Title

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Journal

Gastroenterology, 158(7)

ISSN

0016-5085

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Publication Date

2020-05-01

DOI

10.1053/j.gastro.2020.01.050

Peer reviewed

Published in final edited form as:

Gastroenterology. 2020 May ; 158(7): 1948–1966.e1. doi:10.1053/j.gastro.2020.01.050.

Circadian Rhythms in the Pathogenesis and Treatment of Fatty Liver Disease

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Abstract

Circadian clock proteins are endogenous timing mechanisms that control the transcription of hundreds of genes. Their integral role in coordinating metabolism has led to their scrutiny in a number of diseases, including NAFLD. Discoordination between central and peripheral circadian rhythms is a core feature of nearly every genetic, dietary, or environmental model of metabolic syndrome and NAFLD. Restricting feeding to a defined daily interval (time-restricted feeding) can synchronize the central and peripheral circadian rhythms, which in turn can prevent or even treat the metabolic syndrome and hepatic steatosis. Importantly, a number of proteins currently under study as drug targets in NAFLD (SREBP, ACC, PPARs, and incretins) are modulated by circadian proteins. Thus, the clock can be used to maximize the benefits and minimize the side effects of pharmaceutical agents for NAFLD. The circadian clock itself has the potential for use as a target for the treatment of NAFLD.

Keywords

Dyssynchrony; gut microbiome; steatohepatitis; lipogenesis; bile acids

The 2017 Nobel Prize in Medicine signaled a recognition of the fundamental role that circadian clock proteins play in physiology and disease. It was awarded to Jeffrey C. Hall, Michael Rosbash, and Michael W. Young for their discovery of molecular mechanisms

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Author Contributions: ARS, SD, and AZ drafted the manuscript, AZ revised the manuscript for important intellectual content and provided supervision.

Disclosures: All authors have no relevant disclosures. Dr. Zarrinpar is a co-founder and “equity holder” of Tortuga Biosciences, a company that engineers native bacteria to functionally manipulate the gut microbiome, and wholly unrelated to the circadian research described in this review.

controlling circadian rhythms in *Drosophila.* Clock proteins are ubiquitous endogenous timing mechanisms that help maintain energy homeostasis by coordinating cellular processes within and between organ systems. They do so by regulating the transcription of hundreds of genes, including themselves. Their integral role in coordinating metabolism has led to closer scrutiny of their contribution to a number of diseases including non-alcoholic fatty liver disease (NAFLD).

Though the term “circadian rhythms” usually conjures thoughts of jet lag and sleep deprivation, physiologically they are core cellular processes that affect every organ system. The pervasiveness of clock proteins, which are found in diverse organisms from cyanobacteria to mammals, shows their evolutionary importance to life. Development of a molecular program to anticipate and prepare for periodic, predictable changes gives a tremendous adaptative advantage. Organisms have developed specialized sensory cells that allow them to adjust their own rhythmicity to external cues (called zeitgebers – German for “time-giver”). The daily light-dark cycle on earth governs rhythmic changes in the behavior and physiology of most species, including humans. Almost all organisms consolidate their biological processes (e.g. sleeping, feeding) to particular times of day. To acknowledge that the periodicity of these oscillations stays approximately 24 hours, Franz Halberg, a pioneer in the field of biological rhythms, introduced the term “circadian rhythms” (from Latin, *circa* – “about”, *diem* – “a day”) in 1959.

In mammals, the circadian pacemaker resides in hypothalamic structures called the suprachiasmatic nuclei (SCN), and it is entrained by specialized retinal ganglion cells that measure ambient light (Figure 1). However, circadian clock proteins are not restricted to the SCN. Explants from lung, liver, and other organs exhibit sustained oscillations *in vitro*.[1, 2] Thus, endogenous functional clocks are also active in peripheral tissues.[3] Temporal signals such as timing of food intake can reset the hepatic clock without affecting the SCN central clock rhythms. Consequently, different entrainment signals can activate peripheral and central circadian clocks separately.[4, 5] However, this does not undermine the importance of the SCN in coordinating physiological processes across multiple tissues.

In this review, we will describe the molecular organization of the circadian clock proteins, the relationship between circadian clock and metabolic master-regulators, and clock regulation of metabolic processes important to the pathophysiology of NAFLD. Environmental cues that entrain peripheral circadian rhythms will be also discussed in detail. The effects of circadian dyssynchrony on host metabolism will be summarized next along with recent preclinical and clinical studies which show that maintaining peripheral circadian rhythms by consolidating feeding within a narrow time frame (i.e. time-restricted feeding [TRF]) can restore metabolic health. Over the last five years, there have been many excellent reviews of pre-clinical data regarding of the role of circadian clock in hepatic metabolic processes specifically,[6–11] and metabolic syndrome in general.[12–16] Ours will build on this strong body of work by focusing on key regulatory pathways that play an important role in the treatment of steatohepatitis and fibrosis, thus demonstrating the vital role of circadian machinery in helping our understanding of NAFLD.

MOLECULAR ORGANIZATION OF THE CLOCK

The circadian clock in mammals, expressed in nearly every cell, is comprised of a series of transcription-translation feedback loops (Figure 2). These include circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) as transcriptional activators. These are opposed by period (PER) and cryptochrome (CRY) as transcriptional repressors, (Figure 3A). CLOCK and BMAL1 are two central components that make up the positive (or activation) limb of the molecular clock. The CLOCK:BMAL1 heterodimer then translocates into the nucleus to initiate transcription at the E-box (5′-CACGTG-3′), a specific DNA binding sequence (DBS) on the promoters of its target genes.[17] The main downstream targets of CLOCK:BMAL1 include their own repressors, period (PER1, PER2 and PER3) and cryptochrome (CRY1, CRY2), in addition to over 300 other genes.[18] Over time, PERs and CRYs accumulate in the cytoplasm, where they are further regulated by casein kinase 1 (CK1) ε, CK1δ, and F-box/LRR-repeat protein 3 (FBXL3). CK1ε and CK1δ phosphorylate PER for degradation; FBXL3 promotes the degradation of CRY.[19, 20] However, if Ck^ phosphorylates PER after it is bound to CRY, the three-protein complex migrates into the nucleus and inhibits the CLOCK:BMAL1 heterodimer.[21] By inhibiting their own activators, PERs and CRYs repress the transcription of their own genes. Post-translational modification of PERs and CRYs leads to their degradation. This then initiates a new circadian cycle with increased binding of CLOCK:BMAL1 to the now open E-Box binding sites.

In a second regulatory loop, CLOCK:BMAL1 drives the transcription of the nuclear hormone receptors retinoic acid receptor-related orphan receptor (*Ror*) α and *Rory* and *Rev-Erbα* and *Rev-Erbβ* by binding E-box elements on their promoters (Figure 3B).[22–24] Both of these receptors can bind the ROR-responsive element (RORE) promoter DNA binding sequence (5'-AGGTCA-3'), but with opposite effects; REV-ERBα/β suppresses expression at the site, where as RORα/γ promotes expression. [25, 26] Together, REV-ERBs and RORs generate large scale cyclical fluctuations in transcription of hundreds of clock-controlled genes, including regulation of the transcription of *Bmal1*.[27]

Recent studies have revealed an additional autonomous feedback loop involving basic helix-loop-helix family member e40 (BHLHE40, more commonly DEC1) and BHLHE41 (more commonly DEC2) which also suppress CLOCK:BMAL1 activity (Figure 3C).[28, 29] CLOCK:BMAL1 induces transcription of *Dec1* and *Dec2* by binding E-box elements on their promoters. Unlike PERs and CRYs which act directly on the CLOCK:BMAL1 heterodimer, DECs compete with CLOCK:BMAL1 for the E-box, thus suppressing all E-box genes, including transcription of their own genes as well as that of PERs and CRYs.

Other circadian clock-controlled genes bring additional refinement to this complex interplay of feedback loops. CLOCK:BMAL1 modulates the expression of hundreds of other genes by promoting the transcription of D-box binding protein (DBP) by binding E-box elements on its promoter (Figure 3D).[11, 30] DBP rhythmically activates transcription of genes that have D-box elements in their promoter regions. Nuclear factor interleukin 3 (NFIL3, also E4BP4), which is regulated by a RORE promoter region (and thus susceptible to activation by RORs and suppression by REV-ERBs), represses DBP-dependent transactivation by

competing for the D-box. Computational genomic analysis of D-box elements has identified nearly 1500 regions that can be recognized by DBP and NFIL3.[31]

The transcription-translation feedback loops generate large scale rhythms in the activities and levels of downstream clock-controlled genes.[32] Since these processes are temporally controlled, it is important to regulate the abundance and activity of each component to ensure it is in the right place at the right time. This is achieved by post-translational modifications of clock components such as phosphorylation, acetylation, SUMOylation, O-GlcNAcylation, and ubiquitylation. Gene expression studies show that approximately 10-40% of the rodent genome exhibits a 24-hour rhythm in a highly tissue-specific manner.[33] However, humans are diurnal animals that feed during the day whereas most rodents are nocturnal animals that feed primarily at night. The recent publication of a transcriptome atlas of a baboon, a diurnal primate, shows that the aforementioned percentages from rodent studies do not convey the full picture.[3] Of the approximately 11,000 genes that are commonly expressed in all tissues in the baboon, 96.6% have 24-hour rhythmicity in at least one tissue. Greater than 80% of the 18,000 protein-coding baboon genes were circadian. More importantly, more than 80% of genes encoding proteins targeted by pharmaceuticals are transcribed cyclically. This implies that most physiological processes are under circadian control, and thus the disruption of circadian rhythms can result in widespread downstream pathology.

THE CIRCADIAN CLOCK REGULATES METABOLIC PROCESSES

Hepatic metabolic processes, including glucose, lipid, and cholesterol/bile acid metabolism are highly dynamic, influenced by feeding/fasting and circadian rhythms. Data supporting these relationships come from several lines of research, including metabolic phenotyping data from circadian clock transgenic mouse lines, molecular biology studies showing direct interactions between regulators of nutrient homeostasis and circadian clock proteins, and the relationship of feeding/fasting cycles and metabolic gene expression. In this section, we will discuss the relationship between circadian clock machinery with various aspects of metabolism that are dysfunctional in patients with NAFLD.

Circadian Clock and Regulators of Nutrient Homeostasis and Autophagy

The liver contains several master metabolic regulators that sense feeding/fasting states and control the expression of hundreds of downstream metabolic targets. These include AMP-activated protein kinase (AMPK), cAMP response element-binding protein (CREB), v-Akt murine thymoma viral oncogene homolog 1 (AKT/PKB), and sirtuin 1 (SIRT1). All have cyclical activity mainly driven by feeding behavior and can regulate, and are regulated by, circadian clock proteins.

AMPK, a fasting-sensitive protein kinase that regulates cellular energy homeostasis, can modulate, and is modulated by, the circadian clock machinery. AMPK can affect the length of circadian cycling by phosphorylating CRY and targeting it for degradation.[34] Moreover, RORα activation can modulate lipid metabolism and prevent hepatic steatosis by activating AMPK which controls key lipid regulators (discussed below).[35] Feeding, on the other hand, activates CREB, which upregulates *Per1* transcription.[36] This relationship connects G

protein coupled receptors (GPCRs) that use cAMP as a second messenger to transcription of circadian clock genes.[37] Several metabolic hormones including glucagon and epinephrine, both of which activate hepatic gluconeogenesis, can influence circadian clock machinery through CREB activation.[38–40] In addition, CRYs can independently suppress CREB thereby not only affecting hepatic gluconeogenesis but also modulating *Per* transcription.[39] Finally, AKT, a protein kinase that regulates glucose metabolism among other cellular processes (e.g. cell proliferation, apoptosis), and is also activated by feeding, can modulate the circadian clock machinery by phosphorylating BMAL1 and CLOCK in the cytosol and preventing their translocation across the nuclear membrane.[41]

Sensing the nutrient status is a critical regulation step in the maintenance of homeostasis. Metabolic stressors such as starvation, induce autophagy to utilize glycogen and lipid components for generating fuel.[42] Circadian induction of autophagy during low nutrient states (e.g. inactive state) allows efficient coordination of these cellular processes. AMPK, under glucose starvation conditions, phosphorylates and activates the autophagy-initiating kinase ULK1 (Unc-51 like autophagy activating kinase 1), thus promoting autophagy. However, under nutrient sufficiency, mammalian target of rapamycin (mTOR), another metabolic master-regulator, phosphorylates ULK1 and disrupts its interaction with AMPK, thus inhibiting autophagy.[43] Interestingly, liver specific *Bmal1-null* mice display dampened rhythms of autophagy gene expression, along with increased mTOR activation.[44] Thus, autophagy has multiple layers of regulation from metabolic regulators and the circadian clock.

SIRT1, an anti-aging and nutrient sensing protein, can upregulate the expression of a number of genes involved in autophagy and energy metabolism by binding to the E-box elements in a complex with the CLOCK:BMAL1 heterodimer.[45, 46] This complex in particular creates daily oscillations in nicotinamide phosphoribosyltransferase (NAMPT), which is the rate-limiting enzyme in NAD^+ (nicotinamide adenine dinucleotide) salvage pathway, thus inducing circadian oscillation of intracellular NAD^+.[47, 48] SIRT1 can further act on circadian clock proteins by deacetylating BMAL1 and PER2, as well as other key metabolic regulators, through its regulation of NAD^+.[48] SIRT1 histone deacetylation leads to a repressive chromatin configuration, essentially down regulating their downstream targets.[49] As a result of SIRT1 interaction with CLOCK:BMAL1 and deacetylation of key regulator proteins, these genes have day-night rhythmicity to their transcriptional activity and temporal regulation of their target genes. Thus, the role of autophagy in the pathogenesis of NAFLD is well described and compounds that target autophagy pathways have been proposed to have therapeutic potential.[50–52]

Circadian Clock and Glucose Homeostasis

Glucose metabolism is regulated by the circadian clock machinery. The relationship between circadian changes in metabolically important hormones (i.e. glucocorticoids, insulin, ghrelin, incretins, adiponectin, and leptin) and ambient light provided the initial clues that the central clock plays an important role in glucose and nutrient homeostasis.[53, 54] Moreover, these hormones can directly affect circadian clock in their target cells. Insulin is a regulator of *Per2* and *Rev-erbα*, and both glucose and insulin can affect the expression of

Dec1 and *Dec2*.[55, 56] Low glucose/fasting induction of AMPK can repress the cryptochromes as described in the previous section. Thus, the relationship between circadian clock and glucose homeostasis is bidirectional.

Of particular interest, incretins, which are already the target of FDA approved pharmaceuticals aimed to treat type 2 diabetes (T2D) and obesity, are being investigated as therapeutic agents in NAFLD.[57] Glucagon-like peptide 1 (GLP-1), an incretin secreted by the intestinal L-cells located primarily in the ileum, is an insulin sensitizer.[58] GLP-1 release correlates with the pattern of insulin secretion, demonstrating an increase immediately preceding the feeding period. Thus, GLP-1 contributes to the decrease in blood sugar levels by enhancing the glucose-dependent insulin secretion.[57, 59] Patients with NAFLD display diminished concentrations of active incretins.[60–62] GLP-1's release is circadian in rodents[59] and humans,[63] and this rhythmicity is lost with the consumption of a high fat/palmitate diet[64, 65] and sleep deprivation.[66] Knockdown of *Bmal1* or *Clock* results in impairment of insulin release from β-cells in response to exendin-4.[67, 68] Thus, circadian clock control of glucose-modifying hormones is not limited to the hypothalamic-pituitary axis.

Circadian clock machinery regulates glucose at a cellular level as well. Glucose homeostasis is altered in nearly all transgenic lines where the circadian clock is perturbed (Table 1).[12] The liver specific *Bmal1* knockout (KO) mice helped elucidate the relationship between circadian clock and GLUT2, the main hepatic glucose transporter.[69–71] The expression of *Glut2* in mice is characterized by the peak and trough of expression corresponding to the inactive/daytime and active/nighttime states, respectively. This homeostatic stage is disrupted with loss of *Bmal1*, which results in constitutively low expression of both transcript and protein levels of *Glut2.* Hepatocyte-specific deletion of *Bmal1* in mice led to fasting hypoglycemia, hypoinsulinemia, and loss of rhythmicity in the expression of hepatic glucose metabolic genes.[69–71]

The influence of the circadian clock is not limited to glucose transport. BMAL1 also regulates the expression of *Pgc1α* (peroxisome proliferator-activated receptor-gamma coactivator 1α), a co-activator of gluconeogenesis.[72] Hepatic glycogenesis is controlled by CLOCK through transcriptional activation of glycogen synthase 2 *(Gys2)*, the rate limiting enzyme in glycogen synthesis.[70] The increased expression of *Gys2* is temporally timed with surge of post-prandial glucose. *Clock* mutant mice display severely disturbed feeding rhythms and glucose dysregulation (i.e hyperglycemia, hypoinsulinemia).[71, 73] In addition, negative mutations in *Clock* and *Bmal1* lead to impaired gluconeogenesis.[74] *Per1/Per2* double KO mice have severe hypoglycemia in the fasting/inactive period and impaired glucose tolerance.[69] *Cry1/Cry2* double KO mice also show glucose intolerance with elevated circulating corticosterone levels.[75] CRYs are transcriptional repressors that regulate gluconeogenic enzymes through the repression of glucocorticoid receptors[75] as well as CREB.[39, 76]

Not all circadian clock transgenic mice have unfavorable glucose homeostatic responses. Liver-specific KO of *Rory* are protected against insulin resistance and hyperglycemia.[77] Incidentally, RorY KO are also protected against diet-induced hepatic steatosis.[35] Likewise, mice with hepatic overexpression of *Cry1* had normoglycemia and improved insulin

sensitivity in response to dietary challenge.[39] These findings have bolstered the argument for therapeutic agents targeting circadian clock components as potential therapeutic target for T2D and

Circadian Clock and Lipid and Bile Acid Homeostasis

In addition to glucose homeostasis, the circadian clock regulates lipid and bile acid metabolism. This includes regulatory transcription factors such as peroxisome proliferator-activated receptor (PPARs) and sterol regulatory element-binding proteins (SREBP) 1c (lipid metabolism) or rate limiting enzymes such as acetyl-CoA carboxylase (ACC; fatty acid biosynthesis) and cholesterol 7 alpha-hydroxylase (CYP7A1; cholesterol and bile acid metabolism). Dysfunction in these regulatory pathways are part of the pathophysiology of NAFLD.

One of the main reasons for lipid accumulation in hepatocytes of patients with NAFLD is dysregulation of *de novo* lipogenesis. SREBP1c is one of several transcription factors that can drive the induction of lipogenic genes. Overexpression of SREBP1c in the liver is associated with a two-fold increase in ALT, AST, hepatic triglyceride levels, and serum free fatty acids.[78] Transgenic mice that overexpress SREBP1c displayed a fatty liver phenotype along with hyperglycemia and hyperinsulinemia.[78] The activity of this regulator is dependent on the fastingfeeding cycle and nutritional status.[79] However, more recent studies demonstrate that SREBP1c, and its targets, are also regulated by the circadian clock machinery. SREBP1c transcripts, as well as protein levels, display daily rhythmicity.[80] This rhythmicity is also reflected in the binding pattern of SREBP1c to some of its canonical target genes.[81] SREBP1c is regulated by BMAL1, REV-ERBα, RORα, RORγ, and DEC1.[80, 82, 83] For example, REV-ERBα can control hepatic cholesterol and lipid metabolism through the controlled expression of *Insig2* (insulin induced gene 2) which is an inhibitor of SREBP1c.[83]

Cyp7a1, the gene for the rate limiting enzyme that converts cholesterol to bile acids, is also regulated by the circadian clock and expressed cyclically. REV-ERBα [84] and DBP [85] regulate its transcription. *Per1/Per2* double KO mice have abnormal expression of *Cyp7a1* and other key bile acid enzymes, suggesting that they have at least indirect regulation of bile acid synthesis.[86] Moreover, *Cyp7a1* transcription is regulated by hepatic farnesoid X receptor (FXR) and small heterodimer partner (SHP), as well as fibroblast growth factor (FGF) 15/19 which is released as a result of FXR activation in the ileum. *Fxr* and *Shp* transcription (and thus, also transcription of *Fgf15/19)* are also clock-gated.[87, 88] FXR agonists, such as obeticholic acid are currently being investigated as a potential therapeutic agent for NAFLD.[89] Finally, it should be noted that BMAL1 can further influence triglyceride metabolism through PGC1α regulation of *Fxr* further demonstrating circadian clock's multiple layers of regulatory control.[90] Altogether, cyclical activity of CYP7A1 and its regulating enzymes generate circadian fluctuation of serum and fecal bile acids which has implications on lipid absorption and microbiome composition.[91, 92]

Another important regulator of lipogenesis that participates in clock controlled metabolic processes is ACC. Phosphorylation and inactivation of ACC is driven by feeding and follows a 24-hour rhythm.[11, 93] Circadian clock regulation of AMPK (e.g. with RORα) represses

ACC and provides temporal control of lipogenesis.[34] Mice with liver-specific deletion of ACC1 exhibit lowered accumulation of hepatic triglycerides and decreased *de novo* fatty acid synthesis.[94] Similarly, ACC2 KO mice fed a high fat high carbohydrate diet display lower body weight and less epididymal fat compared to wild type mice.[95] Recently an allosteric inhibitor of ACC1/2 showed promising liver specific effects, including decreased lipogenesis, hepatic steatosis, and improved insulin sensitivity.[96]

Genes involved in lipid metabolism and lipogenic processes are regulated by PPARs, a class of ligand-activated transcription factors.[97] PPARα KO mice fed a high fat diet (HFD) accumulate more hepatic triglycerides and show a much higher NAFLD activity score.[98, 99] Conversely, activation of PPARs with fenofibrate (PPARα) and rosiglitazone (PPARγ) leads to amelioration of certain parameters of NAFLD, including decreased ALT, AST, steatosis, and inflammation in both mouse and clinical studies. Several randomized controlled trials have demonstrated improvement in liver inflammation in both diabetic and non-diabetic patients with pioglitazone, another PPARγ agonist.[100–102] BMAL1 and CLOCK transcriptionally regulate the circadian rhythmicity of *Pparα*. PPARα in turn directly binds to PPAR response elements (PPRE) on the *Bmal1* promoter and promotes its expression in a positive feedback loop.[103, 104] In addition, PPARα directly interacts with PER2 in hepatic tissue to modulate transcription of their target genes.[105] Since PPARS are highly circadian proteins, it is unclear whether dosing at different times of day can alter the bioavailability and effectiveness of the drug in treating NAFLD.

Circadian Clock and Endoplasmic Reticulum Stress

One of the major functions of the endoplasmic reticulum (ER) is to facilitate the correct folding of proteins. In addition, ER is the site of lipid synthesis in hepatocytes.[106] Stressors such as changes in redox states or high-protein demands lead to an accumulation of unfolded or mis-folded proteins, ultimately leading to ER stress. The risk of ER stress is higher at certain times of the day; thus, many of its regulatory proteins are under circadian control. As an adaptation response, the unfolded protein response (UPR), along with its component proteins, such as inositol-requiring enzyme 1α (IRE1α), is triggered to restore cellular homeostasis.[107, 108] The UPR plays a critical role in the pathogenesis of NAFLD.[109–111] In mice, UPR-regulated genes exhibit a biphasic 12-hour periodicity which is driven by circadian clock control of IRE1α as well XBP1 (X-box binding protein 1), a UPR master regulator.[112] In arrhythmic *Cry1/Cry2* double KO mice, hepatic *Ire1α* is constitutively expressed, suggesting that this pathway is under clock control. These mice also had perturbed levels of hepatic and serum triglycerides.[112]

Finally, the ER-associated transcription factor CREBH (hepatocyte specific CREB) regulates stress-related lipogenesis and fatty acid oxidation in mice.[113–115] CREBH is an important mediator of the circadian oscillator in the liver and exhibits rhythmicity in its proteolytic cleavage but not mRNA level, indicating that circadian clock regulates it through posttranslational modification.[115, 116] CREBH is regulated indirectly by BMAL1 and AKT, and its activity is modified by DBP and NFIL3.[116, 117] Mice with CREBH deficiency leads to reduced levels of genes involved in fatty acid metabolism. These mice display

hypertriglyceridemia, increased fatty acids, and are more susceptible to diet-induce hepatitis and hepatic fibrosis, whereas CREBH activation protects against steatosis.[118–120]

LUMINAL CONTENT ENTRAINS HEPATIC CLOCK

The SCN functions as the master pacemaker and is sensitive to light signals but largely unresponsive to feeding patterns. On the other hand, the peripheral clock in the liver is dependent on feeding pattern for the amplitude and phase of oscillation of its transcripts.[4, 121] Restricting feeding in mice to only the daytime (when they normally sleep) results in a phase shift between the central and peripheral clocks.[81] Recent studies show that feeding is a stronger driver of rhythmic gene expression than circadian clock proteins with 70% of hepatic transcripts losing rhythmicity under arrhythmic feeding.[122] Thus, feeding is an important cue (i.e. zeitgeber) for hepatic circadian clock function. It should be noted that the impetus for feeding comes from the hypothalamus, where the SCN is located. Therefore, dyssynchrony in feeding and light cycles can be considered root disorders of the SCN/hypothalamus.[12]

The gut microbiome is widely recognized for its importance in regulating metabolic physiology, and as playing an important role in the pathophysiology of metabolic syndrome and NAFLD.[123–125] Recent studies show that the gut microbiome can contribute to dysmetabolism by disruption of epithelial and hepatic circadian rhythms. Though the gut microbiome is not exposed to light, it exhibits cyclical variations that are closely intertwined to host gene expression, feeding pattern, and gender.[126–130] Indeed, circadian disruption induced by jet-lag,[126] genetic mutation of host circadian genes,[126–128] or by diet[126, 129, 130] are associated with disruptions in microbe-host circadian dynamics that can result in increased adiposity, insulin resistance, and inflammation.[12] Luminal secondary (bacterially-produced) metabolites demonstrate cyclical rhythms over a 24-hour period.[126, 127, 129] The cyclical variations of the bacterial taxa is one of the most robustly reproducible characteristics of the gut microbiome in murine models.[131] However, the cyclical dynamics of the human gut microbiome have been difficult to study. Nevertheless, salivary samples collected over a 24-hour period[132, 133] and analysis of stool microbiome collected at different times[134, 135] suggest that the human microbiome is also highly dynamic.

Gut microbes can drive host circadian rhythms.[12, 125] Germ-free mice, antibiotic-induced microbiome depleted mice, or mice who lose cyclical oscillation of their gut microbiome through dietary interventions lack hepatic and intestinal circadian rhythms.[129, 136] Circadian variation of the gut microbiome can cyclically activate luminal toll-like receptors (TLRs).[136] During the rest phase, intestinal TLRs are inactive and there is an increase in the transcription factor PPARα, which promotes the transcription of *Rev-Erbα*. During the active phase, TLRs respond to bacterial proteins and suppress *Pparα* transcription. With microbiome depletion, TLRs are less activated and both PPARα and REV-ERBα are elevated regardless of time. Though others have reported an increase in TLR expression[137] and no changes in *Pparα* expression,[137, 138] likely due to differences in antibiotic depletion protocols, the observation that REV-ERBα does not cycle in microbiome-depleted mice has been reproduced in multiple studies.[138, 139] The circadian relationship between the gut microbiome and REV-ERBα was recently reproduced in another study investigating the role

of microbiome on circadian clock.[139] Cyclical expression of REV-ERBα leads to cyclical transcription of *Nfil3* (See Figure 3D). *Nfil3* can regulate body composition through circadian expression of the intestinal lipid metabolic program, regulating lipid absorption and its export from intestinal epithelial cells.[139] Germ-free and antibiotic treated mice have low expression of *Nfil3*, and, thus, much lower body lipid absorption.

Though these studies show a relationship between cyclical fluctuations of the gut microbiome and the circadian machinery of the host intestinal epithelial cells, it is not clear which microbially-derived mediators are responsible for changes in hepatic oscillations and, thus, metabolic functions. Two potential mediators are bile acids and short-chain fatty acids (SCFAs) (Supplemental Figure 1).[125] As discussed above, bile acids and key enzymes in their production and uptake are cyclically expressed and regulated by circadian clock genes.[84, 91, 140] Though incompletely characterized, secondary bile acids can affect intestinal and hepatic circadian gene expression *in vivo*.[141] Increased expression of bile salt hydrolase, a bacterial enzyme that deconjugates bile acids and allows for the production of secondary bile acids, induces a shift in ileal and hepatic circadian gene expression in antibiotic treated mice.[142] Moreover, sleep disruption, altered feeding pattern, and circadian gene KOs alter the expression of key bile acid regulatory genes.[85, 91] Cecal SCFAs have cyclical fluctuations that are perturbed with a HFD.[129] Though the mechanism is incompletely understood, supplementation with SCFAs can cause changes in *Bmal1* and *Per2* gene expression. Interestingly, *Bmal1* KO mice lose cyclical fluctuation of their fecal SCFAs.[143] Overall, these studies suggest a reciprocal interaction between the circadian clock and luminal bile acids and SCFAs.

DYSSYNCHRONY AND METABOLIC SYNDROME

Synchrony between the SCN and hepatic circadian clock temporally organizes the expression of a large number of metabolic regulatory genes to the daily pattern of food availability. In the absence of a robust hepatic circadian clock, the organism becomes susceptible to various metabolic disorders, including increased adiposity, ectopic steatosis, and insulin resistance.[12] Transgenic mouse lines affecting various clock genes demonstrate the interconnectedness between circadian rhythms and metabolic regulators. Circadian gene transgenic lines nearly universally have perturbed metabolic phenotypes (e.g. insulin resistance, increased adiposity) (Table 1).[12] Moreover, nearly all dietary-induced dysmetabolic mouse models have perturbed feeding patterns, often characterized by the loss of discrete meals and the spread of caloric intake throughout the day and night.[12] Thus, circadian dyssynchrony, characterized by phase shifts and dampening of circadian gene oscillations that lead to misalignment of peripheral and central circadian rhythms, is common to nearly all models of metabolic syndrome, including NAFLD.

Murine Models

Efforts to decipher the mechanistic relationship between circadian clock and host metabolism have been quite fruitful, especially over the last two decades. In general, decreased expression of the clock genes in the positive limb result in worse metabolic outcomes. Conversely, decreased expression of the negative limb (see Figure 2) clock genes

leads to improved metabolic outcomes and protection from hepatic steatosis. A summary of selected studies of clock transgenic mice and their metabolic characteristics are presented in Table 1.

Environmental stressors, such as changes in diet, sleeping pattern, or lighting conditions can also cause circadian dyssynchrony. For example, in the diet-induced obesity (DIO) model – where mice are given a HFD and develop increased adiposity, hyperlipidemia, steatosis, and insulin resistance – mice spread their caloric intake throughout the day and night, thereby doubling the percentage of food eaten during the day/inactive period.[144] This altered feeding pattern causes a disruption of the hepatic circadian clock. *Ob/Ob* mice, a genetic model of obesity characterized by hyperphagia due to leptin deficiency, also have dampened oscillation of their circadian clock prior to weight gain.[145, 146] In addition to feeding, changes to light (e.g. light at night) or disruption of sleep in mice also leads to dysmetabolic states characterized by increased features of metabolic syndrome including steatosis.[16]

Human Studies

As in mice, human studies reveal a strong relationship between the circadian dyssynchrony and dysmetabolism. Genetic studies have been instrumental in showing this relationship. Haplotype analysis of patients with NAFLD revealed an association between steatosis and CLOCK, where gene variants have a potential role in the susceptibility, pathogenesis, and disease progression.[147] A similar study reported the putative role of CLOCK polymorphisms which was associated with a 1.8-fold increase in susceptibility to obesity.[148] SNPs in CLOCK have also been associated with metabolic syndrome, hypoglycemia, obesity, and T2D.[149] Genome wide association studies provide strong evidence for the role of circadian gene variants in susceptibility to metabolic syndrome. They have demonstrated a link between PER3 and BMAL1 to T2D, BMAL1 to hypertension, and CRY2 with insulin resistance.[150]

Environmental and behavioral effects on the circadian clock also have metabolic consequences, which are summarized in Table 2. Though human studies show significant association between sleep disturbances, shift-work, and genetic predispositions to metabolic syndrome, it is not clear whether the mechanisms driving these metabolic phenotypes are similar to what has been observed in mice. Provocatively, one of the studies that investigated the circadian dynamics of the gut microbiome suggests that the microbiota could be mediating the dysmetabolic phenotype of circadian dyssynchrony in humans.[126] Gnotobiotic mice that received a fecal transplant from jet-lagged individuals had increased adiposity and insulin resistance compared to gnotobiotic mice who received pre-jet lag and post-jet lag stool from the same individuals.[126] However, only two patients were used in this small cohort and more vigorous investigation of the role of the gut microbiome in circadian dyssynchrony is necessary.

CORRECTION OF DYSSYNCHRONY WITH TIME RESTRICTED FEEDING

It was not clear until recently whether correcting circadian dyssynchrony is sufficient to reverse the dysmetabolic effects of these various insults. Time-restricted feeding (TRF), a behavioral paradigm where feeding is consolidated to the active period, aligns peripheral and

central circadian rhythms.[151] Correction of circadian dyssynchrony with TRF prevents and treats the metabolic consequences of a large variety of insults.[12, 152–155] It should be noted that TRF is not synonymous with intermittent fasting. The former is focused on aligning peripheral and central circadian rhythms. When applied to human populations, the number of calories consumed is irrelevant in the time-restricted diet, which is part of its appeal. Intermittent fasting, on the other hand, focuses on creating periods of fasting, usually in alternate days, to induce changes to metabolism. Caloric intake is strictly regulated on fasting days.

Mice in the TRF condition only have access to food at the nocturnal (active) phase, which promotes natural feeding rhythms and restores the amplitude and phase of oscillations of peripheral circadian clock genes, which are obliterated in DIO mice.[151] TRF also restores cycling of metabolic regulators (e.g. AMPK, mTOR, CREB) as well as their downstream targets. The metabolic effects of restoring synchrony can be profound. Though TRF mice consume the same number of calories and have similar activity levels, they do not have the dysmetabolic phenotype of DIO mice. TRF prevents and treats the increased adiposity, insulin and leptin resistance, steatohepatitis, and dyslipidemia that occurs with the DIO condition.[130, 151, 156, 157] Subsequent studies show that TRF protects against the metabolic effects of a variety of dysmetabolic diets, including the Western diet, high fructose, and high sucrose diets.[156]

TRF also protects against the dysmetabolism induced by circadian gene KOs. *Per1*, hepatic *Bmal1*, hepatic *Rev-Erbα/β, Cry1/Cry2* KOs all have dysmetabolic phenotypes when given free access to HFD. In all cases, TRF prevented the dysmetabolic phenotype.[82] TRF reduces hepatic steatosis and enhances cellular response to metabolic stress. Hence, the benefits from TRF likely arise from restoring daily feeding/fasting rhythms and balancing nutrient absorptions with cellular stress response. TRF also protected against the dysmetabolic phenotypes due to aging, obesity, and central circadian rhythm dysfunction in *Drosophila*.[158, 159]

Contrary to popular belief, instead of consuming three distinct meals, humans consume calories sporadically and over a large range of time within a 24-hour period.[160] Thus, TRF could potentially be an easily adoptable behavioral intervention that improves outcomes in patients with metabolic syndrome. Several small-scale human TRF studies have demonstrated interesting, though sometimes contradictory results. In one randomized crossover trial, healthy volunteers were first either assigned to a standard three meals per day diet or one meal in the evening, both without caloric restriction.[161] The two groups did not have a difference in the number of calories consumed. The one-meal a day group had higher morning fasting plasma glucose levels and a delayed insulin response in an oral glucose tolerance test (GTT). A different study compared healthy males consuming a standard three meals per day diet with TRF in the afternoon-to-evening group. In this case, the TRF group had decreased fat mass, without any difference in triglycerides, total cholesterol, or LDL.[162] A subsequent randomized, supervised controlled feeding trial in humans demonstrated that participants in the early TRF group had improved insulin sensitivity, appetite, and blood but had higher levels of triglycerides and total cholesterol.[163] A more recent study aimed to compare early vs. late TRF among men at risk for T2D (monitored with continuous glucose

monitors) found that the early group had lower mean fasting glucose whereas both TRF groups had an improvement in glucose response to meals.[164] However, it is not clear whether the investigators controlled for the length of time the subjects were fasting during their sample collection, necessitating additional larger studies. A recent study investigated the effects of TRF on patients with metabolic syndrome undergoing pharmacotherapy. In this single-arm, paired-sample trial of 19 patients who had an initial eating window of >14 hours and were undergoing statin or antihypertensive therapy, imposing a 10-hour feeding window (monitored with continuous glucose monitors) led to improved cardiometabolic health. Though these studies are promising, a study investigating the effects of TRF on NAFLD endpoints have not yet been investigated.

CONCLUSION

NAFLD is a complex disease that is associated with a multitude of metabolic perturbations.[7, 165] Since many circadian clock controlled genes are vital participants in metabolic processes of the body, it is not surprising that some of these rhythmic genes can be potential targets for therapy. Behavioral interventions, such as TRF, may have benefits in NAFLD that is independent of its weight loss effects. TRF may be easier for patients to adopt since it does not restrict calories nor require a specific macronutrient dietary profile.[160]

Chemical modulators of clock-regulated processes such as bile acid metabolism (FXR agonist obeticholic acid, FGF15/19 analogue NGM282), incretin response (GLP-1 receptor agonist liraglutide), PPAR-controlled lipogenic mechanisms (PPARα activator Elafibranor) are under various stages of the clinical trial process.[11, 166, 167] However, since some components of the core clock directly participate in metabolic processes and the oscillator itself is responsive to resetting stimuli, small molecules directed towards clock components might provide alternative targets for therapeutic intervention.[47, 168] In this light, administration of compound KL001, an activator of CRY proteins showed improved glucose tolerance in DIO mice.[169, 170] This is consistent with the known role of CRYs in the inhibition of gluconeogenesis.[75] Recent studies show that CRY1 is targeted for degradation by autophagy, thus depressing hepatic gluconeogenesis in a temporal manner.[76] Further research into these sites on CRY1 might direct researchers towards novel drug targets for the treatment of hyperglycemia and insulin resistance. Nobiletin, a drug that enhances the amplitude of *Ror* expression, prevents weight gain and improves metabolic parameters in DIO and *Ob/Ob* mice.[171, 172] Additionally, REV-ERB agonists SR9009 and SR9011 treatment led to improved metabolic endpoints in DIO mice.[173, 174] These drugs could potentially be beneficial in NAFLD.

Furthermore, the circadian clock extends its functional network to the control of global physiological processes including the targets of nearly all drugs affecting metabolic syndrome spectrum disorders.[175] The rhythmicity imparted by the circadian clock in drug metabolic and detoxification processes can be a potential target for the treatment of NAFLD.[10, 11] By better accounting for the circadian cycling of their targets, new drugs can be administered in a way that maximizes benefits, and minimizes side effects, potentially arriving at a therapeutic dose with fewer administrations and lower dosages.[176] Further study of the chronopharmacology and circadian xenobiotics of small molecule therapeutics

might provide better insight into the proper temporal profile and contribute to improved efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support: AZ is supported by K08 DK102902, R03 DK114536, R21 MH117780, R01 HL148801. All authors receive institutional support from P30 DK120515, P30 DK063491, and UL1 TR001442.

Biographies

Abbreviations:

ACC	acetyl-CoA carboxylase
AKT (or PKB)	v-Akt murine thymoma viral oncogene homolog 1 or (protein kinase B)
ALAN	artificial light at night
AMPK	AMP-activated protein kinase
BMAL1	brain and muscle ARNTL-like protein 1
CCGs	clock-controlled genes
CCl_4	carbon tetrachloride
CK1	casein kinase 1

CLOCK	circadian locomotor output cycles kaput
CREB	cAMP response element-binding protein
CREBH	cAMP response element binding protein hepatocyte specific
CRY	cryptochrome
CYP7A1	cholesterol 7 alpha-hydroxylase
DBP	D-box binding protein
DEC (or BHLH)	basic helix-loop-helix family member
DIO	diet-induced obesity
dTRF	delayed time-restricted feeding
eTRF	early time-restricted feeding
FBXL3	F-box/LRR-repeat protein 3
FGF	fibroblast growth factor 15
FXR	farnesoid X receptor
GLP-1	glucagon-like peptide
GLUT2	glucose transporter 2
GPCRs	G-protein coupled receptors
GTT	glucose tolerance test
GYS2	glycogen synthase 2
HFD	high fat diet
INSIG2	insulin induced gene 2
IRE1α	inositol-requiring 1α
ITT	insulin tolerance test
KO	knockout
LDLR	low-density lipoprotein receptor
mTOR	mammalian target of rapamycin
NAD^+	nicotinamide adenine dinucleotide
NAFLD	non-alcoholic fatty liver disease
NAMPT	nicotinamide phosphoribosyltransferase

NASH	non-alcoholic steatohepatitis
NCD	normal chow diet
NFIL3 (or E4BP4)	nuclear factor, interleukin 3 regulated
Ob/Ob	leptin knockout mouse
OR	Odds ratio
OSA	obstructive sleep apnea
PER	period
PGC1α	PPAR-gamma coactivator 1α
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response elements
ROR	retinoic acid receptor-related orphan receptors
RORE	ROR-responsive element
SCFA	short-chain fatty acid
SCN	suprachiasmatic nucleus
SHP	small heterodimer partner
SIRT1	sirtuin 1
SREBP	sterol regulatory element-binding proteins
T2D	type 2 diabetes
TGs	triglycerides
TLR	toll-like receptor
TRF	time-restricted feeding
Ub	ubiquitylation
ULK1	unc-51 like autophagy activating kinase 1
UPR	unfolded protein response
WT	wild type
XBP1	X-box binding protein 1

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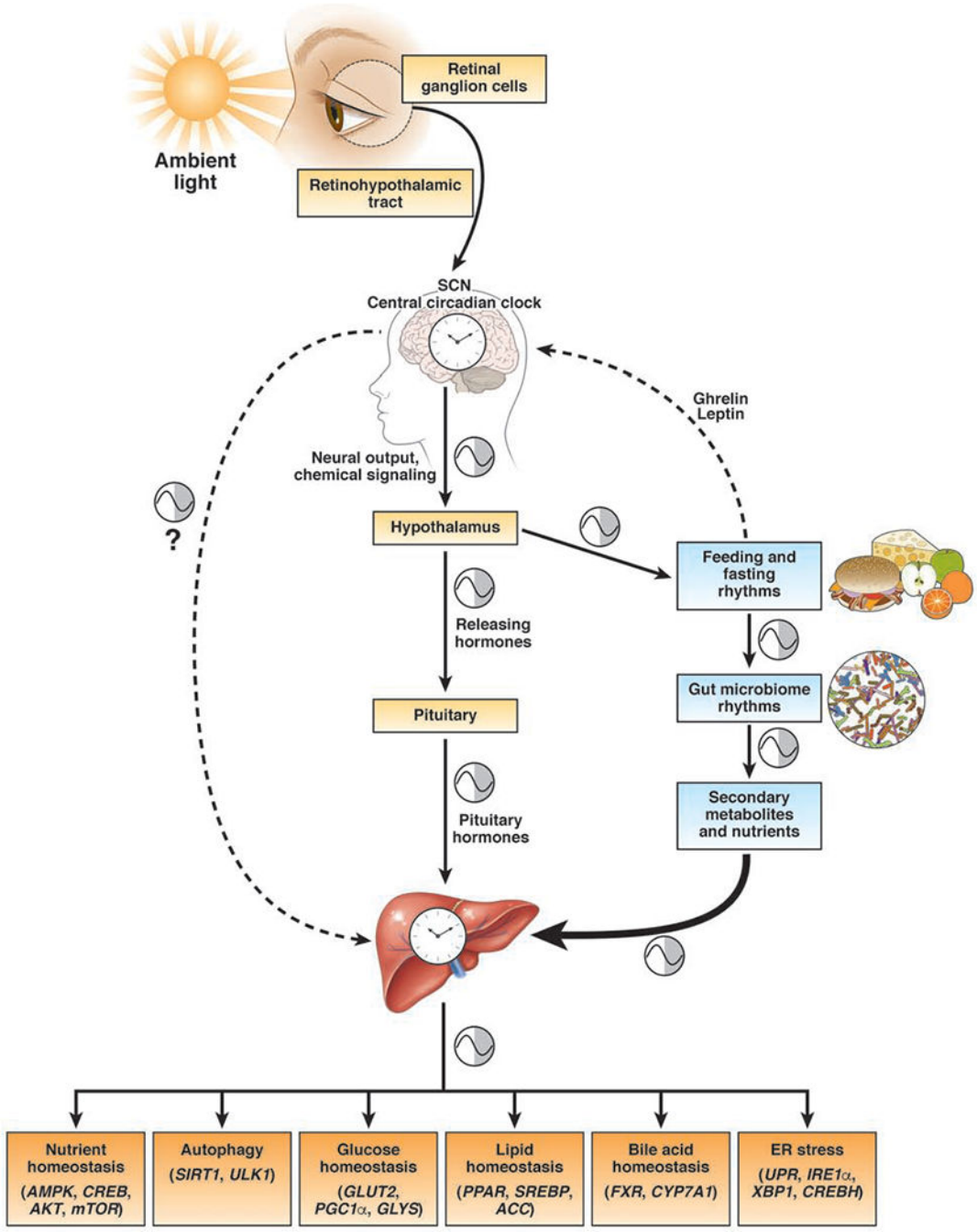

Figure 1: Central and Peripheral Circadian Rhythms.

Retinal ganglion cells detect ambient light and entrain the suprachiasmatic nucleus (SCN). Their projects travel through the retinohypothalamic tract, though indirect signals are also received from the thalamus. The SCN contains the central circadian clock which can modulate other oscillatory networks including sleep/wake, temperature, and feeding/fasting homeostasis through the hypothalamus. In addition, the SCN regulates the circadian release of hypothalamic releasing hormones (e.g. CRH), and thus, pituitary hormones (e.g. ACTH). Hormones released from pituitary gland are one way that the central clock can regulate the

peripheral organs. The SCN also regulates peripheral clock through poorly understood neurohumoral mechanisms. SCN can drive feeding/rhythms through the hypothalamus. Fasting induces the release of ghrelin which promotes feeding while feeding induces the release of leptin which promotes satiety, and thus affecting feeding/fasting rhythms. Diet and feeding/fasting rhythms modulate cyclical fluctuations in the gut microbiome, which then creates cyclical changes in secondary metabolite production, such as SCFAs and bile acids. Secondary metabolites and/or bacterial products are necessary for the maintenance of peripheral circadian rhythms. Feeding/fasting rhythms are powerful drivers of hepatic circadian clock. The rhythmic expression of genes in the liver generates rhythmicity in the transcripts and activity of genes involved in critical metabolic processes such as glucose, lipid, and bile acid metabolism, nutrient homeostasis, autophagy and ER stress. Metabolic syndrome occurs in the setting of central and peripheral circadian rhythm dyssynchrony.

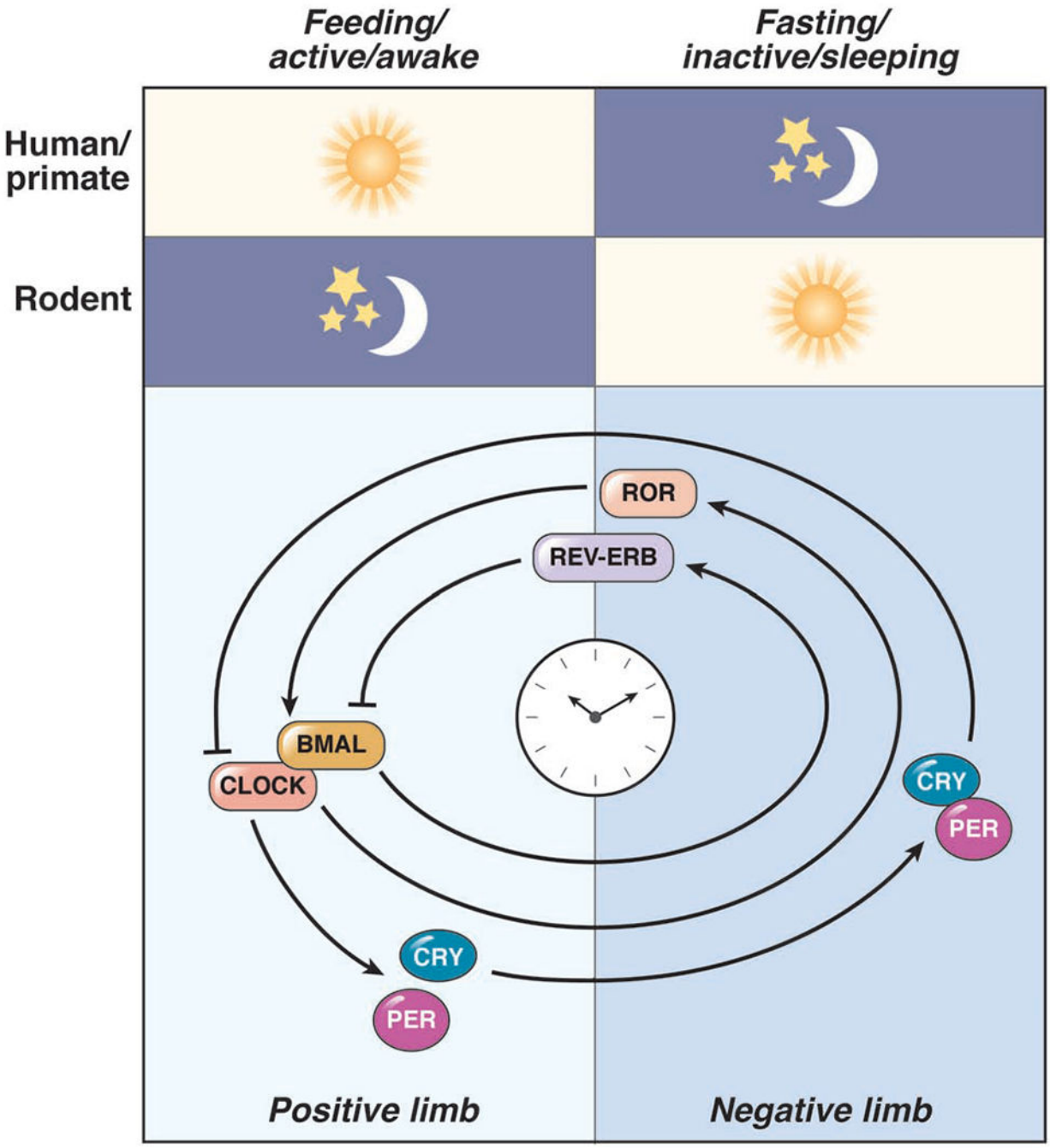

Figure 2: Overview of Circadian Clock Machinery.

Humans/primates are diurnal animals with the feeding/active period occurs during the light hours. However, mice (and most rodents) are nocturnal, with the feeding/active period occurring in the dark hours. The cellular molecular clock is a series of transcription-translation feedback loops that include the transcriptional acti vators (CLOCK and BMAL1; the positive limb) and repressors (PER and CRY; the negative limb). The PER:CRY complex repress their own activators CLOCK:BMAL1. CLOCK:BMAL1 also promote REV-ERBs

and RORs which add another layer of regulation over the circadian clock and regulate hundreds of additional genes.

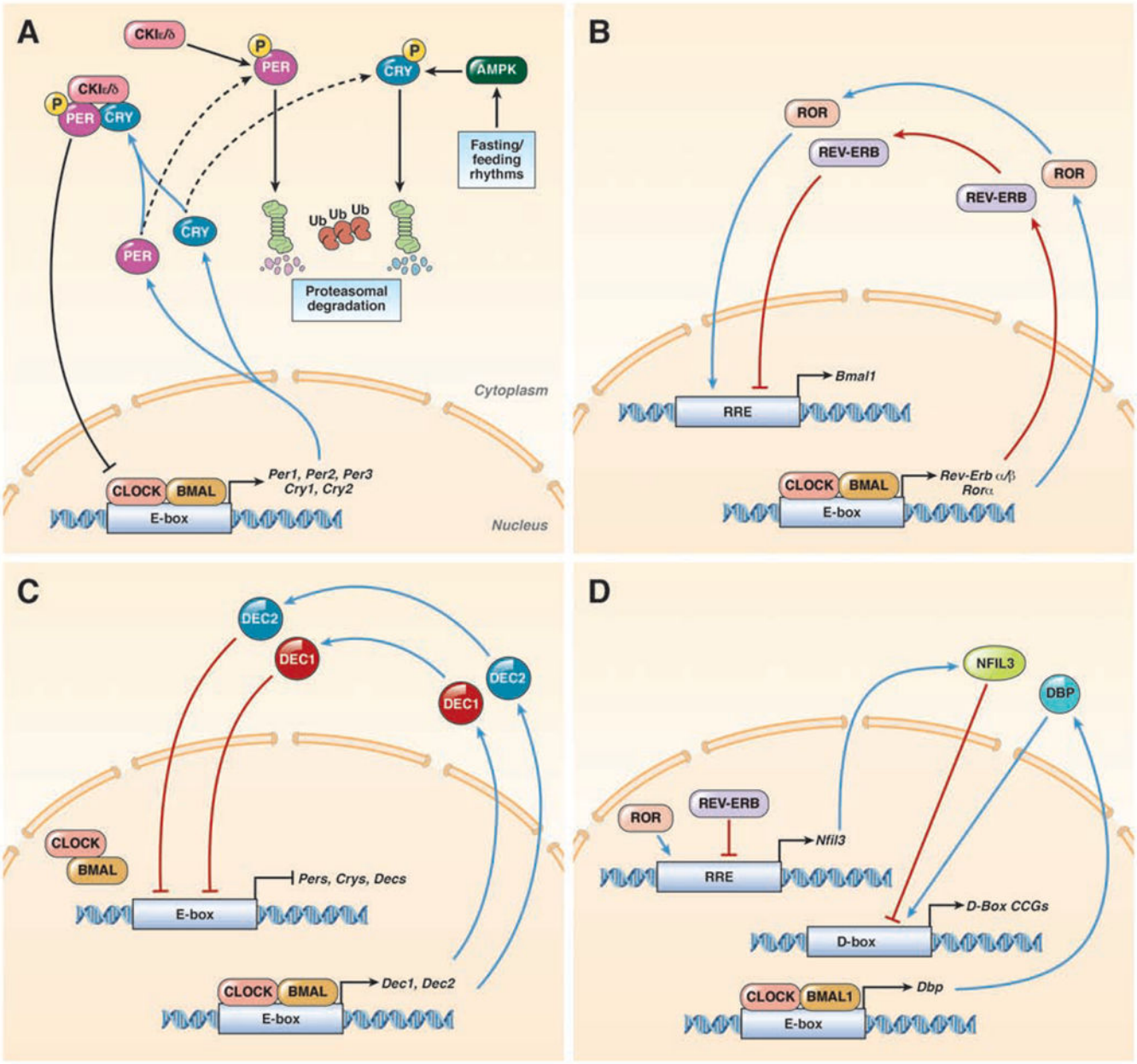

Figure 3: Circadian Clock Machinery.

(**A**) In mammals, the core molecular clock is comprised of a series of transcription-translation feedback loops that include the transcriptional activators (CLOCK and BMAL1). The CLOCK:BMAL1 heterodimer regulate genes at a specific DNA binding sequence, the E-box to regulate expression of hundreds of genes, including their own repressors (*Per1, Per2, Per3, Cry1*, and *Cry2*). Once translated, PERs and CRYs accumulate in the cytoplasm, where they are regulated by CK1ε/δ and AMPK, respectively. If these PER or CRY are individually phosphorylated, they will undergo ubiquitylation (Ub) and proteasomal degradation. However, if PER and CRY form a heterodimer prior to phosphorylation by CK1ε/δ, the three protein complex is transported to the nucleus, where it directly inhibits the CLOCK:BMAL1 heterodimer. (**B**) CLOCK:BMAL1 also regulates the expression of Rev-

Erbs and Rors. Once translated REV-ERBs and RORs bind to the RRE DNA binding sequence but with opposite effects. RORs promote, while REV-ERBs suppress at this DNA binding sequence. Together these clock proteins control the regulation of hundreds of genes including the expression of *Bmal1*. (**C**) A third loop of the circadian clock involves DEC1 and DEC2 that are transcriptionally activated by CLOCK:BMAL1. DEC1 and DEC2 proteins migrate back into the nucleus and inhibit Per1 transactivation by competing for E-Box binding. (**D**) CLOCK:BMAL1 promote the transcription of *Dbp*, and REV-ERB/ROR regulate the expression of *Nfil3*. Once translated, DBP and NFIL3 promote or suppress, respectively, gene expression at the D-Box DNA binding sequence. The D-box DNA binding sequence controls expression of hundreds of clock-controlled genes (CCGs).

TABLE 1:

Circadian Clock Transgenic Lines and their Effects on NAFLD

Mouse Strain	Diet/ Intervention	Liver Phenotype	Δ Glucose	Δ Insulin	Δ serum TGs	Δ Weight	Δ Adiposity	Refs
Rev-erbα KO + Hepatic Reverb β KO	NCD	Marked hepatic steatosis with deficiency of both Reverbs, but only subtle changes with loss of either subtype alone	↓					177
Rev-erbα KO	NCD	Higher hepatic TG (trend)	↑	↑ **Normal ITT**		↔	↑	178
	HFD (53%)	Higher hepatic TG (trend)	↑	↑		↑	↑	
Per1/2 KO	NCD	Lower hepatic TG depending on what time of day they were eating (oscillating)						179
Cry1/2 double KO	NCD		↑	↔		↓		180
	HFD (45%)	Hepatic steatosis	**Impaired GTT**	↑ **Impaired ITT**		↑	↑	
Bmal1 KO	NCD				↑	↓		181
	HFD (60%)	Hepatic steatosis in global (not tissue-specific) Bmal1 KO mice, but not in control mice			↑	↓	↓	
ClockΔ19 KO	NCD		↑	↔	↑	↑	↑	73
	HFD (45%)	Lipid engorgement of hepatocytes in Clock KO mice compared to WT				↑	↑	
ClockΔ19 KO	NCD	Normal				↔		182
	HFD	Lower hepatic TG				↔		
mPer2−/− KO	NCD & CCl_4	Increased hepatic fibrosis in KO mice						183
Rev-erbα KO (and HDCA3 KO)	NCD	Increased hepatic TGs/ steatosis in KO mice						184

ALAN: artificial light at night; **CCl_4:** carbon tetrachloride; **DIO:** diet-induced obesity; **GTT:** glucose tolerance test; **HFD:** high fat diet (with percentage of fat in diet); **ITT:** insulin tolerance test; **NCD:** normal chow diet; **TGs:** triglycerides; **WT:** wild type; **KO**: knockout;

Table 2:

Selected studies investigating associations between sleep or food behaviors and metabolic syndrome or NAFLD

Article	Study Type	Population	Control	Comparator	Key findings
Selected studies investigating associations between sleep disturbance or time-restricted feeding and metabolic syndrome					
Spiegel 1999 [185]	Observation study	Healthy volunteers (n=11)	n/a	Sleep deprivation	• Sleep-deprivation reduced glucose tolerance and evening cortisol concentrations
Wang 2014 [186]	Meta-analysis of 13 observational studies	Workers exposed to night shift (n=2,286)	Day shift work	Night shift workers	• Higher risk of metabolic syndrome in workers exposed to night shift (OR 1.5-1.6)
Vyas 2012 [187]	Meta-analysis of 9 case control studies, 11 prospective cohort studies, and 6 retrospective cohort studies	Workers exposed to shift work (n=2,000,000)	Shit workers	Day workers	• Shift work is associated with an increased risk of myocardial infarction and coronary events (OR 1.23)
Karlsson 2001 [188]	Observational study	Working adults (n=27,485)	Day workers	Shift workers	• OR of obesity ~1.3 higher in shift workers compared to day workers • Dyslipidemia clustered with shit workers compared to day workers ($p<0.05$)
Wilkinson 2019 [189]	Paired-sample trial	Patients with metabolic syndrome (n=19)	2-week baseline of eating interval of ≥14 h per day	12-weeks of time-restricted eating (TRE) to a 10-hour interval	• After 12-weeks of TRF, there was a 3% reduction in weight ($p=0.0003$), 11% reduction in LDL-C (=0.016), and 8% reduction in estimated calorie intake ($p=0.007$) , but no difference in insulin resistance ($p=0.107$)
Selected studies investigating associations between sleep disturbances and/or shiftwork and NAFLD					
Hsieh 2011 [190]	Observational	All-male Tokyo office workers (n=8157)	Sleep >7hours	Sleep <7 hours	• Rates of NAFLD were higher in patients who slept <7 hours (OR 1.23)
Kim 2013 [191]	Observational	Workers and spouses at a Korean conglomerate (n= 69,463)	Sleep >5 hours	Sleep <5 hours	• Rates of NAFLD were higher among women (but not men) who slept 5 hours (OR 1.59 in women; 1.03 in men)
Imaizumi 2015 [192]	Observational studies	General patients (n=3986)	Sleep duration 6 to ≤7 h	Sleep duration ≤ 6 hours	• Rates of NAFLD were higher among women (but not men) who slept ≤ 6hours (OR 1.44 in women, 0.98 in men)
Bernsmeier 2015 [193]	Case-control study	Cohort in Basel (n= 68)	Healthy volunteers (n=22)	Biopsy-proven NAFLD group (n=46)	• NAFLD group had shorter sleep duration and poorer sleep quality compared to controls ($p<0.01$). In NAFLD patients, but not in controls, daytime sleepiness was correlated with higher liver enzymes, and insulin resistance. • NAFLD patients with fibrosis had stronger correlation to daytime sleepiness ($p=0.02$)
Marin-Alejandre 2019 [194]	Cross-sectional study	Fatty Liver in Obesity (FLiO) participants in Spain (n=134)	Normal weight non-NAFLD patients (n=40)	Overweight patients with NAFLD (n=94)	• Adjusted odds of sleep disturbance was higher in NAFLD group (OR 1.59) • Regression models predicted ~20% of variability in liver stiffness could be attributed to

Article	Study Type	Population	Control	Comparator	Key findings
					sleep disturbance or sleep quality
Liu 2016 [195]	Cohort study	Patients who developed NAFLD after 5 years of follow-up (n=2197)	Sleep duration 7-8 hours	Sleep duration 8-9 hours; sleep duration >9 hours	• Adjusted odds of NAFLD was higher with longer sleep durations (OR 1.21 for 8-9 hours group 1.31 in >9 hours group)
Balakrishnan 2017 [196]	Observational study	General workers (n=8159)	Non-shift workers	Shift workers	• Adjusted odds of NAFLD did not differ between shift-workers and non-shift workers (OR 1.11)
Katsagoni 2018 [197]	Randomized controlled trial	Patients with NAFLD (n=63)	Controls with NAFLD without intervention	Mediterranean lifestyle (adequate sleep and activity)	• Mediterranean lifestyle was associated with a decrease in liver stiffness (adjusted OR 0.78)
Katsagoni 2017[198]	Case Control Study	Patients with NAFLD and controls (n=155)	Controls without NAFLD (n=55)	Patients with NAFLD (n=100)	• Optimal sleep duration was inversely associated with NAFLD presence (OR = 0.38, p=.0.05)
Selected studies investigating link between OSA and NAFLD					
Musso 2013 [199]	Meta-analysis of 18 cross-sectional studies	18 cross-sectional studies (n=2, 183) of patients with OSA who were assessed for NAFLD	OSA patients without NAFLD	OSA patients with NAFLD	• OSA was associated with NASH (OR 2.37), and advanced fibrosis (OR 2.3) in biopsy proven NAFLD patients
Pamidi 2012[200]	Observational	Healthy and young OSA patients	Control subjects without OSA (n=20)	Patients with OSA (n=12)	• OSA was associated with a 27% lower insulin sensitivity compared to controls