Title: "A novel female-specific circadian clock mechanism regulating metabolism"

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Acknowledgements: We wish to thank Drs. David Bowtell (Peter MacCallum Cancer Centre) and Andreas Möller (QIMR Berghofer) for providing Siah2 KO breeders. We also want to express strong appreciation to Dr. Zach Hall and members of the DeBruyne, Ehlen and Tosini labs, as well as members of the Neuroscience Institute for valuable feedback and advice for preparing this manuscript. This work was funded by NIH NIGMS grants 1SC1GM109861 and 1R35GM127044 to JPD, as well as in part by NIH NIMHD grants 8G12MD007602, 8U54MD00758, 1G20RR031196, S21MD000101, and C06RR18386 to Morehouse School of Medicine. GT is supported by NIH NEI grant R01EY026291. JCE is also supported by NIH NIGMS grant SC1 1GM127260, JCE, GT and JPD are also supported by NIH NINDS grant U54 NS083932, and JCE and JPD are also supported by IOS NSF grant No. 1832069.

Contributions: JEB, JPD, and, TCS conceived and developed the idea. AA, JEB, JPD, CG, ARH, TM, KS, TCS developed experimental and analytical methodologies. AA, JEB, KB, JPD, CG, ARH, TM, SO, KS, and TCS performed experiments. AA, JPD, TM, KS, and TCS analyzed the data. JPD, JCE, GT provided resources and acquired project funding. JPD supervised and directed the project. AA, JEB, KB, JPD, CG, ARH, TM, SO, KS, and TCS contributed to the final manuscript.

Signficance statement:

Circadian clocks drive daily rhythms in many aspects of our physiology, optimally aligning functions across the day-night cycle. How circadian clocks drives these rhythms is thought to be due to largely similar transcriptional pathways and mechanisms in males and females, although some rhythms are modulated by sex and growth hormones. In this study, we present data that uncover the surprising existence of a *female-specific* transcriptional mechanism that is essential for the proper rhythmic control of gene expression in the liver. Disrupting this mechanism substantially impairs the circadian regulation of lipid and cholesterol metabolism selectively in females, impairing their resistance to diet-induced obesity. These results reveal that circadian clocks may be broadly coupled to physiological rhythms using unexpected sex-specific mechanisms.

Abstract:

Circadian clocks enable organisms to predict and align their behaviors and physiologies to constant daily day-night environmental cycle. Because the ubiquitin ligase Siah2 has been identified as a potential regulator of circadian clock function in cultured cells, we have used Siah2-deficient mice to examine its function *in vivo*. Our experiments demonstrate a striking and unexpected sexually dimorphic effect of *Siah2* deficiency on the regulation of rhythmically expressed genes. The absence of Siah2 in females, but not in males, altered the expression of core circadian clock genes and drastically remodeled the rhythmic hepatic transcriptome. Siah2 loss, only in females, increased the expression of 100's of genes selectively at mid-day, resulting in a ~70% increase in the number of rhythmically expressed genes, and shifted the expression of 100's of other genes from a mid-night peak, to a mid-day peak. The combined result is a near inversion of overall rhythmicity in gene expression selectively in Siah2-deficient females. This dramatic reorganization created a substantial misalignment between rhythmic liver functions and feeding/behavioral rhythms, and consequently impaired daily patterns of lipid/lipoprotein metabolism and metabolic responses to high-fat diet. Collectively, our results suggest that Siah2 is part of a female-specific circadian mechanism important for maintaining metabolic homeostasis and may play a key role in the establishing sexual dimorphisms in metabolism.

Circadian rhythms in physiology and behavior are driven by transcriptional feed-back loop timing mechanism that drives ~24hr rhythms in expression of 1,000's of target genes (1, 2) thoughout the body. Disruption of these rhythms, or more commonly, misalignent of these rhythms with the environmental day-night cycle or within the organism causes a wide-range of health consequences, including metabolic dysfunction and obesity (3-5).

Siah2 is a Ring-type E3 ligase whose role in regulating the hypoxia pathway (6) and tumorogenesis is well known (7, 8). We have recently reported evidence suggesting that Siah2 is also a regulator of circadian clock function (9). We identified Siah2 in a screen for ubiquitin ligases that mediate degradation of RevErbα/β, heme-sensitive transcriptional repressors that regulate circadian rhythms and lipid metabolism (10-16). Suppressing *Siah2* expression in a cellular clock model (genetically female U2OS cells (17) both altered RevErbα stability and lengthened periodicity, thus directly implicating Siah2 in the regulation of circadian rhythms (9). To explore this possibility further, we have now examined the effect of Siah2 deletion on clock function in a mouse model (4). Surprisingly, our experiments demonstrate that Siah2 regulates clock function and lipid metabolism in female, but not male mice.

Results:

To determine the effect of *Siah2* deletion on circadian clock function, we examined the expression of five core rhythmic genes (*Bmal1, Cry1, Per2, RevErbα* and *Dbp*) (2, 18, 19) in the livers of both male and female Siah2-deficient and wild type mice. Expression was examined at 3-hour intervals throughout a 24-hour period. We found a striking difference in the effect of Siah2 gene deletion in female and male mice (Fig1A). In male, loss of Siah2 has no detectable effect on the expression of these genes. In female mice, however, Siah2 loss increased the peak expression of all but one (*RevErbα*) of the rhythmic genes and delayed the expression of the repressors *Per2* and *Cry1* (Fig 1A). These results suggest that Siah2 regulates the

amplitude and timing dynamics of the circadian clock gene expression *in vivo*, but only in females.

Based on these studies, we then examined the effect of Siah2 loss on the entire rhythmic hepatic transcriptome. In females, Siah2 loss resulted in a significant reorganization of the timing of rhythmic gene expression in livers (Fig.1B). This reorganization was expressed in two ways.

First, Siah2 loss in females resulted in a dramatic net increase (ca. 34%) in the number of rhythmically expressed transcripts (Fig 1B-C). This was due to a much larger population of genes that gained rhythmicity in Siah2-deficient females over genes that lost rhythmicity (Fig 1D). In contrast, Siah2-deficiency in males caused a small net decrease (ca. 7%) in the overall number of rhythmic genes (Fig.1B-D). Strikingly, most of the genes that gained rhythmicity in Siah2 KO females did so with peak expression strongly clustered around mid-day (ZT6, Fig 1E); males lacked phase clustering (Fig 1E). This large bias in the timing of expression of these genes suggests that Siah2 regulates a circadian transcriptional mechanism that represses the expression of a large number of genes around mid-day in females.

Second, Siah2 loss caused large shifts in when rhythmic genes peak, predominantly altering expression of nocturnally expressed genes. Examination of the patterns of genes that were rhythmically expressed in both genotypes revealed that Siah2 loss changed the time of peak expression levels of individual genes in both sexes (Fig 2A). In males, Siah2-deficiency shifted expression of genes that are expressed at all times of day, and did not alter the liver's overall gene expression profile (Fig 2A-B). In surprising contrast, Siah2 loss in females predominantly shifted genes expressed during the night in wild type livers, resulting in a dramatic increase in the number of genes expressed during the day and reduction in the number of genes expressed during the night (Fig 2A-B). These results are exemplified in genes that were shifted by 6-hours or more by Siah2 loss (see also Fig S1), which demonstrate that Siah2 loss in females, but not males, has a strong bias towards shifting the rhythms of nocturnally expressed genes to the daytime (Fig 2C). These results therefore suggest that Siah2 also regulates a female-specific circadian transcriptional mechanism that drives rhythmic nighttime gene expression.

Overall, our data indicate that Siah2 has a profoundly sex-specific role in regulating rhythmic gene expression selectively around mid-day and mid-night. This role appears to be specific to the 'rhythmicity' of gene expression, as we found only a few genes whose expression was altered by Siah2 loss, independent of time-of-day (Fig S2A). The consequence of the changes in rhythmic gene expression is a substantial alteration in the overall landscape of circadian rhythmicity in Siah2 deficient female livers, shifting it from predominantly nocturnal pattern in wild type to a diurnal (i.e. daytime) pattern in Siah2 deficient females (Fig 3A-B).

We found very little overlap between males and females in the genes impacted by Siah2 loss (Fig 3C). Likewise, there was little overlap in genes altered by Siah2 loss with genes regulated by estrogen (Fig S3) or those that otherwise display sexually dimorphic expression in wild type mice (Fig S2C-D, see also Supplemental Datasets 1-4). Siah2 expression itself is not different between males and females (Fig S4), and its loss had little effect on rhythms of REVERBα protein abundance in livers of either sex (Fig S5), possibly due to compensation by other ubiquitin ligases (20-22). Combined, these results suggest Siah2 may be regulating one or more unknown female-specific clockwork factors to modulate clock gene expression and direct the timing of its rhythmic outputs (Fig S6).

The shift in overall gene expression patterns caused by Siah2-deficiency in females creates a prominent misalignment between the timing of gene expression with the normal locomotor and feeding rhythms of *Siah2* KO females (Fig S7). Circadian misalignment between feeding and hepatic gene expression rhythms is known to lead to obesity and metabolic disorders(3, 23-25). Of note, Siah2 loss in females, but not in males, altered the rhythmic expression of a preponderance of genes involved in lipid metabolism (Fig 3D-F, Fig S8). Thus, the female-specific change in circadian landscape in Siah2-deficient mice led us to predict that these mice would have sex-specific deficits in metabolic homeostasis.

We tested this prediction two ways. First, we challenged Siah2-deficient and normal young adult mice (8 weeks) with a diet rich in fats (high-fat diet, HFD; 45 kCal from fat) to examine their overall metabolic homeostasis. Compared to males, females are resistant to HFD induced obesity and overall metabolic syndrome(26-28). The overall effects of the metabolic changes were most clearly seen in terms of weight gain. Female, but not male, *Siah2* KO mice gained substantially more weight when fed HFD than did wild type controls (Fig 4A-B). The weight gain in *Siah2* KO females was significant by 7 weeks of HFD, and by 13 weeks, resulted in an overall ~20% increase in weight compared to wild type females. Overall, weight gain in Siah2 KO females very comparable to males of both genotypes (Fig 4C). These results suggest that Siah2 plays an important role in maintaining sexual dimorphism in diet-induced obesity.

The weight gain in Siah2-deficient females was predominantly due to an increase in adiposity (Fig 4D-E) and was not associated with a change in food consumption (Fig. S9), sex-hormone production or reproductive capacity (Fig S10). Unexpectedly, glucose homeostasis remained largely intact in obese Siah2 KO females (Fig. S11), suggesting that the mechanistic relationship between diet-induced obesity and diabetes may be sex-specific. Juvenile (4 week old) Siah2 KO mice fed HFD have also been shown to have sex-specific metabolic traits(29) that are somewhat different than those we observed, suggesting complex interactions between *Siah2*, diet and puberty. Nonetheless, we also found that female *Siah2* KO mice fed a sucrose matched control diet for 13 weeks (>20 weeks old) also displayed significant increases in adiposity (Fig 4E). Combined, these data suggest that high-fat diet exacerbates an underlying metabolism deficit that is specific to adult Siah2-deficient females.

Siah2 loss altered the regulation of serum cholesterol and triglyceride levels in response to HFD selectively in females (Fig S12) and rendered them susceptible to HFD-induced hepatic steatosis (Fig 4F). Siah2-deficient males were indistinguishable from wild type males in these studies. In contrast, adipocytes of HFD fed Siah2-deficient mice were larger than wild type mice of either sex (Fig S13), suggesting that the sex-specificity of obesity that we observed may not directly involve processes endogenous to adipocytes (i.e. regulation of PPARgamma)(30). Instead, our results point towards a female-specific role of Siah2 in regulating lipid metabolism in the liver.

The second way we tested this prediction was to determine if the time-dependent changes in the expression of lipid-metabolic genes in *Siah2* KO females (Fig 3E, Fig S8) led to alterations in the rhythmic profiles of serum lipoproteins and fatty acids. To do this, we collected serum from a new cohort of females fed normal chow *ad libitum* at 3-hour intervals across the day. Consistent with the overall change in circadian transcriptional regulation of lipid-regulatory genes, female Siah2 KO mice displayed robust day-time specific increases in serum cholesterol and triglyceride levels (Fig 4G). These effects were limited to early daytime and did not appear to disrupt normal nighttime profiles. In addition, serum free fatty acid (FFA) levels were slightly increased during throughout the day and decreased across the night, creating a significant

diurnal rhythm in FFA levels in female Siah2 KO mice (Fig 4H). Thus, changes to rhythmic gene expression in female *Siah2* KO mice markedly alter in the rhythms of lipoprotein/lipid metabolism. Moreover, the early daytime increase in serum lipids occurs when mice are predominantly sleeping, not eating – functionally similar to eating when the body is sleeping/fasting. As such a misalignment has been shown to lead to obesity(3, 23-25), our results suggest that the changes in the rhythmic gene expression patterns are strong contributors to the metabolic phenotypes in Siah2 deficient female mice.

Conclusion:

Taken together, our data suggest that Siah2 is a key component of an unknown female-specific circadian clockwork mechanism. Siah2 is a ubiquitin ligase with no known ability to directly regulate gene expression. Thus, Siah2 likely acts on one or more transcription factors that have sexually dimorphic roles in regulating circadian gene expression (Fig S6). Identifying these unknown transcription factors is of high interest, as they appear to modulate expression of core clock genes in females and have strong roles in regulating rhythmic expression of output genes. Our experiments apparently reveal a novel mechanism, as the consequences of Siah2 loss on gene expression expand, rather than reduce, the overall differences between males and females. In the liver, this mechanism appears to be important for control of lipid metabolism, aligning these metabolic rhythms in females to the correct time of day. In this way, this novel Siah2-dependent circadian mechanism may contribute to metabolic resilience in females and the overall sexual dimorphism in metabolism. Moreover, these results show that circadian clocks can drive rhythms using different mechanisms in males and females, suggesting possibility that each sex may cope differently with circadian clock-related disorders.

Data availability:

The full RNAseq data that support the findings of this study are available from the corresponding author upon reasonable request and have been deposited in Gene Expression Omnibus with the accession codes (to be provided prior to publication).

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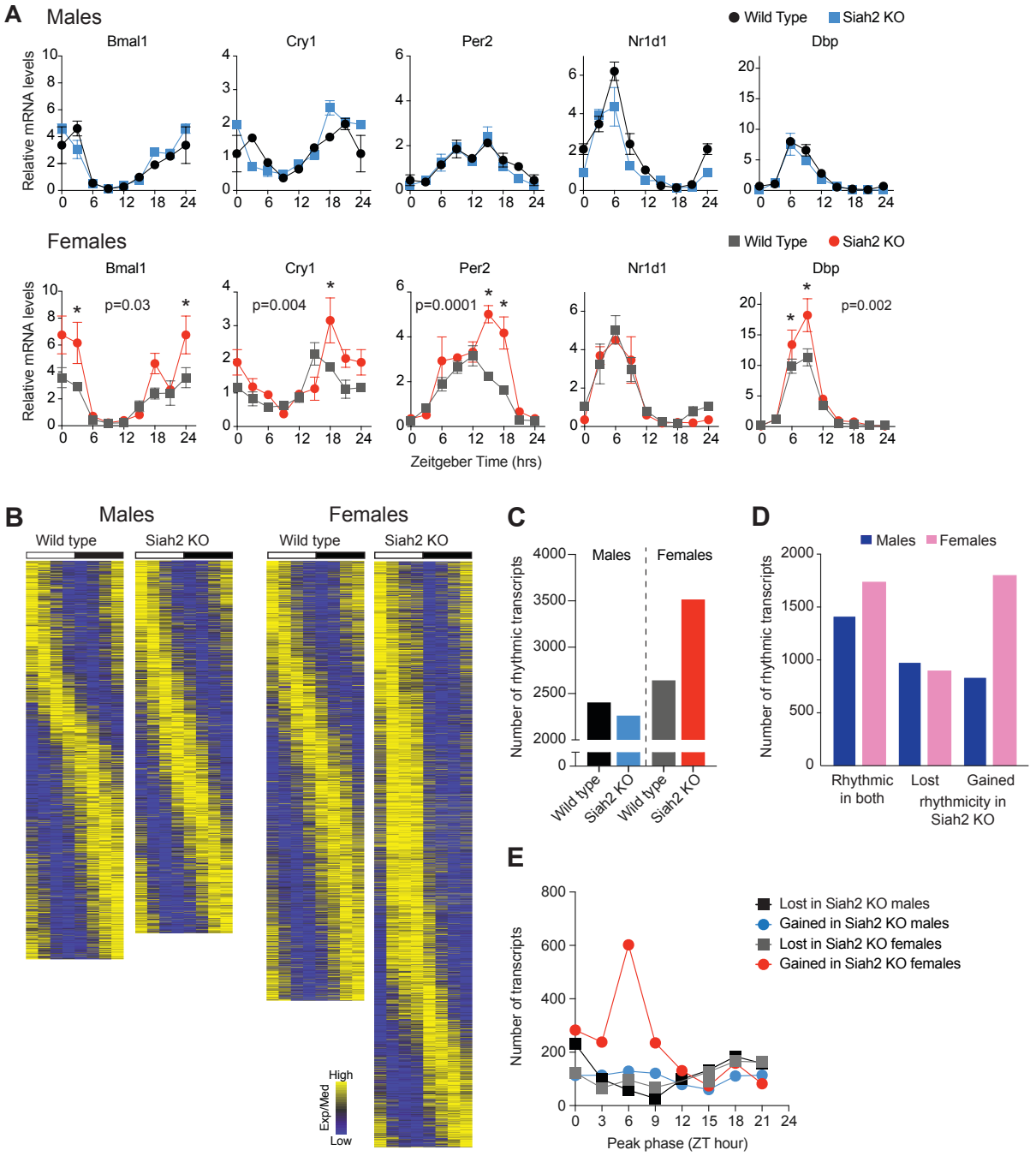

Fig. 1. Siah2 loss increases rhythmic gene expression in females. (**A**) Quantitative RT-PCR profiles of clock gene mRNAs in liver (mean +/- sem, n=3 livers per genotype, sex and time.). The Zeitgeber time (ZT) 0 point is double plotted at ZT24 for clarity. ZT0-12 = lights on, ZT 12-24 = lights off. P-values shown reflect significant time x genotype interactions (two-way ANOVA). * = p<0.05 between genotypes at individual timepoints (Sidak's multiple comparison test). (**B**) Heatmaps of expression profiles of the rhythmically expressed genes (defined in Supplemental Materials and Methods), sorted by peak expression timing/phase for each group independently. White/black bars indicate the light-dark cycle. (**C**) Numbers of rhythmically expressed genes in each group. (**D**) Comparisons of Siah2-induced changes to rhythmicity of genes in each sex. (**E**) Frequency distribution of expression peak timing, across the day, of genes that gained or lost rhythmicity in Siah2-deficient (*Siah2* KO) livers in both sexes.

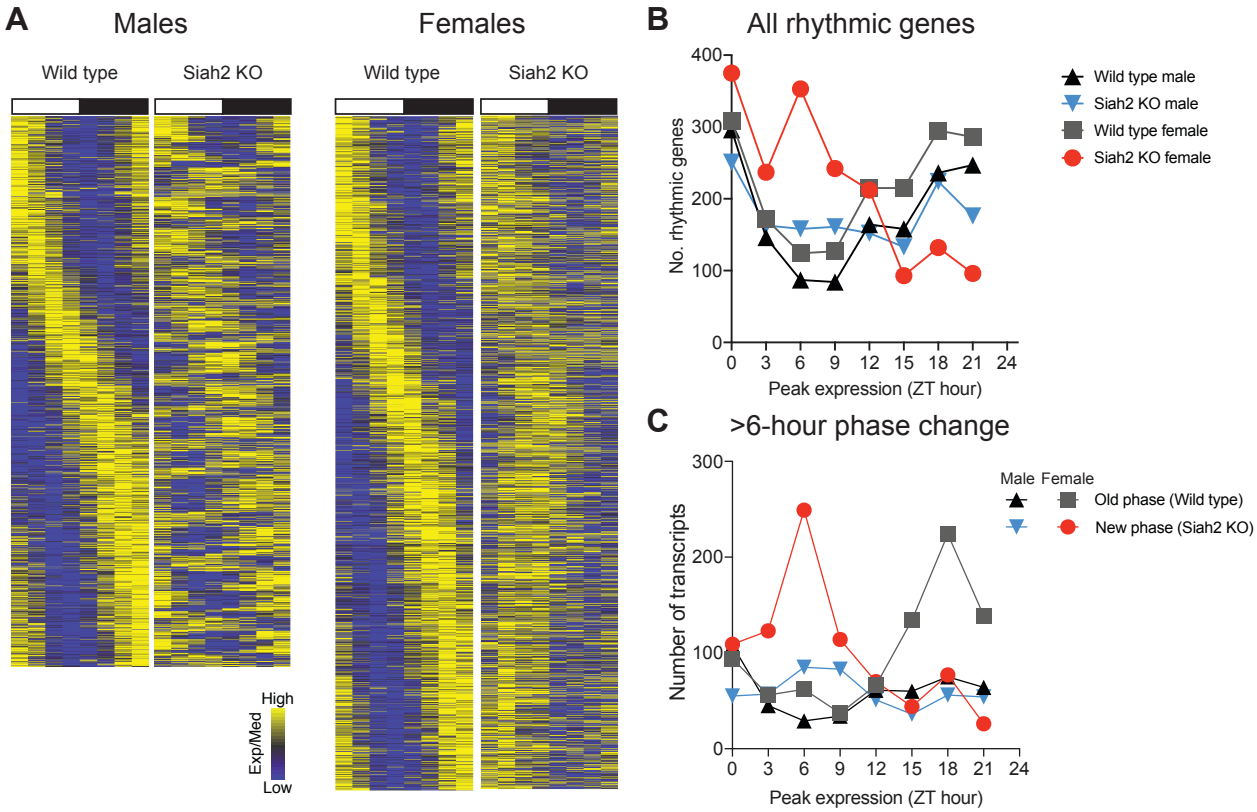

Fig. 2. Siah2 loss in females shifts nocturnally expressed rhythmic genes to the day. (**A**) Heatmaps of genes that were rhythmically expressed in both genotypes; genes are aligned across genotypes. (**B**) Frequency of genes shown in **A** with peak expression occurring at 3-hour intervals across the day. (**C**) Frequency distributions of peak phase across time of day for genes rhythmically expressed in both genotypes but shifted by more than 6 hours. See also **Fig. S1** and **Supplemental Datasets 1-2**.

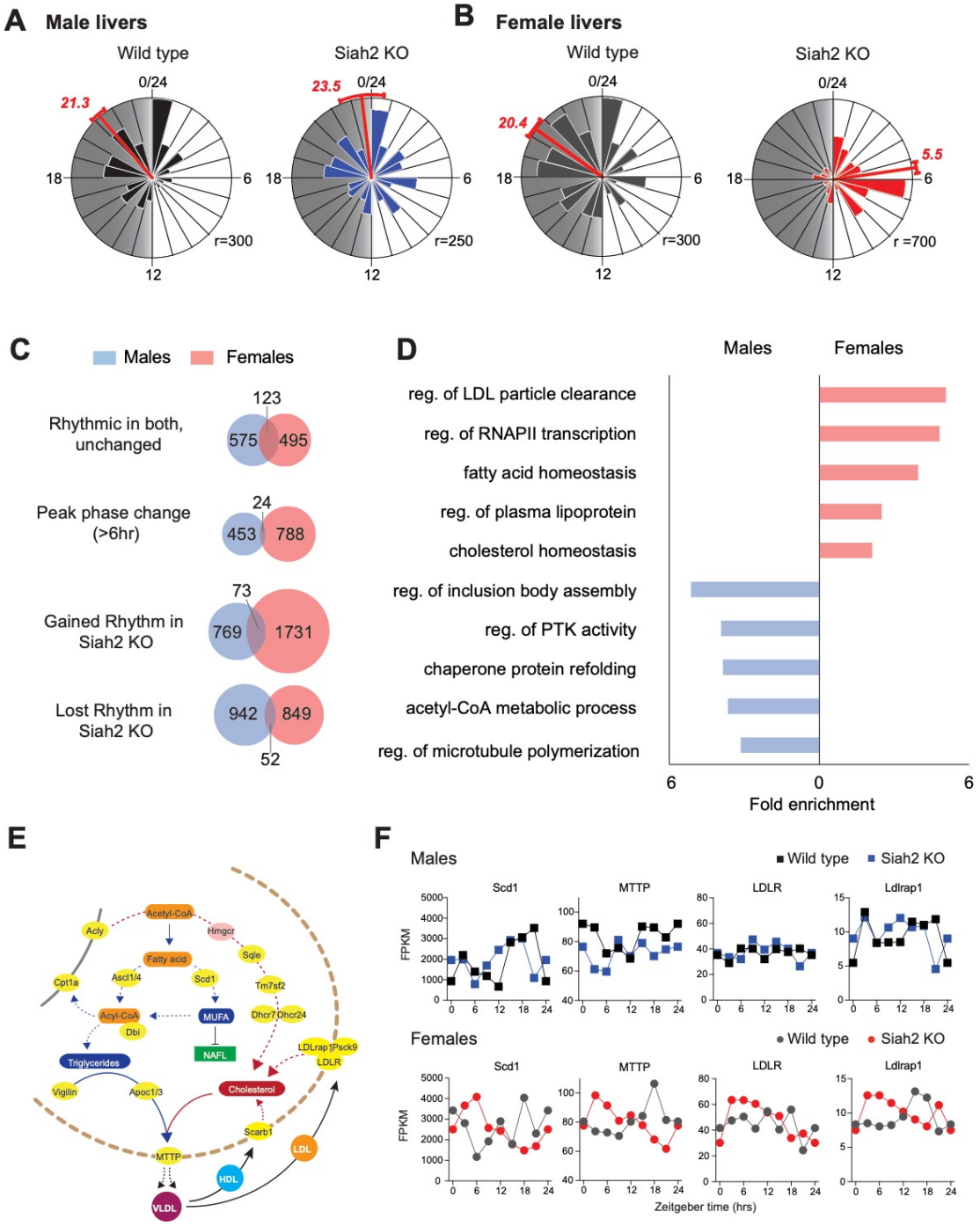

Fig. 3. Sex-specific roles of Siah2 in regulating rhythmic gene expression. (**A- B**) Raleigh plots (circular histograms) of expression peak timing for all rhythmically expressed genes in each group (see Fig. 1B). The numbers in red depict the average peak-time (in ZT hours) indicated by vector analysis (red line, +/- SEM). Gray shading = nighttime, r = radius in number of genes. (**C**) Venn diagrams comparing the effects of Siah2 loss between males and females on rhythmic gene expression. (**D**) Top gene ontogeny terms corresponding to the genes whose expression was affected by Siah2 loss in both sexes. (**E**) Simplified lipid and cholesterol metabolic pathways in the liver, highlighting genes (yellow) uniquely affected by Siah2 loss in females. (**F**) Examples of female-specific effects of Siah2 loss on rhythmic gene expression. See also **Fig. S7** and **Supplemental Datasets 1-2**.

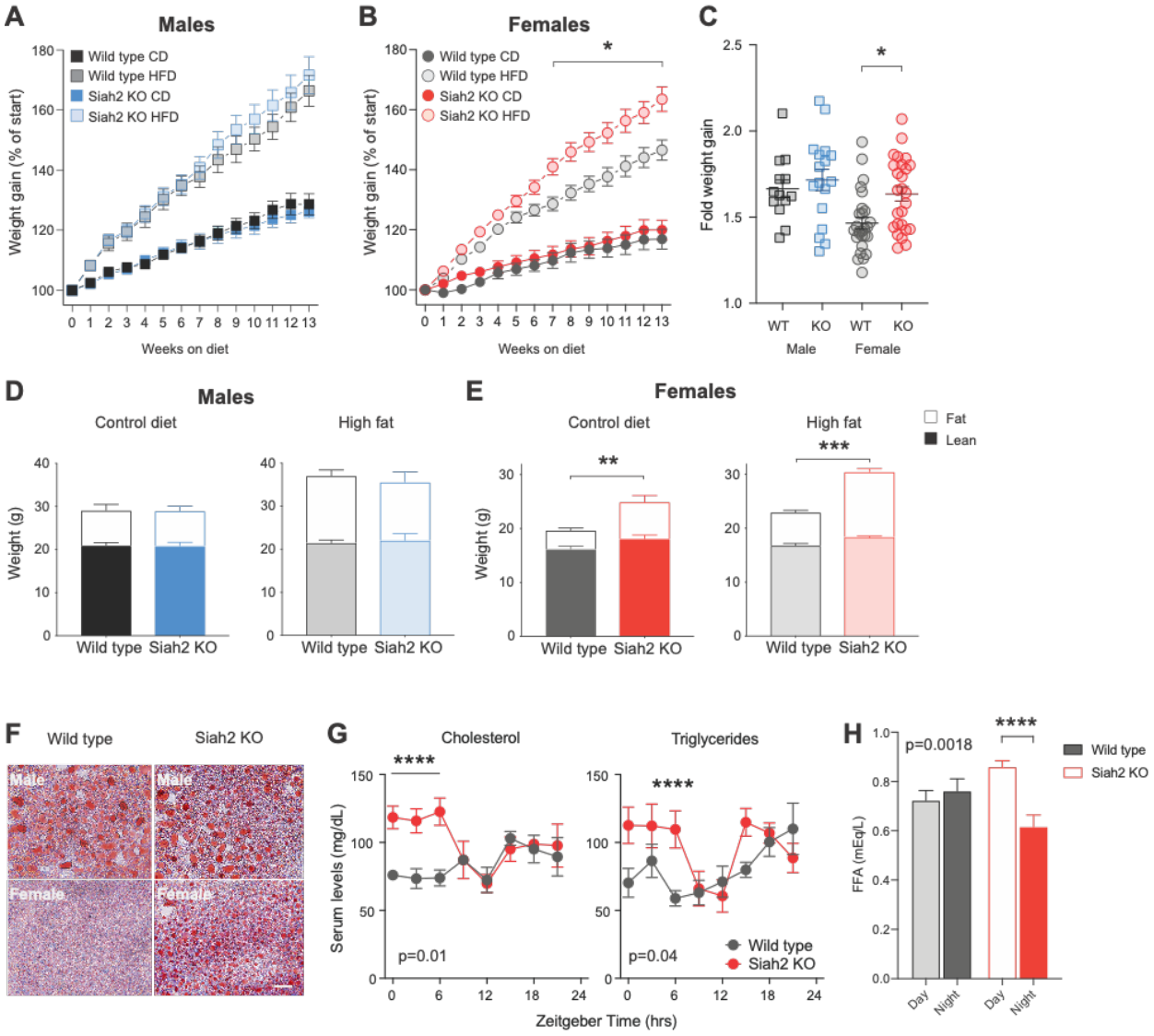

Fig. 4. Siah2 loss alters metabolism and serum lipid levels in females. (**A-B**) Weight gain in mice fed control diet (CD) or 45% fat diet (HFD). Mean +/- SEM, n= 8-10 for CD (combined from two independent trials), n = 13-17 male mice, combined from two trials, or 26-27 female mice from 4 trials. Two-way ANOVA indicated a significant interaction between genotype x time on diet in females only ($p<0.0001$, $F(13, 663) = 6.484$), with significant differences in weight gain by week 7 (* = $p <0.01$, Sidak's multiple comparison test). (**C**). Fold weight gained at 13 weeks for the HFD mice in **A-B** (* $p <0.0001$, $t = 4.809$, $df = 714$) same as in **A**). (**D-E**) Body composition analysis. Mean +/- sem, n=4-5 mice/sex and genotype. Loss of Siah2 significantly increased adiposity in females on either diet for 13 weeks (** = $p=0.0247$, $t=3.039$, $DF=15$, *** $p=0.0044$, $t=4.13$, $DF=15$). (**F**) Oil Red O staining of liver from mice fed HFD for >13 weeks. Red staining = fat. Scale bar = 50 microns. (**G-H**) Serum lipid levels across the day in female mice fed normal chow *ad libitum*. In G, each point is the mean +/- SEM (n=3 mice, except ZT 9, n=4). In H, times 0-9 levels were averaged for day (+/- SEM, n = 13 each), times 12-21 averaged for night (+/- SEM, n=12 each). The p-values shown denote significant genotype x time interactions from two-way ANOVA (cholesterol = $F(7, 34) = 3.071$, triglycerides = $F(7, 34) = 2.355$), FFA = $F(1, 46) = 11.01$). **** = $p<0.05$ between genotypes (G) or times of day (H) (Sidak's multiple comparison test).

Supplementary Materials for

"A novel female-specific circadian clock mechanism regulating metabolism".

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This file includes:

Materials and Methods

Figs. S1 to S13

Captions for Datasets S1 to S4

Materials and Methods

Animals and diets: Wild type and *Siah2* KO mice maintained on a C57Bl6 background (1), kindly provided by Dr. Andreas Moller (QIMR Berghofer) and David Bowtell (Peter MacCallum Cancer Institute) were bred and maintained in our animal facility at Morehouse School of Medicine. After an initial cross to C57Bl6 mice, heterozygotes were intercrossed, and homozygous wild type and *Siah2* KO mice were used to establish related lines. All mice had *ad libitum* access to normal chow (PicoLab Laboratory Rodent Diet 5L0D, LabDiet, St. Louis MO, USA) and water and were housed in a 12-hour light:12-hour dark (12:12 LD) cycle unless otherwise stated. Breeding mice were fed breeding chow (PicoLab Mouse Diet 20 5058, LabDiet, St. Louis MO, USA) and all other mice were fed normal rodent chow unless otherwise indicated. For the metabolic studies, mice were individually housed and provided Research Diets D12451 (45% kCal fat) as a high-fat det (HFD) or the sucrose matched control diet (CD) D12450H, starting at 7-8 weeks of age; body weights and food consumption were monitored weekly thereafter. After 13 weeks on these diets, mice were subjected to additional tests (see below). At the indicated times, mice were euthanized using CO_2 followed by decapitation to collect blood and tissues. All animal studies were approved by the Institutional Animal Care and Use Committee of Morehouse School of Medicine, and in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Body composition and serum analyses: Body composition was performed on mice that were fasted for 6 hours (starting at ZT0, lights off) prior to sacrifice. Body composition was performed post-mortem using MRI at the NIH University of Cincinnati Mouse Metabolic Phenotyping Center (MMPC). Serum was collected following euthanasia with CO_2 and decapitation and samples were sent for analysis of insulin, total cholesterol, triglyceride, phospholipid and non-esterified fatty acid levels at the same facility. Sex hormones were measured from serum collected single-housed females in proestrus, as determined by cytological examination of vaginal lavages (2) and submitted to Ligand Assay & Analysis Core at the University of Virginia (https://med.virginia.edu/research-in-reproduction/ligand-assay-analysis-core) for measurements of LH/FSH (mouse/rat multiplex), estradiol (mouse/rat) and progesterone (mouse/rat) levels.

Glucose and insulin tolerance tests: Mice were weighed at ZT0 and then fasted for 4 hours after which the fasting glucose levels were taken. Mice were then injected intraperitoneally with either 1.5mg/gram body weight glucose solution (20% in 0.9% NaCl) or 0.75 IU of insulin per gram body weight (in 0.9% NaCl) and blood glucose levels were measured by drawing blood from the tail 20, 40, and 100 minutes post-injection using a Contour Blood glucose monitoring system (Bayer) as we have done previously(3). Mice were first subjected to glucose tolerance tested, allowed to recover for 1 week before insulin tolerance testing.

Liver and adipose staining: Oil Red O staining was performed on 16-micron frozen liver sections from wild type or *Siah2* KO, male or female mice fed HFD using the Abcam Oil Red O Kit (ab150678), according to the manufacturer's instructions. The stained sections were mounted and imaged at a magnification of 40x. Perigonadal white adipose tissue was dissected from the indicated mice, stored in a 15% sucrose at 4°C, and prepared for whole-mount staining(4). Briefly, approximately 4mm x 4mm x 2mm sections of adipose tissue were washed in PBS for 10 minutes then stained sequentially with DAPI (Thermofisher; D1306) and Cell Mask Orange (ThermoFisher; C10045) for 1 hour each, with PBS washes between. Images and

measurements were obtained using a spinning disc confocal microscopy system and Slidebook 6 (Intelligent Imaging Innovation, Denver CO, USA).

RNA isolation and quantitative real-time PCR: Liver samples from *Siah2* KO and wild type mice were collected every 3 hours over 24 hours (n=3 per genotype and time point). Total RNA was extracted from livers using Trizol reagent (Invitrogen) per manufacturer's instructions. Approximately one microgram aliquots of total RNA were reverse transcribed and subjected to quantitative PCR using Applied Biosystems cDNA kit (Thermo Fisher) and SsoAdvanced SYBR GREEN qPCR mix (Bio-Rad) respectively. Samples were run using the CFX96 Touch Real-Time PCR Detection System (running version 3.1 of the CFX Manager Software; Bio-Rad) and data all normalized to Gapdh and plotted relative to the time-independent average of the wild type samples using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used were as follows:

m*GAPDH*: forward: AGACAGCCGCATCTTCTTGT, reverse: CTTGCCGTGGGTAGAGTCAT; mNr1d1: forward: CCCTGGACTCCAATAACAACACA, reverse: GCCATTGGAGCTGTCACTGTAG; *mArntl*: forward: AACCTTCCCGCAGCTAACAG, reverse: AGTCCTCTTTGGGCCACCTT; *mPer2:* forward: GAAAGCTGTCACCACCATAGA, reverse: AACTCGCACTTCCTTTTCAGG; *mDbp:* forward: GGAACTGAAGCCTCAACCAAT, reverse: CTCCGGCTCCAGTACTTCTCA; *mCry1:* forward: TGAGGCAAGCAGACTGAATATTG, reverse: CCTCTGTACCGGGAAAGCTG.

Transcript profiling: Total RNA was extracted from the *Siah2* KO and wild type livers harvested from mice maintained on normal chow at 3-hour intervals across a 12:12 LD cycle. Total RNA was isolated as above, and equal amounts were pooled from livers from 3 mice/time/genotype/sex for RNAseq. These RNA pools were converted into sequencing libraries by using Illumina TruSeq stranded mRNA Library Prep kits and sequenced by Omega BioServices (Norcross, GA) using the Illumina HiSeqX10 platform. Samples were sequenced to a depth > 37 million 150bp X 150bp paired end reads. The reads were mapped to the mouse MGSCv39-mm9 genome using Tophat 2.1.0. Expression levels were assessed using Cufflinks 2.2.1, which calculates raw counts and the number of fragments per kilobase per million (FPKM). Low expression genes were filtered out if the sum of their raw count was less than 100 across the time points in all 4 groups (male wild type, male *Siah2* KO, female wild type and female *Siah2* KO). Count data was used for DeSeq2 (https://yanli.shinyapps.io/DEApp/) (Fig. S2), and FPKM was used for all other analyses.

Time Series Analysis for Circadian Cycling: MetaCycle::meta2d (ver.1.2.0; https://CRAN.R-project.org/package=MetaCycle) was used to detect circadian transcripts with the default settings and period length set to 20 for 'minper' and 28 for 'maxper'(5). Data from one cycle was concatenated to create a 48 hour time series for analyses using JTK cycle(6) to identify cycling transcripts and their peak expression phases and DODR ver.0.99.2(7) (https://CRAN.R-project.org/package=DODR) to directly compare gene expression profiles between sexes/genotypes. We acknowledge that concatenating the data like this likely increases the false-positive rates in both algorithms, but it also greatly reduces the false-negative rate; the latter is of greater value when trying to identify potential underlying mechanisms that differentiate two groups. To help limit the overall effect of false-positives, we combined results from 2-3 measures (i.e. rhythmicity, phase, meta.p) from both analyses to define differences (described below). It should be noted that performing these analyses on non-concatenated data produces the same overall proportionality in the results – *Siah2* KO females still have ~60% more rhythmically expressed genes than the other groups, with similar changes in phase etc,

just with fewer numbers of genes in each category. The peak times (i.e. phase) were derived using JTK cycle and imported into Oriana (version 3, Kovach Computing, Anglesey, Wales UK) to produce Raleigh plots and GraphPad Prism (v7 or later; GraphPad Software, SanDiego CA, USA) for further analyses. Heat maps were generated using an R code (https://github.com/gangwug/SRBR_SMTSAworkshop/blob/master/R/fig.R).

The goal of JTK-cycle is to identify rhythmic transcripts within a dataset but does not perform direct comparisons between datasets. DODR identifies overall differences between time-series datasets, including phase, amplitude and overall abundance, but does not assess circadian-like rhythmicity per se, thus can identify changes even in transcripts that are not rhythmic in either dataset being compared. Therefore, we used JTK cycle parameters to define rhythmicity and peak phase, and DODR to substantiate differences/lack of differences between groups, as depicted in the table below (bold font indicates key difference).

	Group 1 JTK adjP value	**Group 2 JTK adjP value**	**DODR meta.p**	**Phase difference**
Rhythmic in both, unchanged	< 0.05	< 0.1	< 0.01	**< 6 hours**
Rhythmic in both, phase change	< 0.05	< 0.1	Not used	**> 6 hours**
Rhythmic in both, other difference	< 0.05	< 0.1	**> 0.01**	< 6 hours
Gained Rhythm/ Females only	< 0.05	**> 0.1**	**> 0.01**	< 6 hours
Lost Rhythm/ Males only	**> 0.1**	< 0.05	**> 0.01**	< 6 hours

We chose these JTK adjP value cutoffs in attempt prevent overestimating differences between groups, while still including genes with less robust rhythms. Using the same cutoffs for both groups increases the number of different genes between groups, but reducing the cutoffs proportionally reduces the numbers of genes in each category, but the differences between groups remain proportional (i.e. there are still more differences in females than males). DODR was not used to support phase changes, but meta.p > 0.01 for 85% of genes with Siah2-loss induced phases differences in females (Fig 4a), 75% of those changes in males (Fig 4a) and 82% of sexually dimorphic phase changes (Fig S11) genes (**see Supplemental datasets 1-3**). In addition, we found small subsets of genes we classified as rhythmic in across both groups, but DODR identified a difference other than a 6-hour phase change were not closely examined but are denoted in **Supplemental datasets 1-3**. All of the differentially expressed genes were combined and subjected to Gene Ontology analyses (http://geneontology.org). Top relevant child terms for biological processes were selected after sorting by decreasing order of fold enrichment and increasing order of p-values.

Locomotor and feeding behavior: Wheel running locomotor activity was recorded and analyzed as described previously (8). For feeding behavior, mice were fed ad-libitum with normal chow and eating behavior was recorded for 8 days using infrared video cameras (ZOSI 720p CVI TVI, ZosiTech, Zhuhai city, China) connected to a TigerSecu 8 Channel DVR Security Video Recording System. Videos were analyzed using Noldus EthoVision XT (v14, Noldus, Leesburg VA, USA), and feeding behavior was coded in 1 min bins using the following criteria:

1) the mouse took food from the feeder with its mouth or, 2) the mouse moved food in the feeder with its mouth; either behavior having persisted for 3 seconds or more. When eating behavior occurred, that 1 min bin was coded as “1”. One-minute bins without any feeding behavior were coded as “0”. One-minute bins were summed for each hour and averaged across the 8-day recording according to ZT hour in 1-hour intervals for each mouse, and then normalized to the within animal mean of its total daily feeding activity.

Statistical Analyses: Except where noted above, all graphs and statistical analyses were generated using Graphpad Prism (v7 or later; GraphPad Software, SanDiego CA, USA). Statistical analyses performed were typically two-way ANOVAs, examining *sex* x *genotype* or *genotype* x *diet* interactions, followed by Sidak’s multiple comparison test, unless otherwise indicated. Relevant F values and degrees of freedom are also reported as ‘F (DFn, DFd) = [value]’ or t=[value], df=[value]. Differences were considered significant if $p<0.05$, unless otherwise indicated.

Supplemental References

1. Frew IJ*, et al.* (2003) Generation and analysis of Siah2 mutant mice. *Mol Cell Biol* 23(24):9150-9161.
2. Byers SL, Wiles MV, Dunn SL, & Taft RA (2012) Mouse estrous cycle identification tool and images. *PLoS One* 7(4):e35538-e35538.
3. Owino S*, et al.* (2018) Nocturnal activation of melatonin receptor type 1 signaling modulates diurnal insulin sensitivity via regulation of PI3K activity. *J Pineal Res* 64(3):10.1111/jpi.12462.
4. Berry R*, et al.* (2014) Imaging of adipose tissue. *Methods Enzymol* 537:47-73.
5. Wu G, Anafi RC, Hughes ME, Kornacker K, & Hogenesch JB (2016) MetaCycle: an integrated R package to evaluate periodicity in large scale data. *Bioinformatics* 32(21):3351-3353.
6. Hughes ME, Hogenesch JB, & Kornacker K (2010) JTK_CYCLE: An Efficient Nonparametric Algorithm for Detecting Rhythmic Components in Genome-Scale Data Sets. *Journal of Biological Rhythms* 25(5):372-380.
7. Thaben PF & Westermark PO (2016) Differential rhythmicity: detecting altered rhythmicity in biological data. *Bioinformatics* 32(18):2800-2808.
8. Ehlen JC*, et al.* (2015) Maternal Ube3a Loss Disrupts Sleep Homeostasis But Leaves Circadian Rhythmicity Largely Intact. *J Neurosci* 35(40):13587-13598.
9. Palierne G*, et al.* (2016) Changes in Gene Expression and Estrogen Receptor Cistrome in Mouse Liver Upon Acute E2 Treatment. *Molecular Endocrinology* 30(7):709-732.

Datasets S1-S4. (separate files)

Supplemental datasets 1-3 list the genes/NM identifiers and FPKM data, and the JTK-cycle and DODR results from comparing wildtype and *Siah2* KO females (**Dataset 1**), wild type and *Siah2* KO males (**Dataset 2**) or wild type males and wild type females (**Dataset 3**); a 'key' for the column labels is provided in each. These data are parsed according to difference within the comparison (described below). **Dataset 4** lists the genes with time-independent changes in gene expression, due to either Siah2 loss (in males and females) or sex. DeSeq2 results are included. The full RNAseq datasets have been deposited in NCBI GEO, under the identifier (provided prior to publication).

Fig. S1.

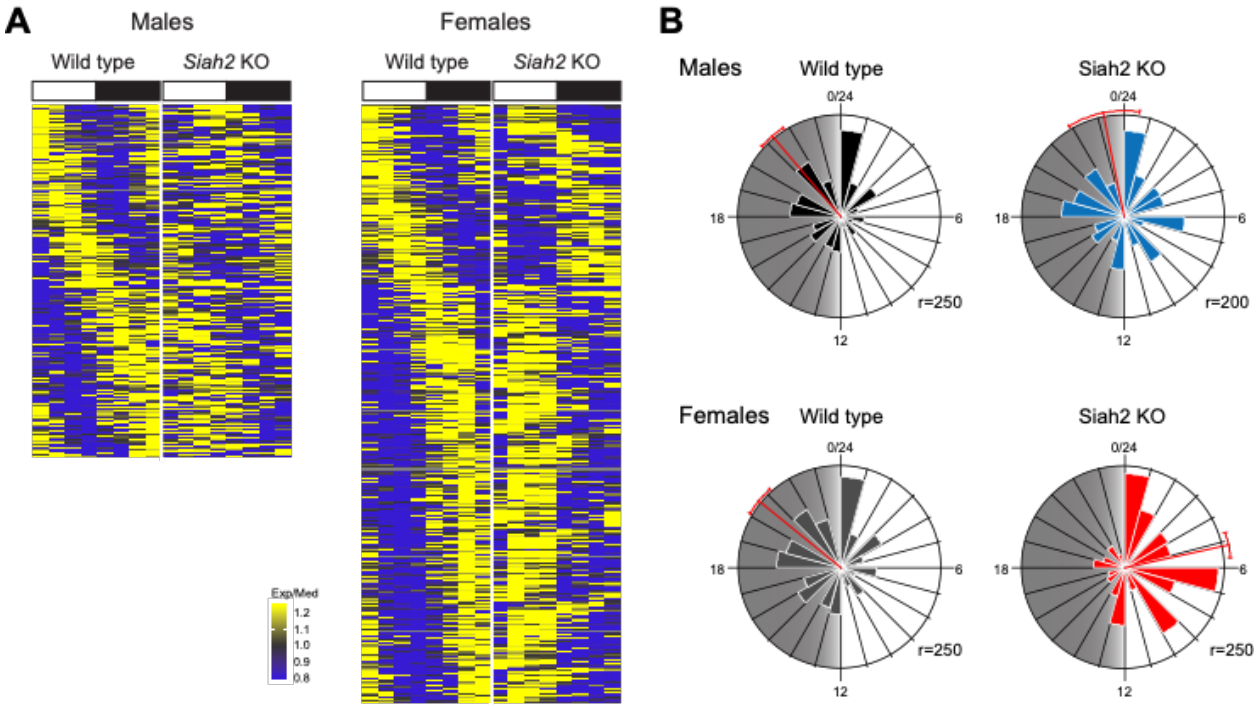

Fig. S1. Siah2 loss preferentially shifts nocturnally expressed genes in females

(A) Heatmaps depicting the expression of the genes that were rhythmically expressed in both genotypes but displayed a 6-hour (or greater) change in peak expression between genotypes. Genes are aligned across genotypes (separate for each sex) and sorted vertically by peak phase in wild type. Genes are also plotted on the same heatmap temperature scale across genotypes (separate for each sex), to enable comparisons of expression levels. The white-dark bar across the top of each indicates the light-cycle. **(B)** Raleigh plots (circular histograms) of peak phase for the genes shown in A. r = radius in number of genes. The red lines indicate mean overall peak phase, +/- sem.

Fig. S2.

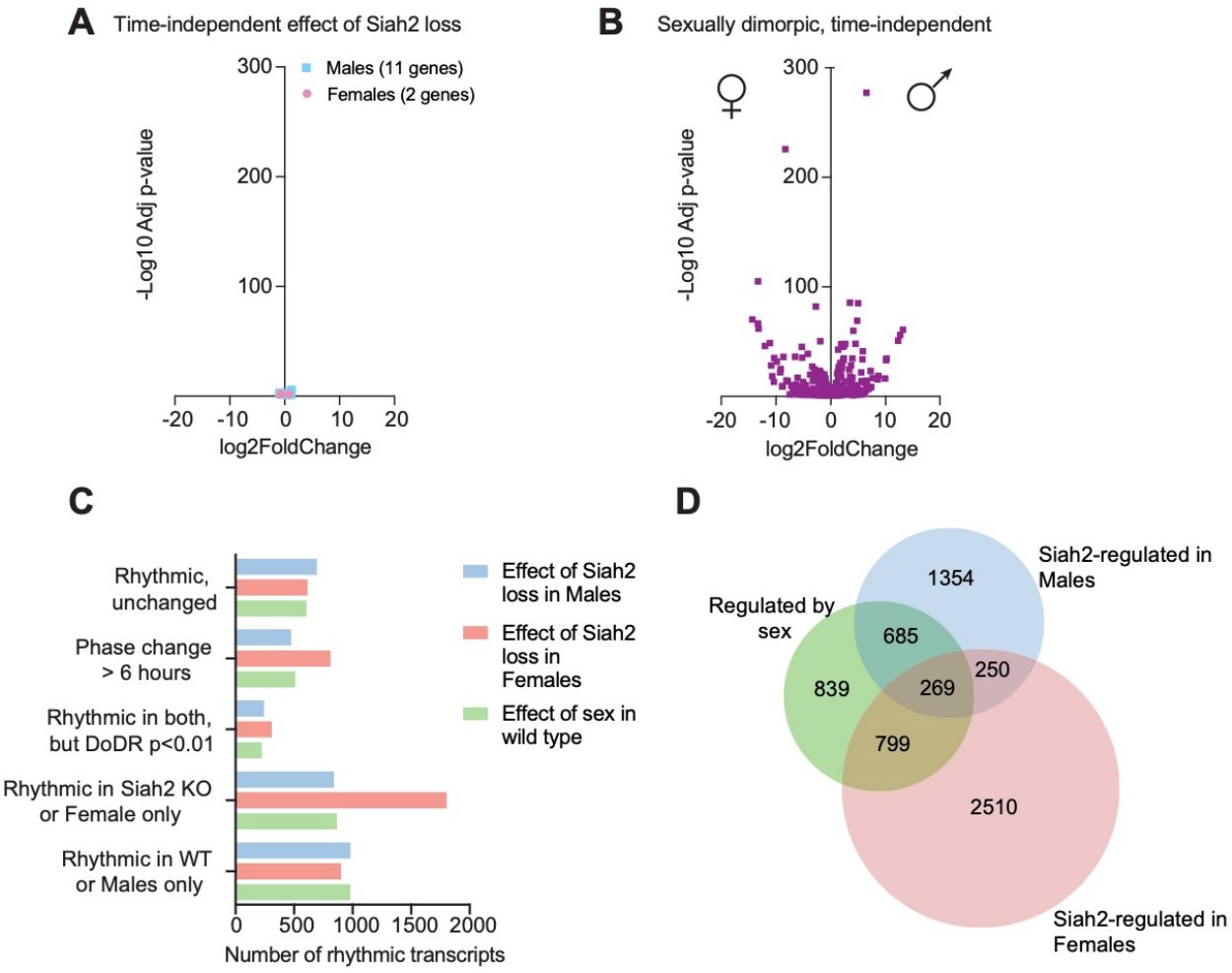

Fig. S2. Siah2 loss does not drastically alter time-independent gene expression or preferentially target sexually dimorphic gene expression profiles.

(A) RNAseq data were combined across timepoints according to sex/genotype, treating timepoints as biological replicates (n=8 per sex/genotype) and the effects on Siah2 loss on gene expression in each sex were compared using DeSeq2. This volcano plot shows the few genes that were significantly different (pAdj <0.05), which are plotted scale identical to those shown in **B** for comparison. **(B)** Time-independent gene expression comparison between wild type males and females, as described for A. Only the significantly different (pAdj <0.05) transcripts are shown for clarity. The sex symbols indicate higher expression in female or male livers, respectively. Genes for both A and B are listed in **Supplemental Dataset 4. (C).** Comparison of the effects on diurnal gene expression of Siah2 loss in males and females and the effect of sex in wild type mice, categorized as described in the **Supplemental Materials and Methods**. Some of these data are replotted from **Figure 1** for comparison. See also **Supplemental Datasets 1-3**. **(D)** Venn diagrams depicting the overlap in genes with dimorphic rhythmic expression with those differentially expressed by Siah2 loss in male or female livers, across the categories in **C**. See also **Supplemental Datasets 1-3.**

Fig. S3.

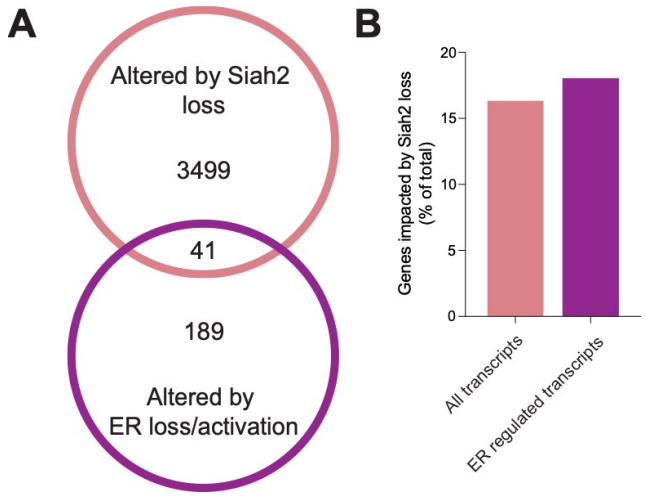

Fig. S3. Estrogen responsive genes are not enriched among those altered by Siah2 loss in female livers.

(A) Venn diagram depicting the overlap between genes regulated by Siah2 loss and estrogen signaling in female livers (9). **(B)** Proportions of transcripts whose expression was altered by Siah2 loss in female livers. Siah2 loss altered the expression of 3,540/22,002 total genes, or ca. 16% of the total genes examined. Siah2 loss altered a similar proportion (ca. 18%) of estrogen responsive genes suggesting that Siah2 loss is not selectively impacting estrogen-signaling. Thus, the effects of Siah2 loss on rhythms is unlikely to occur via mis-regulation of estrogen signaling.

Fig. S4.

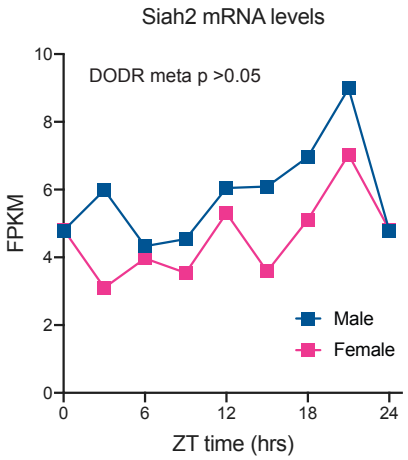

Fig. S4. *Siah2* mRNA expression itself is not sexually dimorphic. *Siah2* mRNA levels in wild type male and female livers. Overall there was no detectable difference in expression (DODR meta p >0.05, DESeq2 after combining timepoints padj >0.05).

Fig. S5.

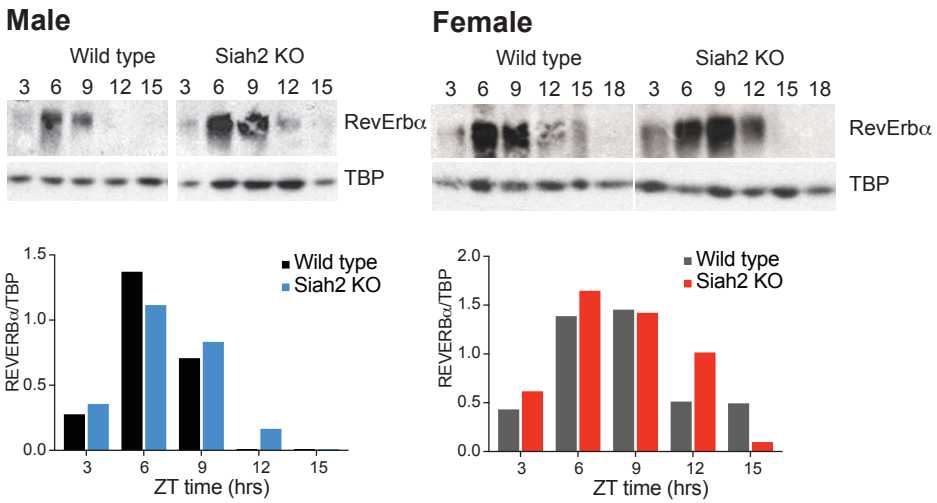

Fig. S5. Siah2 loss does not significantly alter RevErbα protein abundance rhythms in the liver. Livers (n=3 per timepoint) were harvested each group, equal amounts of total proteins were pooled and examined by immunoblot for RevErbα abundance (using antisera EY16D, Cell Signaling), normalized by Tata-Binding Protein (TBP, D5C9H, Cell Signaling). ImageJ quantifications of the blots above are shown below. This result is representative of two independent experiments (i.e. tissue collections) covering the interval from ZT 7-14 at hourly intervals.

Fig S6

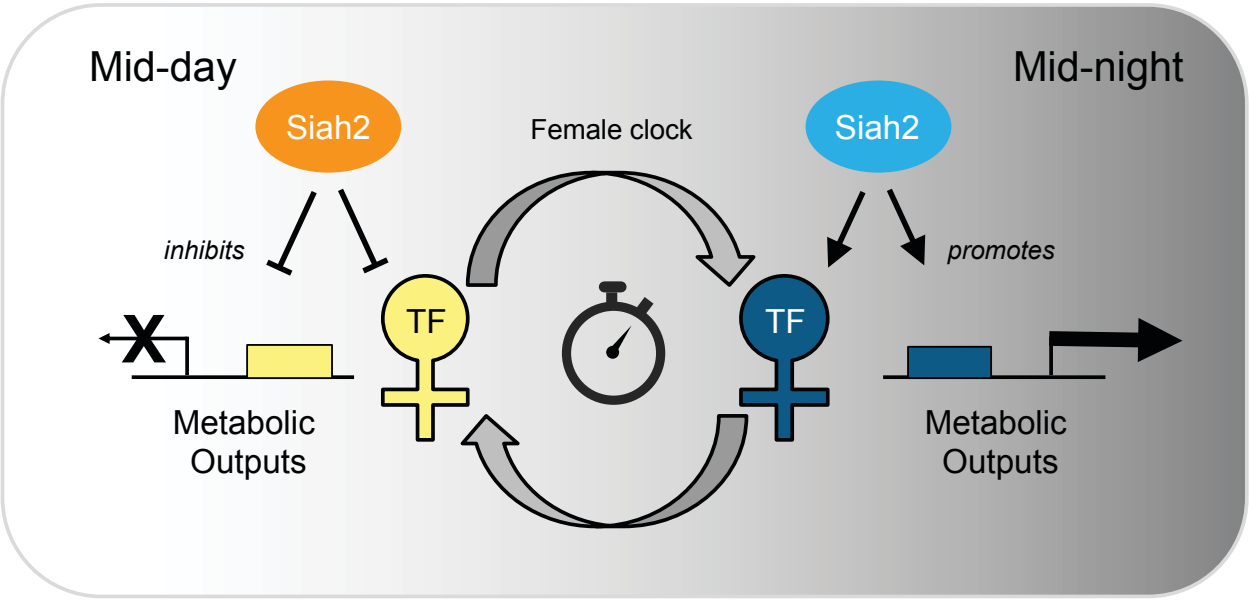

Fig. S6. Model depicting likely intersecting points of Siah2 with the female circadian clockwork.

Siah2 appears to have opposing roles on regulating rhythmic gene expression in the female liver. Our data suggest Siah2 plays a strong role in repressing gene expression at mid-day, while also promoting expression of many genes during the night. In addition, for some targets, Siah2 appears to advance/delay normal nocturnal expression to the daytime. Since Siah2 is not known to directly bind DNA, we propose that Siah2 is mediating these effects via regulating unknown transcription factors that play a female-specific role in overall circadian regulation.

Fig. S7.

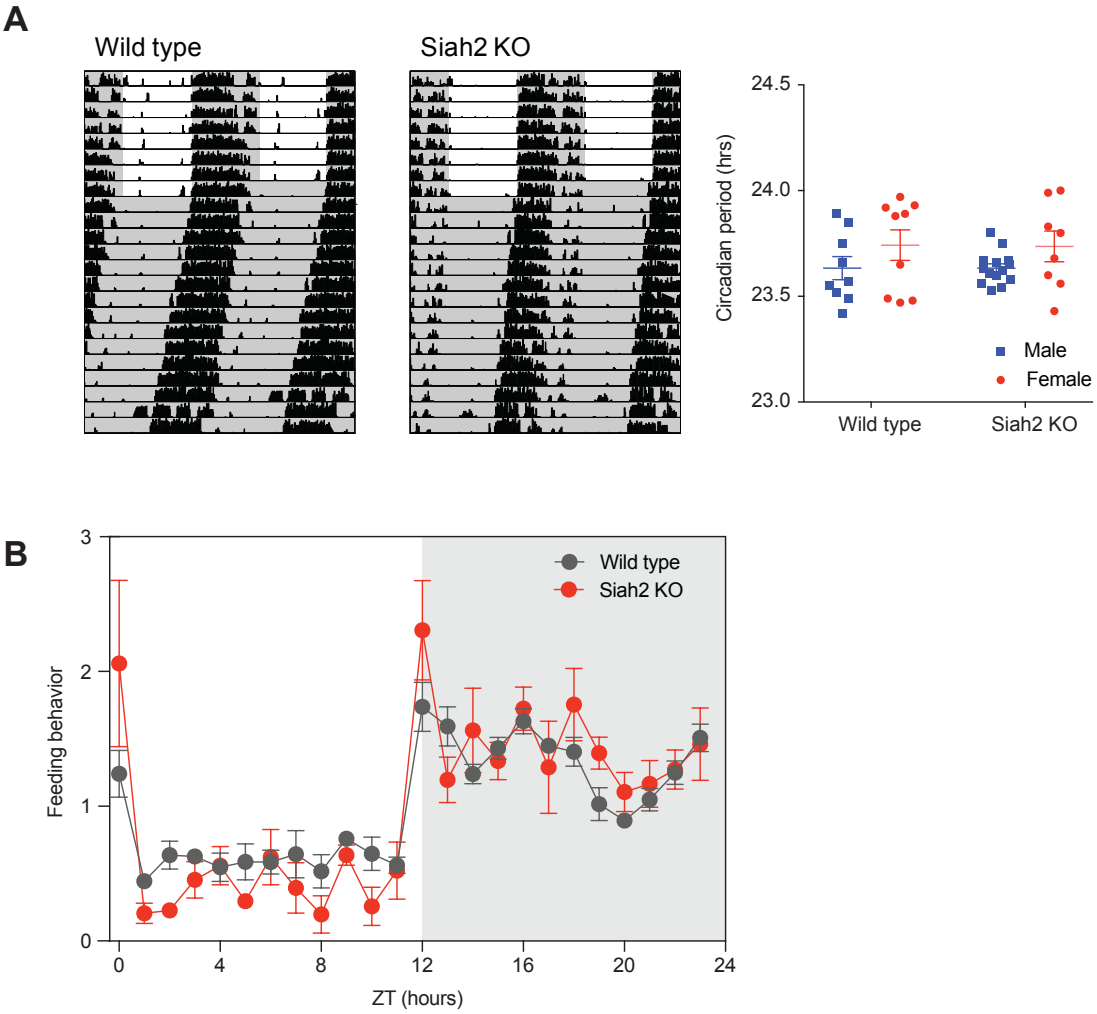

Fig. S7. Locomotor and feeding behavior are unaffected by Siah2 loss.

(A) Representative double-plotted actograms of wheel running behavior in wild type or *Siah2* KO mice. The first 8 days of the recordings (indicated by the alternating white/gray shading) were done on a 12:12LD cycle, followed by constant darkness (solid gray shading). Individual and mean +/- SEM data obtained from mice of both genotypes and sexes are shown on the right, combined from animals of both sexes and genotypes run in two independent experiments (n = 9 wild type males, 9 wild type females, 14 Siah2 KO males, and 8 Siah2 KO females). Siah2 loss did not significantly alter the behavioral circadian periods within either sex ($p>0.9$, two-tailed t-test, effect sizes of either genotype was than 0.006 hours). **(B)** Diurnal feeding behavior of wild type (n=5) and *Siah2* KO (n=4) female mice on normal chow. Each point is the mean of the average 8-day profile produced by each animal, and the error bars (SEM) indicate the inter-animal variability at each time point. Two-way ANOVA did not detect a significant interaction between genotype and time that would indicate a change in the daily feeding pattern ($p = 0.4751$ for interaction, $F (23, 161) = 0.9947$).

Fig. S8.

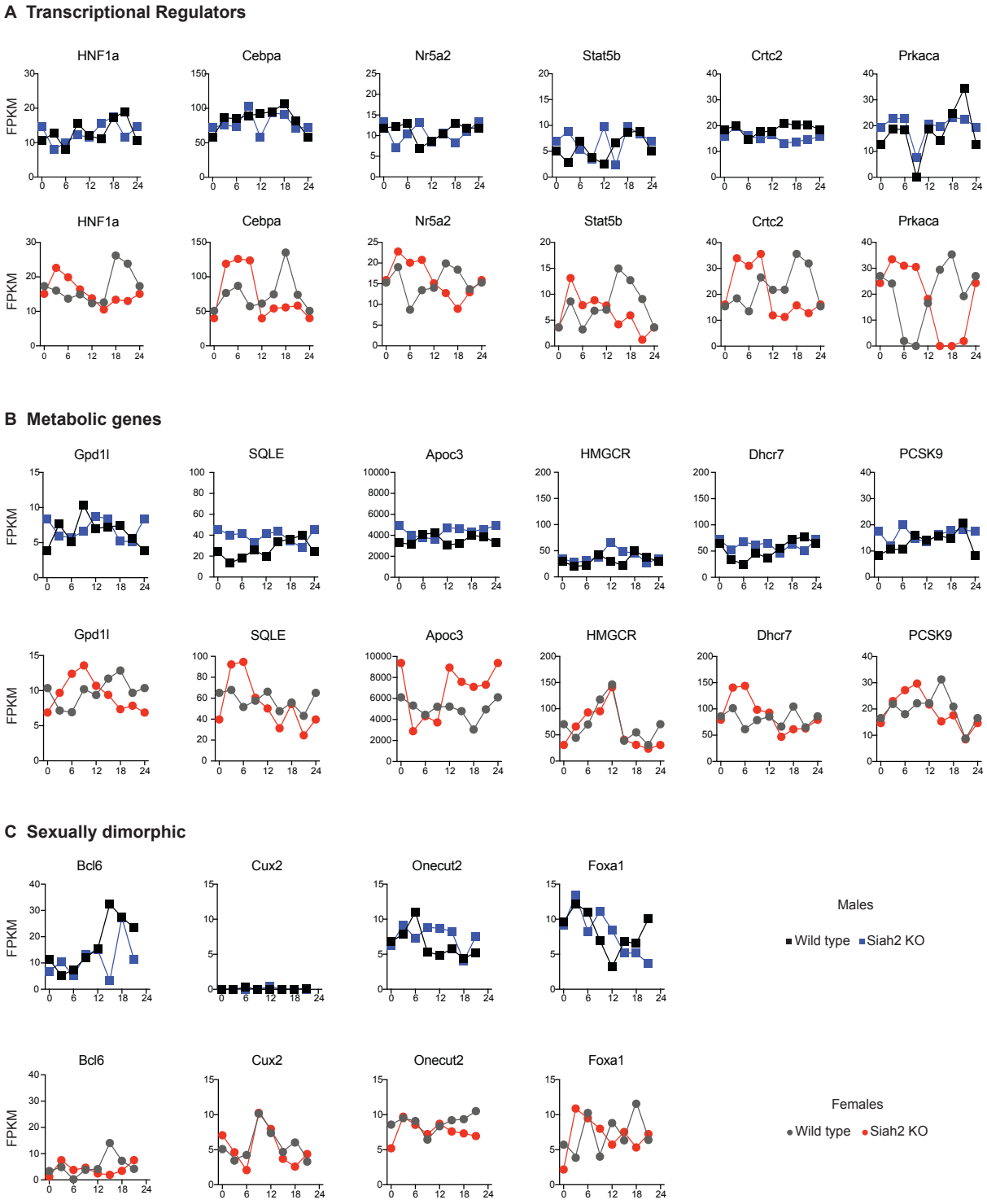

Fig. S8. Siah2 loss does not induce male-like gene expression profiles. RNAseq profiles for representative genes encoding transcriptional regulators **(A)**, lipid metabolic **(B)**, or factors involved in mediating sexual-dimorphic gene expression **(C)** in the liver.

Fig. S9.

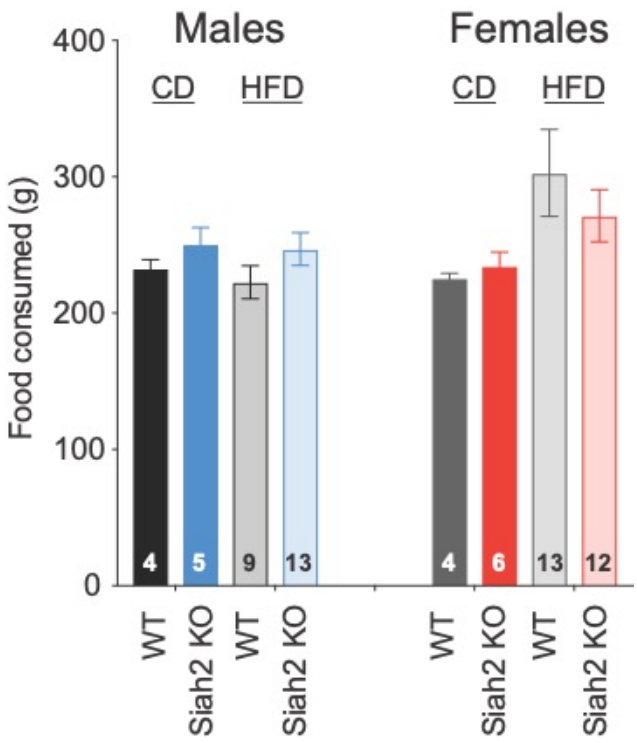

Fig. S9. Cumulative food consumption after 13 weeks on diet. Bars = means +/- sem, n's for each group are indicated within each bar. CD = control diet, HFD = high-fat diet fed mice. Intake of either diet was not significantly altered by genotype (two tailed t-tests, $p > 0.18$ between genotypes, for all diets and sexes (CD males $t=1.187$, $df=7$, CD females $t=0.6856$, $df=8$, HFD males $t=1.388$, $df=20$, HFD females $t=0.8299$, $df=23$).

Fig. S10.

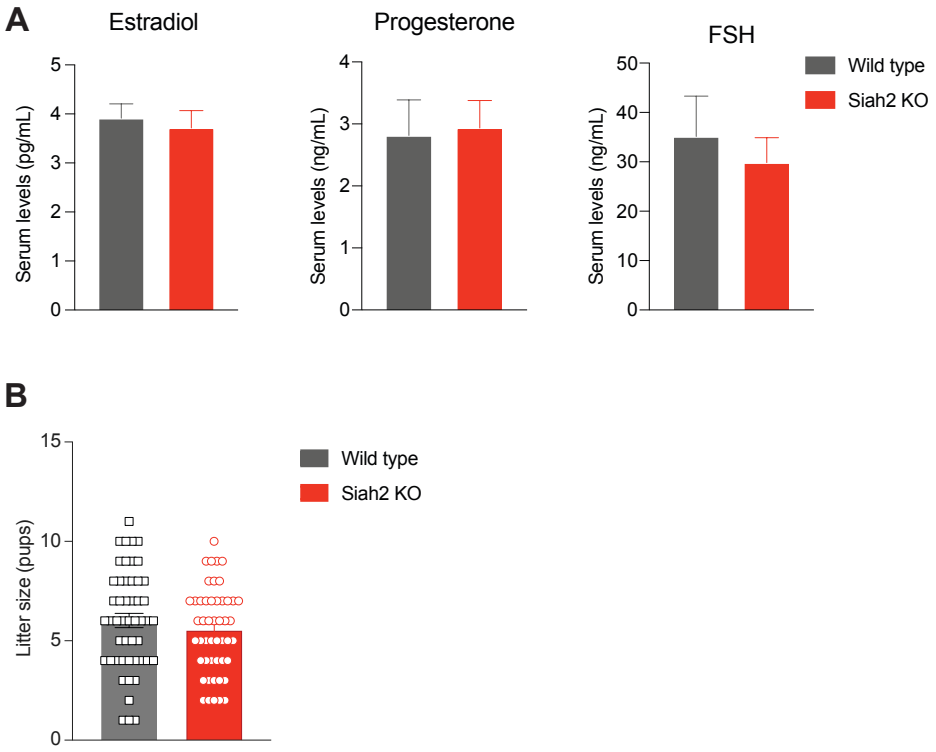

Fig. S10. Circulating female hormones and litter sizes are not impaired by Siah2 loss.

(A) We collected serum from females in proestrus sacrificed between ZT 7-14 for measurement of the indicated hormones (LH levels were below level of detection). Mean +/- SEM (n=7 each, except n= 5 for wild type progesterone) hormone levels are shown. $p > 0.5$ for each, unpaired two-tailed t-tests. (**B)** Litter sizes for last 50/49 litters obtained from 18/19 homozygous wildtype/homozygous *Siah2* KO females (respectively), bred with males of like genotypes. Individual data points and mean +/- SEM are shown ($p = 0.3$, $t=1.038$, $df=97$, two-tailed t-test).

Fig. S11.

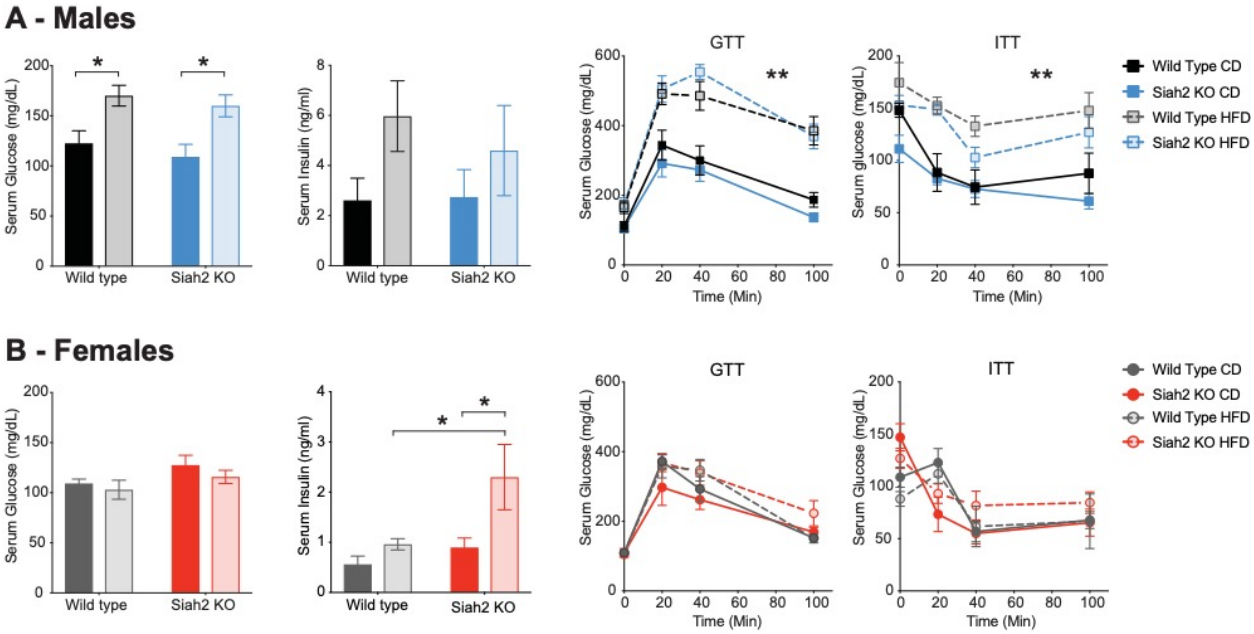

Fig. S11. Obese *Siah2* KO females do not show strong diabetic-like phenotypes.

Fasting serum glucose and insulin levels, as well as glucose and insulin tolerance (GTT and ITT, respectively) in males **(A)** and females **(B)**. All animals were fasted for 4 hours (starting at ZT0) before testing. Data are all means +/- sem, n=5-7 mice per group, except n = 4 for wild type CD males and wild type HFD females. * = $p<0.03$, ** $p=0.0009$ for time x diet interaction ($F(3, 72) = 6.140$), three-way ANOVA), and main effect of diet ($p<0.0001$, $F(1, 72) = 121.0$) but no three-way interaction ($p=0.79$, $F(3, 72) = 0.3480$), *** main effects of diet ($p<0.0001$, $F(1, 72) = 68.48$") and genotype ($p=0.0043$, $F(1, 72) = 8.688$) only, no three-way interaction ($p=0.6376$, $F(3, 72) = 0.5684$). # $p=0.039$, $t=2.605$, $df=15$ and ## $p=0.037$, $t=2.64$, $df=15$ using Sidak's multiple comparison test.

Fig. S12.

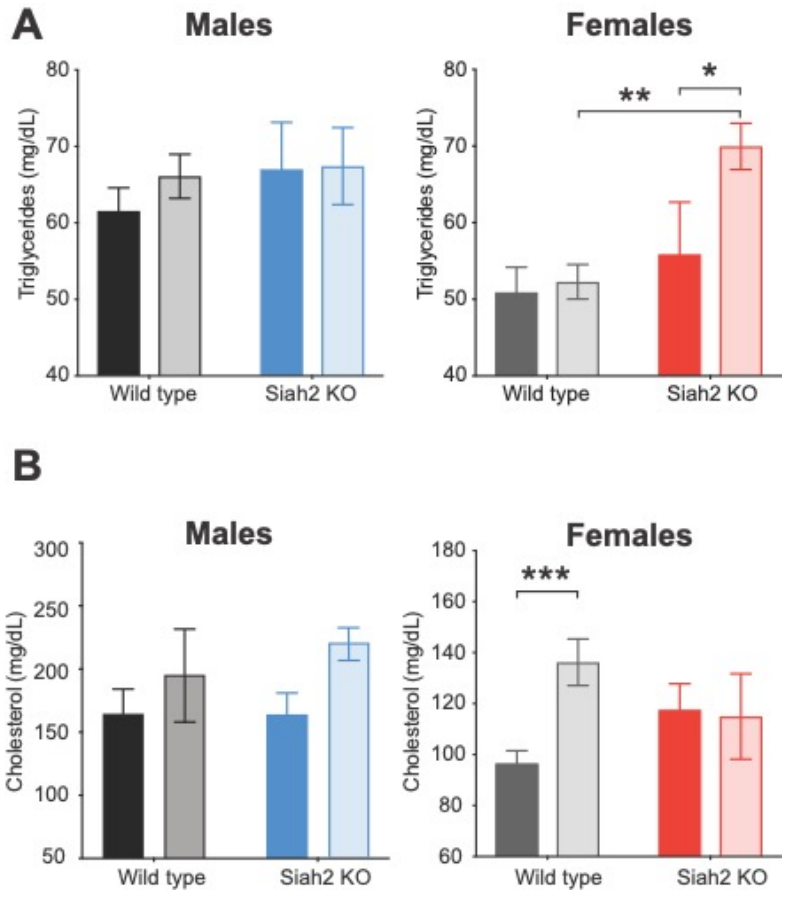

Fig. S12. Siah2 differentially regulates lipoprotein responses to diet in females.

Serum triglycerides **(A)** or total cholesterol **(B)** in response to 13 weeks of feeding CD (dark bars) or HFD (light bars). Sample were collected at ZT10. Mean +/- SEM are shown (n=5 mice sampled per group, except n= 4 for Siah2 KO males or females fed CD). * p = 0.048 (t=2.05, df = 15), ** p = 0.009 (t=30342, df = 15), *** p = 0.043 (t=2.563, df=15), Sidak's multiple comparisons test.

Fig. S13.

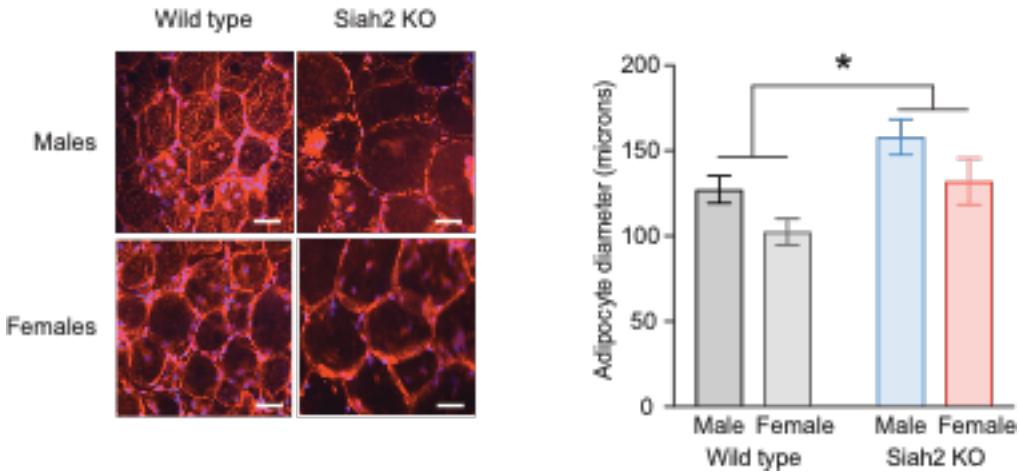

Fig. S13. Sex-independent effect of Siah2 loss on adipocyte size on HFD.

Left – Representative images of adipose samples obtained from mice fed HFD for >13 weeks and stained with Cell Mask Orange (red) and DAPI (blue) to visualize cell membranes and nuclei, respectively. Scale bar = 50 microns. Right – quantitation of 100 cell diameters measured per mouse (mean +/- sem, n=3 mice/group). * p=0.018 ($F(1, 8) = 8.836$), main effect of genotype, two-way ANOVA.