

Zinc finger homeobox-3 (ZFHX3) orchestrates genome-wide daily gene expression in the suprachiasmatic nucleus

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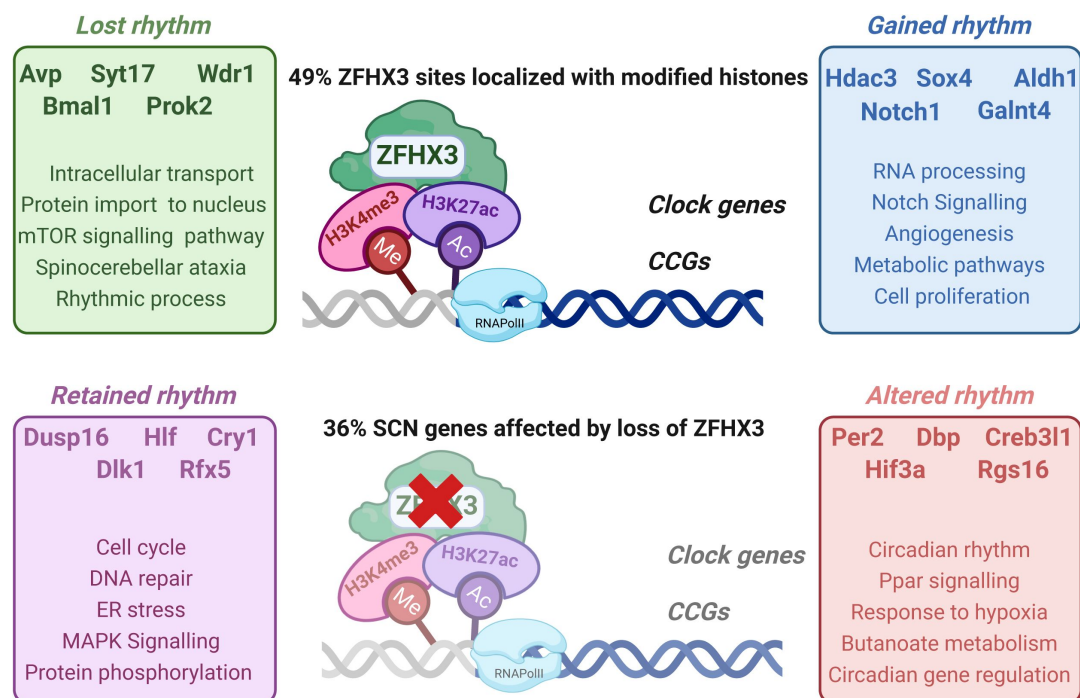
This is an **important** study that generates an inventory of accessible genomic regions bound by a transcription factor ZFHX3 within the suprachiasmatic nucleus in the hypothalamus and details the impact of its depletion on daily rhythms in behavior and gene expression patterns. Analysis using circadian phase-estimation algorithms makes the argument that gene regulatory networks are at play and changes in gene expression of a few clock genes cannot account for the observed animal behaviour. While the transcriptome analysis is **compelling**, the data on the activity of the TF in rhythmic gene expression is **solid**, and interpretations that allow for direct and/or indirect roles have been incorporated.

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Abstract

The mammalian suprachiasmatic nucleus (SCN), situated in the ventral hypothalamus, directs daily cellular and physiological rhythms across the body. The SCN clockwork is a self-sustaining transcriptional-translational feedback loop (TTFL) that in turn co-ordinates the expression of clock-controlled genes (CCGs) directing circadian programmes of SCN cellular activity. In the mouse, the transcription factor, ZFHX3 (zinc finger homeobox-3), is necessary for the development of the SCN and influences circadian behaviour in the adult. The molecular mechanisms by which ZFHX3 affects the SCN at transcriptomic and genomic levels are, however, poorly defined. Here, we used chromatin immunoprecipitation sequencing (ChIP-seq) to map the genomic localization of ZFHX3 binding sites in SCN chromatin. To test for function, we then conducted comprehensive RNA sequencing at six distinct times-of-day to compare the SCN transcriptional profiles of control and ZFHX3-conditional null mutants.

We show that the genome-wide occupancy of ZFHX3 occurs predominantly around gene transcription start sites (TSS), co-localizing with known histone modifications, and preferentially partnering with clock transcription factors (CLOCK, BMAL1) to regulate clock gene(s) transcription. Correspondingly, we show that the conditional loss of ZFHX3 in the adult has a dramatic effect on the SCN transcriptome, including changes in the levels of transcripts encoding elements of numerous neuropeptide neurotransmitter systems while attenuating the daily oscillation of the clock TF *Bmal1*. Furthermore, various TTFL genes and CCGs exhibited altered circadian expression profiles, consistent with an advanced in daily behavioural rhythms under 12h light-12h dark conditions. Together, these findings reveal the extensive genome-wide regulation mediated by ZFHX3 in the central clock that orchestrates daily timekeeping in mammals.



Introduction

Circadian (approximately one day) clocks are internal biological time-keepers co-ordinating coherent 24-h molecular, behavioural and physiological rhythms that are synchronized with changing environmental conditions across day and night. In mammals, the circadian clock mechanism consists of cell-autonomous transcription-translation feedback loops (TTFLs) that drive rhythmic, ~24-h expression patterns of canonical clock components (Takahashi et al., 2008 [↗](#)). The primary TTFL involves rhythmic transcription of period (*Per1*, *Per2*, and *Per3*) and cryptochrome genes (*Cry1* and *Cry2*). Thereafter, PER and CRY proteins form heterodimers that act on the dimeric complex of CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-Like 1) to repress their own transcription, which is mediated by E-box (CACGTG) cis-regulatory sequences at their gene promoters. A secondary feedback loop is driven by *Rev-erba* (*Nr1d1*), which is also directly transcribed through binding of CLOCK:BMAL1 at E-boxes. REV-ERBA feeds back to repress *Bmal1* transcription by competing with a retinoic acid-related orphan receptor (ROR) activator to bind ROR response elements (RREs) in the *Bmal1* promoter. Along with this, CLOCK:BMAL1 heterodimers also trans-activate a series of clock-controlled gene (CCGs), many of which encode transcription factors (TFs) that regulate additional downstream programmes of gene expression (Herzog et al., 2017 [↗](#); Hogenesch et al., 2003 [↗](#)).

At an organismal level, mammalian circadian rhythms are directed by the suprachiasmatic nucleus (SCN) of the hypothalamus, which synchronizes robust 24-h oscillations across peripheral tissues (Hastings et al., 2018 [↗](#), 2019 [↗](#); Schibler et al., 2015 [↗](#)). In the SCN, the cell-autonomous TTFL molecular clocks in individual cells are synchronized through intercellular coupling to form a coherent oscillatory network (Herzog et al., 2017 [↗](#); Maywood et al., 2021 [↗](#)).

This intercellular coupling is maintained through several neuropeptide neurotransmitter systems, including vasoactive intestinal peptide (VIP), arginine vasopressin (AVP) and gastrin-releasing peptide (GRP) systems (Brown et al., 2005 [↗](#)), while Prokinetin2 (PROK2) serves as a pace-making element in the SCN (Morris et al., 2021 [↗](#)). Moreover, zinc finger homeobox-3, ZFH3, is a key transcription factor in the SCN. It is highly expressed in discrete populations of adult SCN cells and exerts robust control over circadian behavioural rhythms. Intriguingly, while a dominant missense mutation in *Zfhx3* (short circuit, *Zfhx3*^{Sci/+}) results in a short circadian period in mice (Parsons et al., 2015 [↗](#)), a conditional null *Zfhx3* mutation results in more severe behaviour with short circadian period and arrhythmic mice (Wilcox et al., 2017 [↗](#)).

Given the crucial role of the SCN in orchestrating daily timekeeping in mammals, it is imperative to understand the role of ZFH3 in regulating the molecular circadian clock, but although the effects of mutations in *Zfhx3* are known at the level of circadian behaviour, its molecular functions that underpin daily timekeeping are not well-characterized. Therefore, we set out to assess systematically the function of ZFH3 by employing a multi-omics approach in the SCN. First, we analysed the genomic binding pattern of ZFH3 by conducting ZFH3 targeted chromatin immunoprecipitation (ChIP-seq) of SCN tissue to identify the genomic scope of ZFH3 and its potential mechanistic roles. Second, we implemented a detailed transcriptional profiling of the SCN in control and *Zfhx3* conditional null mutants, using vibratome based microdissection (Bafna, Lau, et al., 2023). This allowed us to map the effect of ZFH3 on tissue-specific expression of both rhythmic and non-rhythmic genes.

Overall, we have defined genome-wide occupancy of ZFH3 in the SCN, showing that it is found predominantly around actively transcribed genes and is accompanied by modified histones and other SCN TFs. We noted that conditional (adult) deletion of ZFH3 drastically modifies the SCN transcriptome by affecting genes important for circadian timekeeping, neuropeptide signalling and synaptic processes. An in-depth investigation on time-of-day dependent gene expression clearly highlighted loss of *Bmal1* rhythmic transcription along with aberrant 24-h oscillations for TTFL and non-TTFL genes. This change in rhythmic clock gene transcriptional profiles contributed towards a phase-advanced internal central clock mirroring the advance seen in the daily behavioural activity patterns of the null mutant mice. These findings reveal a pivotal and genome-wide physiological role for ZFH3 in the maintenance of robust circadian timekeeping in the SCN and identify molecular substrates that underpin this role.

Results

ZFH3 binds to active promoter sites in the SCN

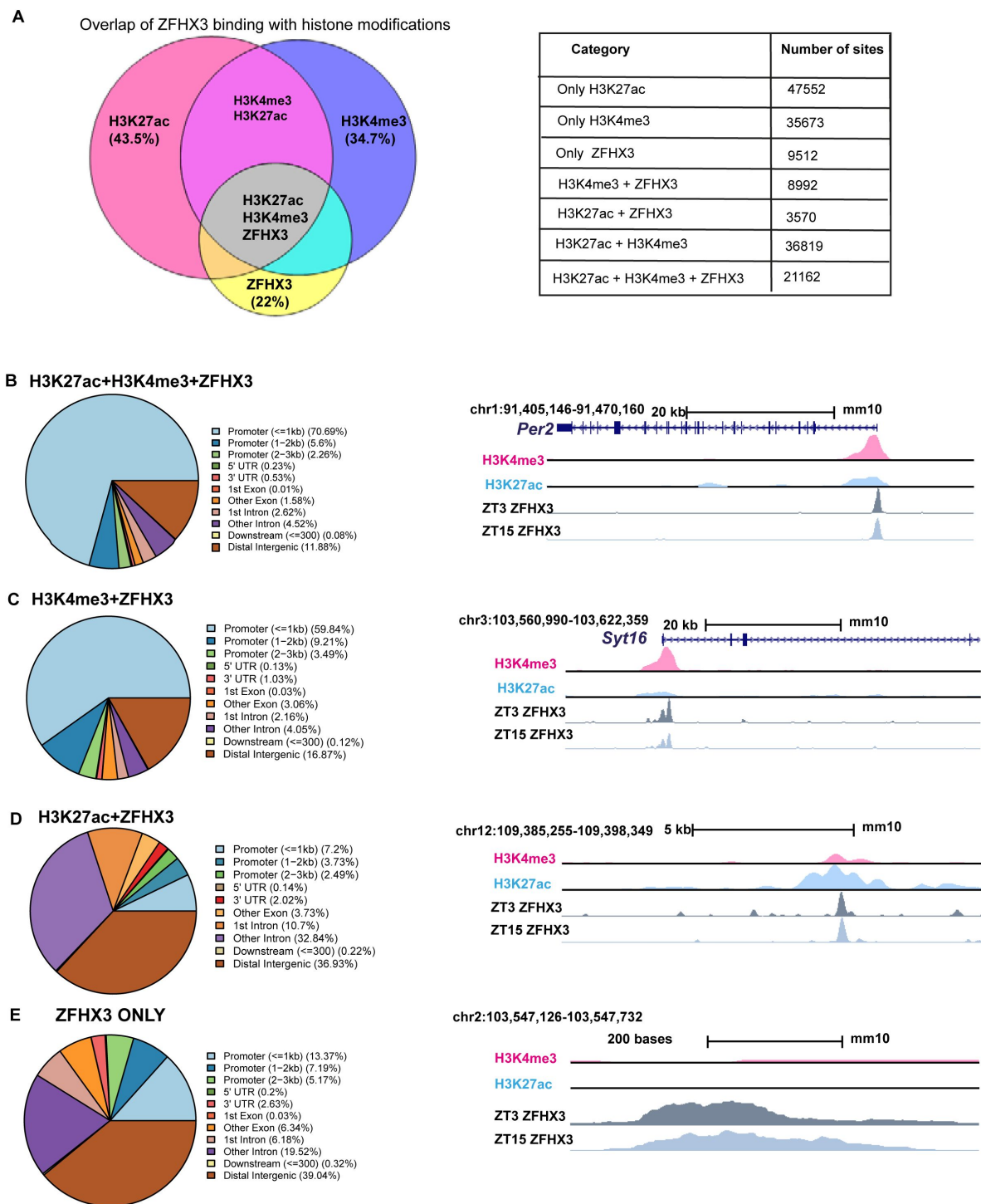
Genome-wide binding of ZFH3 was profiled in the SCN using ChIP-seq. Briefly, genomic DNA was extracted from C57BL/6J mice raised in standard 12-h/12-h light-dark conditions at two distinct time-points; *zeitgeber* time (ZT) 03 (3 hours after lights on) and ZT15, and subjected to ChIP-seq (Methods). The sequenced reads were aligned to the mouse genome and significant sites of ZFH3 occupancy were assessed in the context of previously identified modified histone binding regions (Bafna, Banks, et al., 2023) in the SCN. We found a total of 43,236 ZFH3-bound sites, overlapping with approximately 22% of total modified histone regions in the SCN genome. About 60% of the

ZFHX3 bound sites were found near promoters (≤ 3 Kb of TSS) and so would be likely to identify direct transcriptional targets of ZFHX3, whilst ca. 21% were in distal intergenic regions potentially involved in other modes of gene regulation.

When assessed in relation to modified histone binding, ZFHX3 sites fell into four categories. First, the majority of ZFHX3 peaks coincided with both H3K4me3 (trimethylation of Histone H3 at Lysine 4) and H3K27ac (Histone 3 lysine 27 acetylation) occupancy (48.9% of total ZFHX3 peaks at ZT3, Supplementary Table 1), and were focussed near transcription start sites ($\text{TSS} \pm 3\text{kb}$) (**Fig. 1B** [↗](#)). Since such sites are likely responsible for active transcription (Beacon et al., 2021 [↗](#)), the ZFHX3 occupancy in these sites highlighted probable transcriptional regulation by ZFHX3 in the SCN. Next, a subset of ZFHX3 sites (20.7%) were bound only by H3K4me3 (no H3K27ac) as illustrated at **Fig. 1C** [↗](#). These are indicative of poised transcriptional regulation (Calo & Wysocka, 2013 [↗](#)) and were also enriched near the TSS. About 8.2 % of ZFHX3 sites were bound by H3K27ac exclusively (no H3K4me3) (**Fig. 1D** [↗](#)). These sites were commonly found at intergenic and intronic regions and suggestive of enhancer-mediated gene regulation (Won et al., 2008 [↗](#)). Finally, 22% of ZFHX3 sites were found to be devoid of modified histone binding and were also concentrated at distal intergenic regions (39.04%) (**Fig. 1d** [↗](#)). Overall, the broad occurrence of ZFHX3 alongside histone modifiers near the gene TSS and at distal intergenic regions in the SCN genome suggests its crucial role in gene regulation potentially via promoter-enhancer interaction.

Next, we assigned each ZFHX3 site to its closest transcribed gene ($\pm 3\text{Kb}$) and performed functional annotation upon these genes. The functional annotation of genes which were associated with ZFHX3 and both the H3K4me3 and H3K27ac histone modifications were found to be associated with neurodegeneration and axonal guidance, synaptic processes known to be important in the SCN (glutamatergic, GABAergic and dopaminergic systems) and various signalling pathways. Importantly, they also included pathways involved with circadian entrainment and timekeeping (**Supplementary Fig. 1A** [↗](#)), highlighting the reciprocal intersection between ZFHX3 and the SCN cell-autonomous clock. In contrast, genomic regions co-operatively bound by ZFHX3 and H3K4me3 but with no H3K27ac, or sites with ZFHX3 alone, were found to be associated with genes involved in several fundamental biological processes such as DNA replication or MAPK signalling, but notably not circadian rhythm (**Supplementary Fig. 1B, C** [↗](#)). Therefore, it is plausible that ZFHX3 binds at the TSS and regulates actively transcribing genes, particularly those involved in tissue-specific functions such as daily timekeeping, while its occupancy without histone modifiers and/or at poised state regulates genes responsible for other basal functions. Interestingly, it is worth noting that ZFHX3 occupancy did not differ appreciably between the two time-points tested (ZT03 vs. ZT15): ZFHX3 was seen to bind consistently at the same genomic loci (Supplementary Table 1). This indicates that other factors/ mechanisms confer temporal control to these sites whilst the presence of ZFHX3 is permissive to such cis-and trans-regulatory cues.

We next examined the signature DNA binding motifs/ cis-regulatory elements occupied by ZFHX3, either alone or with specific histone modifications (H3K4me3, H3K27ac). As anticipated, this revealed an over-representation of AT-rich motifs (Parsons et al., 2015 [↗](#)). Notably, it also suggested co-occurrence of ZFHX3 with SCN developmental and maintenance TFs such as DLX (distal-less homeobox) and LHX (LIM homeobox) (VanDunk et al., 2011 [↗](#)) across the tested categories (**Supplementary Fig. 1D** [↗](#)). Furthermore, ZFHX3-bound sites were also enriched for non-AT-rich motifs such as the RFX family of TFs, which have been implicated in transcriptional responses to light in the SCN (Araki et al., 2004 [↗](#)). Interestingly, we noted enrichment of ARNT (bHLH) domains but only where ZFHX3 peaks were seen adjacent to H3K27ac and H3K4me3 (i.e., active promoters). This clearly points towards the possibility of shared binding of ZFHX3 with the circadian clock TFs BMAL1:CLOCK at E-boxes and highlights a point of interaction between the core TTFL and ZFHX3 in the regulation of circadian gene transcription in the central pacemaker.



***Zfhx3* knock-out affects circadian behaviour and entrainment of mice**

To explore the contribution of *Zfhx3* to SCN function in the adult animal, we compared circadian wheel-running in cohorts of *Zfhx3*^{Flox/Flox};UBC-mice that had been dosed with tamoxifen (gene deletion, +Tamoxifen group) with mice that had not been dosed with tamoxifen (no deletion genetic control, -Tamoxifen group). Comparisons of gene expression between the -Tamoxifen and +Tamoxifen groups by real time PCR confirmed loss of *Zfhx3* expression in the +Tamoxifen group. (Supplementary Fig. 2A). Behavioural analysis was performed in conditions of constant darkness and in a 12 h light/12 h dark cycle. Consistent with previous studies, this analysis showed that the free-running circadian period was significantly shortened by conditional deletion of *Zfhx3* (Fig. 2A). Furthermore, the overall activity rhythm was less well defined with significantly reduced amplitude, increased intra-daily variability and lower inter-daily stability in the deleted mice (Supplementary Fig. 2B, C, D). The full analysis is given in Supplementary Table 2. The loss of ZFH3 therefore compromised free-running circadian behaviour.

Visual inspection of the actograms whilst the animals were in light/dark cycles suggested that animals with gene deletion showed poor entrainment to the light cycle, with animals showing increased activity in the hours prior to the light to dark transition (Fig. 2A, right). To quantify this, we analysed the distribution of wheel running activity across 24-h, by expressing wheel running activity within each 30 minute segment of the day as a proportion of the total daily wheel running activity for that animal (Fig. 2C). Repeated Measures ANOVA demonstrated a significant interaction between tamoxifen dosing and time of day (tamoxifen dosing x time: $F(47, 1316) = 9.013$; $p < 0.0001$). Pairwise analysis demonstrated that, compared to genetic control animals, gene deleted animals showed a significant increase in wheel running activity in the hours prior to lights off ($p < 0.05$ for ZT 10.5-12.5) and a significant reduction in wheel running activity in the hours prior to lights on ($p < 0.05$ for ZT 20-23). Consistent with this, analysis of the activity onset demonstrated that gene deleted animals showed an earlier onset of activity than controls (Supplementary Fig. 2E). This phase advance under 12 h L/12 h D is consistent with the shortening of circadian period seen under DD. Thus, ZFH3 contributes to circadian time-keeping and entrainment in the adult SCN, independently of any developmental roles.

ZFH3 knock-out (KO) results in extensive effects across the SCN transcriptome

Having mapped the genomic binding sites of ZFH3 and validated the behavioural effects of deletion of ZFH3, we then employed total directional RNA sequencing to define the impact of *Zfhx3* deletion on the SCN transcriptome. Adult SCN tissues were collected from tamoxifen-treated *Zfhx3*^{Flox/Flox};UBC-Cre⁻ (Cre-neg) and *Zfhx3*^{Flox/Flox};UBC-Cre⁺ mice that were not treated with tamoxifen (-Tamoxifen, genetic control), both serving as independent control groups, and tamoxifen treated *Zfhx3*^{Flox/Flox};UBC-Cre⁺ (+Tamoxifen) mice i.e., *Zfhx3*-deleted, at six distinct times of light/dark cycle (Methods). As an initial analysis we compared overall (i.e., time-independent) gene expression levels between the groups (Cre-neg vs. +Tamoxifen) to examine the global effect of *Zfhx3* deletion on the SCN transcriptome, and found that almost 36% ($n = 5,637$) of total assessed genes were affected by the acute loss of *Zfhx3* in the SCN. Of these, 2,725 were down-regulated and 2,912 up-regulated in +Tamoxifen animals compared to Cre-neg controls. (Supplementary Table 3, Fig. 3A). Furthermore, as expected, both the control groups (Cre-neg and -Tamoxifen) showed consistent differential gene expression levels when compared to the mutant which mitigated any potential downstream effect of tamoxifen dosing *per se* (Fig. 3B, C).

To better understand the relationship between ZFH3 occupancy on the genome and changes in gene expression, we compared the results of our initial CHIP-Seq with the RNA-Seq described above. Of the total differentially expressed genes, 80% ($n = 4,543$) revealed ZFH3 occupancy at

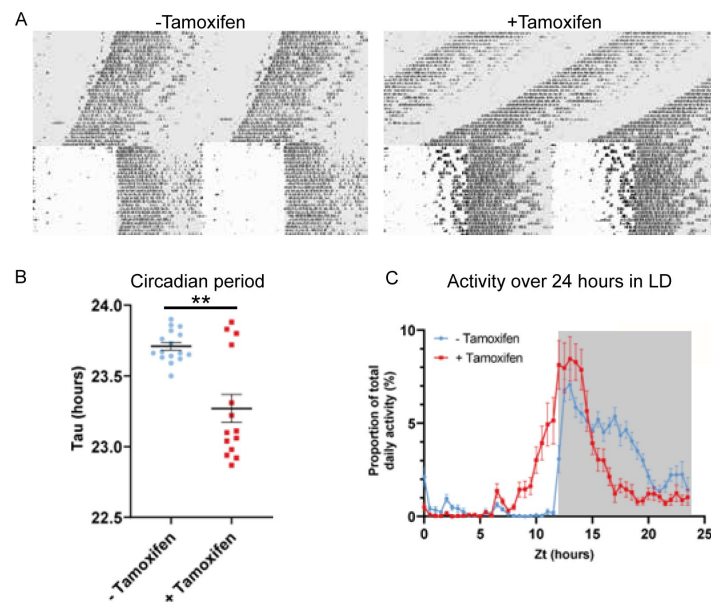


Figure 2.

Circadian wheel running analysis of control and mutant animals.

A. Double plotted actograms showing wheel running activity of -Tamoxifen and +Tamoxifen animals through 32 days of constant darkness and 24 days under a 12h light/dark cycle. Regions in grey denote periods in darkness. B. +Tamoxifen animals show a reduced circadian period C: Graph showing the distribution of activity in -Tamoxifen and +Tamoxifen animals over 24 hours in a 12h light/dark cycle. Data is taken from the final seven days the animals were in the light/dark cycle shown in A. Grey denotes the dark period of the cycle.

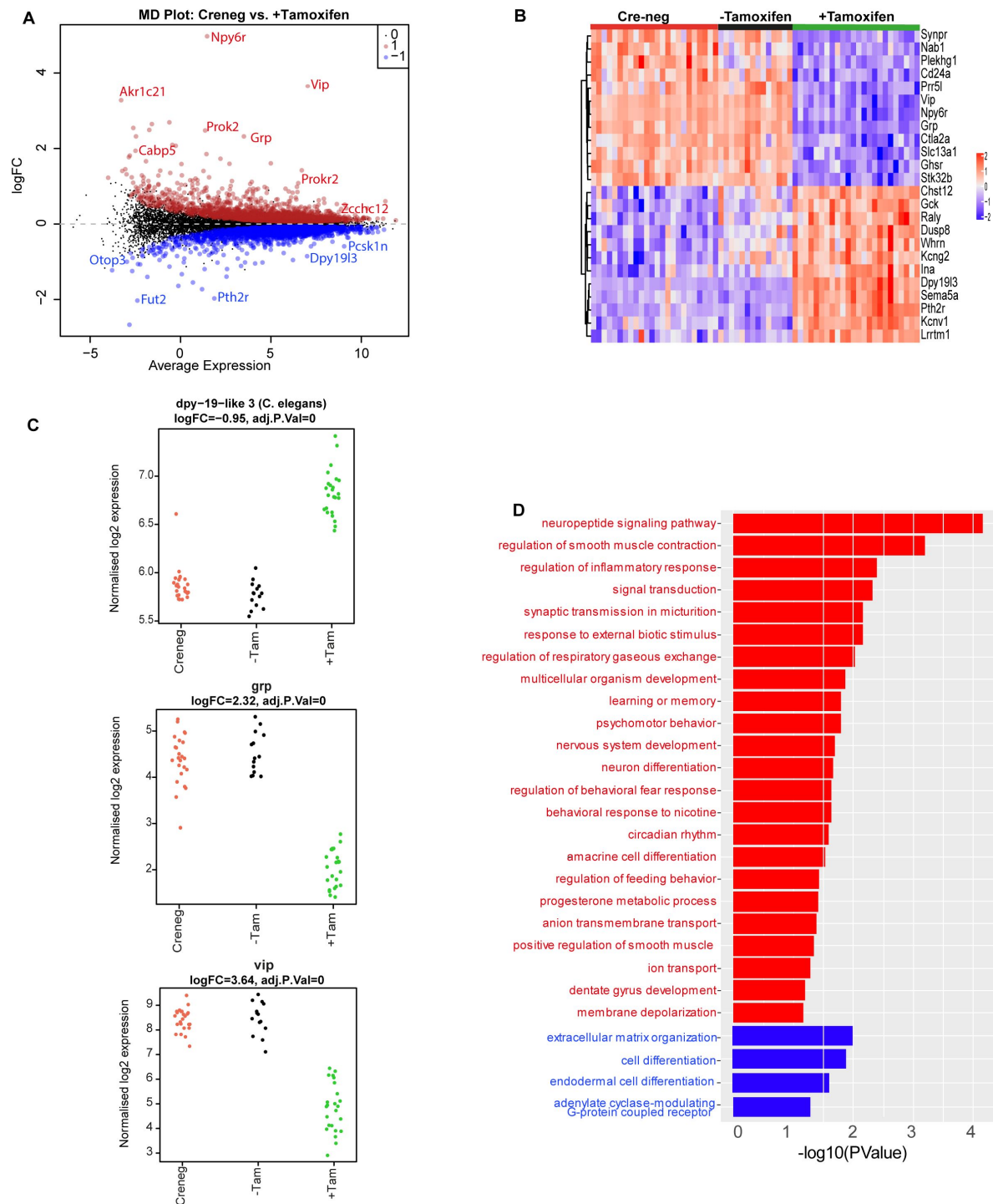


Figure 3.

Effect of ZFH3-KO on SCN transcriptome.

(A) Mean-difference plot (MD-plot) showing downregulated (red) and upregulated (blue) gene expression after the loss of ZFH3. (B) Heatmap showing normalized expression of top 25 (by adj pvalue) differential genes for the compared groups. (C) Stripcharts of differentially expressed genes (*Dpy19l3*, *Grp*, *Vip*). (D) Functional annotation of down-regulated (red) and upregulated (blue) genes after ZFH3-KO using the GO::BP (biological processes) terms by DAVID.

their promoter sites along with histone modifications (H3K4me3 and H3K27ac), showing a direct interaction between ZFH3 bound TSS ($\pm$ 3Kb) and gene expression ($p = 0$, odds ratio = 6.3, Jaccard index = 0.4, signifying strong association). In contrast, only 7% of differentially expressed genes ($n = 381$) were solely bound by ZFH3 at the TSS ($p = 0.37$, odds ratio = 1, Jaccard Index = 0.1, signifying weak association), indicating the effect of ZFH3 on gene transcription is predominantly observed when jointly occupied by histone modification at gene TSS.

We then sub-selected highly differential genes ($\log_2FC \geq 1$, $FDR \leq 0.05$) and conducted functional gene enrichment analysis. Consistent with previous studies (Hughes et al., 2021 [\[1\]](#); Parsons et al., 2015 [\[2\]](#); Wilcox et al., 2021 [\[3\]](#); Wilcox et al., 2017 [\[4\]](#)), genes that were downregulated were seen to be associated with neuropeptide signalling pathways (*Vip*, *Grp*), circadian rhythm, neuron differentiation etc., while those that were upregulated showed functional enrichment for cell differentiation and extracellular matrix organization processes (**Fig. 3D** [\[5\]](#)). Interestingly, our comprehensive assessment also highlighted an effect of loss of ZFH3 on signal transduction by affecting cholinergic receptor (*Chrna3*, *Chrn4*), melatonin receptor (*Mtnr1a*), prokineticin receptor (*Prokr2*), GRP receptor (*Grpr*), and the SCN peptide *Nmu*. Therefore, it is likely that loss of ZFH3 leads to physiological impairment by affecting a diversity of neuropeptide neurotransmitter systems in the SCN and more generally, circadian-relevant transcripts.

Loss of *Zfh3* affects rhythmic transcription of TTFL and clock-controlled genes

The present and recent studies have demonstrated severe disturbances of daily and circadian behaviour in mice carrying a missense mutation (*Zfh3*^{Sci}) (Parsons et al., 2015 [\[2\]](#)) or knock-out (*Zfh3*^{Flox/Flox};UBC-Cre⁺) (Wilcox et al., 2017 [\[4\]](#)) of ZFH3. In addition to assessing general gene up- or down-regulation in mutants (above), we next wanted to assess how deletion of ZFH3 might alter transcriptional rhythms. For this, we investigated gene expression levels in the SCN at six distinct times-of-day (Hughes et al., 2017 [\[6\]](#)), starting at ZT02, at 4-h intervals (Methods). In total, we analysed the daily transcriptional profile of 20,521 genes in the SCN and compared the rhythmicity index between the control (Cre-neg) and mutant (+Tamoxifen) groups using statistical framework *dryR* (Weger et al., 2021 [\[7\]](#)). This enabled us to categorise the genes into one of 5 distinct modules (Methods): Module 1 refers to genes with no detected 24-h rhythm in their expression levels in either condition ($n = 16832$, **Supplementary Fig. 3A** [\[8\]](#)); Module 2 consists of genes that were rhythmic in control SCN but lost rhythmicity in the ZFH3-KO ($n = 1566$, **Fig. 4A** [\[9\]](#)); Module 3 consists of genes that gained daily rhythmicity after ZFH3-KO ($n = 403$, **Supplementary Fig. 3B** [\[10\]](#)); Module 4 was composed of rhythmic genes that exhibited no change between control and mutants ($n = 1653$, **Fig. 4B** [\[11\]](#)); Module 5 included genes that retained rhythmic expression but with a change in either amplitude or phase (peak expression) in the mutant group ($n = 67$) (Supplementary Table 4, **Fig. 5** [\[12\]](#)).

Thus, we noted ~82% of compared genes did not show a daily rhythm (module 1). Conversely, ~18% of gene expressed in the SCN exhibited daily rhythmicity under one or other condition. Of these, ~45% were not affected by loss of ZFH3, whereas ~55% of rhythmic transcripts showed alteration in their daily pattern following loss of ZFH3. Indeed, ~42% of rhythmic genes required ZFH3 to maintain that rhythm, whereas ~2% of genes (403/20521) gained rhythmicity in the absence of ZFH3. Thus, ZFH3 contributes, directly or indirectly, to a significant part of the daily rhythmic transcriptome of the SCN.

For each module, we compiled the gene list and examined the functional enrichment pathways. The genes that lost rhythmic transcription after the deletion of ZFH3 (module 2) were seen to be involved in intracellular protein transport, signal transduction such as WNT-pathway linking circadian and cell-cycle processes (Matsu-Ura et al., 2018 [\[13\]](#)), oxidative stress response etc. (**Supplementary Fig. 4A** [\[14\]](#)). On the contrary, genes that gain rhythmicity (module 3) were associated with RNA processing, stress and metabolic pathways. The genes that did not show any

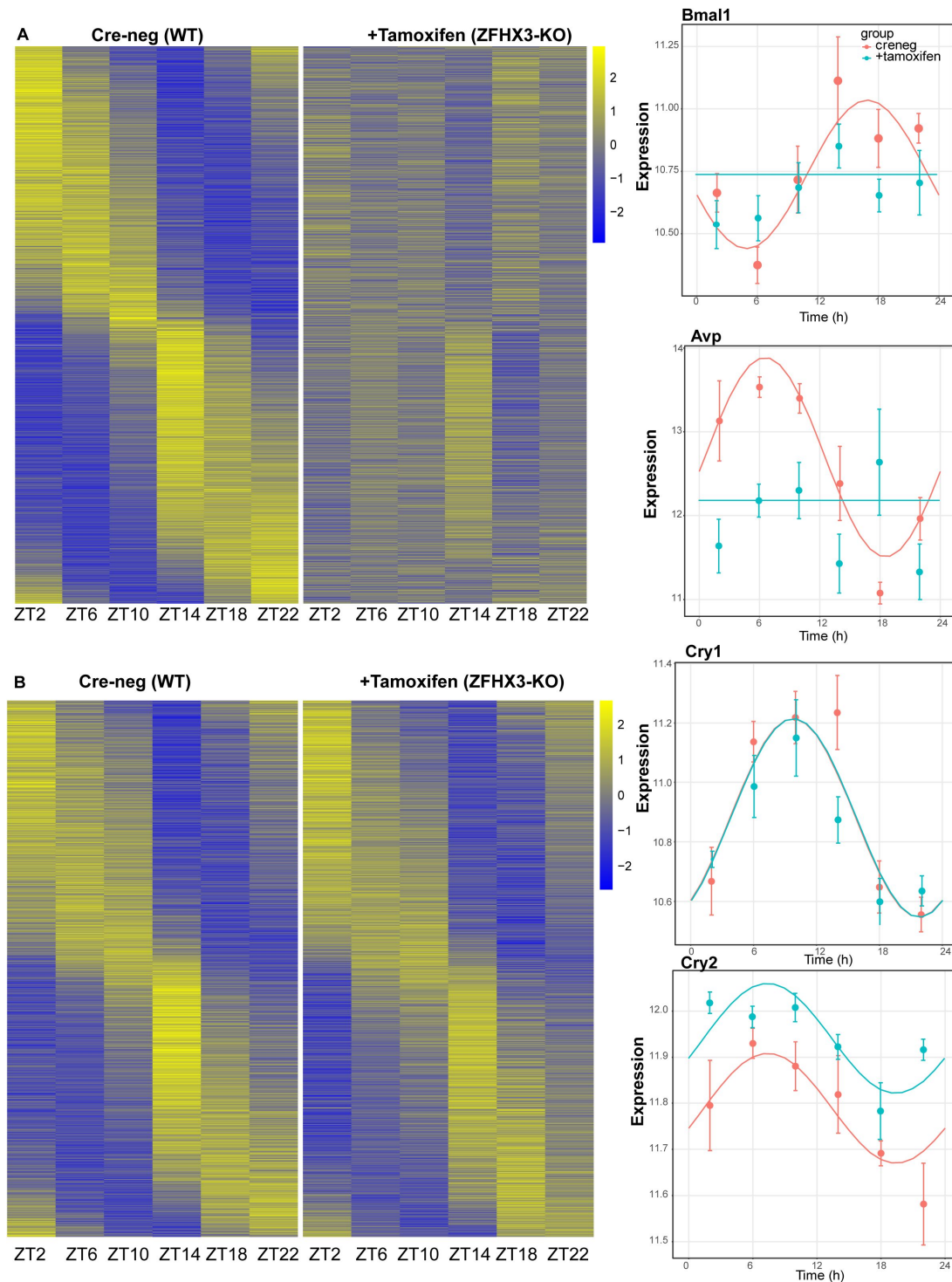


Figure 4.

Effect of ZFH3-KO on rhythmic gene expression.

(A) Left-Heatmap showing loss of rhythmic gene expression after *Zfhx3*-KO (module 2) as computed by dryR statistical framework, Right-Illustrative examples of daily abundance of module 2 genes, *Bmal1* and *Avp*, in Cre-neg and +Tamoxifen conditions. (B) Left-Heatmap showing no effect on rhythmic gene expression after *Zfhx3*-KO (module 4), Right-Illustrative examples of module 4 genes, *Cry1* and *Cry2*, in Cre-neg and +Tamoxifen conditions.

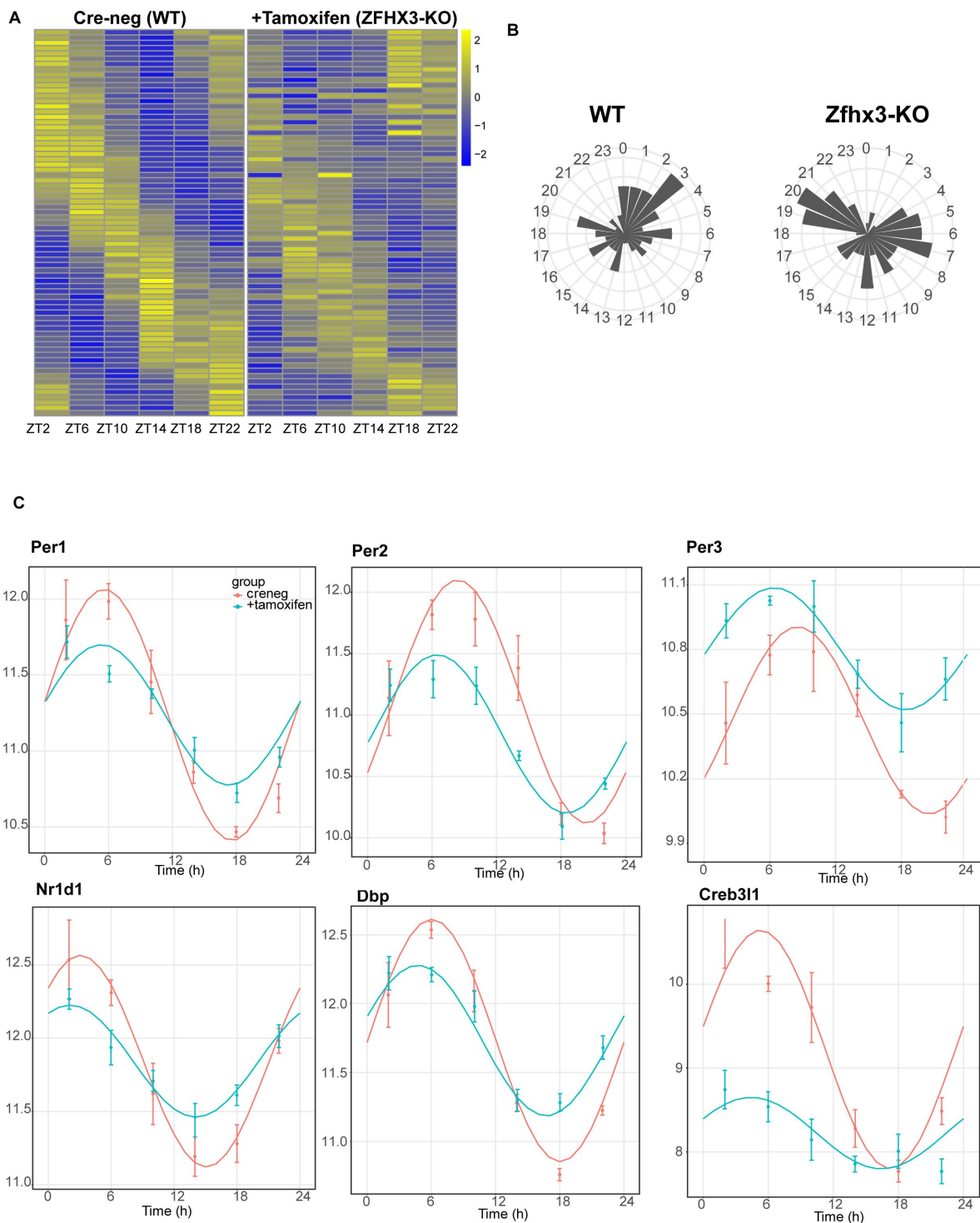


Figure 5.

Effect of ZFH3-KO on clock gene expression clustered in module 5.

(A) Heatmap showing change in rhythmic gene expression after *Zfhx3-KO* (module 5) as computed by dryR statistical framework, (B) 24-h radial plots showing effect on phase (peak expression) of rhythmic genes in Cre-neg (WT) vs. +Tamoxifen (ZFH3-KO) conditions (C) Illustrative examples of daily abundance of module 5 genes, showing change in phase and amplitude in ZFH3-KO condition (blue) as compared to control (pink). The y-axis values represent normalised expression in the SCN.

change in rhythmic expression (module 4) were found to be enriched for functional processes such as protein phosphorylation and de-phosphorylation, cytoskeleton organization, DNA repair etc. Interestingly, only a tiny proportion (~1.8%) of the rhythmic genes showed substantial changes in their daily cyclic gene expression profiles (phase and amplitude, module 5), but these were primarily involved in circadian rhythm generation, entrainment and clock-controlled gene expression (**Supplementary Fig. 4B**). This suggests that in the absence of ZFH3 the core circadian mechanism of the SCN is disrupted but is not ablated.

Indeed, almost all the genes driving the cell-autonomous TTFL were severely affected by *Zfh3* perturbation with *Bmal1* demonstrating a complete loss of 24-h rhythm (**Fig. 4A**), and its counterpart *Clock* mRNA showing overall reduced expression levels (Supplementary Table 3). PER family genes (*Per1-3*) showed a significant advancement in peak expression with low amplitude (**Fig. 5C**). Specifically, we observed a phase advance of ca. 2-h for *Per2* and *Per3*, with *Per1* (Kuhlman et al., 2003) which is also known to mediate light-responsive pathways, advancing by only ~0.3-h. Strikingly, there was no obvious change in the rhythmic levels or phase for CRY family genes (*Cry1, 2*) (**Fig. 4B**), indicating that ZFH3 acts only on select genes driving the cell-autonomous clock in the SCN. Given that *Bmal1* transcription is under the control of ROR (*Rora, b, c*) and REV-ERB (*Rev-erb a, b*) factors (Liu et al., 2008), we also noted loss of the rhythm in *Rorb* expression, whilst *Rev-erba* showed a phase advance by almost one hour (Table1).

Next, we examined the effect of loss of ZFH3 on rhythmic expression of the clock-controlled genes (CCGs). Clock-controlled genes are basically genes whose expression is regulated by circadian clock factors (such as CLOCK:BMAL1) that are directly associated with the TTFL. Several CCGs like DBP (D site albumin promoter binding protein) show rhythmic transcription (Lopez-Molina et al., 1997), and in turn control expression of a repertoire of genes, generating a cascade of 24-h cycling genes in the SCN (Herzog et al., 2017). In our mutant group, *Dbp* mRNA levels also presented an advanced peak (~1.66 h) with reduced amplitude (**Fig. 5C**). Another such TF-*Creb3l1* (cAMP responsive element binding protein 3-like 1), that has been implicated in intercellular coupling (Bedont et al., 2017), showed a distinctive change in phase and amplitude. Therefore, the alteration seen in the rhythmicity index at the level of TTFL genes due to the loss of ZFH3 was recapitulated in the diurnal expression of certain CCGs in the SCN.

Finally, we studied the effect of ZFH3 deletion on the rhythmic pattern of intercellular coupling agents (neuropeptides), whose overall mean expression levels were significantly reduced (**Fig. 3**, *Vip, Grp*). Along with lowered baseline expression, we noted severe loss in 24-h rhythm for crucial SCN neuropeptides such as *Avp* (**Fig. 4a**), *Prok2* and *Npy* (**Fig. 5c**). For *Avp*, there was no apparent increase in daytime mRNA expression (Arima et al., 2002), and the levels remained intermediate throughout the day. Conversely, for both *Prok2* and *Npy*, transcript levels were reduced to minimal levels, possibly resulting in compromised intercellular-coupling in the SCN.

The effects of ZFH3 on daily transcriptional profiles therefore varied between different gene categories. Whereas rhythmicity of downstream, clock-controlled genes was either independent of ZFH3 or required ZFH3 to be sustained, core circadian function was sustained but with altered dynamics. Intriguingly, this systematic approach not only highlighted the genes that lost 24-h rhythmic oscillations, such as *Bmal1* and the neuropeptide *Avp* (module 2, **Fig. 4A**), but also identified genes that retained rhythmicity with changes in their waveform (module 5). Given the fact that ZFH3 deficient mice do not lose rhythmic behaviour in a daily LD cycle (Wilcox et al., 2017) (**Fig. 2**), but do exhibit a short circadian period or arrhythmicity in free-running (complete darkness: DD) conditions, we believe our targeted SCN-specific assessment unravels the vital role of ZFH3 in the mechanistic regulation of the daily molecular clock.

Gene	Rhythm	Phase	Amplitude
<i>Per1</i>	Altered	-0.36	0.72 (low)
<i>Per2</i>	Altered	-2.0	0.69 (low)
<i>Per3</i>	Altered	-2.1	0.30 (low)
<i>Cry1</i>	No change	-	-
<i>Cry2</i>	No change	-	-
<i>Bmal1</i>	Lost	-	-
<i>Clock</i>	No change	-	-
<i>Nr1d1</i>	Altered	-0.99	0.67 (low)
<i>Nr1d2</i>	No change	-	-
<i>Rorb</i>	Lost	-	-

Table 1

Effect of loss of ZFH3 on core-clock genes in the SCN

The state of the daily SCN clock in ZFH3 deficient mice

In order to link the perturbations of the core molecular clock in the SCN of ZFH3 deficient mice with their altered behavioural profiles, we used TimeTeller, a recently established method to predict circadian clock phase alongside the parameter ‘Theta’ which provides an index of clock disruption from transcriptomic data (Vlachou et al., 2024). This approach allows one to assess the clock globally as a system instead of gene by gene. For this, an initial training model was built using the available Cre-neg and-Tamoxifen (n = 35, control) mice data (Fig. 6A). Cross-validation for the training model suggested that the model is informative and predicts the control SCN time well with a median prediction error of 0.08 hours (95% CI: -0.83; 1.33) and 0.50 hours (95% CI: -1.67; 3.00), i.e., the difference between the TimeTeller predicted time and the known ZT of SCN collection (Supplementary Fig. 5A). This was further assessed by predicting SCN timing in an independent dataset of SCN RNA-Seq (Deota et al., 2023). Similar to the TimeTeller predictions for the control group, median prediction error was 0.00 hours (95% CI: -2.00; 1.33) for the *ad libitum* fed and -0.66 hours (95% CI: -1.83; 1.00) for the temporary night-time restricted feeding groups. These are not distinguishable from the control SCN in the current study (Fig. 6B). Similarly, the clock disruption parameter theta is low (Medians: 0.03, 0.03 and 0.04) as is expected for a fully functional and well predictable circadian clock in all three control groups. Furthermore, TT clock functionality parameter theta suggests that +Tamoxifen SCN have a similarly functional daily clock compared to the control SCN (Median 0.04 vs. 0.03 in controls (Supplementary Fig. 5B)).

While TT’s theta metric suggests the clock in the ZFH3-deleted SCN is not disrupted as it is indistinguishable from controls, the phase of the +Tamoxifen SCN was consistently advanced by a median 1.83 hours (95.0% CI 0.585, 2.67), when compared to the current controls and the published data (Deota et al., 2023) as illustrated in Fig. 6B. Of note, detecting this ~2-h phase difference from data sampled at 4-h intervals showcases the advantages in robustness and sensitivity of assessing the clock as a system rather than by a gene-by-gene approach, and TimeTeller as a tool of inquiry. In summary, according to TimeTeller, the SCN daily clock is intact but advanced by approximately 2 h in comparison to the control groups after loss of ZFH3. This is consistent with the observed daily behavioural activity in mutant mice as shown in Fig. 2.

Discussion

The SCN is distinctive in its ability to sustain autonomous rhythmicity which is driven by circadian cascades of transcription that direct metabolic, electrophysiological and signalling rhythms. Our current findings highlight the instrumental role of the transcription factor, ZFH3, in driving the daily timekeeping mechanism. By comparing the daily behavioural activity of ZFH3-KO mice with the genetic matched control, we clearly noted an advancement in LD activity and shortening of circadian period in mutants. This finding is consistent with previous studies (Parsons et al., 2015; Wilcox et al., 2021; Wilcox et al., 2017) and prompted a detailed molecular investigation in the SCN. Through targeted ChIP-seq we successfully demonstrated the genome-wide occupancy of ZFH3 in SCN chromatin with 78% of ZFH3 bound sites found to be co-localized with modified histones (H3K4me3, H3K27ac), and a vast majority of these concentrated near gene TSS. This provides strong evidence in support of the observed pervasive regulation conferred by ZFH3 on the SCN transcriptome. That said, a considerable proportion of ZFH3 sites were also seen devoid of joint histone modifications (H3K4me3 + H3K27ac) and enriched for CTCF binding sites. CTCF is a ubiquitous DNA binding protein that has exceptional roles in genomic organization, chromatin looping and gene regulation (Holwerda & de Laat, 2013; Kim et al., 2015; Mach et al., 2022). This co-occurrence hints on the potential accessory role of ZFH3 in modifying genome topology and chromosomal looping to bring about changes in target gene expression by regulating promoter-enhancer interactions (Pugacheva et al., 2020). Interestingly,

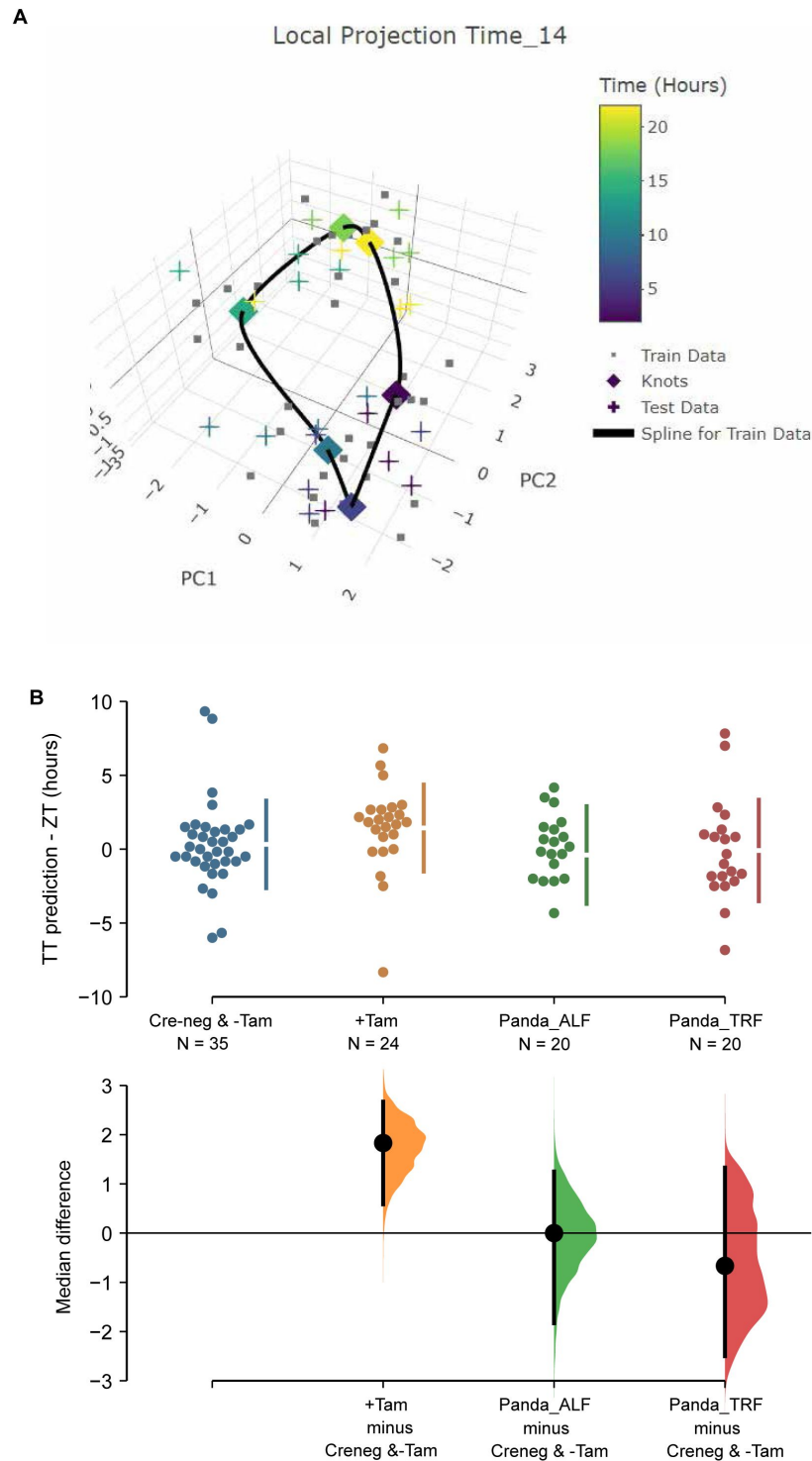


Figure 6.

TimeTeller model SCN circadian clock in control and ZFH3-KO mice.

(A) TimeTeller (TT) model build with the data of all 35 available SCN from Cre-neg and -Tam mice. Only the knots of the model at the collected ZTs, and predictions for test data of +Tam SCN according to when they were collected are shown. (B) Quantitation of SCN phases as difference between ZT to TT prediction for all genotypes of this study and previously published wild-type SCNs from ad libitum or night fed mice (Panda_ALF and Panda_TRF). Only the +Tam group is different from Cre-neg & -Tam control training data ($p=0.0028$).

along with CTCF binding sites, ZFH3 sites were also enriched for other SCN enriched regulatory factors; RFX, LHX, ZIC (Araki et al., 2004 [↗](#); Hatori et al., 2014 [↗](#)) (**Supplementary fig. 1D** [↗](#)), suggesting well-coordinated tissue-specific gene regulation.

Given the crucial role of ZFH3 in daily timekeeping, we speculated a dynamic change in its genomic occupancy and compared the binding intensity of the transcription factor at two anti-phasic (12-h apart, ZT3 vs. ZT15) time-points (Methods). Surprisingly, we did not detect any differential binding site(s) between the tested time-points, but rather noticed a uniform and coherent localization. This suggests that ZFH3 belongs to a family of poised transcription factors, whose occupancy at regulatory sites does not change during the day, but could potentially act as a co-activator/mediator to modulate oscillating gene transcription. Indeed, in a recent unpublished study (Del Rocío Pérez Baca et al., 2023 [↗](#)), ZFH3 was shown to be associated with chromatin remodelling and mRNA processing factors, strengthening the viewpoint as a co-regulator. It is, therefore, highly plausible that ZFH3 occupies the targeted TSS and partners with clock TFs such as BMAL1 (*CACGTG*), as observed around active sites, to regulate rhythmic gene transcription.

Along with ChIP-seq, we executed detailed RNA sequencing to study the effect of loss of ZFH3 specifically in the SCN. Crucially, ZFH3 affected almost a third of the transcriptome in the SCN. An overall (time-independent) assessment of deletion of *Zfhx3* highlighted a significant impact on expression of genes involved in several biological processes ranging from cellular differentiation to behavioural responses. We then performed a comprehensive time-dependent assessment to delineate the functional role of ZFH3 in regulating the circadian timekeeping mechanism. We observed about 55% of rhythmic genes showed alteration at distinct levels in their daily transcriptional pattern following loss of ZFH3.

At the TTFL level, ZFH3-KO resulted in the loss of rhythmic *Bmal1* gene expression. It remains to be determined whether this is associated with changes at the posttranslational level, given the findings by Abe et al where arrhythmic *Bmal1* transcript did possess posttranslational rhythm (Abe et al., 2022 [↗](#)), and could explain the subsequent 24-h driven *Per* (1-3) and *Cry* (1-2) mRNA rhythms noted in our study. Likewise, in a separate study constitutive *Bmal1* expression has been shown to sustain rhythmic levels of transcriptional and translational products of *Per*, *Cry*, *Clock*, *RORc*, and *Rev-erba* by means of mathematic modelling (Mirsky et al., 2009 [↗](#)). Arguably, clear circadian rhythmicity in BMAL1, CLOCK, or REV-ERBa protein levels were not sustained in association with constitutive *Bmal1* levels under *in-vitro* conditions (Padlom et al., 2022 [↗](#)). Therefore, in future it will be interesting to study the daily abundance of protein levels of the clock genes in *Zfhx3*-deficient SCN, with its associated loss of *Bmal1* mRNA 24-h rhythm, to gain a complete understanding of the clock molecular circuitry.

Broadly speaking, aside from *Bmal1*, the majority of clock genes did not lose 24-h rhythmicity but showed an early peak in expression, ranging between ~0.4 – 2-h that perfectly resonates with the advanced rhythmic behaviour seen in mutants. In this case, it is difficult to delineate a straightforward cause-and-effect axis in terms of the direct targets of the perturbed ZFH3 activity. Based on the current data, ZFH3 is possibly partnering with CLOCK:BMAL1 to regulate the daily transcription of clock genes (*Per*, *Ror*, *Reverb*) and CCGs. Two possible scenarios could explain the noted effect on the TTFL genes due to lack of ZFH3 (**Fig. 5** [↗](#)). First, ZFH3 preferentially controls *Per* family gene transcription (and not *Cry*) by binding at the promoter (**Fig. 1** [↗](#)). Loss of ZFH3 affects the daily rhythm of *Per* gene transcription, and potentially PER protein levels, which in turn disturbs the CLOCK:BMAL1 driven transcription cascades. This leads to an aberrant daily transcriptional profile of the *Ror* (activator)/ *Reverb* (repressor) genes as observed, which are known to control the amplitude and oscillation of *Bmal1* (Liu et al., 2008 [↗](#)), and further results in loss of *Bmal1* transcriptional rhythm. Therefore, the observed change in *Ror* and *Reverb* family gene expression levels could be reminiscent of the effect of ZFH3 on the TTFL. In a second scenario, the constitutive expression of *Bmal1* mRNA could result from the primary effect of ZFH3 on CLOCK:BMAL1 driven *Ror/Reverb* genes (affecting ROR/REVERB translational products),

which does not disrupt the TTFL, but weakens the daily clock. The direct /indirect effect is seen in the cyclic expression of *Per* family genes, with relatively greater phase change in *Per2* and *Per3* than the *Per1* gene (Kuhlman et al., 2003 [DOI](#)) (**Supplementary fig. 6** [DOI](#)). It is, however, noteworthy that the *CRY* family of genes (*Cry1*, *Cry2*) did not show any obvious change in the daily expression and is supposedly shielding the molecular clock, as recently postulated by Abe et al (Abe et al., 2022 [DOI](#)).

As previously reported (Parsons et al., 2015 [DOI](#)), lack of ZFH3 also resulted in severe reduction in the SCN neuropeptides such as *Avp*, *Vip*, *Grp*, *Prok2*, that potentially compromises intercellular coupling and synchronization of circadian timekeeping. This universal effect, where loss of ZFH3 does not deter a particular but all the key SCN neuropeptides, clearly points that 1) ZFH3 is prevalent in almost all neuronal populations within the SCN, that are largely clustered based on their neuropeptide expression (Morris et al., 2021 [DOI](#); Wen et al., 2020 [DOI](#)) 2) potentially results in loosely coupled intercellular clocks that are still able to drive 24-h behavioural and molecular rhythms but with advanced phase. The prevalence of ZFH3 in neuronal populations is also supported by a complete independent assessment in which almost 60% of the top 100 categorised neuronal genes in the SCN (as per single-cell atlas) were seen to be affected by ZFH3 deletion based on the current dataset. In contrast, only 20% of the 100 most expressed genes in the astrocytic cell populations were impacted.

Along with this, lack of *Zfh3* also leads to aberrant daily transcriptional rhythms of several CCGs, with the majority of them seen to lose daily oscillations. This could potentially result in hindrance of clock-regulated vital biological processes such as regulation of cell-cycle, metabolic pathways etc. Indeed, ZFH3 is reported to be crucial for the development of a functional SCN (Wilcox et al., 2021 [DOI](#)), and could act as an ‘integrator’ of afferent signals such as light and feeding to generate timely and robust physiological outputs. Moreover, genetic ablation of *Zfh3* has been shown to result in a compromised light sensing mechanism in retina (Hughes et al., 2021 [DOI](#)), while the missense mutation (*Zfxh3*^{Sci/+}) results in reduced growth and altered metabolic gene expression in the arcuate nucleus of the hypothalamus (Nolan et al., 2023 [DOI](#)) converging on its functional roles in other brain regions. The pleiotropic roles of ZFH3 further extend in non-brain tissues with its recently described crucial involvement in angiogenesis for tumor development (Fu et al., 2020 [DOI](#)), cardiac function (Jameson et al., 2023 [DOI](#)) and spinocerebellar ataxia (Figuerola et al., 2024 [DOI](#); Wallenius et al., 2024 [DOI](#)). It is important to note that although in the present study we used adult-specific *Zfh3* null mutants resulting in global loss of ZFH3, the circadian effects observed both at molecular and behavioural levels are independent of its pleiotropic role(s) in other tissues. In future, however, it will be beneficial to carry out the investigation using an inducible, SCN specific genetically ablated model to circumvent any confounding effects. Regardless, the current research is of great importance as it not only maps the genomic binding of ZFH3, but also demonstrates its critical association with both lineage- and clock-controlling TFs that perfectly resonates with the observed profound effects of ZFH3 on the SCN transcriptome, regulating a vast majority of genes ranging from circadian timing to synaptic transmission and inflammatory responses. This highlights how ZFH3 regulates the core-clock (TTFL) in addition to its control over CCGs in order to drive sustained transcriptional rhythms in the SCN. Given the extensive pleiotropy of ZFH3, it provides a solid substrate to further understand the functional interaction of ZFH3 in distinct tissues indispensable for overall health and fitness.

Methods

Mice

All animal studies were performed under the guidance issued by Medical Research Council in Responsibility in the Use of Animals for Medical Research (July 1993) and Home Office Project Licence 19/0004. WT C57BL/6J were maintained and provided in-house by MRC Harwell. Animals

were group housed (4-5 mice per age) in individually ventilated cages under 12:12h light-dark conditions with food and water available ad libitum. Adult specific knock out *Zfhx3* mice were generated using an inducible Cre line as described by Wilcox et al (Wilcox et al., 2017 [DOI](#)). Briefly, tamoxifen inducible Cre mice (UBC-Cre) were crossed to *Zfhx3* floxed (*Zfhx3*^{Flox}) mice to produce an initial stock of *Zfhx3*^{Flox/+};UBC-Cre⁺ mice. These were subsequently crossed to *Zfhx3*^{Flox/+} mice to generate experimental cohorts (Sun et al., 2012 [DOI](#)). Out of the 6 possible genotype combinations, 2 genotypes; *Zfhx3*^{Flox/Flox};UBC-Cre⁻ (mice homozygous for *Zfhx3*^{Flox} allele but not carrying the Cre allele) and *Zfhx3*^{Flox/Flox};UBC-Cre⁺ (mice homozygous for *Zfhx3*^{Flox} allele and hemizygous for Cre allele) were assigned as “control” (Cre-neg) and “experimental” (+Tamoxifen) groups, respectively, and dosed with tamoxifen. In addition, we also included a cohort of *Zfhx3*^{Flox/Flox};UBC-Cre⁺ mice without tamoxifen treatment as a secondary control (-Tamoxifen). Animals were group housed (4-5 mice per age) in individually ventilated cages under 12:12h light-dark conditions with food and water available ad libitum.

Experimental Design

WT C57BL/6J mice (aged between 8 to 12 weeks) were used for SCN tissue punch collection as described by Jagannath et al. (Jagannath et al., 2013 [DOI](#)) at ZT3 and ZT15, where lights on at 7 am (ZT0) and lights off at 7 pm (ZT12), for ZFHx3-ChIP-seq. For SCN RNA-Seq, mice from control (Cre-neg and-Tamoxifen) and *Zfhx3* knock out (+Tamoxifen) cohorts were used for SCN tissue collection (Bafna, Lau, et al., 2023) at 6 distinct time-points starting from ZT2 at every 4h. To achieve high resolution transcriptomic dataset, four biological replicates per time-point were collected independently from the control (Cre-neg) and experimental (+Tamoxifen) groups. For the second control group viz.-Tamoxifen; 2-4 biological replicates were collected due to time constraints. Furthermore, each biological replicate for all the three tested condition constituted 3 individual SCN samples.

Animal behaviour

Circadian wheel running was performed as previously described (Banks & Nolan, 2011 [DOI](#)). Briefly, mice were singly housed in cages containing running wheels, placed in light controlled chambers and wheel running activity monitored via ClockLab (Actimetrics). Animals were given a five-day acclimatisation period in a 12-h light/dark cycle (100 lux light intensity) before their activity was monitored for 32 days in constant darkness and 24 days under a 12-h light/dark cycle (100 lux light intensity). Circadian activity analysis was performed upon the entire of the period in constant darkness and upon the final seven days in the light/dark cycle. Circadian analysis was performed using ClockLab Version 6 (<https://actimetrics.com/products/clocklab/clocklab-analysis-version-6/> [DOI](#)). *Zfhx3*^{Flox/Flox};UBC-Cre⁺ animals were used for this analysis, with animals being randomly assigned into tamoxifen un-dosed (-Tamoxifen) or tamoxifen dosed (+Tamoxifen) cohorts. The numbers of animals per cohort were: male-Tamoxifen = 8; male +Tamoxifen = 8; female-Tamoxifen = 8; female +Tamoxifen = 8. Statistical analysis and graphing of circadian data was performed using GraphPad Prism 8.

SCN tissue collection for RNA-seq

Animals were sacrificed at the aforementioned time-points by cervical dislocation and brains were removed and flash frozen on dry ice. The frozen brains were placed on dry ice along with the brain matrix (Kent Scientific, Torrington CT, USA) and razor blades to chill. The brain dissection was performed on a vibrating microtome (7000smz-2 Vibrotome, Campden Instruments) under chilled conditions (Bafna, Lau, et al., 2023). First, the frozen brain was placed on the chilled brain matrix and a ca. 3mm thick region was removed from the caudal end using a razor blade to discard the cerebellum and attain flat surface. Then, the dissected brain was super-glued to a chilled (2 –3°C) metal chuck and placed into the metal specimen tray of the Vibratome. The metal specimen tray was filled with pre-chilled 1X RNase free PBS (phosphate buffered saline, ThermoFisher Scientific, #AM9625). Coronal slices measuring 250 μ m in thickness were cut from

rostral to caudal at slow speed (0.07mm/s) and pre-tested specifications; Frequency: 70Hz, Amplitude: 1.00mm. Once cut, the slice with intact optic chiasm, third ventricle and bilateral SCN tissues (Fig. S1) was selected and transferred on to pre-chilled glass slides (ThermoFisher Scientific SuperFrost Plus Adhesion slides) with the help of paint brush. The glass slide was placed on top of cold block and SCN tissues were collected by pre-chilled forceps and dissection needles under dissection microscope (Nikon SMZ645 Stereo Microscope).

ZFH3 ChIP and sequencing

ChIP was conducted using the Active Motif ChIPOIT enzymatic kit (<https://www.activemotif.com/catalog/868/chip-it-high-sensitivity>) according to the manufacturer's instructions. Briefly, SCN punches were pooled from 80 mice at each designated times (ZT3, ZT15) corresponding to one biological replicate per timepoint, and fixed for 15 min at room temperature with gentle shaking in 1% formaldehyde. The crosslinking reaction was stopped by addition of glycine to a final concentration of 0.125 M. Crosslinked chromatin was sheared by with Active Motif's EpiShear probe sonicator (cat# 53051). Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by SPRI beads clean up (Beckman Coulter) and quantitation by Clariostar (BMG Labtech).

24 ug sheared chromatin sample collected from each time point (ZT3, ZT15) was incubated with 12ul of rabbit polyclonal antibody against ZFH3 (Parsons et al., 2015), together with magnetic beads, on a rotator at 4°C overnight. This was followed by crosslinking reversal and proteinaseOK digestion as per the manufacturer's protocol. The recovered DNA was purified using PCR purification reagents and subjected to quantitative PCR for quality inspection. ZFH3 immunoprecipitated genomic DNA along with their corresponding input samples was sequenced on Illumina (75-bp, single end reads: ~30 million reads/sample) NextSeq 500 sequencing platform by Active Motif Epigenomic Services.

ZFH3 ChIP-seq peak calling and data analysis

Raw sequence data was quality assessed by removing low-quality bases (Phred < 20) and trimmed using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and Trimmomatic v0.36 (<http://www.usadellab.org/cms/index.php?page=trimmomatic>), respectively. FASTQ files containing trimmed sequences were then aligned to mm10 genome assembly to generate binary alignment map (BAM) files and used for peak calling by MACS algorithm v2.1.0 (Zhang et al., 2008) at a threshold of $q < 0.01$. Finally, peaks from ZT3 and ZT15 were analysed for differential binding by a Bioconductor package Diffbind v2.10.0 (<http://bioconductor.org/packages/release/bioc/vignettes/DiffBind/inst/doc/DiffBind.pdf>) at default settings with $p < 0.05$ and fold change $> \pm 2$. No significant differential ZFH3 bound peaks were detected between ZT3 and ZT15. In order to investigate the ZFH3 peaks overlapping with histone modifications (H3K4me3, H3K27ac) in the SCN, data was downloaded from NCBI Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) accession number GSE217943. The data was normalized using bamCoverage tools and bigwig files were generated for visual inspection using UCSC Genome Browser and processed using BEDTools suite (Quinlan & Hall, 2010). ChIPSeeker package (Yu et al., 2015) was used to retrieve the nearest gene around resultant peaks and to annotate the genomic region of the peak.

RNA extraction

Total RNA from each sample replicate was extracted using RNeasy Micro Kit (QIAGEN, #74004) following the protocol for microdissected cryosections as per manufacturer's guidelines and stored at -80°C. Quality and quantity of RNA were measured using an Agilent Bioanalyzer (Pico chip) and a Nanodrop1000 (Thermo Fisher Scientific, Waltham, MA USA), respectively.

RNA Sequencing and data analysis

PolyA RNA-Seq libraries were prepared by Oxford Genomics Centre, University of Oxford using NEBNext Ultra II Directional RNA Library Prep Kit (NEB, #E7760) and sequenced on NovaSeq6000_150PE (150 bp paired-end directional reads: ~40 million reads/sample) platform. Paired-end FASTQ files were quality assessed (Phred<20 removed) with FastQC and Illumina adapters were trimmed with TrimGalore (v0.4.3). Then, the reads were aligned to mm10 genome assembly using STAR (v2.7.8a) with MAPQ value for unique mappers set to 60. Binary alignment map (BAM) files were used to generate read counts per gene by FeatureCounts via Samtools (v1.11). Finally, limma-voom method (Liu et al. 2015) from the Bioconductor package-limma (v3.48.0) was adopted to quantify differential gene expression and normalised logarithmic CPM (counts per million) values were generated for downstream analysis. Genes without more than 0.5 CPM in at least 2 samples are insignificant and filtered out. 8582 of 24421 (35.14%) genes were filtered out for low expression. TMM was the method used to normalise library sizes.

To study the overlap between genes with ZFH3 occupancy at TSS and those impacted by *Zfh3* deletion, R-package GeneOverlap (<https://bioconductor.org/packages/release/bioc/html/GeneOverlap.html>) was adopted where overlap between two gene lists was tested using Fisher's exact test with P-value = 0 meaning the overlap is highly significant. The Fisher's exact test also gives an odds ratio which represents the strength of association. If an odds ratio is equal to or less than 1, there is no association between the two lists. If the odds ratio is much larger than 1, then the association is strong. The class also calculates the Jaccard index which measures the similarity between two lists. The Jaccard index varies between 0 and 1, with 0 meaning there is no similarity between the two and 1 meaning the two are identical.

Differential rhythm analysis

The read counts per gene (n = 24,421) for each analysed sample was obtained by FeatureCounts and arranged in sample matrix by using coreutils (v8.25). The matrix file was used as an input for *dryR* (R package for Differential Rhythmicity Analysis) available at <https://github.com/neaf-lab/dryR>. Normalized gene expression from “control” (Cre-neg) and “experimental” (+Tamoxifen) groups were categorized into 5 modules with a threshold of BICW (Bayes information criterion weight) > 0.4 based on their time-resolved expression pattern.

RT-qPCR

Total RNA was extracted from the SCN tissue as described above from three/ four biological replicates per genotype (-Tamoxifen vs. +Tamoxifen), where each biological replicate constitute one independent SCN tissue. 10 ng RNA per sample replicate was tested by one-step RT_qPCR reagent (PrecisionPLUS OneStep RT-qPCR Master Mix with ROX at a reduced level premixed with SYBRgreen, PrimerDesign) using an Applied Biosystems 7500 realtime PCR instrument (10 min at 55°C, 8 min at 95°C, 40× 10 sec at 95°C, and 60 sec at 60°C, followed by meltcurve analysis 65°C–95°C in 0.5°C increments at 5 sec per step) in triplicates for *Zfh3* expression in control and mutant groups. 7500 Applied Biosystems Software v2.3 (<https://www.thermofisher.com/uk/en/home/technical-resources/software-downloads/applied-biosystems-7500-real-time-pcr-system.html>) was used to obtain the relative quantification values and examine melt curves. All RT-qPCR data was normalized to the *Rpl32* reference control gene and analysed using the standard curve method (Larionov et al., 2005). The primers used for *Zfh3* (CTGTGCCAAGACATGCTCAAC [forward] and CAGCAGGGAGGAACATGCTA [reverse]) and *Rpl32* (AGGCACCAGTCAGACCGATA [forward] and TGTGTTGGCATCAGGATCTGG [reverse]) were designed and screened for target specificity with the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/>). The primer sets were validated before use (requirements: no predicted off-targets, primer efficiency 85-120%, no significant signal in nontemplate control, single peak in melt curve, and single band at the predicted size when separated via agarose gel

electrophoresis). ZFHX3 gene expression differences from qPCR were plotted and analyzed with GraphPad Prism v9.5.1 for Windows, GraphPad Software (www.graphpad.com). Unpaired-two tailed t-test between control and mutant group across the tested time points was set at $\alpha = 0.05$.

Gene annotation

The gene list derived from Bioconductor-based package ChIPseeker v1.28.3 (Yu et al., 2015) was fed into the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool (Dennis et al., 2003). The functional annotation chart based on KEGG pathway, Gene Ontology (GO): biological processes (BP) was plotted with the help of the ggplot2 package in Rv4.0.5 (<https://ggplot2.tidyverse.org/>).

HOMER motif analysis

The findMotifsGenome.pl function within the HOMER v4.11 package (Heinz et al., 2010) was used to identify enriched motifs and their corresponding TFs with options size 200 –len 8, 10, 12 –mask –prepare –dumpfasta, with default background regions.

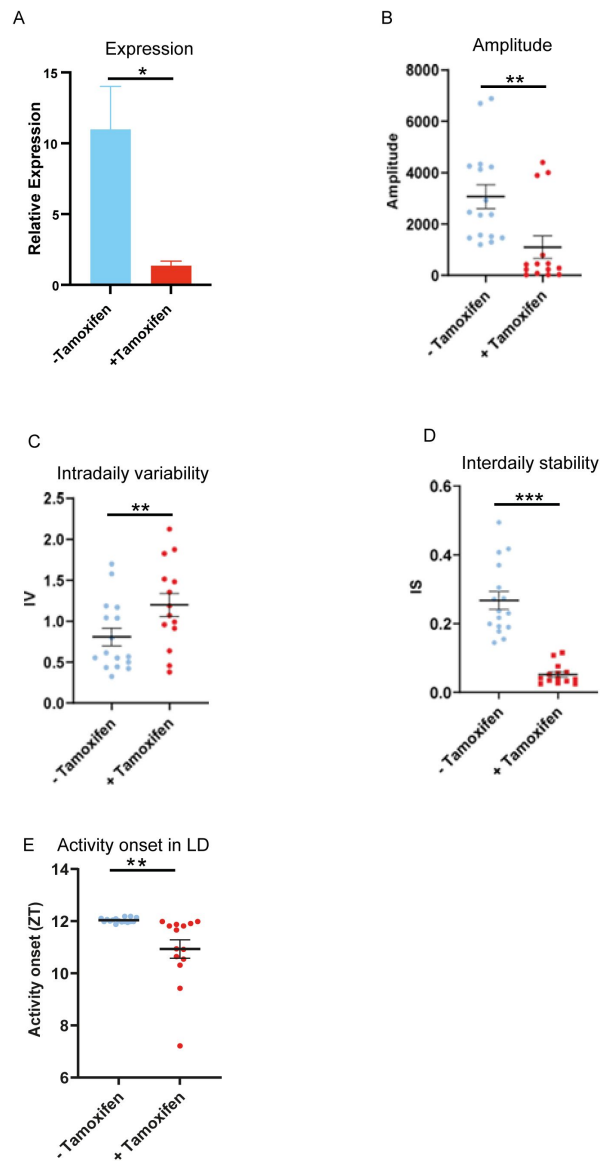
TimeTeller modelling of the SCN clock

TimeTeller (TT) analysis was conducted using all 35 available SCN time-course RNA-Seq samples from control (Cre-neg and-Tamoxifen) mice to train the model (Vlachou et al., 2024). Data from +Tamoxifen SCN were then projected into the model as test samples. Based on the available training data, we selected the 10 most rhythmic and consistent genes to build the TimeTeller model, in this case: *Bmal1*, *Per2*, *Cry1*, *Tef*, *Hlf*, *Dbp*, *Nr1d2*, *Npas2*, *Ciart*, and *Bhlhe41*. As a first step to cross-validate the model, we used a “leave-one-out” approach. Iteratively, 5-6 randomly chosen samples from all Cre-neg and-Tamoxifen SCN were left out from the training data to build the TT model, and the left out samples were treated as test samples to determine the TT prediction for all Cre-neg and-Tamoxifen SCN. In addition, we used published SCN RNA-Seq data to independently validate the full model with all 35 control SCN from this study (Deota et al., 2023). TT analysis predicts phase and clock functionality (theta) based on the averaged likelihood for the projected test sample. For the SCN model, we used time-course normalization with log threshold selected at 6. Further, to compare TT phase predictions between genotypes, we used estimation statistics (Ho et al., 2019) and p-values were generated using a permutation testing with 5000 bootstrap samples.

Data availability

All raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE261795>) under accession number GSE261795.

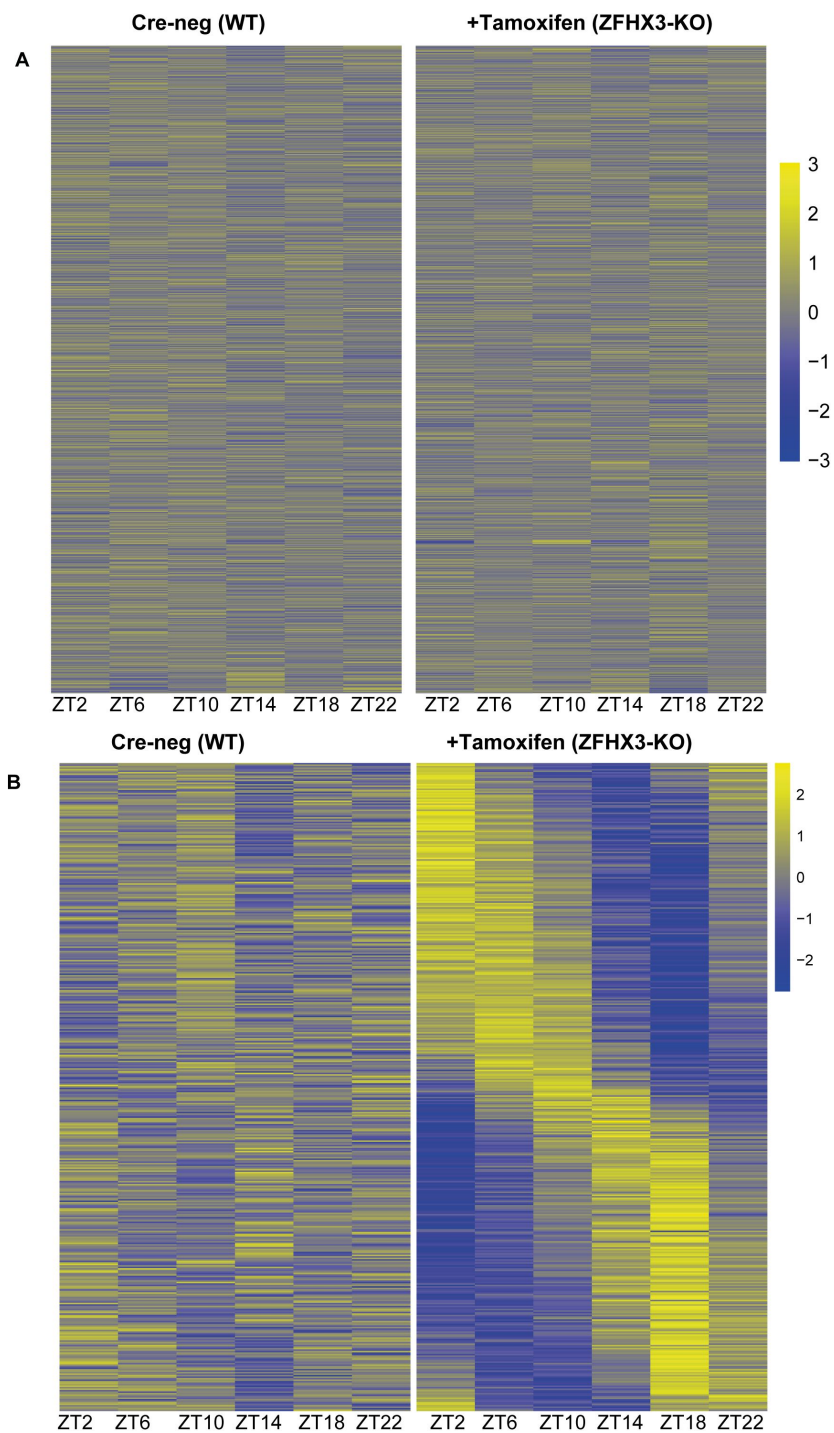
Supplementary figures



Supplementary Figure 2.

+Tamoxifen animals show changes in circadian wheel running parameters in conditions of constant darkness.

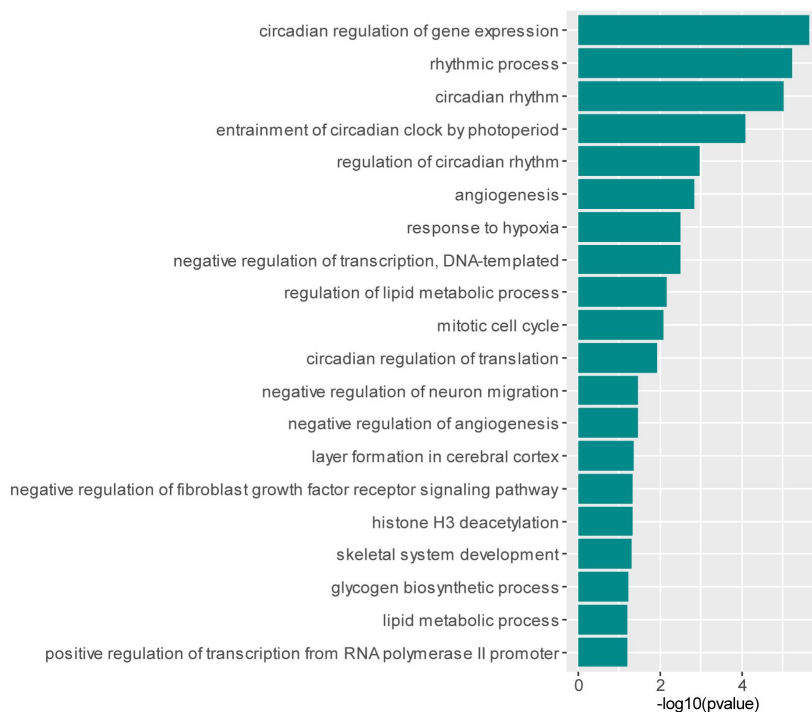
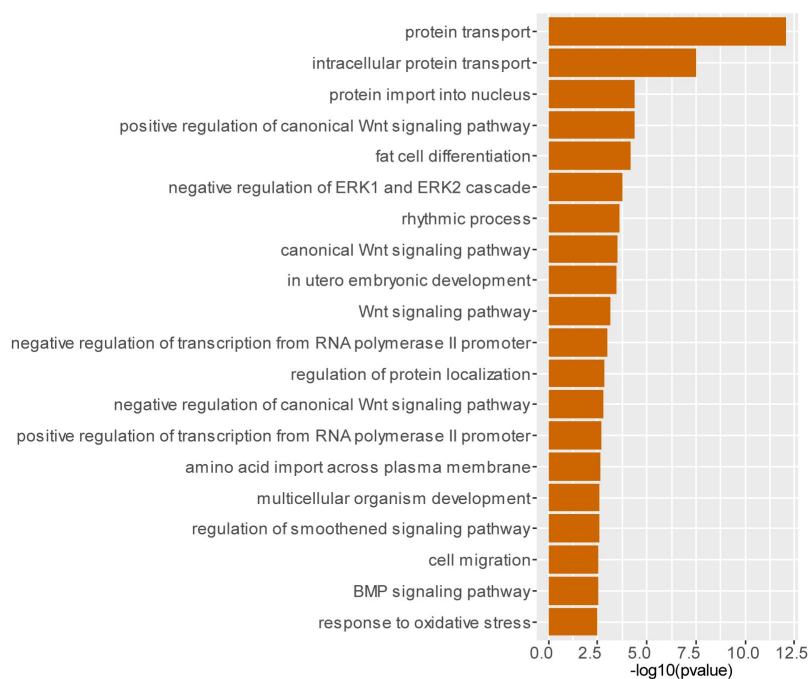
Compared to -Tamoxifen animals, +Tamoxifen show a significantly (A) reduced *Zfhx3* gene expression ($p < 0.02$, unpaired t-test) (B) reduced circadian amplitude, (C) increased Intradaily variability (IV), (D) reduced Interdaily stability (IS) and (E) advanced activity onset in LD.



Supplementary Figure 3.

Effect of *Zfhx3*-KO on SCN transcriptome.

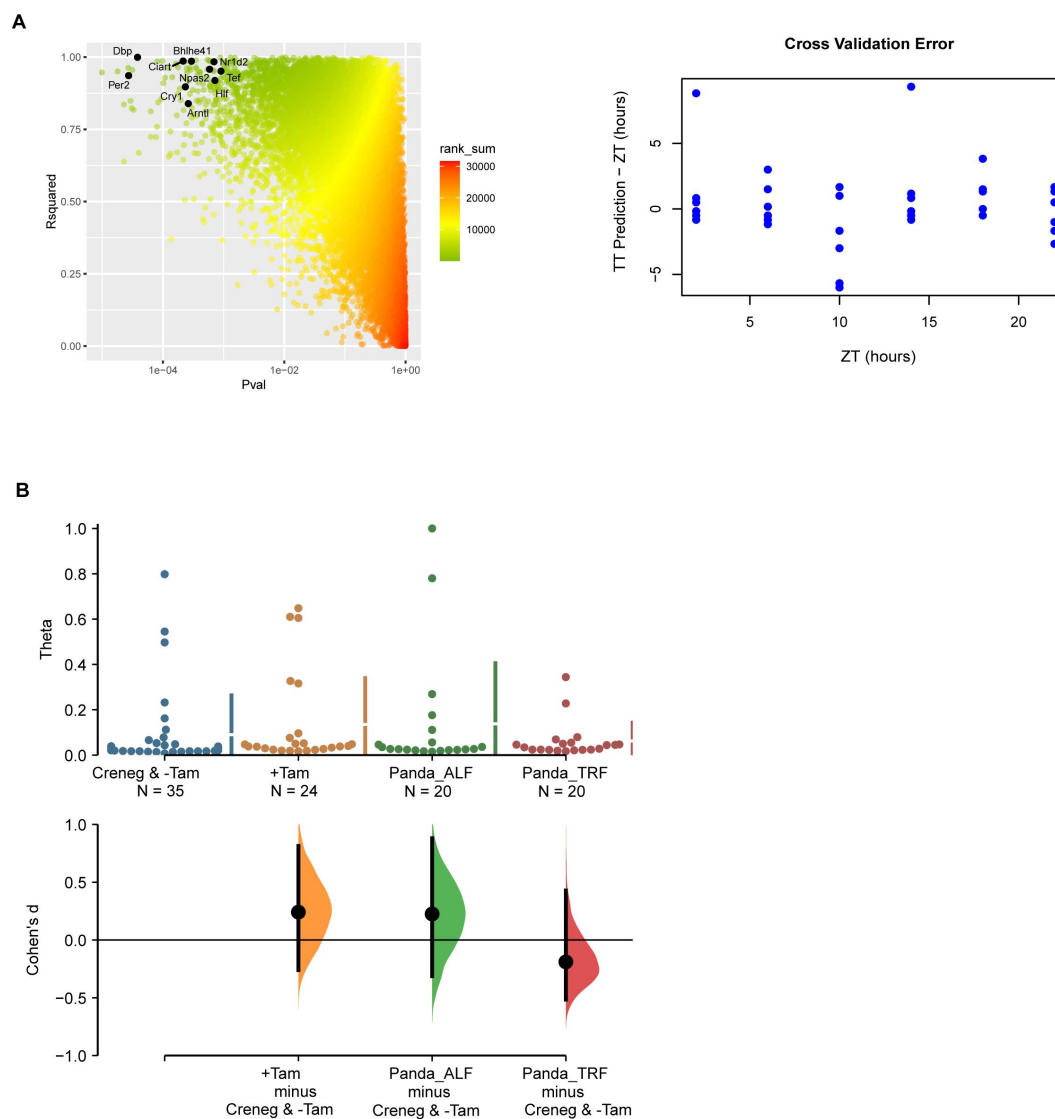
(A) Heatmap showing module 1 genes (n = 16832) that show no rhythmic expression before and after *Zfhx3*-KO as computed by dryR statistical framework (B) Heatmap showing module 3 genes (n = 403) that showed rhythmic expression after *Zfhx3*-KO.



Supplementary Figure 4.

Functional annotation of rhythmic genes in the SCN.

(A) Top 20 enriched functional pathways from genes that lost rhythmic transcription after gene deletion (module 2) and (B) genes that showed change in phase and/or amplitude (module 5) using the GO::BP (biological processes) terms by DAVID.



Supplementary Figure 5.

SCN circadian clock using TimeTeller.

(A) (Left) Choosing genes for the TimeTeller model. Looking for good rhythmicity p-val and high R squared in population cosinor analysis leads to selection of a limited panel of well-behaved clock genes (named), i.e., clock genes with adjusted pval < 0.10 and R squared > 0.80. (Right) Results of the cross-validation of the TT model using the indicated genes in the left panel. (B) TimeTeller theta parameter is indistinguishable between the genotypes. Theta is a measure of certainty for phase prediction in TimeTeller, i.e., how closely the test samples can be projected into the training model.

Acknowledgements

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Additional information

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Author contribution

AB, GB and PMN conceived the study. AB and GB were involved in sample collection and processing. AB and PMN carried out the investigation with AB conducting the formal data analysis. Data visualization was performed by AB, GB, MHH and PMN. RD and VV carried the data modelling and TT prediction. Supervision of the research was carried out by MHH and PMN. The original draft was written by AB. Manuscript was reviewed, edited and finalised by AB, GB, RD, VV, MHH and PMN.

Additional files

Supplementary Table 1 [↗](#)

Supplementary Table 2 [↗](#)

Supplementary Table 3 [↗](#)

Supplementary Table 4 [↗](#)

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Reviewer #1 (Public review):

Summary:

Authors of this article have previously shown the involvement of the transcription factor Zinc finger homeobox-3 (ZFHX3) in the function of the circadian clock and the development/differentiation of the central circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. Here, they show that ZFHX3 plays a critical role in the transcriptional regulation of numerous genes in the SCN. Using inducible knockout mice, they further demonstrate that the deletion of *Zfhx3* induces a phase advance of the circadian clock, both at the molecular and behavioral levels.

Strengths:

- Inducible deletion of *Zfhx3* in adults
- Behavioral analysis
- Properly designed and analyzed ChIP-Seq and RNA-Seq supporting the conclusion of the behavioral analysis

Comments on revisions:

The authors have properly addressed reviewers' issues.

<https://doi.org/10.7554/eLife.102019.2.sa2>

Reviewer #2 (Public review):

Summary

ZFHX3 is a transcription factor expressed in discrete populations of adult SCN and was shown by the authors previously to control circadian behavioral rhythms using either a dominant missense mutation in *Zfhx3* or conditional null *Zfhx3* mutation using the Ubc-Cre line (Wilcox et al., 2017). In the current manuscript, the authors assess the function of ZFHX3 by using a multi-omics approach including ChIPseq in wildtype SCNs and RNAseq of SCN tissues from both wildtype and conditional null mice. RNAseq analysis showed a loss of oscillation in *Bmal1* and changes in expression levels of other clock output genes. Moreover, a phase advance gene transcriptional profile using the TimeTeller algorithm suggests the presence of a regulatory network that could underlie the observed pattern of advanced activity onset in locomotor behavior in knockout mice.

In Figure 1, the authors identified the ZFHX3 bound sites using ChIPseq and compared the loci with other histone marks that occur at promoters, TSS, enhancers and intergenic regions. And the analysis broadly points to a role for ZFHX3 in transcriptional regulation. The vast

majority of nearly 40000 peaks overlapped H3K4me3 and K27ac marks, active promoters which also included genes falling under the GO category circadian rhythms. However, no significant differential ZFH3 bound peaks were detected between ZT3 and ZT15. In these experiments, it is not clear if and how the different ChIP samples (ZFH3 and histone PTM ChIPs) were normalized/downsampled for analysis. Moreover, it seems that ZFH3 binding or recruitment has little to do with whether the promoters are active.

Based on an enrichment of ARNT domains next to K4Me3 and K27ac PTMs, the authors propose a model where the core-clock TFs and ZFH3 interact. If the authors develop other assays beyond just predictions to test their hypothesis, it would strengthen the argument for a role in circadian transcription in the SCN. It would be important in this context to perform a ChIP-seq experiment for ZFH3 in the knockout animal (described from Figure 2 onwards) to eliminate the possibility of non-specific enrichment of signal from 'open chromatin'. Alternatively, a ChIPseq analysis for BMAL1 or CLOCK could also strengthen this argument to identify the sites co-occupied by ZFH3 and core-clock TFs.

Next, they compared locomotor activity rhythms in floxed mice with or without tamoxifen treatment. As reported before in Wilcox et al 2017, the loss of ZFH3 led to a shorter free running period and reduced amplitude and earlier onset of activity. Overall, the behavioral data in Figure 2 and supplementary figure 2 has been reported before and are not novel.

Next, the authors performed RNAseq at 4hr intervals on wildtype and knockout animals maintained in light/dark cycles to determine the impact of loss of ZFH3. Overall transcriptomic analysis indicated changes in gene expression in nearly 36% of expressed genes, with nearly half being upregulated while an equal fraction was downregulated. Pathways affected included mostly neuropeptide neurotransmitter pathways. Surprisingly, there was no correlation between the direction in change in expression and TF binding since nearly all the sites were bound by ZFH3 and the active histone PTMs. The ChIP-seq experiment for ZFH3 in the UBC-Cre+Tam mice again could help resolve the real targets of ZFH3 and the transcriptional state in knockout animals.

To determine the fraction of rhythmic transcripts, Using dryR, the authors categorise the rhythmic transcriptome (about 7% in all) into modules that include genes that lose rhythmicity in the KO, gain rhythmicity in the KO or remain unaffected or partially affected. The analysis indicates that a large fraction of the rhythmic transcriptome is affected in the KO model. However, among core-clock genes only *Bmal1* expression is affected showing a complete loss of rhythm. The authors state a decrease in *Clock* mRNA expression (line 294) but the panel figure 4A does not show this data. Instead it depicts the loss in *Avp* expression - misstated in line 321 (we noted severe loss in 24-h rhythm for crucial SCN neuropeptides such as *Avp* (Fig. 3a)).

However, core-clock genes such as *Pers* and *Crys* show minor or no change in expression patterns while *Per2* and *Per3* show a ~2hr phase advance. While these could only weakly account for the behavioral phase advance, the authors used TimeTeller to assess circadian phase in wildtype and ZFH3 deficient mice. This approach clearly indicated that while the clock is not disrupted in the knockout animals, the phase advance can be correctly predicted from a network of gene expression patterns.

Strengths

The authors use a multiomic strategy in order to reveal the role of the ZFH3 transcription factor with a combination of TF and histone PTM ChIPseq, time-resolved RNAseq from wildtype and knockout mice and modeling the transcriptomic data using TimeTeller. The RNAseq experiments are nicely controlled and the analysis of the data indicates a clear impact on gene-expression levels in the knockout mice and the presence of a regulatory network that could underlie the advanced activity onset behavior.

Weaknesses

It is not clear whether ZFHX3 has a direct role in any of the processes and seems to be a general factor that marks H3K4me3 and K27ac marked chromatin. Why it would specifically impact the core-clock TTFL clock gene expression or indeed daily gene expression rhythms is not clear either. Details for treatment of different ChIP samples (ZFHX3 and histone PTM ChIPs) on data normalization for analysis are needed. The loss of complete rhythmicity of Avp and other neuropeptides or indeed other TFs could instead account for the transcriptional deregulation noted in the knockout mice.

Comments on revisions:

The authors addressed the majority of my criticisms. They also explained that some requested experiments are beyond the scope of the current manuscript, while others are technically not feasible. I do not have any further concerns.

<https://doi.org/10.7554/eLife.102019.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

Authors of this article have previously shown the involvement of the transcription factor Zinc finger homeobox-3 (ZFHX3) in the function of the circadian clock and the development/differentiation of the central circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. Here, they show that ZFHX3 plays a critical role in the transcriptional regulation of numerous genes in the SCN. Using inducible knockout mice, they further demonstrate that the deletion Of Zfhx3 induces a phase advance of the circadian clock, both at the molecular and behavioral levels.

Strengths:

- Inducible deletion of Zfhx3 in adults
- Behavioral analysis
- Properly designed and analyzed ChIP-Seq and RNA-Seq supporting the conclusion of the behavioral analysis

Weaknesses:

- Further characterization of the disruption of the activity of the SCN is required.

(1) We thank the reviewer for their valuable inputs. Indeed, a comprehensive behavioral assessment of mice of this genotype was executed in Wilcox et al. ;2017 study. In Wilcox et al.; 2017, Figure 4, 6-h phase advance (jetlag) clearly showed faster reentrainment in ZFHX3-KO mice when compared to the controls.

- The description of the controls needs some clarification.

(2) We agree with the reviewer and have modified the text at line 211-212 to clearly describe the controls.

Reviewer #2 (Public review):

Summary:

*ZFH3 is a transcription factor expressed in discrete populations of adult SCN and was shown by the authors previously to control circadian behavioral rhythms using either a dominant missense mutation in *Zfhx3* or conditional null *Zfhx3* mutation using the Ubc-Cre line (Wilcox et al., 2017). In the current manuscript, the authors assess the function of ZFH3 by using a multi-omics approach including ChIPseq in wildtype SCNs and RNAseq of SCN tissues from both wildtype and conditional null mice. RNAseq analysis showed a loss of oscillation in *Bmal1* and changes in expression levels of other clock output genes. Moreover, a phase advance gene transcriptional profile using the TimeTeller algorithm suggests the presence of a regulatory network that could underlie the observed pattern of advanced activity onset in locomotor behavior in knockout mice.*

In figure1, the authors identified the ZFH3 bound sites using ChIPseq and compared the loci with other histone marks that occur at promoters, TSS, enhancers and intergenic regions. And the analysis broadly points to a role for ZFH3 in transcriptional regulation. The vast majority of nearly 40000 peaks overlapped H3K4me3 and K27ac marks, active promoters which also included genes falling under the GO category circadian rhythms. However, no significant differential ZFH3 bound peaks were detected between ZT3 and ZT15. In these experiments, it is not clear if and how the different ChIP samples (ZFH3 and histone PTM ChIPs) were normalized/downsampled for analysis. Moreover, it seems that ZFH3 binding or recruitment has little to do with whether the promoters are active.

(3) We thank the reviewer for their valuable comment. Different ChIP samples (ZFH3 and histone PTM ChIPs) were treated in the same manner from preprocessing (quality control by FastQC, adapter trimming, alignment to mm10 genome) and peak calling was performed using respective input samples as control using MACS2 as mentioned in Methods. The data was normalized using bamCoverage tools and bigwig files were generated for visual inspection using UCSC Genome Browser. These additional details are added to Methods at line 592. Finally, BEDTools was employed to study overlapping peaks between ZFH3 and histone PTMs.

We agree that, alone, the current data does not make any claim for ZFH3 being crucial for promoter to be active. Our data clearly suggests that a vast majority of ZFH3 genomic binding in the SCN was observed at active promoters marked by H3K4me3 and H3K27ac and potentially regulating gene transcription.

Based on an enrichment of ARNT domains next to K4Me3 and K27ac PTMs, the authors propose a model where the core-clock TFs and ZFH3 interact. If the authors develop other assays beyond just predictions to test their hypothesis, it would strengthen the argument for role in circadian transcription in the SCN. It would be important in this context to perform a ChIP-seq experiment for ZFH3 in the knockout animal (described from Figure 2 onwards) to eliminate the possibility of non-specific enrichment of signal from 'open chromatin'. Alternatively, a ChIPseq analysis for BMAL1 or CLOCK could also strengthen this argument to identify the sites co-occupied by ZFH3 and core-clock TFs.

(4a) We agree that follow-up experiments such as BMAL1/CLOCK ChIPseq suggested by the reviewer will further confirm the proposed interaction of ZFH3 with core-clock TFs. However, this is beyond the scope of the current study.

(4b) Again, conducting complementary ChIPseq in ZFH3 knockout mice will strengthen the findings, but conducting TF-ChIPseq in a specific brain tissue such as the SCN (unlike peripheral tissues such as liver) does not only warrant use of multiple animals per sample but is also technically challenging and time-consuming to ensure specificity of the sample. For these reasons, datasets such as ours on the SCN are uncommon. Furthermore, in this particular context, we are certain that, based on current dataset, the ZFH3 peaks (narrow) we observed were well-defined and met the specified statistical criteria mitigating any risk of signal arising from non-specific enrichment from open-chromatin regions.

Next, they compared locomotor activity rhythms in floxed mice with or without tamoxifen treatment. As reported before in Wilcox et al 2017, the loss of ZFH3 led to a shorter free running period and reduced amplitude and earlier onset of activity. Overall, the behavioral data in Figure 2 and supplementary figure 2 has been reported before and are not novel.

(5) We recognise that a detailed circadian behavior assessment from adult mice lacking ZFH3 has been conducted previously by Nolan lab (Wilcox et al; 2017). In the current study, however, we used a separate cohort of mice, to focus on the behavioral advance noted in 24-h LD cycle and generated a more refined assessment. Importantly, these mice were also used for transcriptomic studies as detailed in Figure 3, which we consider to be a positive feature of our experimental design: behavior and molecular analyses were performed on the same animals.

Next, the authors performed RNAseq at 4hr intervals on wildtype and knockout animals maintained in light/dark cycles to determine the impact of loss of ZFH3. Overall transcriptomic analysis indicated changes in gene expression in nearly 36% of expressed genes, with nearly half being upregulated while an equal fraction was downregulated. Pathways affected included mostly neuropeptide neurotransmitter pathways. Surprisingly, there was no correlation between the direction in change in expression and TF binding since nearly all the sites were bound by ZFH3 and the active histone PTMs. The ChIP-seq experiment for ZFH3 in the UBC-Cre+Tam mice again could help resolve the real targets of ZFH3 and the transcriptional state in knockout animals.

(6) We agree with the reviewer that most of the differentially expressed genes showed ZFH3 binding at active promoter sites. That said, the current dataset is in line with recently published ZFH3-CHIPseq data by Baca et al; 2024 [PMID: 38412861] in human neural stem cells and Hu et al; 2024 [PMID: 38871709] in human prostate cancer cells that clearly suggests ZFH3 binds at active promoters and act as chromatin remodellers/mediators that modulate gene transcription depending on the accessory TFs assembled at target genes. Therefore, finding no correlation in the direction of change in expression is not striking.

To determine the fraction of rhythmic transcripts, Using dryR, the authors categorise the rhythmic transcriptome into modules that include genes that lose rhythmicity in the KO, gain rhythmicity in the KO or remain unaffected or partially affected. The analysis indicates that a large fraction of the rhythmic transcriptome is affected in the KO model. However, among core-clock genes only Bmal1 expression is affected showing a complete loss of rhythm. The authors state a decrease in Clock mRNA expression (line 294) but the panel figure 4A does not show this data. Instead it depicts the loss in Avp expression - misstated in line 321 (we noted severe loss in 24-h rhythm for crucial SCN neuropeptides such as Avp (Fig. 3a)).

(7a) Indeed, among the core-clock genes rhythmic expression is lost after ZFH3 knockout only for Bmal1. However, given the mice were rhythmic (as assessed by wheel-running

activity) in LD conditions, the observed 24-h gene expression rhythm in the majority of core-clock genes (Pers and Crys) is consistent with behavior data, and suggests towards an altered molecular clock with plausible scenarios as explained at line 439. That said, the unique and well-defined changes (amplitude and phase) observed as demonstrated in Figure 5 highlights a model in which ZFHX3 exerts differential control, for example in case of Per2 noted advance in molecular rhythm (~2-h), but no such change in Cry, presents an opportunity to delineate further the regulation of TTFL genes.

(7b) Line 294 revised as – “Bmal1 demonstrating a complete loss of 24-h rhythm (Fig. 4A), and its counterpart Clock mRNA showing overall reduced expression levels (Supplementary Table 3)”.

7c) Line 321 is referring to loss of Avp expression and the typo has been corrected from “Figure 3a to 4a”. Thank you.

However, core-clock genes such as Pers and Crys show minor or no change in expression patterns while Per2 and Per3 show a ~2hr phase advance. While these could only weakly account for the behavioral phase advance, the authors used TimeTeller to assess circadian phase in wildtype and ZFHX3 deficient mice. This approach clearly indicated that while the clock is not disrupted in the knockout animals, the phase advance can be correctly predicted from a network of gene expression patterns.

Strengths:

The authors use a multiomic strategy in order to reveal the role of the ZFHX3 transcription factor with a combination of TF and histone PTM ChIPseq, time-resolved RNAseq from wildtype and knockout mice and modeling the transcriptomic data using TimeTeller. The RNAseq experiments are nicely controlled and the analysis of the data indicates a clear impact on gene-expression levels in the knockout mice and the presence of a regulatory network that could underlie the advanced activity onset behavior.

Weaknesses:

It is not clear whether ZFHX3 has a direct role in any of the processes and seems to be a general factor that marks H3K4me3 and K27ac marked chromatin. Why it would specifically impact the core-clock TTFL clock gene expression or indeed daily gene expression rhythms is not clear either. Details for treatment of different ChIP samples (ZFHX3 and histone PTM ChIPs) on data normalization for analysis are needed. The loss of complete rhythmicity of Avp and other neuropeptides or indeed other TFs could instead account for the transcriptional deregulation noted in the knockout mice.

(8) We thank the reviewer for the constructive feedback. The current data suggests ZFHX3 acts as a mediating factor, occupying targeted active promoter sites and regulating gene expression by partnering with other key TFs in the SCN. Please see point 6 for clarification. The binding sites of ZFHX3 clearly showed enrichment for E-box(CACGTG) motif bound by CLOCK/BMAL1 along with binding sites for key SCN-specific TFs such as RFX (please see Supplementary Fig1). Our data thereby shows that it affects both core-clock and clock output genes (at varied levels) thereby exercising a pervasive control over the SCN transcriptome.

For treatment of ChIP samples please see point 3. We followed ENCODE guidelines strictly.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

- The early activity onset associated with a short photoperiod is a phenotype found in mice with a perturbed function of the SCN like Per2 mutant (PMID: 17218255), or Clock

KO (PMID: 22431615). Such disruption of the SCN function also leads to a faster synchronization to day feeding (PMID: 23824542) or jetlag (PMID: 25063847; PMID: 24092737). Therefore, authors should study the synchronizing function of these mice to day feeding and/or jetlag.

(9) Please see our response to point 1.

- The description of the negative controls needs clarification. While the "Method" suggests that both Cre- and Cre+ mice are treated with Tamoxifen, the text rather suggest that the controls are Cre- and Cre+ animals non-treated by Tamoxifen. Because of the potential effect of Tamoxifen on gene expression, Cre- treated animals are a required control.

(10) We thank the reviewer. As detailed in Methods, both Cre- and Cre+ mice were treated with Tamoxifen and compared. The text had been revised at line 212. In addition to this, another genetic control (-Tamoxifen) was also used (Figure 2 and 3).

- On line 486, authors wrote "It is important to note that although in the present study we used adult-specific *Zfhx3* null mutants resulting in global loss of ZFH3, the effects observed both at molecular and behavioural levels are independent of its functional role(s) in other tissues." On what evidence is this statement based? Using global KO rather suggest a potential role of other tissues.

(11) We agree with the reviewer, but at line 486 we refer to the effects observed at circadian behavior and daily gene expression in the SCN to be independent of pleiotropic roles of ZFH3 such as involvement in angiogenesis, spinocerebellar ataxia etc. We have revised the text.

Reviewer #2 (Recommendations for the authors):

It is not clear whether the behavioral experiments presented in this study were performed on a new set of animals - different from the cohort used in the Wilcox et al 2017 paper. For example, the proportion of total activity graphed in Figure 2C look strikingly similar to activity counts in Figure 3A in the prior publication (doi: 10.1177/0748730417722631)- down to the small burst in activity after ZT20 in the control (-Tam) group.

(12) The behavioral experiments presented in this study were performed on a completely new cohort of mice to those used in Wilcox et al.; 2017. The mice used for behavioral assessment. In the current study were later used for molecular experiments. Please see point 5.

Information on ChIP-seq such as read length, PE or SE seq, number of reads/replicate/condition/sample is missing. Versions of the softwares used should be indicated if known.

(13) The details are added as:

(13a) "Briefly, SCN punches were pooled from 80 mice at each. designated times (ZT3, ZT15) corresponding to one biological replicate per timepoint" at line 567.

(13b) "24 ug sheared chromatin sample collected from each time point (ZT3, ZT15)" at line 571.

(13c) "75-bp single end sequencing : 30 million reads/sample" at line 577.

(13d) “At line 584 – MACS algorithm v2.1.0 added”

Versions of other softwares used were already mentioned.

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