Serum iron and hepatic transferrin receptor expression are circadian-regulated

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Abstract

Serum iron has long been thought to exhibit diurnal variation and is subsequently considered an unreliable biomarker of systemic iron status. Circadian regulation (endogenous ~24-hour periodic oscillation of a biologic function) governs many critical physiologic processes. It is unknown whether serum iron levels are regulated by circadian machinery; likewise, the circadian nature of key players of iron homeostasis is unstudied. Here we show that serum iron and hepatic transferrin receptor (TfR) gene (*Tfrc*) expression exhibit circadian regulation. Daily oscillations of both serum iron and hepatic *Tfrc* expression are maintained in mice housed in constant darkness, where oscillation reflects an endogenous circadian period. However, oscillations of both serum iron and hepatic *Tfrc* were ablated when circadian machinery was disrupted in *Bmal1* knockout mice. This study provides the first confirmatory evidence that serum iron is circadian regulated and uncovers liver-specific circadian regulation of TfR, a major player in cellular iron uptake.

Main

Introduction

Cellular iron requirements are supplied by plasma iron that circulates bound to transferrin. Circulating iron, typically measured clinically by assaying serum iron levels, has been recognised to exhibit diurnal variation over a 24-hour period since the 1940's, with concentrations higher in the morning and reduced in the afternoon and evening in humans.[1-4] Partly for this reason, serum iron is generally considered an unreliable biomarker of iron status.[5] Plasma iron levels are regulated by the cellular influx and efflux of iron. Iron entry to the plasma from the duodenum (dietary iron) or reticuloendothelial system (iron recycled from senescent red cells) occurs via the sole cellular iron export protein, ferroportin. Ferroportin function is governed by hepcidin, which is expressed by the liver in response to iron loading and inflammation and suppressed during iron deficiency and enhanced erythropoiesis.[6] TfR facilitates uptake of iron from the plasma via receptor-mediated endocytosis of transferrin-bound iron. TfR is post-transcriptionally regulated by cellular iron status via binding of iron-regulatory proteins (IRPs) to response elements (IREs) in its 3' region, allowing increased TfR expression when iron levels are low and reduced TfR expression when iron is replete.[7]

Biological oscillation may reflect behavioural or dietary factors related to the 24-hour sleep-wake cycle or may reflect endogenous cellular or systemic regulation. Circadian regulation (endogenous ~24-hour periodic oscillation of a biologic function) governs many critical physiologic processes, including hepatic metabolic systems.[8] The circadian clock comprises the activating transcription factors CLOCK and its partner BMAL1 (gene: *ARNTL*), which activate transcription of the *PER* and *CRY* genes, whose protein products accumulate over the day and act to repress CLOCK-BMAL1 transcription, establishing oscillation. CLOCK-BMAL1 also activate expression of the repressive transcription factors REV-ERBα (gene: *NR1D1*) and REV-ERBβ,[9] which compete with activating transcription factors RORα and RORγ; this establishes a second oscillation.[10]

Whether serum iron is regulated by endogenous circadian regulation, or simply reflects behavioural (e.g. dietary) factors, remains unknown. Furthermore, circadian oscillation of genes that govern iron homeostasis have not been defined. Here, we provide mechanistic evidence that clinically-observed oscillations in serum iron are governed by endogenous circadian mechanisms, and further, that hepatic TfR gene expression also oscillates in a circadian fashion.

Results

We firstly determined whether serum iron oscillates in wildtype C57Bl/6 mice maintained on a cycle of alternating 12-hours light and 12-hours darkness. After 2 weeks of entrainment, we collected samples every four hours from Zeitgeber time (ZT) 0 (lights turned on) through

ZT12 (lights turned off) to ZT20. We confirmed the expected reciprocal oscillations in hepatic mRNA expression of core circadian genes *Clock, Arntl, Nr1d1, Cry1 and Per2* (Figure 1A-E). In this system, serum iron levels oscillated, with a difference in mean serum iron of 10µmol/L from peak to trough (Figure 1F). We initially hypothesised that variation in serum iron may be driven by oscillation in hepcidin expression but found that hepatic *Hamp1* mRNA was relatively stable across the day (adjusted (adj) p=0.225; Figure 1G).

We then hypothesised that other key players in iron regulation in the liver involved in either iron export – ferroportin (gene: *Slc40a1*) – or iron import – TfR (gene: *Tfrc*) – may have circadian oscillations that drive serum iron variation. We found that *Slc40a1* (adj p=0.763; Figure 1H) mRNA levels were relatively stable across the day, but *Tfrc* mRNA expression had diurnal variation (adj.p=4.01e-7, 1.9-fold change peak to trough; Figure 1I). We next looked to confirm that TfR gene expression, but not hepcidin nor ferroportin gene expression, has daily variation in the liver of humans utilising the CircaDB database (http://circadb.hogeneschlab.org/human). CircaDB contains RNA-sequencing data of 13 human organs, including the liver, collected by the GTEx Consortium[11] following CYCLOPS analysis[12] which allowed reconstruction of temporal order in the absence of time-of-day information. This dataset strongly suggests that *TFRC* (p=0.05*)* but not *HAMP* (p=0.66) nor *SLC40A1* (p=0.33) exhibits oscillation over a 24-hour period in humans.

Tfrc oscillation appears specific to the liver and was not observed in the hematopoietic organs, i.e. bone marrow or spleen (Figure 1 J-K). Corresponding oscillation in serum soluble transferrin receptor concentration (Figure 1L) also occurred. To confirm that oscillations in *Tfrc* and serum iron are circadian, we undertook a free running experiment, in which animals are housed in constant darkness with *ad libitum* access to food and water for 2 weeks. Conditions in which oscillation of parameters can be considered to reflect a true endogenous circadian period.[13] Figure 2A-C demonstrates reciprocal hepatic oscillation of core clock genes *Clock, Arntl* and *Nr1d1*, as expected. In this free running context, we observed oscillation of serum iron (p=3.51e-3; Figure 2D), with a difference in mean serum iron of 13µmol/L from peak to trough. Hepatic *Tfrc* expression appeared to oscillate under free running conditions, however this oscillation did not reach statistical significance when the JTK_Cycle period was set to 24 hours (p=0.124; Figure 2E). We hypothesise this is likely due to the true endogenous period of hepatic *Tfrc* being greater than 24 hours. Indeed, post-hoc testing with the JTK_Cycle period set to 32 hours revealed statistically significant oscillations in *Tfrc* expression (p=9.98e-6). We also observed non-statistically significant oscillations in soluble transferrin receptor levels (p=0.062, mean difference of 212ng/mL from peak to trough; Figure 2F).

Next, we assessed whether circadian genes influence serum iron and hepatic *Tfrc* mRNA oscillation. We utilised a *Bmal1* knockout model,[14] in a 12-hour light/12-hour dark setting. Littermate control (*Bmal1*$^{+/+}$*)* mice were compared to *Bmal1* knockout (*Bmal1*$^{-/-}$*)* animals at ZT4 and ZT16 (the timepoints at which we had initially observed the largest differential *Tfrc* expression). As expected, *Bmal1*$^{+/+}$ mice exhibited oscillation of *Nr1d1* mRNA between these two timepoints, whereas *Nr1d1* expression was ablated at both timepoints in *Bmal1*$^{-/-}$ animals *(*Figure 3A, genotype-time interaction p=1.65e-8), indicative of effective disruption of the circadian machinery*.* Whilst *Bmal1*$^{+/+}$ mice exhibited an increase in serum iron between ZT4 and ZT16 as previously observed, this increase was ablated in *Bmal1*$^{-/-}$ animals (Figure 3B, genotype-time interaction p=0.048). Oscillations of hepatic *Tfrc* were also ablated in *Bmal1*$^{-/-}$ mice (Figure 3C); a finding that is supported by a published liver transcriptomic dataset of *Bmal1*$^{-/-}$ and wildtype mice at ZT4 and ZT16.[15] However, we were unable to confirm a statistically significant interaction between genotype and time for *Tfrc* expression. Interestingly, although soluble transferrin receptor levels were increased at ZT4 compared to ZT16 in *Bmal1*$^{+/+}$ mice, levels were comparable at both timepoints in *Bmal1*$^{-/-}$ animals (Figure 3D). However, similarly to *Tfrc* expression, we were unable to confirm an interaction between genotype and time. These data imply a direct role for hepatic clock transcriptional

machinery in regulation of serum iron and potentially liver *Tfrc* and soluble transferrin receptor levels.

Discussion

The mammalian circadian clock maintains rhythmic physiologic patterns in functions including sleep-wake cycles, feeding/fasting, and hormonal secretion, over a 24-hour period. Rhythms are synchronised across the whole organism via a central mechanism within the suprachiasmatic nucleus, and by peripheral clocks in tissues including the liver.[16] Maintenance of oscillation during free running conditions, coupled with ablation of oscillation following genetic deletion of the afferent arm of the circadian clock, indicate direct circadian regulation of serum iron and potentially hepatic *Tfrc* expression. Transferrin receptor is post-transcriptionally regulated via the IRE-IRP system which stabilises *Tfrc* mRNA in conditions of cellular iron deficiency (to enable further iron uptake). However, we did not observe substantial rhythmic changes in liver non-heme iron over the 24-hour period (Figure 1M). Further, circadian increases in serum iron aligned with timepoints exhibiting higher expression of *Tfrc*, suggesting circadian regulation of *Tfrc* is distinct from IRE-IRP machinery. In keeping with this, we did not observe daily variation of other hepatic IRP-IRE controlled genes under 12-hour light/12-hour dark conditions – 3' IRE, *Slc11a2* (Figure 1N); 5' IRE, *Slc40a1* (Figure 1H) and *Fth1* (Figure 1O).

In humans, ZT0 reflects dawn, a time when serum iron is higher; levels then decline over the course of the day. We observed the opposite phenotype in mice, which has been previously observed in other circadian systems,[17] perhaps consistent with the nocturnal behaviour of rodents. Here, we demonstrate organ-specific oscillation of hepatic, but not haematopoietic TfR gene expression, potentially reflecting differing roles of iron uptake between these organs. Our data provide the first evidence that long recognised oscillations in serum iron are indeed driven by endogenous circadian machinery, potentially linked to variations in liver iron uptake across the day.

Methods

Animals and Ethics

Mice were caged in pathogen free conditions with food (containing 180 mg/kg iron) and water available *ad libitum*. Lights were turned on at 6am (ZT0) and turned off at 6pm (ZT12). For free running experiments, mice were housed in a temperature and humidity controlled dark box that blocked all external light. *Bmal1*$^{-/-}$ mice[14] were acquired from The Jackson Laboratory (strain: 009100) and bred inhouse. For all other experiments, wildtype C57Bl/6 mice were used. Male mice were used with age matched controls. The use of mice was in accordance with requirements set out by WEHI Animal Ethics Committee (approvals 2017.031 and 2020.034).

Gene expression analysis

RT-qPCR from liver, spleen and bone marrow samples was performed as previously described.[18] Data is presented as -ΔΔCt, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{Hprt1}$ and $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \text{mean } \Delta Ct_{\text{control (ZT0; CT0 or } Bmal1\text{+/+ ZT4)}}$. Primers used for RT-qPCR are in the table below:

Gene	Primer (5' - 3')
Arntl	GTAGATCAGAGGGCGACAGC
	CCTGTGACATTCTGCGAGGT
Clock	TCAACTCAGCAGAGTCAACAGA
	GCTCGGTGTCTGACTTGGAA
Cry1	CCGCTGCGTCTATATCCTCG
	TGAAGCAAAAATCGCCACCTG
Fth1	GGGAGCATGCCGAGAAACT

	AGTCATCACGGTCTGGTTTCTTTA
Hamp1	CAGGGCAGACATTGCGATAC
	TGCAACAGATACCACACTGGG
Hprt	ACAGGCCAGACTTTGTTGGA
	ACTTGCGCTCATCTTAGGCT
Nr1d1	GAGAGGCCATCACAACCTCC
	ACACCACCTGTGTTGTTATTGG
Per2	AGCTTCATCAACCCGTGGAG
	GGGACAGGCTGCATCAGTAG
Slc11a2	GGCGGAGCCGAATCCTATT
	CGCCATCGTCTGGCATCTTT
Slc40a1	CGTCATCCTCTGCGGAATCA
	AGCAGACAGTAAGGACCCATC
Tfrc	GGTTATGAGGAACCAGACCGT
	TGTTCCCACACTGGACTTCG

Analysis of iron parameters

Serum iron and liver non-heme iron was measured as previously described.[18] sTfR was measured by ELISA (Abcam).

Statistical analysis

Biological functions were tested for the presence of a circadian oscillation with a period of 24 hours using the JTK_Cycle alrgorithm.[19] For the iron transporters (*Tfrc*, *Hamp1* and *Slc40a1*) we report a bonferonni adjusted p-value. Differences at ZT4 vs ZT16 in *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice were compared by Student's T-test with Welch's correction. A test for a timepoint by genotype interaction was conducted with a likelihood ratio test comparing a two-way ANOVA with an interaction term to a model without the interaction term. Sample size is denoted in the figure legends. Data represents mean$\pm$standard deviation. Statistical testing was performed in Prism 9.3.1; GraphPad Software and Stata v17.0.

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Author Contributions

Conceptualized study – C.B., and S-R.P.; Designed research – C.B., M.E.B., C.S., and S-R.P.; Performed research – C.B., and A.P.; Analyzed data – C.B., A.P., A.R.D.M, R.H.; Wrote the manuscript – C.B., A.P., A.R.D.M, R.H., M.E.B., C.S., and S-R.P.

Conflict of Interest

None

1

Figure Legends

Figure 1 – Expression of circadian and iron homeostatic genes, serum iron and soluble transferrin receptor in mice maintained on a 12-hour light/ 12-hour dark cycle. C57Bl/6 mice were maintained on a 12-hour light / 12-hour dark cycle for two weeks before mice were euthanised and organs harvested every 4 hours. Liver *Clock* (A), *Arntl* (B), *Nr1d1* (C), *Cry1* (D) (N=13 except ZT0 where N=11 and *Cry1* ZT8 where N=12) and *Per2* (E) expression (N=10 except ZT0 where N=8). (F) Serum iron (N=13 except ZT0 and 8 where N=12) (G-I) Liver *Hamp1* (G), *Slc40a*1 (H) and *Tfrc* (I) expression (N=13 except ZT0 where N=11). (J) Bone marrow (BM) *Tfrc* expression (N=12 except ZT4,8 and 16 where N=13). (K) Splenic *Tfrc* expression (N=13 except ZT0 where N=12). (L) Mouse soluble transferrin receptor (sTfR; N=7). (M) Liver non-heme iron (N=8 ZT0/N=9 ZT4, 12 and 16/N=10 ZT8 and 20). (N-O) Liver *Slc11a2* (N) and *Fth1* (O) expression (N=13 except ZT0 where N=11). P values derived from JTK_Cycle algorithm.

Figure 2 – Expression of circadian and iron homeostatic genes, serum iron and soluble transferrin receptor in mice maintained in constant darkness. C57Bl/6 mice were maintained in constant darkness for 2 weeks before mice were euthanised and organs harvested. Liver *Clock* (A), *Arntl* (B) and *Nr1d1* (C) expression. (D) Serum iron. (E). Liver *Tfrc* expression. (F) Soluble transferrin receptor (sTfR). N=6. P values derived from JTK_Cycle algorithm.

Figure 3 – Expression of circadian and iron homeostatic genes, serum iron and soluble transferrin receptor in *Bmal1*$^{-/-}$ mice. *Bmal1* knockout (*Bmal1*$^{-/-}$) and littermate control (*Bmal1*$^{+/+}$) mice were maintained a 12-hour light/12-hour dark cycle for 2 weeks before organs were harvested at zeitgeber time (ZT) 4 and ZT16. (A) Liver *Nr1d1* expression (N=10 *Bmal1*$^{+/+}$ ZT4, 8 *Bmal1*$^{+/+}$ ZT16, 10 *Bmal1*$^{-/-}$ ZT4 and 11 *Bmal1*$^{-/-}$ ZT16). (B) Serum iron (N=5 *Bmal1*$^{+/+}$ ZT4, 4 *Bmal1*$^{+/+}$ ZT16, 3 *Bmal1*$^{-/-}$ ZT4 and 5 *Bmal1*$^{-/-}$ ZT16). (C) Liver *Tfrc* expression (N=10 *Bmal1*$^{+/+}$ ZT4, 9 *Bmal1*$^{+/+}$ ZT16, 10 *Bmal1*$^{-/-}$ ZT4 and 11 *Bmal1*$^{-/-}$ ZT16). (D) Soluble transferrin receptor (sTfR; N=7). P values within genotypes are derived from a Student's T-test with Welch's correction. P values across genotypes are derived from a likelihood ratio test of a genotype-timepoint interaction.

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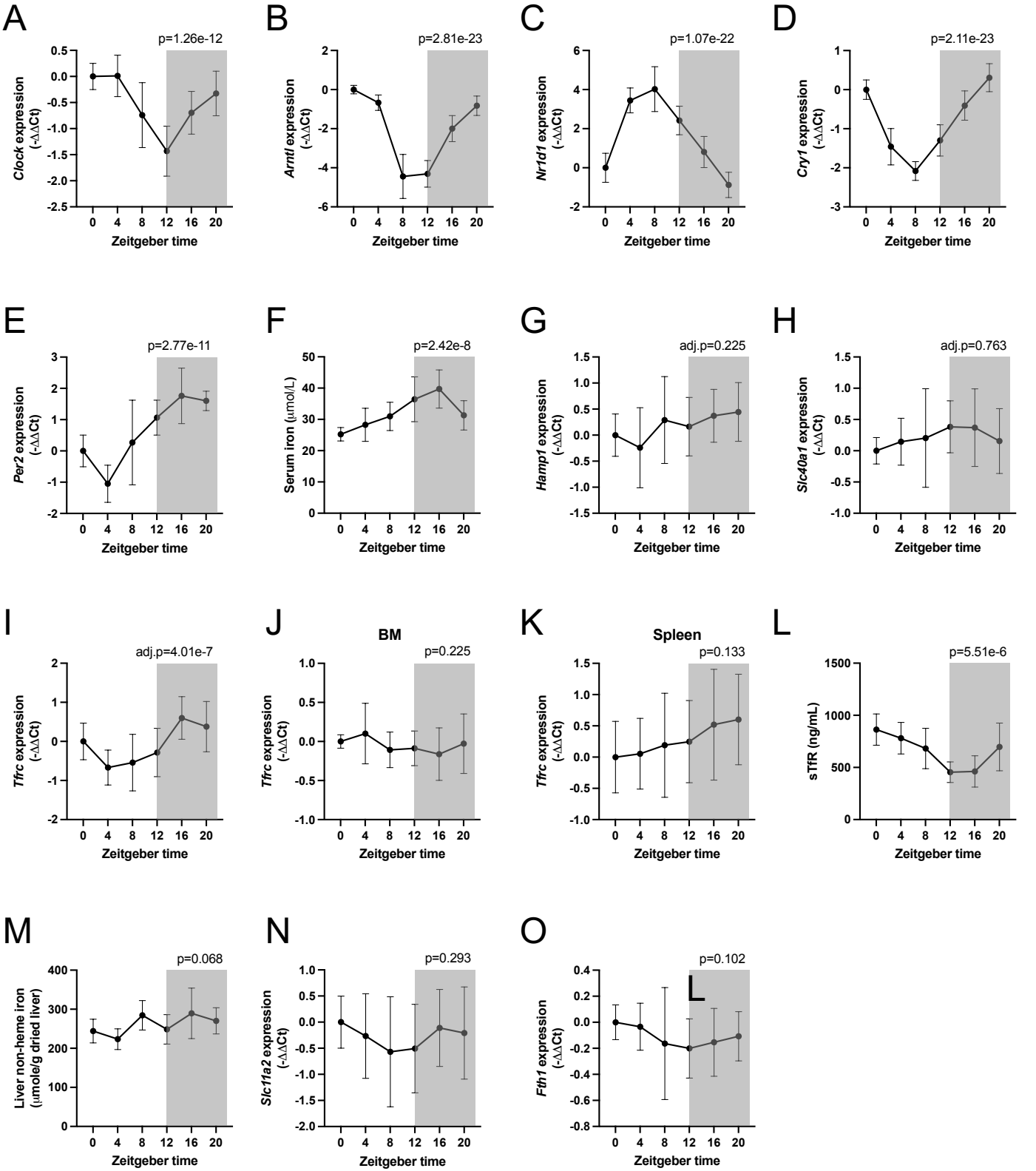
Figure 1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Arntl expression (-ΔΔCt)
C
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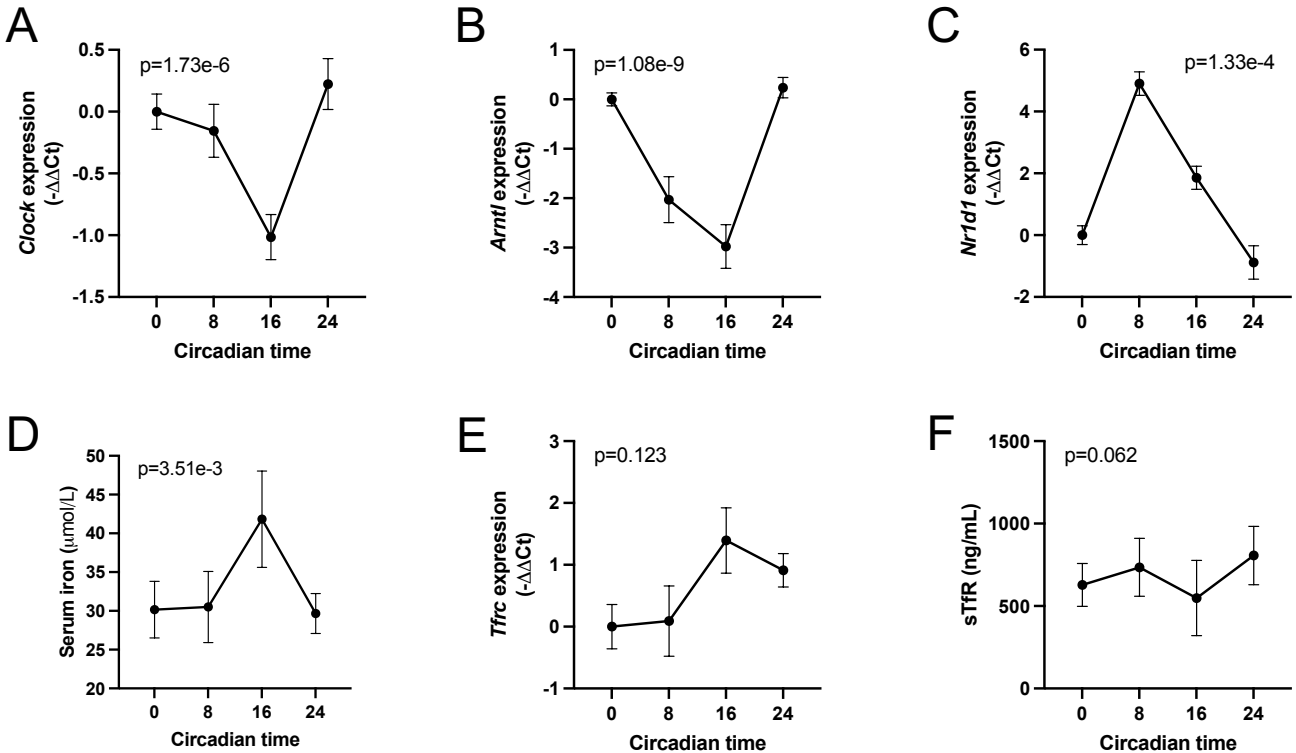
Nr1d1 expression (-ΔΔCt)
D
p=2.11e-23
Cry1 expression (-ΔΔCt)
E
p=2.77e-11
Per2 expression (-ΔΔCt)
F
p=2.42e-8
Serum iron (μmol/L)
G
adj.p=0.225
Hamp1 expression (-ΔΔCt)
H
adj.p=0.763
Slc40a1 expression (-ΔΔCt)
I
adj.p=4.01e-7
Tfrc expression (-ΔΔCt)
J
BM
p=0.225
Tfrc expression (-ΔΔCt)
K
Spleen
p=0.133
Tfrc expression (-ΔΔCt)
L
p=5.51e-6
sTfR (ng/mL)
M
p=0.068
Liver non-heme iron (μmole/g dried liver)
N
p=0.293
Slc11a2 expression (-ΔΔCt)
O
p=0.102
Fth1 expression (-ΔΔCt)
Zeitgeber time

Figure 2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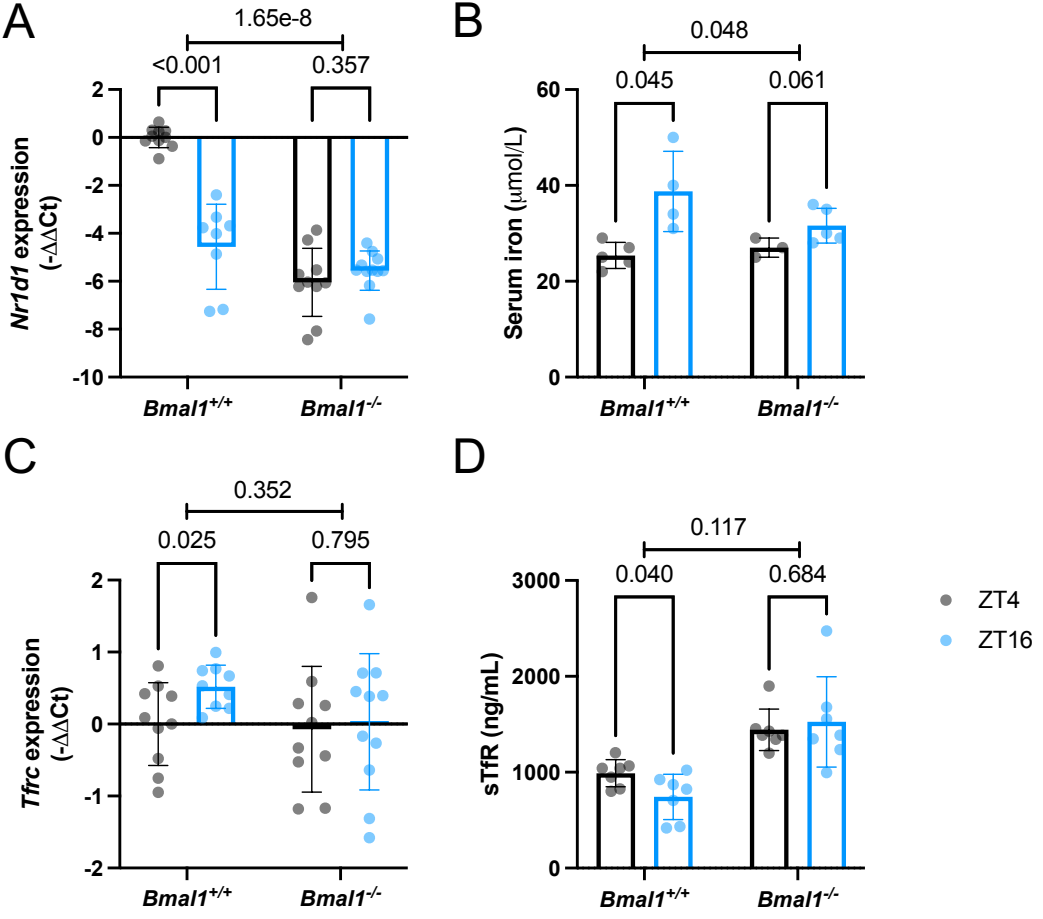
-0.5
-1.0
-1.5
0
8
16
24
Circadian time
B
p=1.08e-9
Arntl expression (-ΔΔCt)
1
0
-1
-2
-3
-4
0
8
16
24
Circadian time
C
p=1.33e-4
Nr1d1 expression (-ΔΔCt)
6
4
2
0
-2
0
8
16
24
Circadian time
D
p=3.51e-3
Serum iron (μmol/L)
50
45
40
35
30
25
20
0
8
16
24
Circadian time
E
p=0.123
Tfrc expression (-ΔΔCt)
3
2
1
0
-1
0
8
16
24
Circadian time
F
p=0.062
sTfR (ng/mL)
1500
1000
500
0
0
8
16
24
Circadian time

Figure 3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-4
-6
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-10
Bmal1+/+
Bmal1-/-
B
0.048
0.045
0.061
Serum iron (μmol/L)
60
40
20
0
Bmal1+/+
Bmal1-/-
C
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0.025
0.795
Tfrc expression (-ΔΔCt)
2
1
0
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-2
Bmal1+/+
Bmal1-/-
D
0.117
0.040
0.684
sTfR (ng/mL)
3000
2000
1000
0
Bmal1+/+
Bmal1-/-
ZT4
ZT16