1; orange bars, fragments around DBE2; black bar, non-DBE fragment. Three primer sets surrounding each DBE were used. The y axis represents relative abundance as fold enrichment (anti-GFP/IgG).

(D) Representative examples of bioluminescence rhythms in NIH 3T3 cells cotransfected with a mBmal1::luciferase reporter construct and siC + empty vector (top, dark blue), siC + Clock (top, light blue), siFoxO3 + empty vector (bottom, yellow), or siFoxO3 + Clock (bottom, orange).

(E) Graphical representation of the amplitude (y axis) and period length (x axis).

(F) Representative examples of bioluminescence rhythms in NIH 3T3 cells cotransfected with a mBmal1::luciferase reporter construct and ev + empty vector (top, dark blue), ev + Clock (top, light blue), FOXO6-EGFP + empty vector (bottom, magenta), or FOXO6-EGFP + Clock (bottom, purple).

(G) Graphical representation of the amplitude (y axis) and period length (x axis). Figure S2 shows the corresponding raw bioluminescence data and RNA quantification data.

Error bars represent SD. *p < 0.05, **p < 0.01.

show that insulin can modulate circadian oscillation via FoxO3 regulation of Clock transcription.

Discussion

The results described here demonstrate that FOXO3 regulates circadian rhythms via *Clock* in response to insulin signaling, identifying a novel transcriptional feedback loop in mammals that links insulin-like metabolic cues and circadian rhythms. As shown, FOXO3 elicits its effect via regulation of *Clock*. We postulate that the *FoxO3* knockout mouse resembles the *Clock* knockout: it is in fact a liver-specific *Clock* knockdown. Whereas circadian amplitude in wild-type animals is robust and tightly regulated, amplitude is dampened in peripheral tissues of $Clock^{-/-}$ [22, 23] and in the liver of $FoxO3^{-/-}$ animals (this study). FOXO3 is critical in the regulation of metabolic processes in the liver, such as gluconeogenesis and lipid metabolism [16, 24, 25], and we now show that FOXO3 is also necessary for regulation of circadian rhythms in this organ. As expression of both core clock and output genes in the liver of FOXO3-deficient mice is deregulated, the data indicate that FOXO3 is crucial for the correct regulation of hepatic circadian oscillations and thus for the intact functioning of the liver, specifically in response to metabolic signals. Remarkably, the
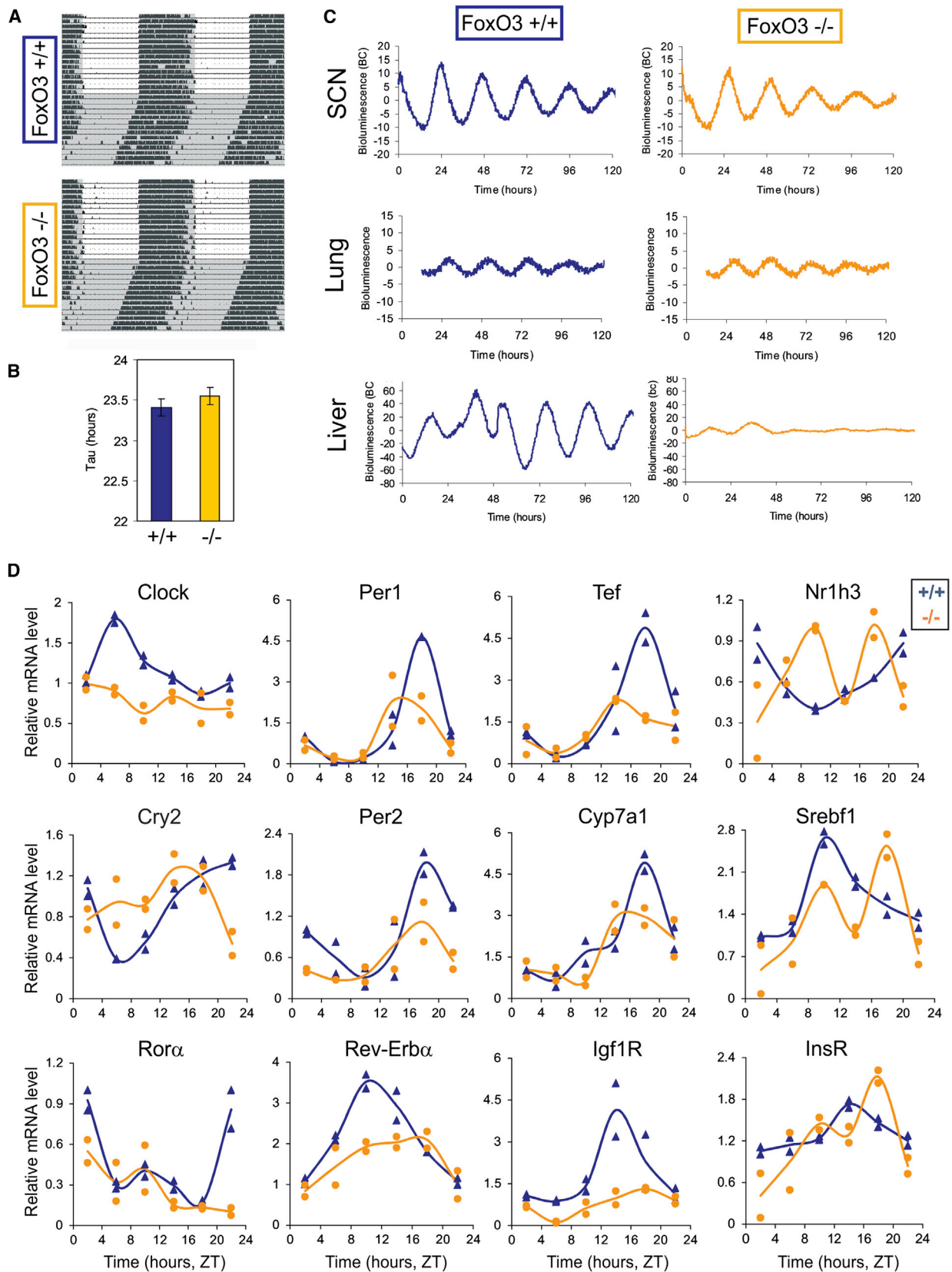
This indicates that the signaling of insulin to *Clock* transcription occurs via the PI3K pathway, which directly signals to FOXO3. Additionally, overexpression of FOXO3 abolished the effect of insulin on *Clock* expression (Figure 4B), which validates that FOXO3 is regulating *Clock* expression in response to insulin/PI3K signaling. Finally, we analyzed the effect of insulin on circadian oscillation of Hepa 1-6 cells. As shown in Figure 4C, insulin treatment reduces the amplitude of oscillation of synchronized Hepa 1-6 (similar effects were observed with the mBmal1::luciferase and the mPer2::luciferase reporter constructs, Figure S4). Together, these data

Figure 3. The Hepatic Circadian Oscillator Is Affected in *FoxO3* Knockout Mice

(A) Circadian behavior of *FoxO3* knockout mice in a Per2::Luc background (bottom, n = 16) and control wild-type littermates (top, n = 12). Animals were kept under normal light conditions (light:dark 12:12 hr) and subsequently exposed to constant darkness (DD) (indicated by gray shade). Representative examples of double-plotted actograms are shown.

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FOXO3-deficient liver is at least partially unresponsive to incoming insulin-like signals, as the expression of the insulin and IGF receptors is disrupted. Taken together, one would expect that this deregulation of hepatic circadian rhythms, as a consequence of FOXO3 deficiency, would lead to physiological effects at the whole-body level. However, Zhang and coworkers [16] observed no apparent phenotype in FOXO3-deficient animals; levels of insulin and hepatic and serum lipids were unaltered. This could be the consequence of a metabolically nonstressed situation. It is conceivable that a phenotype will be observed when FOXO3-deficient animals are metabolically stressed. As shown, insulin regulates *Clock* transcription via FOXO3. As a consequence, transcriptional deregulation of clock-controlled genes was observed in the *FoxO3*$^{-/-}$ mice (Figures 4 and S4; Tables S2 and S3). Both Nampt and Rev-erbα have been shown to form key components in molecular mechanisms linking metabolism and the circadian oscillator, indicating that various signaling pathways, including the insulin-FOXO3-Clock axis, crosstalk in response to metabolic cues [26–29].

In conclusion, the data presented here identify FOXO3 as a crucial component of hepatic circadian rhythmicity exerting its action via the core clock transcription factor *Clock*. Given that the activity of FoxO transcription factors is mainly controlled by the insulin/IGF-1 signal transduction route and that insulin decreases *Clock* expression levels in a PI3K-dependent manner, our data point to a key role of the insulin-FOXO3-*Clock* signaling pathway in conveying external metabolic cues in order to modulate circadian rhythms in the liver. It is likely that other tissues use different mechanisms to respond to the metabolic status that do not require FOXO3. We propose that a FOXO3 deficiency induces disruption of hepatic circadian rhythmicity, and as a consequence a deregulated response to metabolic, insulin-like cues. Further studies will be required to explore the possibility that the insulin-FOXO3-CLOCK axis is necessary for a correct alignment between metabolism and circadian rhythms. This is of major importance in conditions where the circadian system is continuously challenged and becomes deregulated, such as shift work or jet lag.

Experimental Procedures

All experiments involving animals were approved by DEC Consult, an independent Animal Ethical Committee (Dutch equivalent of Institutional Animal Care and Use Committee) under permit numbers EMC2026, EMC2027, EMC2382, and EMC2383. Detailed methods are provided in the Supplemental Experimental Procedures.

Real-Time Bioluminescence Monitoring
Cells were synchronized, and bioluminescence was recorded for several days with a LumiCycle 32-channel automated luminometer (Actimetrics).

Microarray Experimental Setup
RNA was isolated, purified on a column, and analyzed using a 2100 BioAnalyzer (Agilent Technologies). Experimental samples in quadruplicate were hybridized against a common pool of mRNA. Microarrays used were Mouse Whole-Genome Gene Expression Microarrays V1 (Agilent Technologies). RNA amplifications, labeling, hybridization, and data analysis were performed as described previously [30–32].

ChIP Assay
ChIP was performed using a ChIP kit from Upstate (Millipore).

Mouse Lines and Monitoring of Circadian Behavior
FoxO3 knockout mice [15] in a FVB-129-C57BL/6J mixed background and Per2::luciferase mice [17] in a C57BL/6J background were used for wheel-running behavior. The mouse strain FVB;129S6-Foxo3$^{tm1.1Rdp}$/Mmcd was obtained from the Mutant Mouse Regional Resource Center (MMRRC) and was donated to the MMRRC by Ron DePinho of the Dana-Farber Cancer Institute.

Tissue Slicing
Tissues (SCN, lungs, and liver) were processed and sliced as described previously [17, 33].

Accession Numbers

Genome-wide expression profiling data have been submitted to the NCBI Gene Expression Omnibus database under the accession number GSE41566.

Supplemental Information

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.04.018.

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(B) Graphic representation of the free-running period (τ) in constant darkness. Error bars represent SEM.
(C) Representative examples of bioluminescence rhythms in tissue explants obtained from *FoxO3* knockout mice (yellow) and control littermates (blue).
(D) Gene expression analysis (quantitative RT-PCR) of liver mRNA collected at 4 hr intervals around the day. The y axis represents relative mRNA levels relative to the first time point of the control, corrected for a housekeeping gene. Additional data are shown in Figure S3.
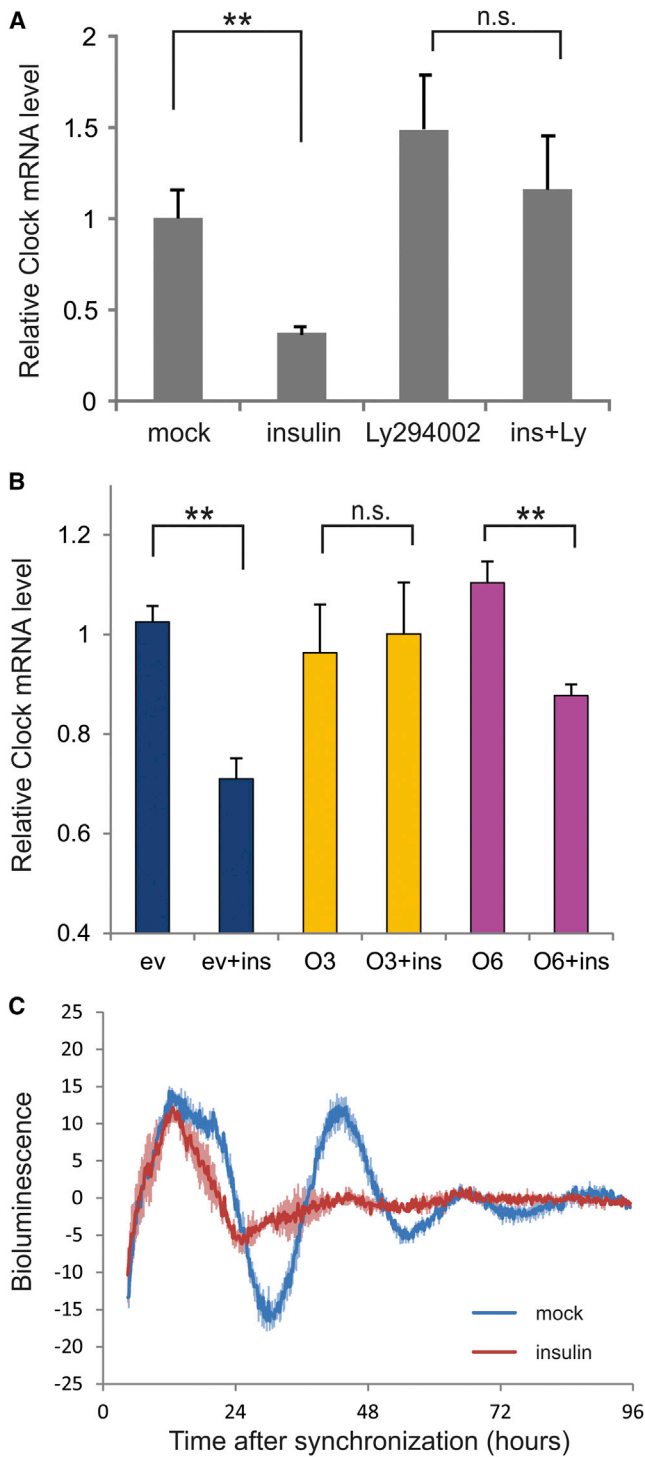
Figure 4. Insulin Modulates *Clock* Expression and Circadian Amplitude in a PI3K- and FOXO3-Dependent Manner

(A and B) Expression analysis (quantitative RT-PCR) of *Clock* mRNA in Hepa 1-6 cells, in response to insulin and PI3K signaling (A) or FOXO3 or FOXO6 overexpression and insulin (B).

(C) Representative example of bioluminescence rhythms in Hepa 1-6 cells transiently expressing the Bmal1::luciferase construct. Cells were either mock treated (blue line) or treated with 700 nM insulin (red line) 3 hr prior to synchronization with dexamethasone. The y axis represents baseline-subtracted bioluminescence. The shaded area represents SEM (light blue, light red). Figure S4 show the effect of insulin using mPer2::luc as a reporter. Error bars represent SD. **$p < 0.01$.

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