Molecular regulation of neural stem cell quiescence by FoxO transcription factors

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Chapter 6

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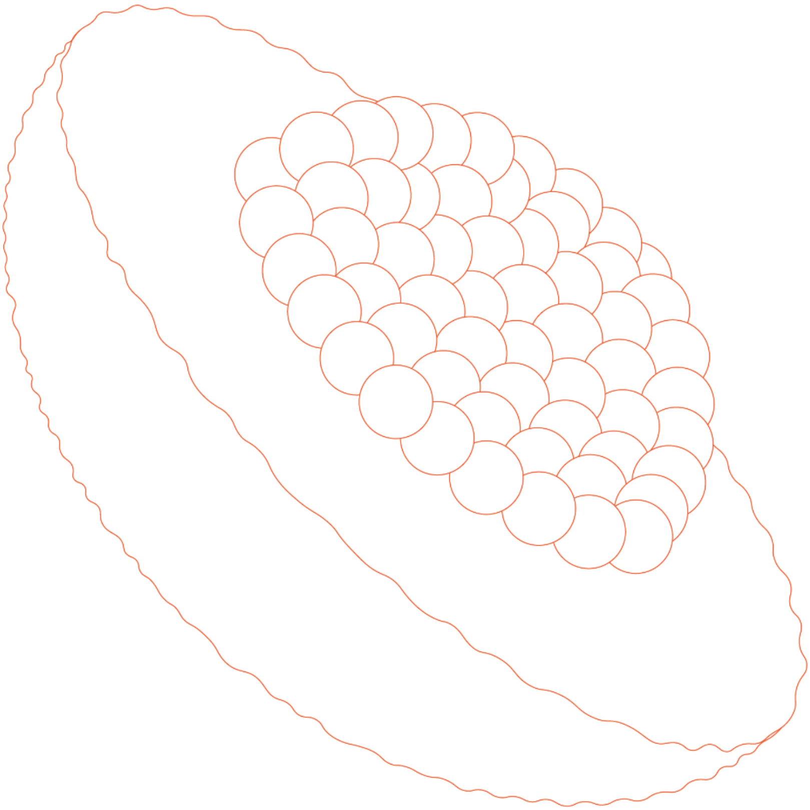

FoxO3 modulates circadian rhythms in adult neural stem cell proliferation

Swip Draijer[1], Raissa Timmerman[1], Jesse Pannekeet[1], Suci Anggriani Pakpahan[1], Alexandra van Harten[1], Aida Farshadi[2], Julius Kemmer[2], Inês Chaves[2] and Marco F.M. Hoekman[1]

[1] Swammerdam Institute of Life Sciences, University of Amsterdam, Amsterdam, the Netherlands, [2] Department of Molecular Genetics, Erasmus University Medical Center, Rotterdam, the Netherlands

Abstract

FoxO transcription factors are essential regulators of stem cell homeostasis. FoxOs act on the interface of metabolism and cell cycle regulation and are important sensors of nutrients and cellular stress. Many metabolic processes and cell cycle transitions show circadian rhythms which are generated by a cellular circadian clock. The circadian clock consists of multiple interlocked transcriptional translational feedback loops that regulate the circadian expression of a wide range of target genes. As such, the circadian clock regulates stem cell function in a range of different tissues, including that of neural stem cells in the adult hippocampus. Loss of the circadian clock disturbs circadian proliferation of neural stem cells and exhausts their number at a premature age. Importantly, FoxO3 preserves the adult neural stem cells population by regulating cell cycle and cellular metabolism. Additionally, FoxO3 regulates circadian rhythms in the liver. However, whether FoxO3 is a regulator of circadian rhythms in neural stem cells remains unknown. Here, we show that loss of FoxO3 disturbs circadian rhythmicity in neural stem cells via regulation of *Clock* expression. We then performed a meta-analysis of published data on the transcriptome and genome-wide binding sites of circadian clock components during a circadian cycle. This revealed the dynamic co-occupancy of multiple circadian clock components within FoxO3 regulatory regions. Finally, we examined proliferation in the hippocampus of FoxO3-deficient mice and found that loss of FoxO3 altered the circadian phase of hippocampal proliferation, indicating that FoxO3 is important in ensuring correct timing of NSC proliferation. Taken together, we suggest that FoxO3 is an integral part of circadian regulation of neural stem cell homeostasis. As such, FoxO3 could preserve NSC populations throughout life by modulating the circadian clock.

Key words: circadian rhythms – FoxO3 – neural stem cell – liver - metabolism – cell cycle

Introduction

The circadian clock is an evolutionary conserved mechanism present in most mammalian tissues generating oscillations in transcription during day and night (Dibner et al., 2010; Zhang et al., 2014). This is the result of the various interlocked transcription-translation feedback loops that constitute the cellular circadian clock, which span approximately 24 hours (reviewed in Buhr & Takahasi, 2013; Takahashi, 2016). Important molecular components of the circadian clock are the bHLH transcription factors CLOCK, its paralogue NPAS2, and BMAL1. The CLOCK-BMAL1 - or NPAS2-BMAL1 - complexes bind to regulatory elements containing an E-box and promote expression of target genes (so-called clock-controlled genes), including genes encoding repressors PER1, PER2, PER3, CRY1 and CRY2. The latter then repress CLOCK-BMAL1 activity, resulting in rhythmic expression. Moreover, CLOCK-BMAL1 activates expression of nuclear receptors REV-ERBα and REV-ERBβ, which repress expression of *Bmal1* and *Clock* (Buhr & Takahasi, 2013; Takahashi, 2016). The suprachiasmatic nucleus (SCN) located in the brain is the light-entrainable master regulator that synchronizes the circadian clock in all peripheral tissues (Dibner et al, 2010). As such, circadian rhythms regulate stem cell fates in many tissues, including the skin, the intestine and the brain (Brown, 2014; Janich et al., 2014; Draijer et al., 2019).

Neural stem cells (NSCs) are the progenitors of the adult brain and have the ability to generate neurons, astrocytes and oligodendrocytes or to self-renew (Taupin & Gage, 2002; Merkle & Alvarez-Buylla, 2006). NSCs reside in mainly two locations in the adult brain, the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus (Basak & Taylor, 2009). A majority of NSCs are dormant, or quiescent, existing in a state outside of the cell cycle, which minimizes accumulation of DNA damage during life, allowing for long-lived populations (Cheung & Rando, 2013; Urbán et al., 2019). Following activation, NSCs enter the cell cycle and give rise to progenitors, which then leave the cell cycle to differentiate into committed cell types. Although NSCs are able to return to quiescence (Urbán et al., 2016), activation of quiescent NSCs results in the gradual decline of NSC populations during aging (Encinas & Sierra, 2012; Ro et al., 2013). A disrupted circadian clock results in increased cell cycle entry of hippocampal NSCs, which ultimately results in exhaustion of the NSC population (reviewed in Draijer et al., 2019). For instance, loss of circadian clock components *Per2, Bmal1 or Rev-Erbα* disrupted circadian proliferation of NSCs and resulted in increased cell cycle entry of NSCs (Borgs et al., 2009; Bouchard-Cannon et al., 2013; Schnell et al., 2014).

Similarly, loss of the transcription factor FoxO3 disrupts quiescence and results in a decrease of the number of NSCs (Renault et al., 2009; Paik et al., 2009). FoxO3 is a transcription factor belonging to the FoxO family, which also includes FoxO1, FoxO4 and FoxO6, and controls expression of many genes involved in the cell cycle and metabolic regulation (Greer & Brunet, 2005; Gross et al., 2008). Moreover, FoxO3 is regulated by the energy-sensing insulin/IGF-1 signalling pathway (Rafalski & Brunet, 2011). Activation of this pathway results in phosphorylation and subsequent nuclear export of FoxO3, inhibiting FoxO3-dependent transcription (Brunet et al., 1999; Van der Heide et al., 2004). Previous studies in our lab demonstrated FoxO3 to regulate hepatic CRs by direct transcriptional control of *Clock* expression (Chaves et al., 2014). Here we show that FoxO3 modulates circadian rhythms of neural stem cells *in vitro* and *in vivo*.

Results

FoxO3 regulates circadian rhythmicity of neural stem cell cultures via *Clock* expression

In the liver, FoxO3 is an important regulator of circadian rhythmicity (Chaves et al., 2014). In order to examine whether FoxO transcription factors modulate circadian rhythms in neural stem cells, we used an *in vitro* neural stem cell model known as multipotent astrocytic stem cells (MASCs), which can be grown in monolayers (Laywell et al., 2000). In these cells, we co-transfected a mBmal1::luciferase reporter construct with either FOXO1-GFP, FOXO3-GFP or FOXO6-GFP (**Figure 1A**) or in combination with either *FoxO1* siRNA, *FoxO3* siRNA or *FoxO6* siRNA (**Figure 1B**) and measured bioluminescence over multiple days in synchronised cultures. We did not interrogate FoxO4 as its expression in the brain is very low (Hoekman et al., 2006; Schäffner et al., 2018). Overexpression of FOXO-GFP fusion constructs all reduced the amplitude of circadian oscillations in mBmal1::luciferase (**Figure 1A, 1C**). Conversely, only knockdown *FoxO3* completely abrogated circadian rhythms in mBmal1::luciferase bioluminescence (Figure 1B, 1D).

As this is in line with the effects of a loss of FoxO3 in the liver, where it directly regulates *Clock* expression (Chaves et al., 2014), we set out to determine whether this is true for neural stem cells as well. To this end, we transiently downregulated FoxO3 using siRNA in MASCs and assessed *Clock* expression 24 hour and 48 hours after synchronization with dexamethasone (**Figure 2A**). This indicated a clear and persisting downregulation of *Clock* expression for at least two days (**Figure 2B,2C**). To verify downregulation of *FoxO3* expression, we assessed its expression over the course of 24 hours. This confirmed its downregulation but also revealed a circadian oscillation of *FoxO3* expression in MASCs (**Figure 2D**).

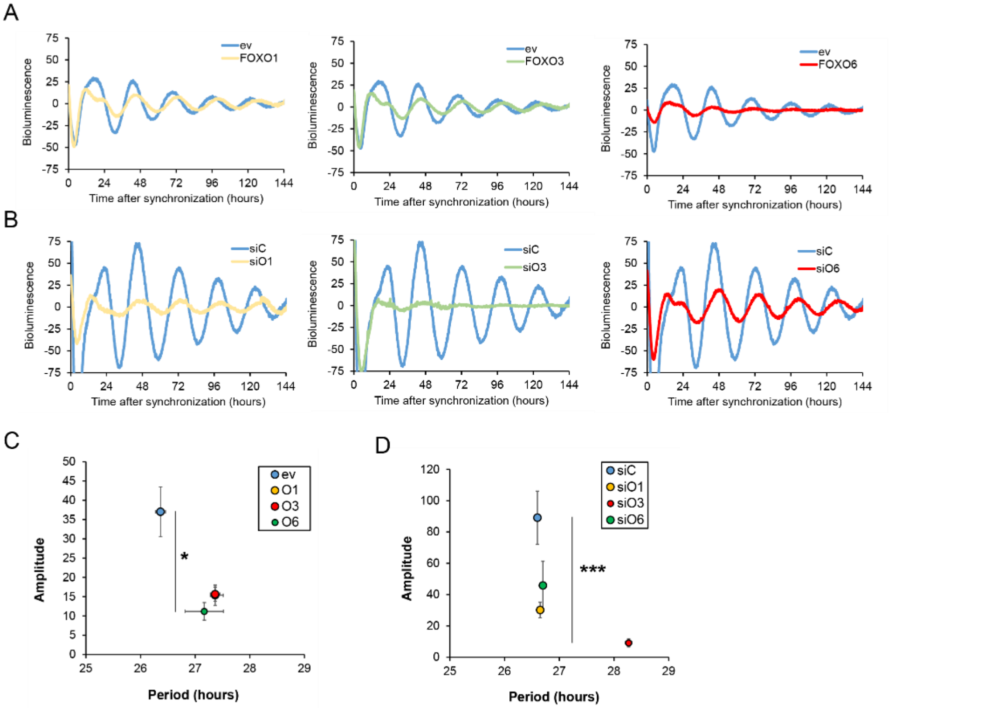

Figure 1. Differential effect of FoxO transcription factors on circadian oscillations in neural stem cells *in vitro*

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

(B) Representative examples of bioluminescence rhythms in cells co-transfected with a mBmal1::luciferase reporter construct and control siRNA (blue line), *FoxO1* siRNA (left, yellow line), *FoxO3* siRNA (middle, red line), or *FoxO6* siRNA (right, blue line).

(C) Graphical representation of the amplitude and period length under FOXO overexpression (left) or *FoxO* knockdown (right). Values are averages of independent experiments (n=3) performed in triplicate. Error bars represent SEM. One-way ANOVA with Dunnett's multiple comparison test. *p<0.05, **; p<0.01; ***, p<0.001.

To determine whether Clock expression could rescue the *FoxO3* knockdown phenotype, we co-transfected a mBmal1::luciferase reporter construct with either siRNA control and empty vector (**Figure 2D**, light blue), siRNA and pFLAG-CLOCK (**Figure 2D**, dark blue), *FoxO3* siRNA and empty vector (**Figure 2E**, red) or *FoxO3* siRNA and pFLAG-CLOCK (**Figure 2E**, orange) and assessed circadian rhythmicity over multiple days. Whereas Clock overexpression did not substantially affect circadian oscillations (**Figure 2D, 2F**), it did rescue the loss of circadian rhythmicity following knockdown of FoxO3 (**Figure 2E, 2F**).

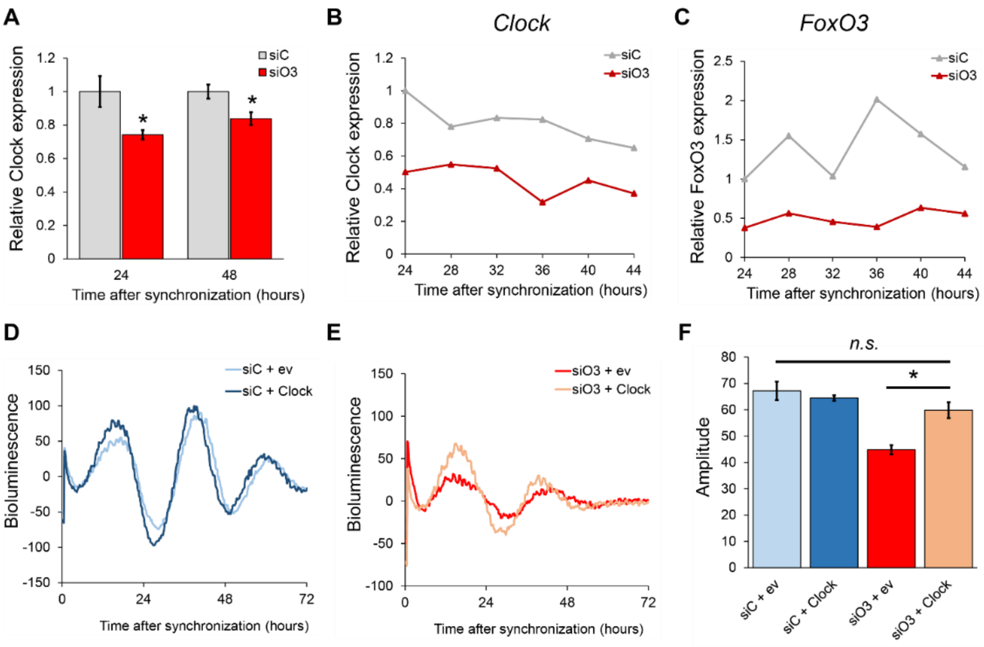

Figure 2. Clock can rescue the FoxO3 siRNA phenotype in neural stem cells

(A) Clock expression in adult neural stem cells following 24 and 48 hours after transfection of siRNA control or FoxO3 siRNA.
(B) FoxO3 expression in adult neural stem cells following siRNA control or FoxO3 siRNA during 24 hours directly following circadian synchronization with dexamethasone.
(C) Clock expression in adult neural stem cells following siRNA control or FoxO3 siRNA during 24 hours directly following circadian synchronization with dexamethasone.
(D) Representative examples of bioluminescence rhythms in cells co-transfected with a mBmal1::luciferase reporter construct and siRNA control plus empty vector (light blue), siRNA control plus pFlag-CLOCK (dark blue)
(E) Representative examples of bioluminescence rhythms in cells co-transfected with a mBmal1::luciferase reporter construct and FoxO3 siRNA plus empty vector (red) or FoxO3 siRNA plus pFlag-CLOCK (orange).
(F) Quantification of amplitudes of bioluminescence rhythms shown in (D). Values are averages of biological replicates (n=2) performed in triplicate. One-way ANOVA with Tukey's multiple comparison test. Error bars represent SD. * $p<0.05$.

FoxO3 is not required for circadian rhythmicity at the single cell level

Recording of circadian bioluminescence requires synchronization of cellular clocks within cell cultures (Nagoshi et al., 2004). As such, knockdown of *FoxO3* could disrupt circadian synchronization rather than rhythmicity itself. To investigate this, we analysed circadian rhythmicity in single cells by using NIH3T3[3C] mouse fibroblast cells expressing Rev-Erbα-VNP fusion protein as a fluorescent marker for the circadian clock (Feillet et al., 2014). We transfected these cells with *FoxO3* siRNA or siRNA control and assessed Rev-Erbα-VNP fluorescence over multiple days (**Figure 3**). Surprisingly, knockdown of FoxO3 did result in a clear reduction in fluorescence level **(Figure 3A, 3B**) but it did not disrupt circadian rhythmicity in single cells (**Figure 3A, 3C**). This suggests that circadian rhythmicity is still present in cells following FoxO3 knockdown but also indicates that FoxO3 regulates the expression of circadian clock components. Since the circadian clock and cell cycle are closely connected (Gaucher et al., 2018), and timing of proliferation of hippocampal neural stem cells is dependent of circadian rhythmicity (Renault et al) loss of FoxO3 could have consequences for the coupling of the circadian clock with the cell cycle.

To explore whether FoxO3 affects cell cycle progression we used NIH3T3[3C] cells transfected with *FoxO3* siRNA or RNA control as, next to expressing Rev-Erbα-VNP, these cells express fluorescent markers for the G1 phase (hCdt1-mKOrange fusion protein) and combined S, G2 and M phases (hGeminin-CFP fusion protein) of the cell cycle (Feillet et al., 2014). We examined cell cycle progression over multiple days and quantified the length of G1 and S/G2/M phase in *siC* (**Figure 3D-G**) and *siO3* (**Figure 3F-G**). This revealed no significant changes in G2/S/M phase or total cell cycle and only a slight trend towards an increased G1 phase length following *FoxO3* knockdown (**Figure 3G)**. FoxO3 knockdown did not disrupt coupling of the circadian clock to the cell cycle as the distribution of circadian cycle length and cell cycle length was similar between *siC* and *siO3* cells (**Figure 3E-G**). Moreover, we transfected NIH3T3[3C] cells with FoxO6 siRNA and analysed simultaneously both circadian clock and cell cycle dynamics. In line with whole cell culture recordings, knockdown of *FoxO6* did not significantly change the circadian magnitude or the circadian amplitude of oscillations in Rev-Erbα fluorescence (**Figure S1A-C**). However, loss of *FoxO6* did increase G1 phase length and reduced G2/S/M phase length while the total cell cycle length was unaffected, indicating an effect on cell cycle dynamics (**Figure S1D-G**). Together, these results indicate that FoxO3 is not required for circadian rhythmicity in single cells.

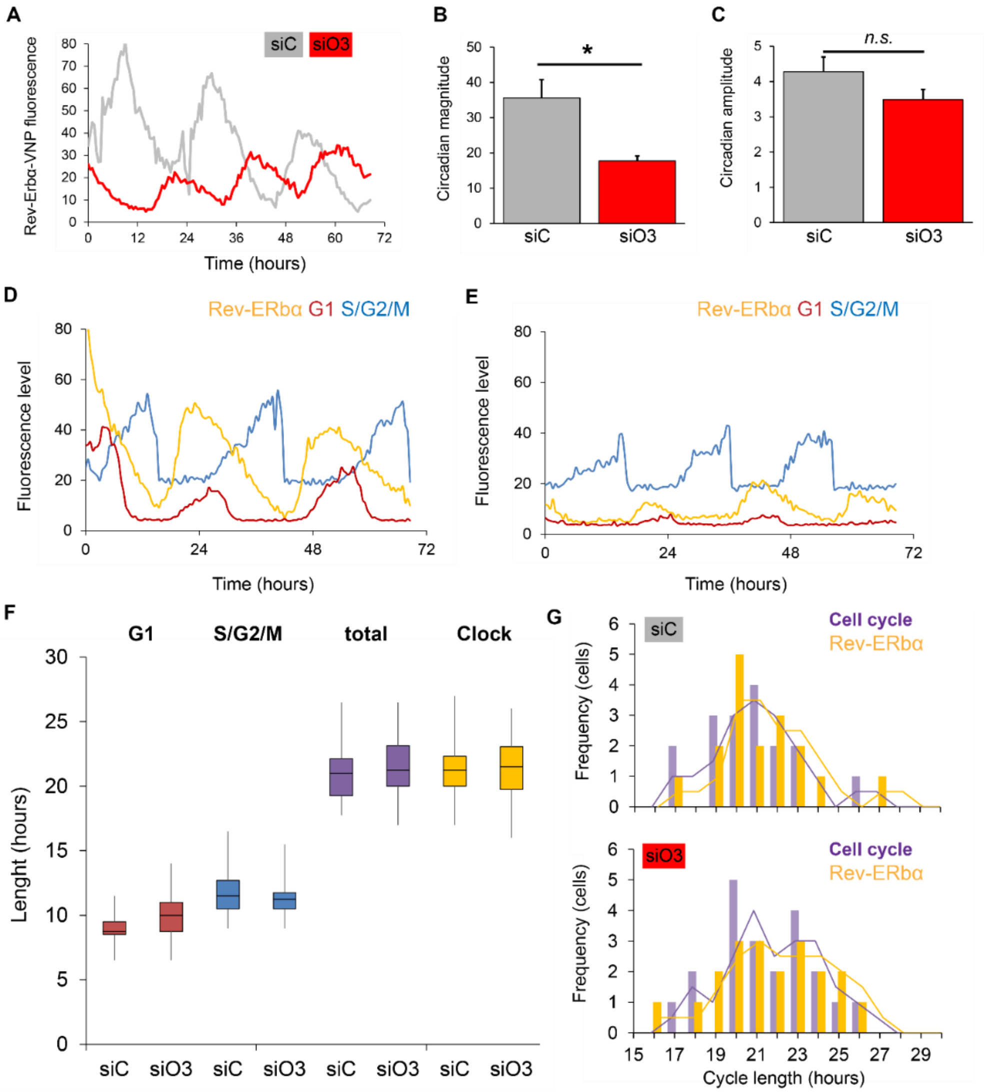

Figure 3. Loss of FoxO3 does not disrupt circadian rhythmicity in single cells

(A) Single cell recordings over multiple days of Rev-ERbα-VNP fluorescence levels in NIH3T3[3C] following transfection with *FoxO3* siRNA (siO3, orange) or control siRNA (siC, grey). Representative examples.

(B) Circadian magnitude of oscillations in Rev-ERbα-VNP fluorescence levels shown in (A). siC, n=13; siO3, n=20. Student's t-test with Bonferroni correction for multiple group comparison. *, $p<0.025$.

(C) Circadian amplitude of oscillations in Rev-ERbα-VNP fluorescence levels shown in (A). siC, n=13; siO3, n=20. Student's t-test with Bonferroni correction for multiple group comparison. Student's t-test with Bonferroni correction for multiple group comparison. n.s., not significant

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FoxO3 is a clock-controlled gene

Our finding that *FoxO3* expression showed circadian oscillations in neural stem cells is in line with previous reports of circadian *FoxO3* expression in the liver (Vollmers et al., 2012; Chaves et al., 2014). This is interesting as this suggests that FoxO3 is a clock-controlled gene. To determine whether expression of FoxO3 is directly regulated by CLOCK-BMAL1, we first searched for occupancy of circadian clock components within gene regulatory regions of all FoxOs (**Figure S2**). Since an extensive analysis of genome-wide circadian clock occupancy in neural stem cells is currently unavailable, we analysed publicly available ChIP-seq data for circadian clock components Bmal1, Clock, Per1, Per2, Npas2, Cry1 and Cry2 in the liver (Koike et al., 2012). As both circadian oscillations of *FoxO3* expression and a functional role for FoxO3 in circadian rhythms have been established in the liver (Vollmers et al., 2012; Chaves et al., 2014), this dataset is valuable in understanding circadian regulation of *FoxO3* expression.

Moreover, Koike et al. (2012) analysed circadian occupancy of transcriptional regulators p300, CBP and circadian recruitment and initiation of RNA polymerase II, assessed using 8WG16 and Ser5P antibodies respectively. This analysis revealed occupancy of circadian clock components on *FoxO1* and *FoxO3* (**Figure S2A,S2B**) but not on *FoxO4* and *FoxO6* (**Figure S2C,S2D**). Importantly, multiple regions within the *FoxO3* gene showed occupancy of circadian clock components at the same genomic positions. This was not the case for FoxO1. Since circadian clock components form regulatory complexes consisting of not only CLOCK:BMAL1 heterodimers but also large quaternary CLOCK:BMAL1/PER:CRY structures (Takahashi, 2016), co-occupancy of multiple circadian clock components is highly indicative of circadian regulation. This supports the notion that FoxO3 is directly regulated by

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(D,E) Single cell recordings of fluorescence marker expression for circadian clock oscillations (Rev-ERbα-VNP, yellow), G1 phase of the cell cycle (hCdt1-mKOrange fusion protein, red) and combined S, G2 and M phases (hGeminin-CFP fusion protein, blue) in NIH3T33C cells transfected with FoxO3 siRNA (D) or siRNA control (E). Representative examples.

(F) Comparison of the average length of cell cycle phase S, G2 and M (S/G2/M, blue), G1 (red), total cell cycle (purple) and circadian rhythm length (yellow) of data shown in (D-G). siC, n=13; siO3, n=20. Student's t-test with Bonferroni correction for multiple group comparison. n.s., not significant.

(G) Frequency histograms of circadian rhythm length (Clock, yellow) and total cell cycle length (purple) in single NIH3T3^{3C} cells transfected with *FoxO3* siRNA (bottom) or siRNA control (top). siC, n=13; siO3, n=20.

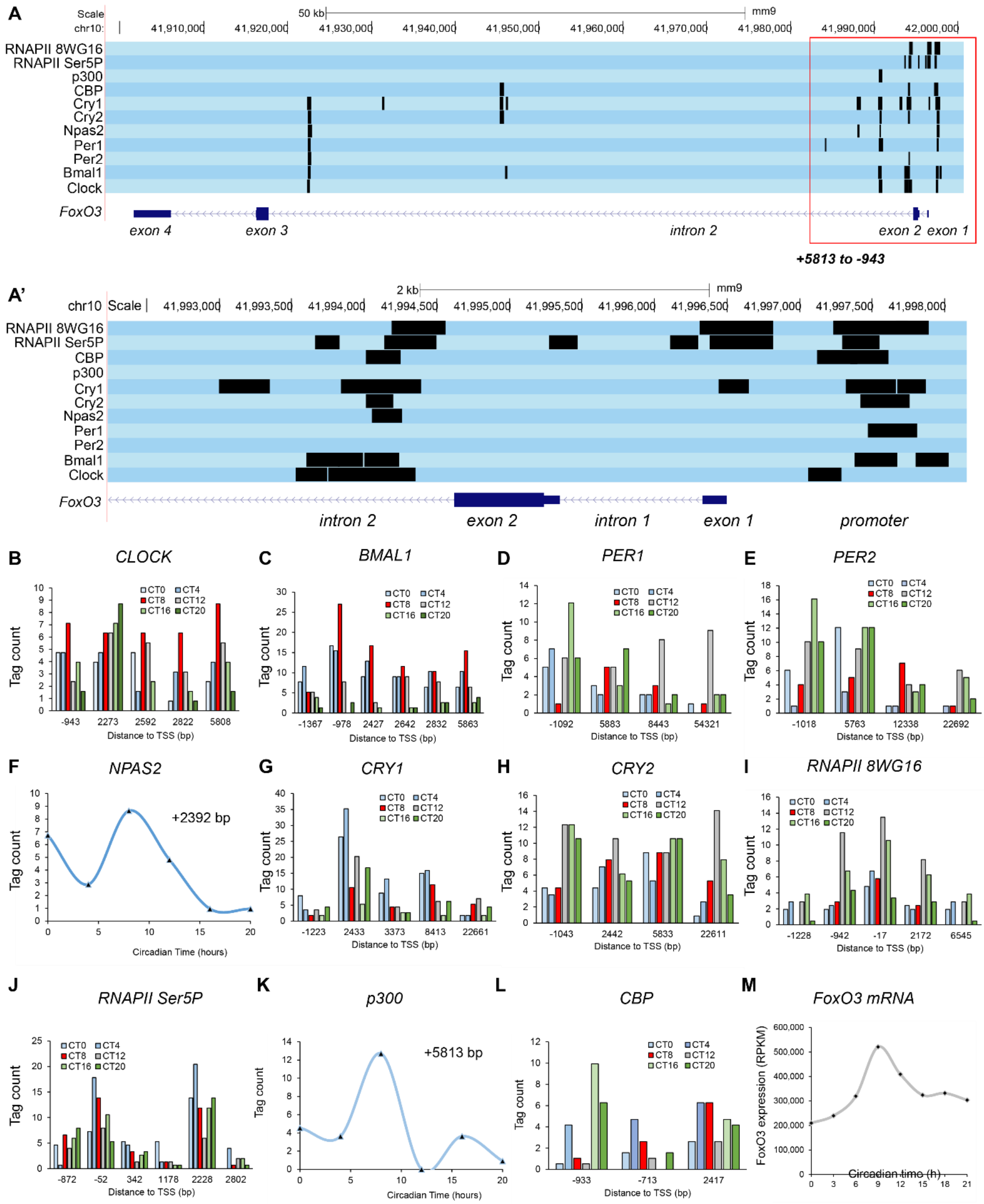

Figure 4. Circadian occupancy of *FoxO3* by circadian clock components and transcriptional regulators in the mouse liver

(A) University of California Santa Cruz (UCSC) genome browser track view of the *FoxO3* gene showing ChIP-seq binding positions for circadian clock components Clock, Bmal1, Per1, Per2, Npas2, Cry1 and Cry2 as well as transcriptional regulators CBP, p300 and recruitment and initiation of RNA polymerase II (8WG16 and Ser5P respectively).

circadian clock components, more so than other FoxO isoforms. Moreover, the co-occupancy of transcriptional activators p300 and CBP as well as the presence of RNA polymerase II at these sites indicates transcriptional regulation of the *FoxO3* gene under circadian control. Next, we examined whether circadian clock components bind to the *FoxO3* gene in a circadian fashion (**Figure 4**).

The core circadian transcriptional system involves rhythmic binding of CLOCK and BMAL1 to regulatory regions within clock-controlled genes (Takahashi et al., 2016). Similarly, we found circadian rhythmicity in the binding of many circadian clock components to multiple regions within the *FoxO3* gene, often in common gene regulatory regions such as the promoter and first introns and close to the transcriptional start site (TSS). We found many of the circadian clock components that were present at the same genomic location to bind in a rhythmic fashion. For instance, CLOCK occupancy oscillated at 943 bp upstream of the TSS (**Figure 4B**), which coincided with rhythmic BMAL1 occupancy at 978 bp upstream of the TSS (**Figure 4C**). Interestingly, both occupancy rhythms had a peak at the same time point, CT8 (**Figure 4B,4C**, red). Since BMAL1 and CLOCK form a regulatory heterodimer, this observation supports coordinated regulation of gene transcription at this site.

To examine circadian transcription, we assessed recruitment (8WG16) and initiation (Ser5P) of RNA polymerase II at the *FoxO3* gene and found it to be rhythmic over a circadian cycle (**Figure 4I, 4J**).

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(A’) Magnification of A showing occupancy of circadian clock components within key regulatory regions of the *FoxO3* gene
(B) Clock occupancy on multiple regions within the *FoxO3* gene during 24 hours
(C) Bmal1 occupancy on multiple regions within the *FoxO3* gene during 24 hours
(D) Per1 occupancy on multiple regions within the *FoxO3* gene during 24 hours
(E) Per2 occupancy on multiple regions within the *FoxO3* gene during 24 hours
(F) Npas2 occupancy on a single region within the *FoxO3* gene during 24 hours
(G) Cry1 occupancy on multiple regions within the *FoxO3* gene during 24 hours
(H) Cry2 occupancy on multiple regions within the *FoxO3* gene during 24 hours
(I) RNA polymerase II 8WG16 (recruitment) occupancy on multiple regions within the *FoxO3* gene during 24 hours
(J) RNA polymerase II Ser5P (initiation) occupancy on multiple regions within the *FoxO3* gene during 24 hours
(K) p300 occupancy on a single region within the *FoxO3* gene during 24 hours
(L) CBP occupancy on multiple regions within the *FoxO3* gene during 24 hours
(M) FoxO3 mRNA expression during 24 hours
Data in (A) – (L) is from Koike et al. (2012). Data in (M) is from Vollmers et al. (2012).

Interestingly, RNA polymerase II was rhythmically recruited to and initiated at sites, respectively 942 bp and 872 upstream of the TSS, overlapping with the putative CLOCK:BMAL1 binding site, which is indicative of transcriptional co-regulation. We also examined whether binding of transcriptional co-activators CBP and p300 was adhering to a circadian rhythm and found multiple regions for CBP and one for p300 where occupancy was rhythmic (**Figure 4K,4L**). This coincided with circadian *FoxO3* mRNA expression in the liver (**Figure 4M**; Vollmers et al., 2012).

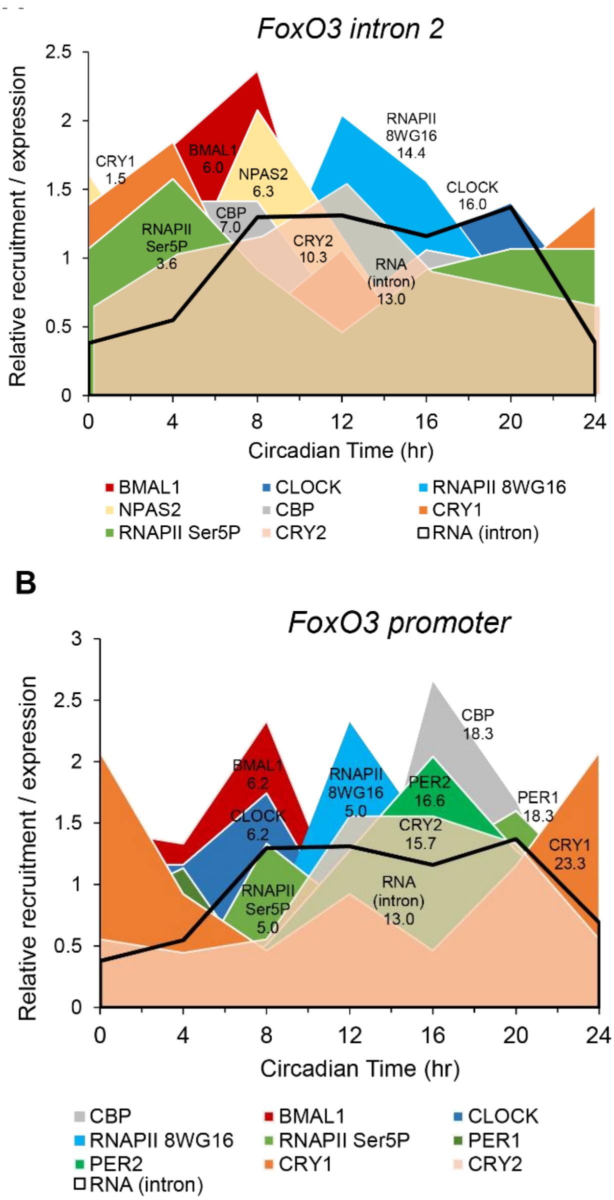

Figure 5. Circadian landscape of the FoxO3 gene

(A) Circadian landscape of the second intron within the *FoxO3* gene showing phase distributions of Bmal1, Clock, Per1, Per2, Npas2, Cry1 and Cry2, CBP, p300 and RNAPII recruitment and initiation. The mean circadian phase of peak binding is indicated under the name. The black line denotes circadian expression of *FoxO3* mRNA.

(B) Circadian landscape of the second intron within the *FoxO3* gene showing phase distributions of Bmal1, Clock, Per1, Per2, Npas2, Cry1 and Cry2, CBP, p300 and RNAPII recruitment and initiation. The mean circadian phase of peak binding is indicated under the name. The black line denotes circadian expression of *FoxO3* mRNA.

Interestingly, this correlated with dynamic histone modifications H3K9ac, H3K27ac, H3K4me3, which are associated with circadian transcription (Masri & Sassone-Corsi, 2013) within the *FoxO3* promoter regions (**Figure S2A**, Vollmers et al., 2012). Together, these results demonstrate that circadian clock components not only bind to the *FoxO3* gene but also do this rhythmically during a circadian cycle. Moreover, multiple circadian clock components as well as transcriptional activators p300 and CBP share rhythmic binding at the same genomic locations and this circadian occupancy coincides with rhythmic recruitment and initiation of RNA polymerase II. As this suggests coordinated regulation by multiple circadian clock components, transcriptional activators and RNA polymerase II, we explored the circadian landscape of the *FoxO3* gene in a publically available dataset of the mouse liver in further detail (Koike et al., 2012) (**Figure 5)**.

We examined two apparent gene regulatory sites: the second intron (**Figure 5A,5B**) and the promoter (**Figure 5A,5C**). Importantly, the circadian phase distributions of circadian clock components and transcriptional regulators occupying the *FoxO3* gene closely followed the genome-wide distribution of these components in a circadian cycle reported by Koike et al. (2012). This circadian genomic landscape is characterized by, first, a transcriptionally poised state with CLOCK:BMAL1 and NPAS2:BMAL1 occupying, respectively, the FoxO3 promoter and second intron while still being bound to CRY1. As occupancy of CRY1 declines, CLOCK:BMAL1 and NPAS2:BMAL1 are activated and coactivators CBP and p300 are recruited, respectively to the FoxO3 promoter to the second intron. This is followed by nascent transcription of FoxO3 mRNA, which peaks around the same time of day as the genome-wide de novo transcription in the liver at CT15 (Koike et al., 20120). Finally, a repressed transcriptional state is created when CLOCK:BMAL1 and NPAS2:BMAL1 occupancy decline and the occupancy of repressors PER1, PER2 and CRY2 occupancy increases. Together, these results suggest that *FoxO3* is a clock-controlled gene, directly regulated by CLOCK-BMAL1.

Whether FoxO3 activity is regulated by the circadian clock, however, remains unknown. Nevertheless, Robles et al. (2017) found rhythmic phosphorylation of FoxO3 serine-283 in the liver (**Figure S2B**). This amino acid is phosphorylated by ERK/p38 signalling, promoting inhibition and degradation of FOXO3 (Wang et al., 2017). As such, this indicates that the circadian clock could regulate FoxO3 activity through both expression and protein stability.

Surprisingly, we found DBEs close to the binding site of the circadian clock components within the FoxO3 regulatory regions (**Figure S2C**).

As this suggests that FoxO3 regulates its own expression in unison with circadian clock components, we examined a publically available dataset of a FoxO3 ChIP-seq in neural stem cells (Webb et al., 2013). Although this is only data of one time point, this revealed a surprising peak of FoxO3 occupancy in the *FoxO3* promoter, close to the genomic location where circadian clock components, CBP and RNAPII bind. Moreover, the summit of the FOXO3 occupancy peak perfectly aligned with a DBE at that location. Interestingly, we also identified FoxO3 occupancy in the promoter region of *Nr1d1*, the gene encoding for Rev-Erbα and a target of CLOCK:BMAL1, close to binding sites of circadian clock components (**Figure S2D**). Together, these results indicate a complex gene regulatory network involving FoxO3 and core circadian clock components in interlocking transcriptional translational feedback loops.

FoxO3 modulates circadian proliferation of hippocampal neural stem cells

The circadian clock is an important regulator of NSC proliferation (Bouchard-Cannon et al, 2012, reviewed in Draijer et al., 2019). As we found FoxO3 to regulate circadian rhythms of neural stem cells *in vitro*, we set out to determine its function in circadian control of NSC proliferation *in vivo*. To this end, we isolated brains of 6-month old FoxO3$^{-/-}$ and FoxO3$^{+/+}$ mice (Hosaka et al., 2004) every four hours during a circadian cycle. We then assessed circadian NSC proliferation by determining the number of Ki67-positive cells in the subgranular zone of the dentate gyrus in the hippocampi of FoxO3$^{-/-}$ and FoxO3$^{+/+}$ mice at each time point (**Figure 6A,6B**). This revealed a clear circadian rhythm in proliferation of SGZ NSCs in FoxO3$^{+/+}$ mice peaking at the end of the light phase (~ZT11, ANOVA: F=17.45, $p<0.001$; CIRCWAVE: $r^2 = 0.85$, $p<0.0001$, **Figure 6C,D**). This is in line with a previously reported circadian proliferation of SGZ NSCs in wild-type mice, which peaked at ZT12-15 (Bouchard-Cannon et al., 2012).

The small difference in peak time could be due to the use of BrdU by Bouchard-Cannon and colleagues as a proliferation marker rather than Ki67. As BrdU needs time to be incorporated into the DNA of dividing cells before it can be detected as a proliferation marker (Taupin, 2007), a delay is expected.

In FoxO3$^{-/-}$ mice, a circadian oscillation in NSC proliferation was also present but peaking at the early dark phase (~ZT14, ANOVA: F=4.58, $p<0.05$; CIRCWAVE, r^2 = 0.72, $p<0.01$; **Figure 6C,D**). As such, the circadian oscillation in proliferation of FoxO3-deficient NSCs was phase-delayed by almost three-hours compared to the circadian rhythm in proliferation exhibited by wild-type NSCs. Also, the total proliferation over 24 hours was clearly reduced in FoxO3$^{-/-}$ mice compared to FoxO3$^{+/+}$ controls (**Figure 6C**). This was expected as FoxO3$^{-/-}$ mice show a reduced number of SGZ NSCs at 6-months old (Renault et al., 2009). These results suggest that FoxO3 is a modulator of circadian dynamics in NSC fates and could be necessary for the correct timing of hippocampal proliferation.

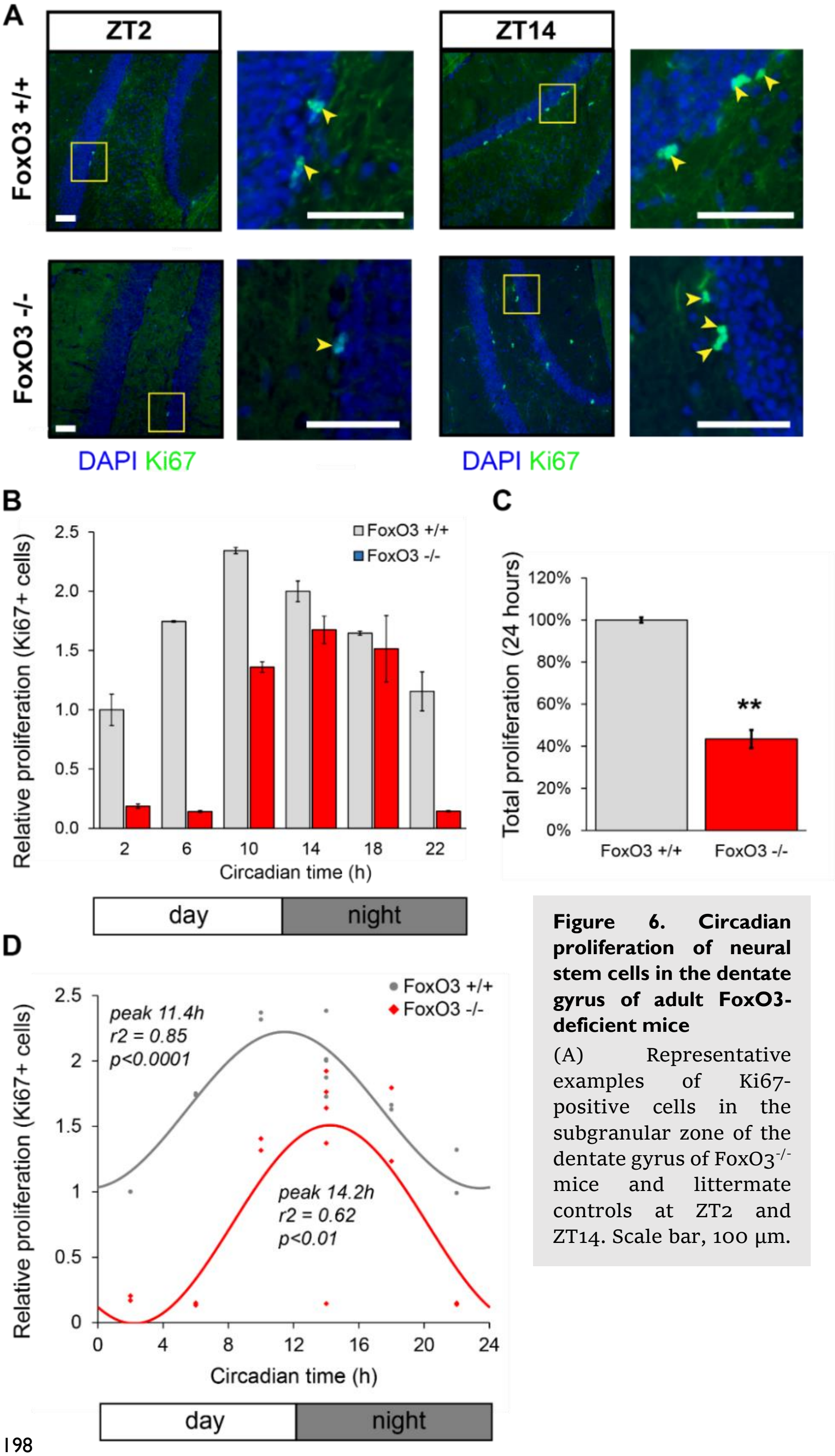

Figure 6. Circadian proliferation of neural stem cells in the dentate gyrus of adult FoxO3-deficient mice

(A) Representative examples of Ki67-positive cells in the subgranular zone of the dentate gyrus of FoxO3$^{-/-}$ mice and littermate controls at ZT2 and ZT14. Scale bar, 100 µm.

Discussion

Neural stem cell maintenance is required for lifelong neurogenesis and governed by circadian rhythms (Obernier et al., 2018; Draijer et al., 2019). FoxO3 is an important regulator of both neural stem cell fates (Renault et al., 2009; Paik et al., 2009) as well as circadian rhythms in the liver (Chaves et al., 2014). Here, we show that loss of *FoxO3* disrupts circadian rhythms in neural stem cell cultures. Moreover, FoxO3 knockdown led to a clear downregulation of *Clock* expression, regardless of circadian time. *Clock* expression, in turn, could partially rescue this phenotype. These findings are similar to the effects of a loss of *FoxO3* in liver cells (Chaves et al., 2014), suggesting a universal function of FoxO3 in circadian regulation across different cell types. To determine whether FoxO3 is required for circadian rhythmicity or for circadian synchronization within cultured cells – loss of either could explain the disruption of circadian rhythms in bulk recordings - we examined circadian rhythmicity in single NIH3T3^{3C} cells containing a Rev-Erbα-VNP fluorescence marker (Feillet et al., 2014). Surprisingly, we still observed circadian rhythms in cells with *FoxO3* siRNA, with no significant changes in amplitude. This demonstrates that FoxO3 is not required for circadian rhythmicity but likely disrupts synchronization between different cells, effectively eliminating circadian oscillations in a bulk recording.

Importantly, dexamethasone, used here to synchronize circadian rhythms in NSCs, is a glucocorticoid that regulates *FoxO3* expression via glucocorticoid response elements within the FoxO3 promoter (Lützner et al., 2012). Whereas ablation of FoxO3 disrupts circadian rhythmicity of NIH3T3 cells synchronized with forskolin as well (Chaves et al., 2014), forskolin-dependent synchronization relies on increased cAMP levels (O'Neill et al., 2008). Increased cAMP levels, in turn, activate AMPK-FoxO3 signalling thereby

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(B) Proliferation rate of neural stem cells in the dentate gyrus of 6-month old FoxO3$^{+/+}$ (grey) and FoxO3$^{-/-}$ mice (blue) over a 24 hour cycle. Values are averages per genotype (n=2-3) and normalized to circadien time (CT2) of controls. Error bars represent SEM.
(C) Data in (B) shown as total proliferation over 24 hours in 6-month old FoxO3$^{+/+}$ and FoxO3$^{-/-}$ mice. Values are average sum of all time points per genotype (n=2). Error bars represent SEM. Student t-test. **, p<0.01.
CT, circadian time.
(D) Data in (B) fitted to a linear harmonic regression model to determine circadian rhythmicity (CircWave).

promoting FoxO3 gene expression and transcriptional activity (Lützner et al., 2012; Herzig & Shaw, 2018). Since both glucocorticoid and cAMP signalling are important for synchronization of circadian oscillators (Dickmeis et al., 2007; O'Neill et al., 2008), FoxO3 could be a key downstream effector of these pathways. Interestingly, circadian glucocorticoid oscillations help to preserve quiescent NSCs in the adult hippocampus (Schouten et al., 2020). As such, FoxO3-dependent quiescence of NSCs could be modulated by glucocorticoid signalling.

Interestingly, loss of FoxO3 clearly reduced Rev-Erbα-VNP fluorescence levels in single $NIH3T3^{3C}$ cells. This is in line with downregulation of *Rev-Erbα (Nrdr1)* expression following FoxO3 knockdown in NIH3T3 cells (Chaves et al., 2014). This could be the result of reduced *Clock* expression, as CLOCK-BMAL1 regulates *Rev-Erbα* expression (Buhr & Takahasi, 2013; Takahashi, 2016). However, this could also be a direct transcriptional regulation by FoxO3 as we identified FoxO3 binding sites in the promoter region of *Rev-Erbα* in NSCs (Webb et al., 2013). Together, these findings suggest that FoxO3, although not required for circadian rhythmicity, does affect the circadian clock by transcriptionally regulating the expression of various parts of the circadian clock machinery, including *Clock*, *Nrdr1* and *Bmal1*.

This is interesting as we found various circadian clock components binding to the sites within the *FoxO3* promoter and its second intron in publicly available ChIP-seq data in the liver (Koike et al., 2012). Not only did circadian clock components bind to *FoxO3*, their binding sites were multiple and overlapping, suggesting the presence of CLOCK-BMAL1 and CLOCK:BMAL1/PER:CRY regulatory complexes (CLOCK-BMAL1-Cry Buhr & Takahasi, 2013; Takahashi, 2016). Importantly, the occupancy of these circadian clock components was dynamic and followed a circadian rhythm with phase-distributions of activators and repressors closely resembling their genome-wide circadian landscape (Koike et al., 2012). Whereas CLOCK and BMAL1 bound to the *FoxO3* promoter region and peaked in their circadian occupancy at the same time, this was not the case for the second intron where BMAL1 and CLOCK occupancy peaked at different times of the day. In the second intron, however, BMAL1 and NPAS2 shared circadian occupancy at the same time of day. Since NPAS2 is the preferred heterodimeric partner of BMAL1 in the forebrain (Reick et al., 2001), it would be interesting to examine circadian regulation of *FoxO3* expression in different cell types.

Interestingly, we discovered that binding of FoxO3 to its own promoter in NSCs (Webb et al., 2013) was close to binding sites of circadian clock components in the liver (Koike et al., 2012). Since FoxO3 is able to regulate its own expression in a positive autoregulatory feedback loop (Lützner et al., 2012; Kannike et al., 2014), it would be interesting to determine whether circadian clock components could act as co-factors to modulate FoxO3-dependent autoregulation, perhaps with FoxO3 overriding circadian clock-dependent regulation in conditions of cellular stress.

Apart from regulation of *FoxO3* expression, FoxO3 is likely to be regulated by the circadian clock on a post-translational level as well. For instance, FoxO3 is rhythmically phosphorylated at serine-283 (Robles et al., 2017). As this amino acid is phosphorylated by ERK/p38 signalling, promoting inhibition and degradation of FOXO3 (Wang et al., 2017), this could result in circadian control over FoxO3 activity and protein stability. Conversely, deacetylation of FoxO3 by Sirt1 promotes FoxO3-dependent cell cycle arrest (Brunet et al., 2004). As Sirt1 activity is circadian and regulates circadian clock gene expression (Asher et al., 2008; Nakahata et al., 2008), it seems likely that SIRT1 could deacetylate FOXO3 in a circadian fashion. Therefore, FoxO3 activity is likely regulated via both circadian clock-dependent transcription and circadian post-translational modifications.

As FoxO3 regulates NSC proliferation (Renault et al., 2009), which is controlled by the circadian clock (reviewed in Draijer et al., 2019), we investigated the consequences of a loss of FoxO3 on circadian rhythmicity in NSC proliferation. Although FoxO3-deficient NSC proliferation adhered to a circadian rhythm, this oscillation was, surprisingly, phase-delayed compared to wild-type controls. This indicates that FoxO3 is not required for circadian rhythmicity of NSC proliferation in contrast to mutant mice lacking Bmal1, Per2 and Reverbα (Bouchard-Cannon et al., 2013; Borgs et al., 2009; Schnell et al., 2014). Instead, FoxO3 appears to coordinate timing of proliferation, likely by controlling cell cycle entry and exit. Future studies should employ markers of proliferation and stem cell identity in combination with pulse-chase experiments using thymidine analogues to fully interrogate the cell cycle dynamics of FoxO3-deficient mice.

In line with the above, it would be interesting to determine the cellular consequences of the phase-shift in circadian hippocampal proliferation. Since FoxO3$^{-/-}$ mice exhibit circadian behaviour as well as circadian rhythmicity in the SCN, FoxO3-deficient NSCs are likely in circadian desynchrony with peripheral tissues. Circadian regulation of cell division is thought to segregate DNA replication away from periods of high metabolic activity as these processes produce mutation-causing free radicals (Chen & McKnight, 2007; Janich et al., 2014). Circadian disruption, characteristic of shift work and sleep disruption, is associated with metabolic pathologies and cancer (Bass & Takahashi, 2010; Sahar & Sassone-Corsi, 2009). Since we found the peak in FoxO3-deficient NSC proliferation to be delayed, it is conceivable that FoxO3-deficient NSCs are exposed to increased levels of oxidative stress, possibly resulting in cell death, senescence, stem cell dysfunction (Le or an accumulation of mutations in their progeny. For SVZ NSCs, such an increase in mutations could increase the frequency of brain tumours as they have been identified as a cell of origin of brain cancer (Altmann et al., 2019).

Whereas loss of FoxO3 did not abolish circadian rhythmicity in NSC proliferation, this is likely to be not fully reflective of circadian rhythmicity in other cellular processes. Considering both the extensive crosstalk between the circadian clock and metabolism (Asher & Schibler, 2011; Gaucher et al., 2018) and the key roles of FoxO3 in NSC metabolism (Rafalski et al., 2013), FoxO3-deficient NSCs could have an increased vulnerability for circadian or metabolic challenges. This is especially relevant to our study as FoxO3 wild type and mutant mice were housed under 12 hour light/12 hour dark conditions and were fed *ad libitum*, which allows for light-induced synchronization of peripheral oscillators and circadian behaviour (Dibner et al., 2010).

Circadian function is also strongly affected by aging (Benitah & Welz, 2020). For instance, the circadian transcriptome has been found to change during aging, in the liver and in epidermal and muscle stem cells (Sato et al., 2017). FoxO3 is a key regulator of aging (Salih & Brunet, 2012) and its targets change with age (Webb et al., 2016), which could be necessary to modulate circadian function throughout life. Interestingly, aging results in desynchrony of SCN neurons (Farajnia et al., 2012) and reduces the robustness of synchronizing cues to peripheral tissues (Benitah & Welz, 2020). The loss of circadian rhythmicity in NSC cultures but not in single cells following knockdown of *FoxO3* indicates a loss of synchrony. It is, therefore, conceivable that FoxO3 acts as a regulator of circadian synchronization during aging to promote cellular homeostasis in response to circadian and metabolic challenges.

This is interesting as there is evidence of circadian heterogeneity in populations of hair-follicle stem cells and epidermal stem cells (Janich et al., 2011, 2013). This affects stem cell function as it creates a heterogeneous stem cell population with individual stem cells differentially responding to cues promoting activation or dormancy. Circadian heterogeneity in NSCs has not yet been demonstrated but could help to explain why some quiescent NSCs become activated and others do not. We observed circadian heterogeneity in FoxO3-deficient NSCs in culture, which not only suggests that circadian heterogeneity is possible within a NSC population but also that FoxO3 could modulate this.

Interestingly, we found extensive occupancy of circadian clock components on FoxO3 but not of FoxO1, FoxO4 or FoxO6 whereas we only found a knockdown of *FoxO3* to result in a loss of circadian rhythmicity of cultured cell populations (this paper, Chaves et al., 2014). This indicates an exclusive role for FoxO3 in circadian regulation. Although all FoxOs recognize the same DNA motifs or DBEs and are partially redundant, FoxOs do show functional diversification (Hosaka et al., 2004). As the effects of FoxO3 appear to be similar across cell types – at least in fibroblasts, hepatocytes (Chaves et al., 2014) and neural stem cells (this study), it would be interesting to find FoxO3-specific targets involved in circadian rhythms. Perhaps FoxO3, but not the other FoxO isoforms, is part of a circadian regulatory complex.

This could be determined in future research using immunoprecipitation combined with mass spectrometry during a circadian cycle. Nevertheless, it is noteworthy that FoxO6 overexpression disturbed circadian rhythmicity in neural stem cells. FoxO6 overexpression had a similar effect on circadian rhythmicity in fibroblasts with considerable overlap in the transcriptome following FoxO3 knockdown or FoxO6 overexpression (Chaves et al., 2014). As we found that FoxO6 activates PI3K-Akt signalling and, consequently, negatively regulates FoxO3 (Draijer et al., *in prep.*), a similar mechanism could explain the effects of FoxO6 on circadian rhythmicity. Interestingly, we observed an increased G1 phase length at the expense of the combined S/G2/M phase lengths following knockdown of *FoxO6*, which is in line with the increased quiescence of FoxO6-deficient NSCs (Draijer et al., *in prep.*).

Unfortunately, G0 and G1 phases cannot be distinguished using the $NIH3T3^{3C}$ model (Feillet et al., 2014). Nevertheless, the relationship between FoxO3 and FoxO6 in regulation of the circadian clock would be an intriguing topic for future research.

To conclude, our results demonstrate that FoxO3 is a regulator of circadian rhythms in NSCs and is itself also regulated by the circadian clock. We suggest that FoxO3 is necessary for the correct timing of hippocampal NSC proliferation to ensure its alignment with cellular metabolism. This could offer valuable insights in understanding the relationship between circadian rhythms, metabolic diseases, cancer and other pathologies.

EXPERIMENTAL PROCEDURES

FoxO3 mutant mice

All experiments involving animals and their care were performed in accordance with the guidelines of the University of Amsterdam, national guidelines and laws. This study was approved by the Dutch Animal Ethics Committee. Mice were housed in standard cages under a 12h light/dark cycle, with ad libitum access to food and water. Wild type mice from strain C57BL6 were used. FoxO3 mutant mice, originally reported by (Hosaka et al. 2004) were used in heterozygous breeding generating wild-type ($^{+/+}$) and mutant progeny ($^{-/-}$). All experimental and control mice were littermates. Both male and female mice were used for all *in vivo* genetic studies.

Genotyping

For genotyping FoxO3 null and wild-type sibling mice, three primers were used in the same PCR reaction: Forward primer 1: 5'-ATTCCTTTGGAAATCAACAAAACT-3'; Reverse primer 1: 5'-TGCTTTGATACTATTCCACAAACCC-3'; Reverse 2: 5'-AGATTTATGTTCCCACTTGCTTCCT-3'. FoxO3 wild type allele produced a band of 100 bp and the FoxO3 null allele produced a band of 186 bp in a Taq DNA polymerase PCR reaction.

Tissue preparation

Mice were anesthetized every 4 hours starting at CT2 (10:00 AM) and brains were fixed in 4% PFA for 8 hours at 4°C and then in 30% sucrose in PBS overnight at 4°C for immunohistochemistry. Coronal and sagittal sections (16 μm) were cut using a cryostat (Leica) and mounted on slides.

Immunohistochemistry

Immunohistochemistry was performed using primary antibodies rabbit anti-Ki67 (1:500, Abcam, ab15580) using heat-induced antigen retrieval. Briefly, sections were incubated in 10 mM sodium citrate (pH 6.0) for 15 minutes at 90 °C. Sections were then blocked with 4% hiFBS (Gibco) in a Tris-buffered saline solution pH 7.6 containing Triton X-100 and incubated with primary antibody overnight at 4°C. After three washes with a Tris-buffered saline solution, sections were incubated with secondary antibodies donkey anti-rabbit IgG Alexa Fluor 488 or 594 or goat anti-mouse IgG Alexa Fluor 555 (Molecular Probes) at 1:1000 for 2 hours at room temperature. Sections were counterstained with DAPI and embed in FluorSave (Sigma).

Image acquisition and quantification

All fluorescent images were taken with a Leica microscope (DFC310FX). All quantifications were done by an observer blinded to the experimental condition. Images of multiple sections were quantified and averaged per animal. The sections spanned the rostrocaudal length of the hippocampus. Only DAPI+ cells were included. Quantifications were done in the subgranular zone of the dentate gyrus only.

Cell culture

Multipotent astrocytic stem cells (MASCs) were cultured in DMEM F-12 (Invitrogen) + 10% hiFBS (Gibco), 200 nM L-Glutamine (Gibco), 1x N2 (Invitrogen), 1x Penicillin-Streptomycin (Gibco) and supplemented with bFGF (10 ng/mL, Sigma), EGF (10 ng/mL, Sigma) and BPE (12.5 mg/mL, Invitrogen). Cells were incubated at 37 °C, 5% CO_2. MASCs were a gift of C. Fitzsimons. For single cell fluorescence microscopy, NIH3T3^{3c} cells containing *Rev-Erbα-VNP* clock reporter and FUCCI *hCdt1-mKOrange* and *hGeminin-CFP* FUCCI cell cycle reporter genes (Feillet et al., 2014) were cultured in DMEM/F10 containing 10% FBS (Gibco), 1x Penicillin-Streptomycin at 37°C and 5% CO_2 (pH7.7).

Plasmids, siRNA oligos and transfection

For overexpression studies, the following plasmids (Jacobs et al., 2003) (600 ng per transfection) were used: pFOXO1-EGFP-N1, pFOXO3-EGFP-N1, pFOXO6-

EGFP-N1 and p-EGFP-N1 as empty vector control. pGL4.11-Bmal1::luciferase (200 ng) was used as circadian reporter, pFlag-CLOCK (600 ng) was used to rescue FoxO3 siRNA with pcDNA-Neo as empty vector control. For FoxO3 promoter studies, two plasmids with 1.4kb and 2.1kb of the *FoxO3* promoter cloned into a pGL3::luciferase vector (see: below) were used with pGL3::luciferase as empty vector control. For knockdown studies, siRNA oligos (50 pmol) against FoxO1 (siO1), FoxO3 (siO3), FoxO6 (siO6) or a scrambled siRNA (siC) were used. Transient transfections were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions unless specified otherwise.

Real time bioluminescence recordings

To monitor circadian oscillations in MASCs, cells were plated in 6mm dishes and transfected the following day. Forty-eight hours later, medium was replaced with medium containing 25 mM HEPES and 0.1 mM luciferin (Sigma) and 100 nM dexamethasone for circadian synchronization. Bioluminescence was then recorded for multiple day. (75 sec measurements at 10 min intervals) with a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37°C. Data was analyzed with the Actimetrics software to calculate circadian amplitude.

RNA isolation and quantitative real-time PCR

RNA was harvested 24 hours and 48 hours after transfection using TriZOL (ThermoFisher) according to manufacturer's instructions. Relative expression levels were determined by quantitative real-time PCR (Lightcycler 480) using the QuantiTect SYBR Green RT PCR Kit (QIAGEN) according to the manufacturer's instructions. Gene expression was calculated relative to endogenous controls B2M, and normalised to the expression of control samples in each group, to give a ΔΔCt value. Primers used were: *B2m* forward: 5'-TTCTGGTGCTTGTCTCACTG-5', *B2m* reverse: 5'-CAGTATGTTCGGCTTCCCATTC-3', *FoxO3* forward: 5'- CCTATGCCGACCTGATCACC-3', *FoxO3* reverse: 5'-ATTCTGAACGCGCATGAAGC'-3', *Clock* forward: 5'-AGGCTATTTGCCATTTGAAGTCT-3' and *Clock* reverse: 5'-GCTCGTGACATTTTGCCAGATTT-3'.

Time-lapse fluorescence microscopy

NIH3T3[3c] cells were plated in 4 well poly-L-lysine coated glass bottom dishes (D141410, Matsunami Glass Ind.) and reverse transfection was performed using Lipofectamine® RNAiMAX (Invitrogen) with *FoxO3* siRNA, *FoxO6* siRNA or siRNA control. After 24 hours, transfection medium was replaced with regular culture medium and placed in a temperature and humidity controlled chamber (37 °C, 5% CO_2) of a live cell imaging Zeiss LSM510/Axiovert 200M confocal microscope. Images were then recorded every 30 minutes for 72 hours or more using a Coolsnap HQ/Andor Neo sCMOS camera. Live cell imaging was conducted and analysed as decribed previously (Farshadi et al., 2019; Feillet et al., 2014).

Briefly, time-series for each of the fluorescent markers (Rev-ERbα-VNP, hCDT1-mKOrange, hGeminin-CFP) were generated and used to assess lengths of the circadian cycle, total cell cycle, G1 phase and combined S, G2 and M phases (S/G2/M). The G1 phase was defined as the interval between the peaks of hGeminin-CFP and hCDT1-mKOrange expression whereas the S/G2/M phase was defined as the interval between the peaks of hCDT1-mKOrange and hGeminin-CFP expression.

Statistical analysis

All error bars represent the mean ± SEM. Significance is stated as follows: $p>0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). Statistical details of each experiment can be found in the figure legend. n represents number of animals *in vivo* or independent biological repeats *in vitro*. Statistical analysis of circadian oscillation was performed with Circwave (courtesy of Dr. Roelof Hut, http://www.euclock.org/).

SUPPLEMENTAL FIGURES

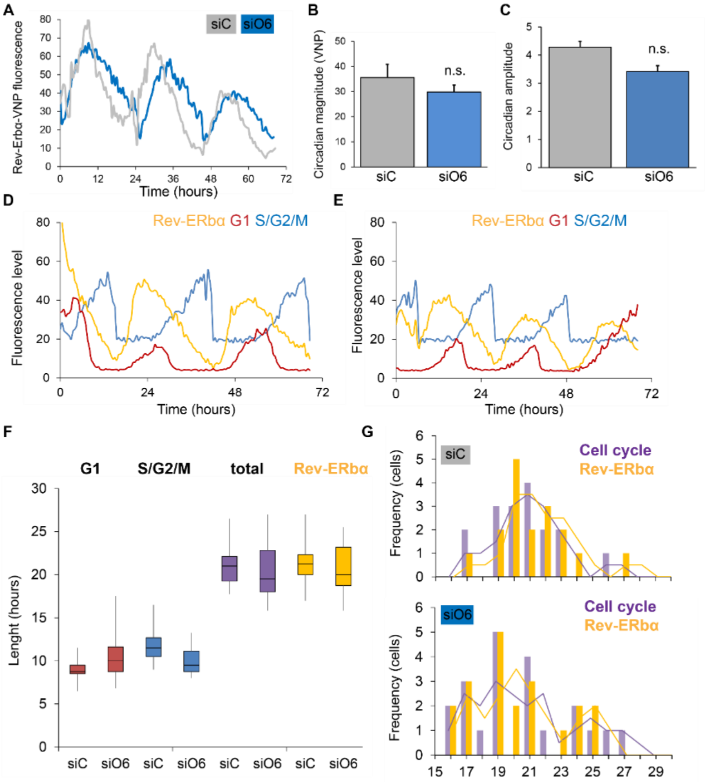

Figure S1. FoxO6 knockdown does not affect circadian clock in single cell NIH3T33C cell but does change cell cycle dynamics

(A) Single cell recordings over multiple days of Rev-ERbα-VNP fluorescence levels in NIH3T33C following transfection with FoxO6 siRNA (siO6, dark blue) or control siRNA (siC, grey). Representative examples.

(B) Circadian magnitude of oscillations in Rev-ERbα-VNP fluorescence levels shown in (A). siC, n=13; siO6, n=26. Student's t-test with Bonferroni correction for multiple group comparison. n.s., not significant

(C) Circadian amplitude of oscillations in Rev-ERbα-VNP fluorescence levels shown in (A). siC, n=13; siO6, n=26. Student's t-test with Bonferroni correction for multiple group comparison. n.s., not significant

(D,E) Single cell recordings of fluorescence marker expression for circadian clock oscillations (Rev-ERbα-VNP, yellow), G1 phase of the cell cycle (hCdt1-mKOrange fusion protein, red) and combined S, G2 and M phases (hGeminin-CFP fusion protein, blue) in NIH3T33C cells transfected with FoxO6 siRNA (D) or siRNA control (E). Representative examples.

(F) Comparison of the average length of cell cycle phase S, G2 and M (S/G2/M, blue), G1 (red), total cell cycle (purple) and circadian rhythm length (yellow) of data shown in (D-E). siC, n=13; siO6, n=26. Student's t-test with Bonferroni correction for multiple group comparison. *, p<0.025; **, p<0.005.

(G) Frequency histograms of circadian rhythm length (Clock, yellow) and total cell cycle length (purple) in single NIH3T33C cells transfected with FoxO6 siRNA (bottom) or siRNA control (top)

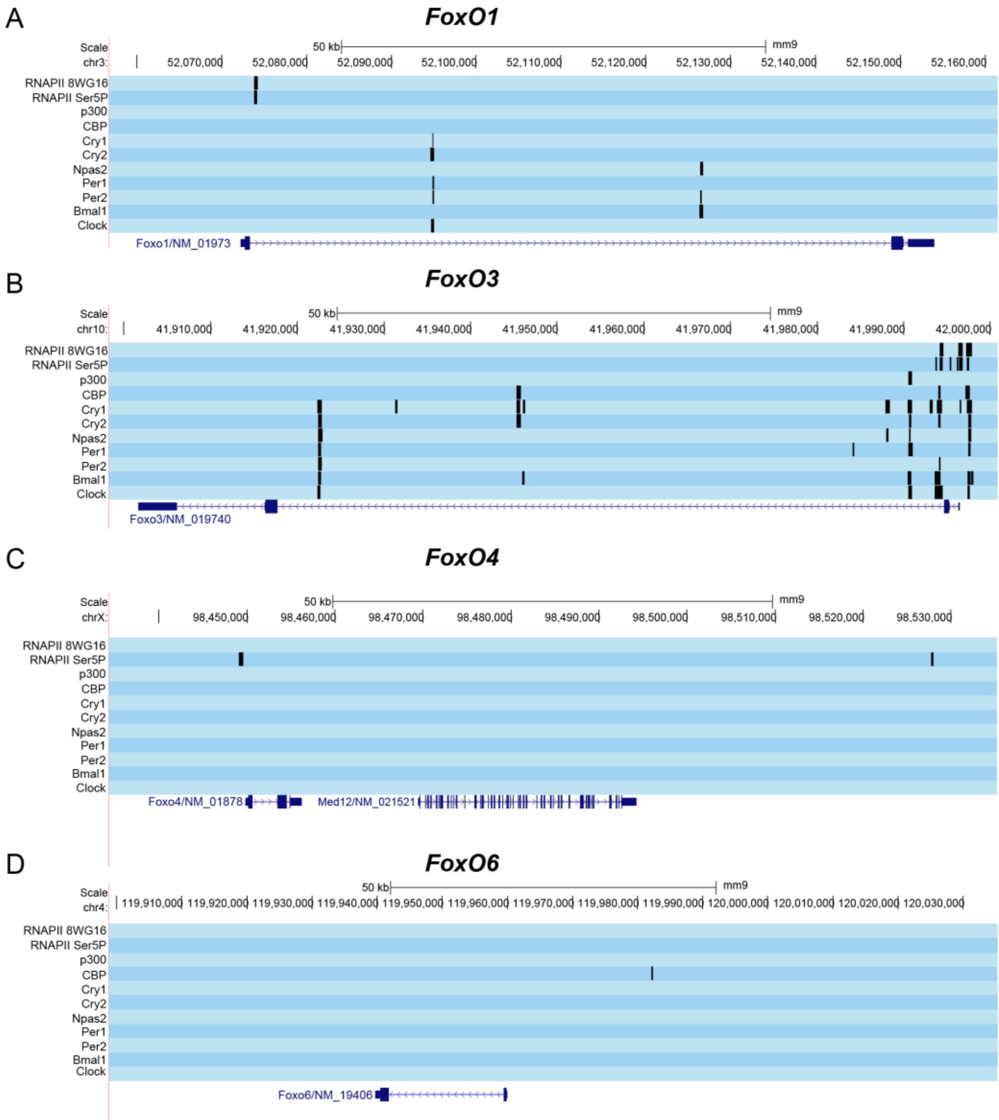

Figure S2. Differential binding of circadian clock components and transcriptional regulators to FoxO transcription factors in the mouse liver

University of California Santa Cruz (UCSC) genome browser track view of the *FoxO1* (A), *FoxO3* (B), *FoxO4* (C) and *FoxO6* (D) genes showing ChIP-seq binding peaks for circadian clock components Clock, Bmal1, Per1, Per2, Npas2, Cry1 and Cry2 as well as transcriptional regulators CBP, p300 and recruitment and initiation of RNA polymerase II (8WG16 and Ser5P respectively). Data from Koike et al. (2012).

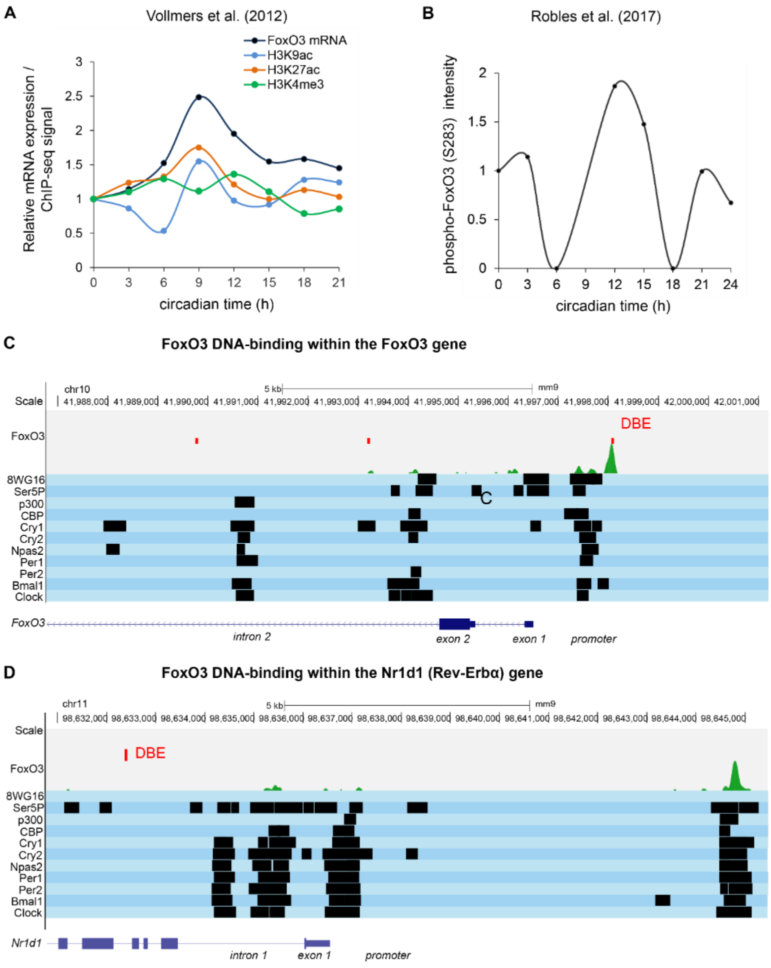

Figure S3. Circadian regulation of FoxO3 mRNA expression and phosphorylation.
(A) Relative *FoxO3* mRNA expression and relative ChIP-seq signal of histone modifications H3K9ac, H3K27ac, H3K4me3 in the *FoxO3* promoter during a circadian cycle (data from (Vollmers et al., 2012) normalized to CT0).
(B) Relative FoxO3 serine-283 phosphorylation during a circadian cycle (data from (Robles et al., 2017), data shown here is time window CT8-39).
(C) UCSC Genome Browser view of *FoxO3 (green)* with circadian clock components binding sites (ChIP-seq data from Koike et al. (2012) of Clock, Bmal1, Per1, Per2, Npas2, Cry1, Cry2 and transcriptional regulators CBP, p300 and RNA polymerase II recruitment (8WG16) and initiation (Ser5P) as well as FoxO3 ChIP-seq signal (Webb et al., 2013). Genomic locations of FoxO3 recognition motif DBE (DAF-16 binding element) are denoted in red.
(D) UCSC Genome Browser view of *Nr1d1* (Rev-erbα) gene with circadian clock components binding sites (ChIP-seq data from Koike et al. (2012) of Clock, Bmal1, Per1, Per2, Npas2, Cry1, Cry2 and transcriptional regulators CBP, p300 and RNA polymerase II recruitment (8WG16) and initiation (Ser5P) as well as FoxO3 ChIP-seq signal (Webb et al., 2013). Genomic location of a FoxO3 recognition motif DBE (DAF-16 binding element) are denoted in red.

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