Development of Novel Circadian Rhythm Reporter

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Abstract:

Adaptations to light and dark cycles on Earth are integral to the survival of all organisms. Indeed, all living organisms retain some form of biological rhythms that ebb and flow with daily environmental stimuli. Importantly, disruptions to circadian rhythms (CRs) are associated with reduction in life/health span and numerous metabolic disorders. As such, there is substantial interest in regulating CRs to improve health outcomes. In mammals, these CRs are generated by a transcriptional-translational feedback loop composed of core clock proteins BMAL1, CLOCK, PERIOD and CRY. Other proteins include RORa and REV-ERBa, which regulate expression of BMAL1 and CLOCK. Alongside light stimuli, CRs are sensitive to environmental cues such as food intake. There has been recent interest in the role that presence of key nutrients, specifically lipid species, plays in regulating CRs. However, the mechanism by which these metabolites act upon the circadian clock is unknown. One limitation of CR-centered research is that it is inherently low-throughput. CR studies typically incur a large financial cost and require large amounts of labor to process and analyze biological samples due to the sheer number of time points and biological replicates. As such, the field would benefit from an accurate circadian reporter that is sensitive to changes to clock gene expression. Here, we present a novel circadian reporter that can streamline circadian rhythm data collection and analysis with the use of PERIOD2-promoter driven fluorescent reporter. We tested the efficacy of the reporter in AML12 cells stably expressing it and treated them with several known circadian inducers to determine complete and robust circadian

profiles. In future directions, we aim to optimize experimental design parameters to enhance performance of the novel circadian reporter.

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Abbreviations:

CR	Circadian Rhythms
SCN	Suprachiasmatic Nucleus
TTFL	Transcription–Translation Feedback Loops
CLOCK	Circadian Locomoter Output Cycles Kaput
ARNTL1/BMAL1	Aryl Hydrocarbon Receptor Nuclear Translocator Like
CRY1	Cryptochrome Circadian Regulator
PER2	Period Circadian Regulator
NR1D1/REV-ERBa	Nuclear Receptor Subfamily 1 Group D Member 1
RORa	Retinoic Acid Recepotr Orphan Receptor Alpha
AMPK	Adenosine Monophosphate activated Protein Kinase
HFD	High Fat Diet
PPARα/γ	Peroxisome Proliferator-Activated Receptor α/γ
Protein Kinase A	PKA
Cyclic Adenosine Monophosphate	cAMP
MUFA	Monounsaturated Fatty Acid
PUFA	Polyunsaturated Fatty Acid
SFA	Saturated Fatty Acid

Chapter 1: Introduction

1.1 Circadian Rhythms

Biological timing is a necessity in all organisms, driven by the need to adapt to changes in the environment. Living species from bacteria to plants to humans exhibit cycles in physiology and behavior over periods as short as seconds (e.g., in the case of cardiac pace-making cells) and up to as long as months (e.g., in the case of seasonal oscillators). The study of circadian rhythms examines 24-h oscillations (also called a *period*) in biological processes at the molecular, cellular, and behavioral levels. These processes have been shown to orchestrate basic cellular functions such as gene expression and protein translation and can even be experienced as the daily rituals of human life (i.e., feeding activity and the onset of sleep). It is widely believed that these mechanisms evolved as a response to the rotation of the planet — cycles of day and night result in changes of ambient light level and temperature in the environment, and therefore evolutionary benefit is conferred to those organisms that can correctly anticipate and synchronize their physiology.

Regulation and entrainment of circadian rhythms is largely determined by external periodic stimuli, more commonly referred to as *zeitgebers*. Zeitgebers encompass a wide variety of environmental cues, such as light, temperature, food intake, and locomotion [1,2]. As such, there is considerable interest in understanding the influence that competing zeitgebers have on circadian rhythms. In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus serves as the “master clock” in that it

coordinates other biological clocks in the peripheral tissues [3]. The SCN's proximity to visual processing centers allows it to rapidly adjust to ambient light cues and fine tune circadian rhythms [4]. However, the effects of zeitgebers differ based on the stimuli and the particular tissue. As an example, the liver and kidney are more sensitive to the timing of food intake than light and thus can be uncoupled from the instruction of the master clock in the SCN via restricted feeding [5]. Ultimately, the hierarchical organization of circadian rhythms is not rigidly linear. Rather, it is a complex, yet flexible, network with multiple signaling nodes that coordinate with one another to produce the result that will enhance fitness/survival.

1.2 Molecular Mechanisms of Circadian Rhythms

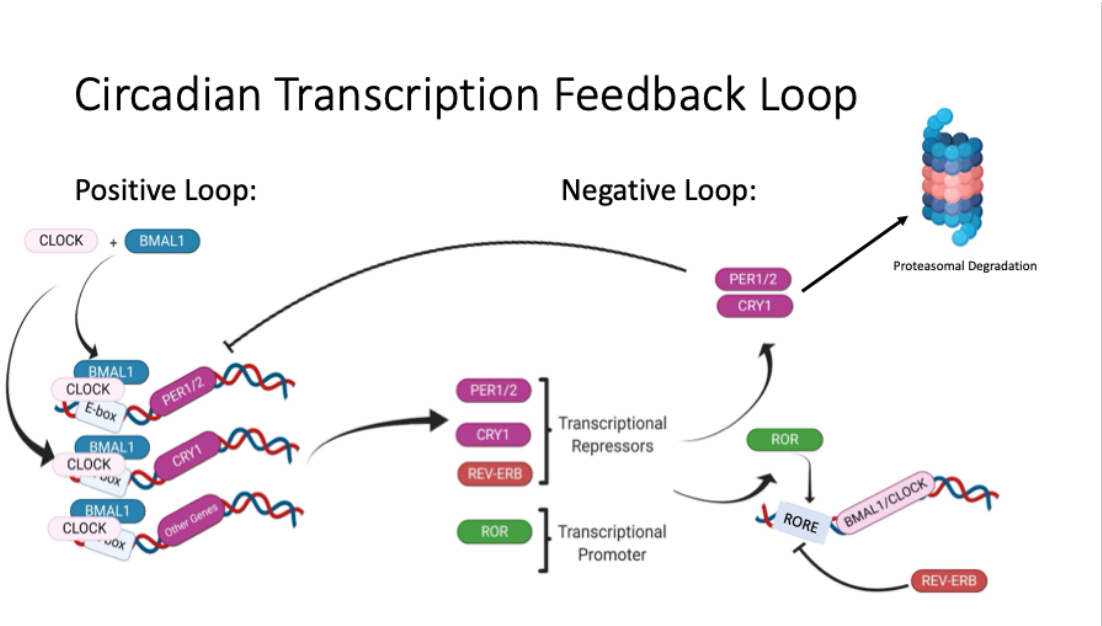


Figure 1. Schematic of Circadian Transcription-Translation Feedback Loop (TTFLs). Upon induction, CLOCK and BMAL1 dimerize and bind to E-box motifs upstream of clock-controlled genes (CCGs) and drive their expression. As PER/CRY proteins accumulate, they also dimerize and inhibit E-box binding of CLOCK/BMAL1, preventing the expression of CCGs. Eventually, the PER/CRY heterocomplex is targeted for degradation, allowing CLOCK/BMAL1 to bind again and resuming the circadian cycle.

Although the biological timing of each tissue type is idiosyncratic, the molecular circadian machinery is conserved in every cell and is cell autonomous. Circadian rhythms are orchestrated by a network of core 'clock genes' that are organized into transcription–translation feedback loops (TTFLs), producing oscillations with a period of approximately 24 hours.

Figure 1 describes the circadian feedback loop in mammals, although many components are largely conserved in other metazoans [6]. The circadian TTFL is composed of six genes: Circadian Locomoter Output Cycles Kaput (CLOCK), Aryl Hydrocarbon Receptor Nuclear Translocator Like (ARTNL; also known as BMAL1), Cryptochrome Circadian Regulator (CRY1-2), Period Circadian Regulator (PER1-3), Nuclear Receptor Subfamily 1 Group D Member 1 (NR1D1; also called REV-ERBa), and Retinoic Acid Receptor-Related (RAR) Orphan Receptor Alpha (RORa). CLOCK and BMAL1 are transcriptional factors that comprise the positive loop. They form a heterodimer that then binds to E-box sequence motifs found upstream of several clock-regulated genes and then recruits RNA polymerase to initiate transcription [7]. CLOCK/BMAL1 also drive the expression of CRY and PER, which themselves are core clock genes and have numerous isoforms. As CRY and PER accumulate in the cytoplasm, PER complexes with CRY and enters the nucleus where they block the binding of CLOCK/BMAL1 binding to E-box motifs, thereby decreasing their own transcription and that of other clock regulated genes. Upon reaching a certain threshold, CRY/PER degrades and competitive inhibition of CLOCK/BMAL1 ceases, thus resuming the circadian cycle [8]. RORa and REV-ERBa, although not a direct element of the TTFL, are responsible for promoting and repressing

the transcription of the BMAL1/CLOCK dimer, respectively, by binding to a conserved ROR response element (RORE) upstream of BMAL1 and CLOCK [9]. Together, these proteins form the ebb and flow of the core circadian TTFL.

For zeitgebers to entrain circadian rhythms, they must target specific signaling pathways upstream of core clock proteins. As mentioned earlier, the circadian clock in SCN is sensitive to light. Indeed, the SCN is located closely to the optic chiasm which contains retinorecipient cells that secrete a neurotransmitter called neuropeptide Y (NPY) when exposed to light [10]. This induces transcription of PER1 and PER2 in the SCN and thus initiates the circadian TTFL [11]. Figure 2 describes how SCN then coordinates biological timing in the peripheral tissues attuned to periodic photic signals. However, cellular context is important. The liver, for example, does not have retinorecipient cells and therefore must rely on other entrainment cues (with some instruction from the SCN) to coordinate its own internal clock. It has been repeatedly demonstrated that the timing of food/energy availability is indispensable to regulating peripheral clocks in both rodents and humans [12, 13]. In fact, food intake can be mimicked in cell culture via serum shock, whereby media composed

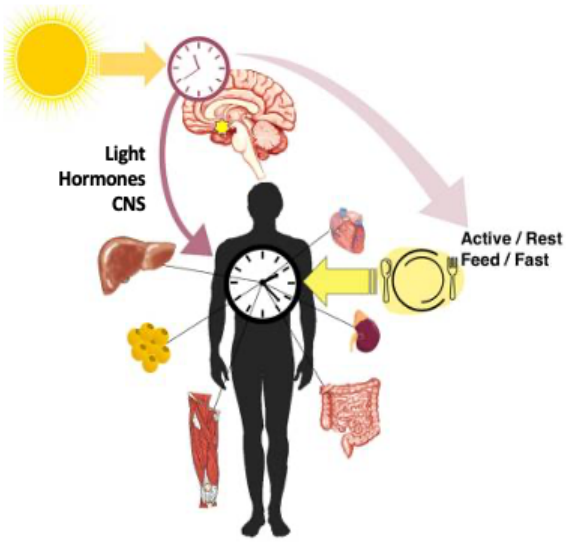


Figure 2: Entrainment and alignment of the SCN and peripheral clocks. **The primary zeitgeber of the SCN oscillator is light, whereas peripheral clocks are strongly entrained by feeding/fasting rhythms. The SCN synchronizes peripheral rhythms directly through hormonal and central nervous system (CNS) signals, as well as indirectly through behavioral feedback from rhythms in activity and feeding.** ***Adapted from Pickel et al. [22]***

of at least 50% serum is applied to the cells [14]. The increase in nutrient availability targets numerous energy and redox sensing pathways that play a major role in regulating circadian rhythms in peripheral tissues [15]. For example, AMP-activated protein kinase (AMPK) is an important energy sensor and is activated by increased levels of AMP due to hydrolysis of ATP. In a low energy state, AMPK also directly phosphorylates CRY1, leading to its interaction with FBXL3, which promotes its proteasomal degradation [16]. AMPK also activates casein kinase I epsilon (CKIe), resulting in more phosphorylation of PER2 by CKIe and subsequent degradation of PER2 [17]. The presence of nicotinamide adenine dinucleotide (NAD) is an important indicator of redox potential. The deacetylase SIRT1 is activated by the presence of elevated oxidized NAD (NAD+). With sufficient NAD+, SIRT1 directly deacetylates PER2, which alters the conformation of the nuclear localization sequence (NLS) [18] . This leaves PER2 unable to reenter the nucleus and results in its eventual proteasomal degradation [19]. Taken together, these examples show that the circadian clock is downstream of numerous sensory and regulatory pathways which allows for flexible adaptation to environmental cues.

1.3 Relationship between Circadian Rhythms and Metabolic Disease

Unsurprisingly, disruptions to circadian rhythms have an enormous physiological impact on healthspan and lifespan. Mice that have had the SCN ablated exhibit alterations to diurnal behaviors and diminished ability to anticipate environmental changes [20]. In humans, similar effects are observed. Shift workers, for example, undergo severe and persistent disruptions to their circadian rhythms. As a result, they are more prone to a wide variety of metabolic disorders and often have weakened

immune systems [21]. These detrimental health effects are attributed, in part, to inconsistent feeding schedules [22]. Consistent with this, the relationship between circadian rhythms and metabolic syndrome-associated conditions has received increasing amounts of attention. For example, the development of insulin resistance and chronic inflammation is more likely to occur following circadian disruption. Gale et al. [23] demonstrated that circadian disruption in rats led to dysregulated glucose levels, lower glucose-stimulated insulin secretion, decreased beta-cell mass/turnover, and increased insulin resistance. Circadian disruptions also result in a decrease in leptin, which resulted in altered feeding behavior, an increase in arterial blood pressure which resulted in poor cardiovascular health, and reduced sleep efficiency [24]. They are also associated with abnormal hepatic lipid, bile acid, and cholesterol metabolism in mice [25]. Interestingly, that same study found that the temporal makeup of the gut microbiome was also impacted. Although not addressed in the following chapters, there is substantial empirical evidence of the impact of the gut microbiome on circadian rhythms [26]. Overall, poor health is intrinsically linked to dysregulated circadian rhythms.

Notably, these metabolic deficiencies are comorbid with circadian disruption when induced by chronic overnutrition. High fat diets (HFD) given to healthy organisms are known to disrupt circadian rhythms. The circadian period of HFD-fed mice was lengthened and diurnal behaviors such as feeding were altered. HFD mice tended to eat more during the light cycle in contrast to their control counterparts. Mice fed the HFD exhibited considerable alterations to core clock gene expression [26]. Interestingly, BMAL1, CLOCK, and PER2 remained largely unchanged in the hypothalamus, but are

markedly more altered in adipose tissue and liver, consistent with previous studies showing decoupling of the central and peripheral clocks. Another important finding was that the *periodic* expression of key lipid metabolism-related pathways are also altered in the mice given a HFD. Peroxisome Proliferator-Activated Receptor Alpha (PPARα) and Gamma (PPARγ) are nuclear receptors that regulate lipid homeostasis and glucose regulation; primarily, initiating transcription of genes involved in fatty acid metabolism in response to excess fatty acids which serve as ligands for the PPARs [27]. However, HFD-mice had arrhythmic circadian expression of both PPAR isoforms, suggesting a diminished ability to sense their energy state and switch between energy substrates. This is, in part, explained by the presence of the E-box motif in the promoter region of PPAR genes [28, 29, 30]. Conversely, PPARs are also known to drive the expression of BMAL1, CLOCK, and REV-ERBa [31,32]. These data highlight the ambiguity as to whether the disruption of circadian rhythms initiates the development of metabolic disorders or vice versa. Likely, the causal event, in this case overnutrition, simultaneously triggers maladaptive measures in both metabolic and circadian pathways via shared upstream regulators.

The harmful effects of disturbing circadian rhythms has naturally inspired investigations into how to target circadian regulated pathways to restore metabolic homeostasis. Due to the important role of food-intake, periodic fasting is a potent method for restoring circadian rhythms [33]. For example, chronic overnutrition promotes activation of the insulin-pAKT-mTOR pathway that drives downstream gene activities that promote anabolic processes. In contrast, a few hours of fasting can activate AMPK, which triggers repair and catabolic processes while also phosphorylating CRY to

promote its degradation. AMPK activates casein kinase I epsilon (CKIe), resulting in more phosphorylation of PER2 by CKIe and subsequent degradation of PER2 [16, 17, 34]. Additionally, NAD+ levels increase with fasting which activates SIRT1, leading to more acetylated PER2 [18, 19, 35]. Fasting also promotes the formation of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase via beta-adrenergic receptor stimulation [36]. cAMP activates protein kinase A (PKA) signaling which triggers an increase in lipolysis and gluconeogenesis to meet energy demands. PKA phosphorylates casein kinase I (CKI), which is then activated and goes on to phosphorylate PER2 [37]. Phosphorylation stabilizes PER2, which enhances the negative arm of the circadian TTFL. This model is further supported by the fact that beta-adrenergic agonists such as isoproterenol increase the expression of PER2 [39]. Finally, glucocorticoid receptors (GRs) are potent regulators of circadian rhythms. Fasting also increases the secretion of glucocorticoids, namely cortisol [47]. Although the understanding of the underlying mechanism is somewhat limited, empirical evidence supports that GR stimulation can robustly trigger the expression of core clock genes [44]. For example, the synthetic GR agonist dexamethasone can trigger the expression of BMAL1, PER1-2, and CRY1 [45, 46] Therefore, fasting can enhance the robustness and amplitude of the oscillation of circadian rhythms.

Another strategy in a similar vein is time-restricted feeding (TRF; also commonly referred to as intermittent fasting), where food availability is limited to a specific window of time to consume. TRF confers many of the same benefits as long term fasting, while being a more sustainable practice than longer fasts [38]. In fact, TRF has been shown to

be more effective in entraining circadian rhythms [40]. A common use for TRF is in aiding recovery from jet-lag and restoring disrupted sleep schedules [41, 42]. In rodent models, the impact of simulated shift work on circadian rhythms was markedly reduced under TRF [43]. Finally, there are also numerous pharmacological methods for synchronizing cell-autonomous clocks. Unsurprisingly, drugs such as forskolin and metformin that target and activate AMPK have been shown to induce circadian rhythms *in vivo* and *in vitro* [48, 49]. As mentioned previously, glucocorticoid stimulation with agonists like dexamethasone and beta-adrenergic agonists like isoproterenol are potent activators of circadian rhythms. These pharmacological methods are incredibly useful for studying circadian rhythms in cells, due to the fact biological clocks in cell cultures are asynchronous and thus it is difficult to tease apart biological factors that influence circadian rhythms. In conclusion, there are numerous methods to restore circadian rhythms which also target symptoms of metabolic syndrome, demonstrating the intimate link between circadian rhythms and metabolism.

1.4 Metabolic Signaling in the Regulation of Circadian Rhythms

1.4a: Lipid metabolism regulates Circadian Rhythms.

While food intake is essential for regulating circadian rhythms, there is less understanding of the role that food composition plays in said role. It is well understood that a high-fat diet mainly rich in saturated fats can disrupt circadian rhythms (see section 1.3). However, recent studies have shown that alterations to lipid composition dampen the damaging effects of a HFD. Omega-3 polyunsaturated fatty acids (PUFAs) (also referred to as ω-3-PUFAs) are an important class of fatty acids commonly found in

the human diet. The three major species of omega−3 fatty acids involved in human physiology are α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Numerous studies have shown that increased intake of ω-3-PUFAs imparts benefits to general health. For example, DHA supplementation protected mice given a HFD were protected from severe non-alcoholic liver disease (NAFLD) and insulin resistance [50]. In human trials, supplementation of DHA and EPA improved sleep quality, which suggests an improvement in rhythmicity of global circadian rhythms [51]. DHA can activate the AMPK signaling pathway [52], which regulates circadian rhythms by regulating CKI and Cry1 phosphorylation and degradation. In addition, DHA can regulate core rhythm gene expression by activating the PPAR signaling pathway [53]. Evidence for direct links between ALA and EPA with circadian rhythms is limited. However, EPA supplementation administered to humans improved sleep quality more effectively than DHA [51] and rodents given a ω-3-PUFA-deficient diet exhibit disrupted circadian rhythms [55, 56]. Together, these findings suggest that ω-3-PUFAs confer protection from the disruptive effects of a HFD on circadian rhythms.

Mono-unsaturated fatty acids (MUFAs) are also known to impart protection from metabolic syndrome related symptoms. One of the most studied MUFAs is oleic acid (C18:1, ω-9), and is the primary lipid species found in “healthy” oils such as olive oil [57], making it very common in modern diets. Oleic acid rich diets, such as the Mediterranean diet, are shown to help reverse the effects of obesity and reduce insulin resistance [58]. Oleic acid also reduces the expression of inflammatory factors such as IL-6, IL-8, and TNFa, which can halt the progression of insulin resistance and diabetes [59, 60]. Oleic

acid has also been shown to reduce hunger and overall food intake [61], which encourages long-term fasting. At the molecular level, MUFAs are potent activators of fatty acid oxidation. MUFAs upregulates the expression of peroxisome proliferator-activated receptor G coactivator 1 alpha (PGC1α), a transcriptional cofactor to PPARg [62]. PGC1α is essential for activating mitochondrial biogenesis and increases the expression of genes involved in oxidative metabolism [63]. PGC1α is also downstream of SIRT1, which deacetylates PGC1α to promote its interaction with PPARγ leading to the transcription of fatty acid oxidation and OXPHOS genes alongside circadian genes such as BMAL1 (see section **1.3**). Furthermore, previous publications from our lab report that MUFAs enhance PGC-1α/PPARα signaling and promote oxidative metabolism in a SIRT1-dependent manner [64]. Because of these findings, It is logical to hypothesize that MUFAs may regulate circadian rhythms via the SIRT1-PGC1α axis. In fact, supplementation with oleic acid is shown to restore circadian rhythms in cells, supporting this hypothesis [65]. In summation, MUFAs directly act on upstream regulators of core clock genes.

Alternatively, chronic exposure to saturated fatty acids (SFAs) such as palmitic acid (C16:0) can have devastating effects on health and contribute substantially to the development of metabolic syndrome. SFAs increase insulin resistance, promote inflammation, and increase oxidative stress [66, 67]. SFAs can also disrupt circadian rhythms. For example, primary hepatocytes and adipocytes treated with palmitic acid had altered expression of circadian genes [68, 69]. In more severe cases, there was complete loss of rhythmicity of CLOCK, PER2, and REV-ERBα. It was found that palmitic

acid treatment destabilized CLOCK/BMAL1 dimerization in a SIRT1 dependent manner. The deleterious effects of a HFD on circadian rhythms can be attributed to SFAs, as HFDs are typically enriched with them. Finally, short chain fatty acids (SCFAs) are fatty acids that have six or less carbons such as acetate, propionate, and butyrate. The role that SCFAs play in regulating circadian rhythms is not well understood, as it was only recently discovered that SCFAs produced by the gut-microbiome can regulate circadian rhythms [70]. Together, these findings demonstrate that fatty acid metabolism plays an enormous signaling role in regulating circadian rhythms.

1.4b: Relationship between Lipid droplets and circadian rhythms

Lipid droplets (LDs) are ubiquitous organelles that store neutral lipids for energy or as substrate for numerous biochemical pathways and act as hubs for metabolic processes in the cell. LDs are composed of a phospholipid monolayer with a neutral lipid core made of triacylglycerol (TAGs) and cholesterol esters (CEs). Long thought to be inert, LDs are now understood to play an important role in metabolic homeostasis [71]. An abundance of proteins sits on the LDs that act as "gate-keepers", controlling the synthesis, catabolism, and transport of TAGs and CEs [72]. Because LDs act as sites for numerous metabolic functions, disruptions to LD biology are heavily tied to the development of metabolic syndrome and associated disorders. There is evidence that LD function is also tied to circadian function. For example, the total volume and average size of LDs rhythmically changes in a diurnal pattern in skeletal muscle [73]. In that study, diurnal patterns in the makeup of key lipid species were observed, with certain species making up the bulk of observed changes. Diacylglycerols (DAGs), although only make up

10% of LD content, comprised ~45% of all the lipid species that exhibited rhythmicity. DAGs act as potent signaling molecules and are intermediates during the esterification of fatty acids to TAGs. DAG signaling broadly regulates the activity of the protein kinase C (PKC) family of enzymes [74]. Upon activation, the PKC family phosphorylates and activates several downstream targets involved in lipogenesis, cholesterol metabolism, and gluconeogenesis, as well as inhibiting insulin receptor function [75]. Notably, PKC has been shown to alter the stability of PER1 proteins in the SCN [76]. Although there isn't an established link between DAG concentration and PKC-dependent phosphorylation of PER1 in mammals, there is evidence of such a phenomenon occurring in *Neurospora* [77]. Ultimately, these data suggest that alterations in LD metabolism and DAG concentrations could regulate circadian patterns. However, it should be noted that these diurnal patterns in DAG metabolism were found in only skeletal muscle and thus may be dependent on cellular context. Further study in other tissues is required.

Another mechanism linking LD function to circadian rhythms is through the ATGL-SIRT1-PGC1a-PPARa signaling axis (described in section 1.4a). Adipose triacylglycerol lipase (ATGL) is responsible for the liberation of acyl chains from the glycerol backbone of TAG to be used for energy or for other purposes. ATGL sits on the LD surface to access TAGs stored in the LD, but it can also exist in the cytosol. As previously mentioned, publications from our lab have shown that the release of MUFAs from LDs is modulated by ATGL, whereby the liberated MUFAs bind the LD surface protein perilipin 5 (PLIN5) and then travel to the nucleus. The MUFA-bonded PLIN5 allosterically activates SIRT1 to deacetylate PGC1a and initiate PPARa-mediated transcription. The ATGL-SIRT1 axis may

also modulate the acetylation state of PER2. However, more studies are needed to validate this hypothesis. In summation, lipids are important signaling molecules that regulate circadian rhythms.

1.4c: Dysfunctional Insulin signaling disrupts circadian rhythms

In 1969, the first clue indicating that the circadian timing system is involved in the pathophysiology of insulin resistance was the observation that patients with type II diabetes had altered daily rhythms in glucose tolerance [78]. Since then, more observations including the development of metabolic syndrome in the transgenic mice lacking core clock genes [79]. We have discussed in previous chapters how disrupted circadian rhythms are typically co-morbid with insulin resistance and that both food intake misaligned with the circadian cycle can progress obesity. In summation, insulin signaling may have the single largest impact on the circadian rhythms. Here, we discuss in more detail how these mechanisms relate to one another.

The primary role of insulin is to promote the influx of extracellular glucose into the cell. When the insulin molecule binds to the insulin receptor (IR), a series of signaling cascades promotes the creation and secretion of GLUT transporters, allowing for the glucose to enter the cell. Because insulin secretion is diurnal, there is an optimal window of time to consume food where insulin is most potent in maintaining euglycemia [80]. The diurnal pattern of insulin-regulated pathways is virtually present in all metabolically active tissues such as the liver and muscle. When food is eaten outside of this window, the pancreas must adjust insulin production to meet the unexpected nutritional challenge. As a result, circadian misalignment manifests, albeit differently in each tissue

type. In the liver, circadian regulation is sensitive to the timing of food intake and circadian misalignment occurs when eating occurs outside of the phase of normal diurnal insulin secretion [81, 82]. Insulin suppresses gluconeogenesis during the feeding period, which is mediated by the interaction of CRY1. Insulin-mediated suppression of gluconeogenesis is partly dependent upon CRY-mediated FOXO1 degradation [83]. Outside of the feeding period, where CRY1 expression is low, the liver is overloaded with glucose and thus must convert it to TAGs for storage. As a result, insulin resistance is more likely to develop in the face of chronic overnutrition. Like the liver, skeletal muscle has an autonomous molecular clock that is sensitive to food intake and notably exercise [85]. CLOCK and BMAL1 regulate muscle insulin sensitivity via changes in production and expression of insulin sensitive glucose transporter GLUT4, as well as through the modulation of the insulin signaling pathway via expression of the deacetylase SIRT1 [86-87]. Likely, this affects the SIRT1-PGC1a-PPARa signaling axis. For example, Gabriel et al. demonstrated that muscle tissue extracted from humans with type II diabetes had irregular rhythms in insulin sensitivity and when treated with resveratrol, a known SIRT1 agonist, insulin resistance was reduced overall [90]. In summation, insulin, and therefore glucose metabolism, is a major regulator of circadian rhythms in numerous cellular contexts.

1.5 Limitations of circadian research

The advent of new analytical technologies was, and still is, critical for the advancement of knowledge in the circadian field. The first inference of the existence of core clock mechanisms was discovered via a mutagenic screening of *Drosophila*

Melanogaster in 1971 by Konopka and Benzer [88]. Since then, approximately 40% of mammalian genes have been identified to be under circadian control [89]. While the knowledge base has grown substantially, circadian research is limited by several factors. The largest barrier to it is ironically time. Both traditional and newer methods for the detection of circadian rhythms are either very labor intensive and time consuming or expensive. Next-generation technologies such as RNA-seq and mass spectrometry are often used to track relative expression of circadian genes and their resulting proteins in an untargeted manner [91, 92]. While both approaches generate massive amounts of holistic data, their cost can be prohibitive and thus are typically performed on fewer biological replicates. The two most used techniques studying expression of circadian rhythms are real-time quantitative polymerase chain reactions (RT-qPCR) and western blotting. These approaches allow for quantification of the expression of numerous clock-controlled genes over time for a much lower cost and can be performed on dozens of samples at a time. However, these techniques are labor-intensive because it requires the harvesting and processing of potentially dozens of samples every few hours. Also, many of the stated techniques require the destruction of the biological sample, whether it be cells or live animals, thus experiments cannot be performed again. As such, collecting and analyzing circadian data is significantly challenging.

An alternative to these approaches which is less cost prohibitive and relatively high throughput is to use fluorescent transcriptional reporters. An example of this is the widely used PER2:LUC reporter [93]. The firefly luciferase (LUC) is an enzyme that oxidizes the compound luciferin, which results in energy being released as light. The gene

that encodes LUC directly follows the PER2 promoter region which contains an E-box motif; thus the luciferase is entirely driven by the same conditions that also drive the expression of the actual PER2 gene. As PER2 transcription changes, so does the level of light emitted by the luciferase reaction. The differential emission of light reflects changes to circadian gene expression over any given course of time. Luciferase reporters are popular because of their ease of use and can even be used in live animal imaging without sacrifice. One limitation of luciferase is that the supply of luciferin must be maintained over the time course, otherwise the initial bolus will be rapidly metabolized. Another confounding issue is that luciferin is shown to lengthen the circadian period in a dose-dependent matter and thus requires optimization to accurately gather data [94]. PER-LUC reporter is therefore limited in ability to produce biologically accurate data. Some researchers have utilized fluorescent proteins (typically GFP and mCherry) as readouts of transcriptional activity. Gabriel et al. transfected recombinant PER2 and CRY1 proteins fused to fluorescent proteins in live cells to track the expression and activity of the circadian proteins in the context of a CRISPR-mediated gene knockout screening to identify potential circadian regulators [95]. However, these transgenic fluorescent proteins are directly fused to native PER2 and CRY1 proteins. PER2 and CRY1 have been shown to rhythmically exit and re-enter the nucleus in order to sequester the proteins and prevent them from competing with CLOCK and BMAL1 [96]. Others have addressed the limitation by utilizing only the promoter region of each gene. Mei et al. utilized this type of reporter to great success in live mice, where they transfected the animals with a vector containing either the PER2 and CRY1 promoter region to drive the expression of

fluorescent protein [97]. This sort of model is prefered because of the inherent disparity between transcription and translation, allowing for more accurate modeling of circadian rhythms. The authors monitored the expression of fluorescent proteins using a unique analytical system whereby optical fibers implanted into the SCN captured changes to fluorescence over time. However, such an approach required the development of a fairly extensive proprietary system and substantial expertise to utilize properly. Inspired by their approach, we aimed to design a novel reporter to be used in cell culture that is much simpler to use and requires little expertise, while still addressing the previously discussed limitations. By using a cell model that stably expresses the reporter, we can streamline hypothesis generation and increase data output in a high-throughput manner, without significantly increasing labor costs and time. Importantly, we now have newer technologies that are specifically designed for live-cell tracking over the course of days and weeks in the form of the Sartorius®Incucyte. Here, we describe the logic and methodology behind the development of the novel circadian reporter, alongside an outline of the analytical pipeline that can be applied to the reporter.

Chapter 2: Results & Discussion

2.1: Development of Circadian Reporter and Analytical Pipeline

In designing an effective fluorescent transcription reporter, we took into consideration the following criteria:

1. The reporter ***must*** contain robust and well-characterized circadian promoter region to drive expression of the fluorescent protein.
2. Fluorescent proteins must have a reduced half-life to not accumulate and mask true circadian transcription activity.
3. There must be an internal transcription control to capture unwanted perturbations to global transcription and to establish a baseline to normalize circadian reporter output. This function can be fulfilled by another fluorescent protein, independent of the circadian reporter.
4. The excitation/emission intensities of candidate fluorescent proteins must have a small, preferably non-existent, spectral overlap.

As mentioned previously, transcription of PER2/CRY1 is regulated by upstream promoter regions containing the E-box motif (canonically CANNTG, where N is any nucleotide), and is also upstream of circadian-regulated genes [7]. Because the E-box motif is essential for amplifying the expression of several core clock genes, their respective promoter regions make excellent candidates for the application of a fluorescent transcription reporter. Therefore, studying the transcriptional dynamics of PER2/CRY1 rhythms offers insight into how environmental conditions ultimately impact circadian rhythms. Interestingly, CLOCK/BMAL1 promoter regions lack an E-box motif as they are transcriptionally regulated by several other unique promoter motifs [98], thus rendering their promoter sequences undesirable. The promoter sequences of PER2 and CRY1 have been extensively characterized and are commonly used in other reporter systems. For the sake of simplicity, we only employed the PER2 promoter region in the

lentiviral construct for testing. The PER2 promoter sequences characterized by Mei et al. can be seen in Table 1. In this design, the PER2 promoter will drive the expression of eGFP. Fluctuations in eGFP expression, and therefore emission intensities, will serve as the readout to be analyzed (Fig. 3).

The half-life of fluorescent proteins is approximately 26 hours while the average mammalian circadian period is 24 hours. Because the fluorescent protein would accumulate and mask minute fluctuations in circadian rhythms, we decided to incorporate a PEST sequence also known as a degron tag or D2 tag. The D2-tag is a peptide sequence rich in proline (P), glutamate (E), serine (S), and threonine (T), which acts as a signal peptide for proteasomal degradation [99]. This prevents the accumulation of fluorescent proteins by reducing the average half-life of a fluorescent protein to approximately 3 hours [102]. Thus, we integrated the D2 tag into our lentiviral construct into the C-terminal end of the eGFP (Fig. 4).

It is typical to normalize fluctuating fluorescence intensities to the initial time point, T_0, or to the lowest value in a cosine wave, also called the trough. While this is sufficient for assessing the relative differences in circadian rhythms, it fails to capture global perturbations in transcriptions that are independent of experimental conditions. To address this limitation, we decided to include the constitutively active cytomegalovirus (CMV) promoter to drive the expression of mCherry. In this design, the mCherry will also be modified with a D2 tag to prevent masking of the signaling due to accumulation of the fluorescent signal. The CMV promoter is a widely used promoter sequence used to enhance expression of recombinant proteins in mammalian systems

and largely independent of host-transcriptional dynamics. As such, it steadily drives the expression of D2-mCherry. The mCherry emission intensities will serve as a transcriptional baseline to normalize eGFP intensities against. mCherry was also chosen as a transcriptional control due to the small excitation-emission overlap it shares with eGFP. Finally, because of variations in transfection efficiency and because where the lentiviral construct will insert into the genome is random, it is much easier to select cells emitting fluorescence in both the red and green channels. Thus, only cells with co-localized eGFP and mCherry emissions will be considered for analysis.

Collection and analysis of fluorescent output will be performed using the Sartorius® Incucyte SX5 microscope. The Incucyte SX5 performs real-time quantitative live-cell imaging and analysis of cell behavior over time, by automatically gathering and analyzing images around the clock within a standard laboratory incubator. This allows for time-lapsed measurements from living cells over days and weeks, thus providing insight into active biological processes in real time while cells remain undisturbed. The Incucyte SX5 allows for image collection in three fluorescence channels (green/orange/near-infrared) as well as in phase contrast in a single experiment. The software package included with the Incucyte (Incucyte v.2020B) natively contains analysis pipelines, capable of performing statistical analysis and creating visual representations of data. The *Cell-by-Cell Analysis* feature can detect and track changes in individual cells according to user-defined metrics using the phase contrast images. In our experiments, we used the Cell-by-Cell feature to only track cells that emit light in the red and green channels at the same time and measure their respective fluorescent intensities. Together with our

reporter, we designed an experimental pipeline that can detect small changes in the rhythmicity of green intensities (normalized to red intensities) within the native software.

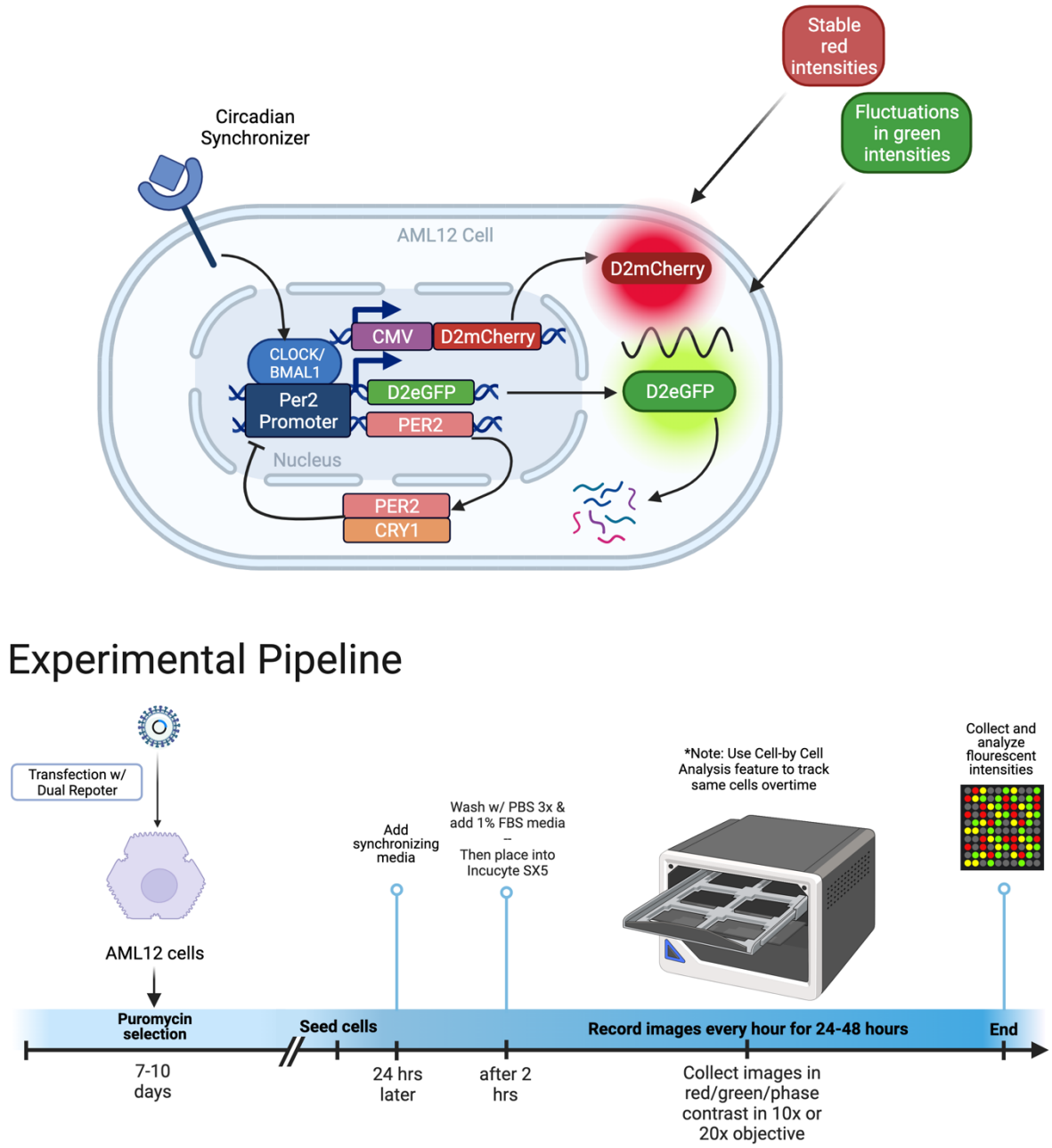


Figure 3. Schematic of Dual Reporter cell model & Experimental Workflow. Upon induction, the CLOCK/BMAL1 heterodimer forms and binds to the PER2 promoter region and drives the expression of D2eGFP as well as the native PER2 protein. With time, the PER/CRY complex forms and competes with the CLOCK/BMAL1, resulting suppression of D2eGFP expression and resuming the circadian cycle. CMV-driven D2mCherry expression is expected to remain steady over time. Cells are then administered the circadian inducer and places into the Incucyte SX5 for time-series microscopy. Single cell intensities are then extracted from the images and analyzed.

Table 1. PER2 Promoter Sequence

PER2	5'- ccgcacgcgtcggattaccgaggctggtcacgtcgtcgcaggtgataggcc gggggccctggtctctgccggctgtgagttgcgcagcggccaagcaccattc ccccgcgccgcagtggtacgcgccactccggggctgcacgagcgggccacc gccgtgccaggtgaatggaagtcccgcaggccggaagtggacgagcctact cgcccgggcgcgggggggcgcaagagcgcgcagcatcttcattgaggaac ccgggcggcgaacatggagttccatgtgcgtcttatgtaaagagagcgacg ggcgtctccaccaattgacgagcgtagctctcaggttccgccccgccagtat gcaaatgaggtggcactccgaccaatggcgcgcgcaggggcgggctcagc gcgcgcggtcacgttttccactatgtgacagcggagggcgacgcggcggca gcggcgctactgggactagcggctccgggcggctgcggcgcaggccgagc gcaccaagtgacgggccgagcaaggga -3'

2.2: Methods & Materials

2.2.1: Cell Culture

AML12 cells were cultured in Dulbecco's modified Eagle's medium/F12 supplemented (DMEM:F12, Gibco) with 10% fetal bovine serum (FBS), 1% insulin-transferrin-selenium, 40 ng/mL dexamethasone, and 1% penicillin/streptomycin (P/S) as described in the ATCC protocol. Transgenic AML12 cell lines were also cultured in the same media with 10 μg/ml puromycin concentration for selection and 4 μg/ml for maintenance. HEK293t cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% FBS and 1% P/S. All cells were maintained at 37 °C under an atmosphere of 5% CO_2. Prior to the start of Incucyte experiments, wild-type and transgenic AML12 cells were triple-washed with phosphate-buffered saline (PBS). Freshly-made media containing known circadian inducers, 50% FBS media or 1 μM Dexamethasone (See section 2.2.3) was applied to both cell lines for two hours as described. Following a two-hour incubation, we aspirated media, washed the cells with

PBS three times, and then added 1% FBS media for the remainder of the experiments. 1% FBS is essential for maintaining the cell population while limiting cell division for the 48-hour duration of the experiment.

2.2.2: Cloning and Transfection

Due to the sheer size of the tentative construct, several rounds of cloning were required to assemble and fuse each sequence of the construct. To avoid this, we employed Gibson assembly to join each fragment together in a single reaction. Rather than insert each fragment one by one via traditional restriction cloning, we designed unique primers with approximately 20 base pair overlapping terminal sequences and then PCR-amplified each sequence in the construct. For DNA fragments over 200 base pairs in size, multiple overlapping DNA fragments can be joined in a single isothermal reaction. Gibson assembly relies on the activities of three different enzymes: a 5'-exonuclease, a DNA polymerase with 3'-extension activity, and a DNA ligase. The 5'-exonuclease chews back the 5' end of each fragment, exposing the 3' end sequence (Fig. 4). Because each hanging 3' end should share the same overlapping sequence, the fragments should anneal with their matching sequence. The DNA polymerase will then fill in any gaps in the annealed DNA molecule, followed by the removal of nicks in the DNA strand by the DNA ligase. This will allow for ordered assembly of the lentiviral backbone containing a native CMV promoter, followed by the mCherry sequence, then the D2 tag, and finally the Per2 promoter region followed by the D2eGFP sequence. (Fig. 4). Once properly assembled, we transformed *E. coli* cells with the newly assembled plasmid and Maxi-prepped the plasmid. We then employed a 2nd generation lentiviral system to

transfect HEK293t cells to generate viral particles. After 2-3 days, the media containing viral particles was applied to AML12 cells. The AML12 cell population was incubated with viral media for 48 hours. `Subsequently, the infected cells were put under 10 µg/mL puromycin selection for 7-10 days to generate a polyclonal population.

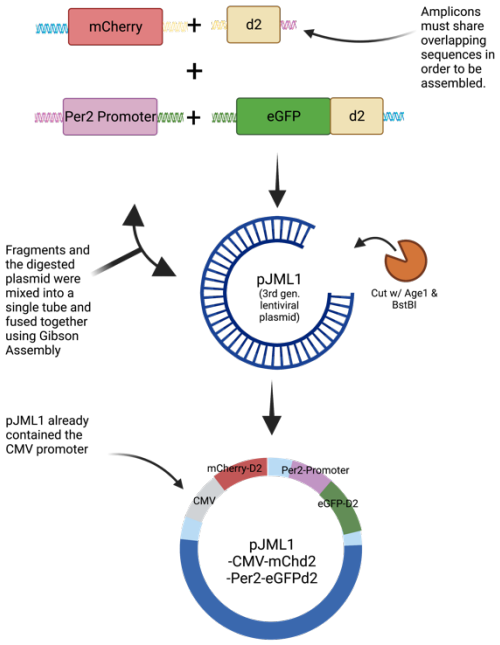


Figure 4: Schematic Gibson assembly cloning protocol. The vector is digested by two restriction enzymes (AgeI & BstBI) at the multiple clonal site located after the native CMV promoter. Then, the PCR-amplified D2-mCherry, PER2 promoter, & D2-eGFP sequences are mixed with the digested backbone and the Gibson assembly master mix. The mixture incubates for 1 hour. *E. coli* cells are transformed with the resulting novel construct and cloned.

2.2.3: Induction of cell-autonomous Circadian Rhythms

While each the circadian clock cell-autonomous, environmental cues and communication between the CNS and peripheral tissues is essential for synchronizing and maintaining the biological clock. Cultured cells are removed from the environmental context of the whole organism; thus, each cellular clock follows its own rhythm. However, there are a myriad of methods available to induce and synchronize the biological clock in cell cultures. To test the efficacy of our newly designed reporter, we utilized the following methods. Serum-shocking [14] is the oldest method for inducing circadian rhythms, whereby cell cultures were administered 50% FBS media. As mentioned in **Section 1.3**, the synthetic glucocorticoid dexamethasone (DEX) is another well-established

pharmacological method for inducing circadian rhythms and we used a 1 μM concentration for the two-hour incubation as described in the literature [100].

2.2.4: Time Series Imaging

Wildtype and transgenic AML12 cells were split into 24-well plates at a concentration of approximately 125,000 cells per well, in the above-described media. After 24hrs, a set of 4 wells were administered with either an empty vehicle or with a vehicle containing either 50% FBS or 1 μM DEX per set. After the two-hour incubation period, cells were washed again with PBS and empty 1% FBS media was added to each well for maintenance for the duration of the experiments. Cells were then place in the IncuCyte SX5 microscope, inside a humidified, 37°C, 5% CO2 incubator.

Time-lapse microscopy imaging was performed with a 20× magnification lens, in three channels: Phase-contrast, Green (excitation: 440–480 nm, emission: 504–544 nm), and Red (excitation: 565–605 nm, emission: 625–705 nm). The green channel captures the PER2-Promoter driven eGFP reporter, while the red channel captures the CMV-driven mCherry reporter. Phase-contrast images were used for general inspection of the population status and for delineation of individual cells. Images were taken every hour for a total duration of 36 hours. Afterwards, the normalized mean fluorescence intensity from each individual cell was captured and used for down-stream analysis.

2.2.5: Statistical Analysis

Cosinor analysis is the traditional method for quantifying the circadian 24-hour cycle, or other types of periodic cycles, by means of examining the degree of “fit” between the time-course experimental data and a cosine-wave function. It allows for

quantification of important cyclical parameters: mesor (M, or baseline), amplitude (A), period (T, or wavelength) and acrophase (Φ, a.k.a. Phase-shift). These parameters are then analyzed and compared between experimental groups to reveal statistically significant differences in circadian attributes.

Both raw fluorescent intensity values in the green and red channel were used for the Cosinor analysis. Red and green values from the wildtype wells were subtracted from the red and green intensities from the transgenic wells to eliminate background/unwanted fluorescence. Then, using the Cell-by-Cell Module, we extracted the individual mean green intensities of each identified cell from each well. Once the mean intensities were normalized to untreated cells at *t=0*, the data set was subjected to Cosinor analysis. Because the CMV-driven mCherry is constitutively expressed, we assumed that each cyclical parameter would effectively be unchanged across all experimental groups, therefore any changes in green intensities are relative to the red intensities. All parameters and subsequent statistical analysis (including Cosinor analysis and One-way ANOVA) were calculated using Graphpad Prism 9©.

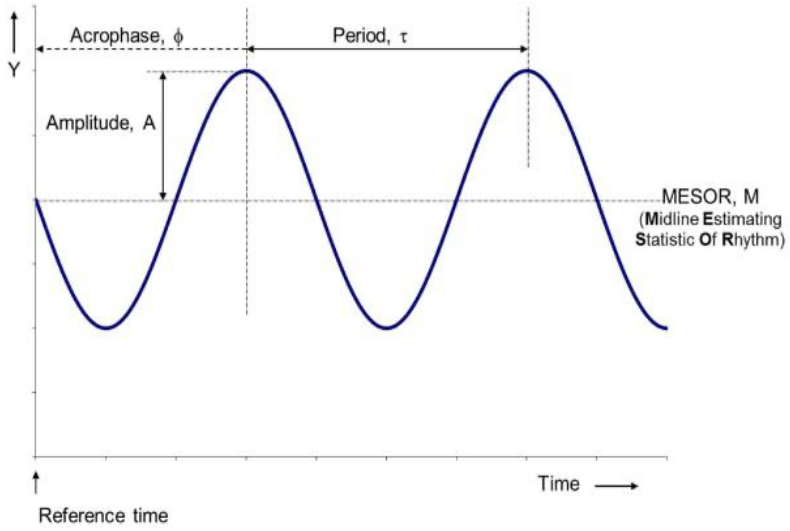


Figure 5: Defined attributes of Periodic Cycles/Circadian Rhythms.

Mesor refers to the rhythm-adjusted mean of the cycle. Amplitude is a measure of the highest extent of predictable change within a cycle. Acrophase is the phase which defines the values at which the function begins at the start of the monitoring (t=0). Finally, Period refers the time for cycle to return to its initial starting point. Adapted from Cornelissen [101].

2.3: Results

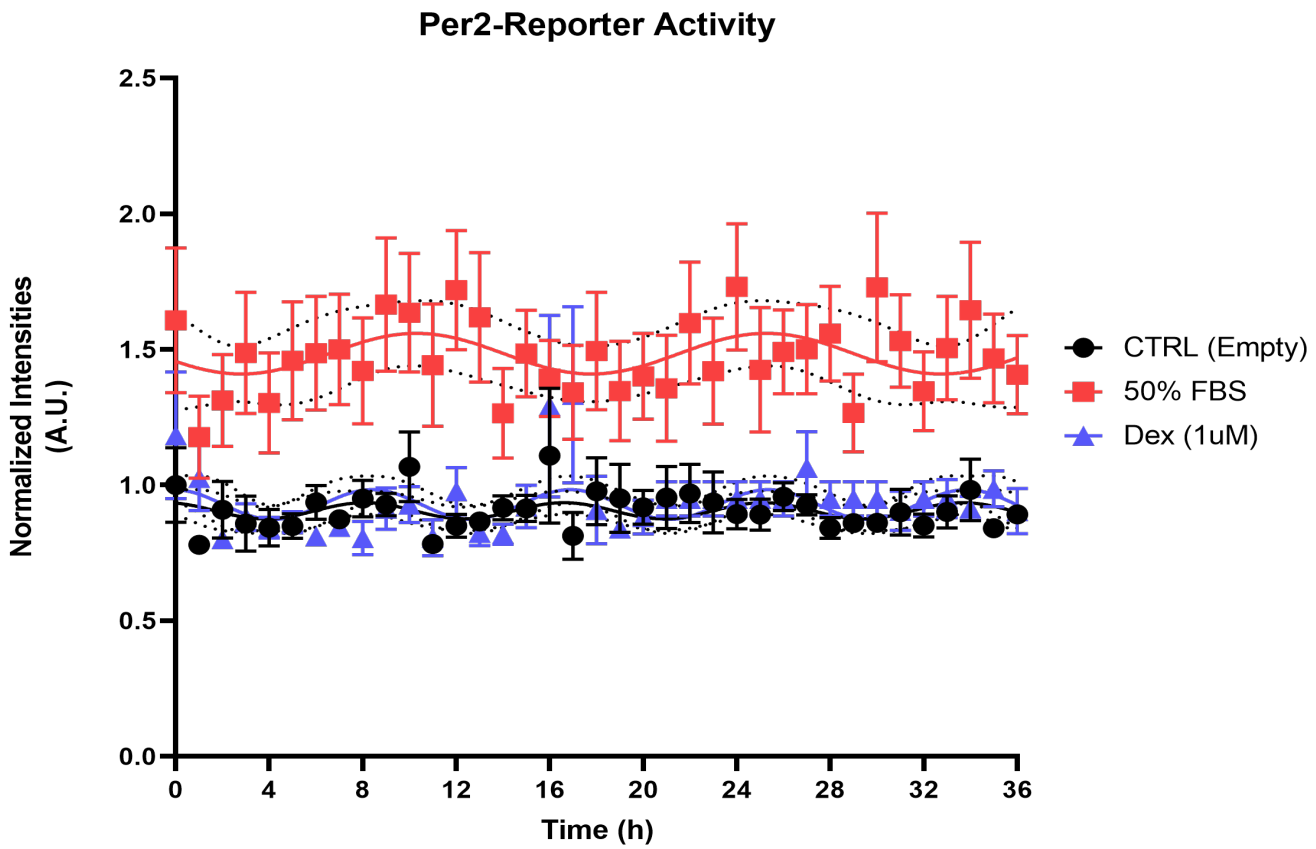


Figure 6: Average fluorescence across wells over time. Rhythmic profile of Per2-Reporter activity in response to circadian synchronizers. Reporter Cells that were given either empty vehicle (black, n=4), vehicle containing 50% FBS (red, n=2), or 1 µM Dexamethasone (blue, n=4). Rhythmicity is represented by sinewave fitted to time series data. Error bars represent standard error of mean (*SEM*). Of all experimental groups, only the FBS-treated cells exhibited rhythmicity.

2.3: Evaluation of Circadian Reporter

After the images of each well were collected, we analyzed the mean intensities of individual cells time-lapse using the native Incucyte software. Fig. 6 visualizes the average changes of fluorescent intensities over time of reporter cells under circadian inducers normalized to untreated cells at *t=0*. As expected, reporter cells treated with empty vehicles exhibited some fluorescence, with minute fluctuations over the 36h period. 50%-FBS-treated reporter cells exhibited a significantly mesor (or baseline) (Fig.7, $p<0.002$) as

well as a much more notable cyclical expression. Interestingly, this phenomenon is not reflected in the amplitude and period parameters. There no statistically significant differences between the untreated and the FBS- and DEX- treated cells. However, there is a general trend towards significance in the amplitude ($p = 0.1734$) and period ($p = 0.3567$) of FBS-treated cells compared to untreated. There was also a significant phase shift in FBS-treated cells compared to the control ($p < 0.05$). A negative shift is phase signifies that the highest amplitude value (peak) was reached sooner in cycle compared to untreated groups. This suggests that the 50%-FBS treatment had indeed triggered the expression of circadian genes. Also, the time-lapse microscopy had occurred 2 hours post-treatment, so there is the possibility that the complete trend was not fully captured. Overall, these data demonstrate that the circadian reporter system is sensitive to FBS-treatment.

The dexamethasone treatment did not elicit the same effect on reporter activity. Across all metrics, 1 µM DEX-treated cells had no significant differences with the

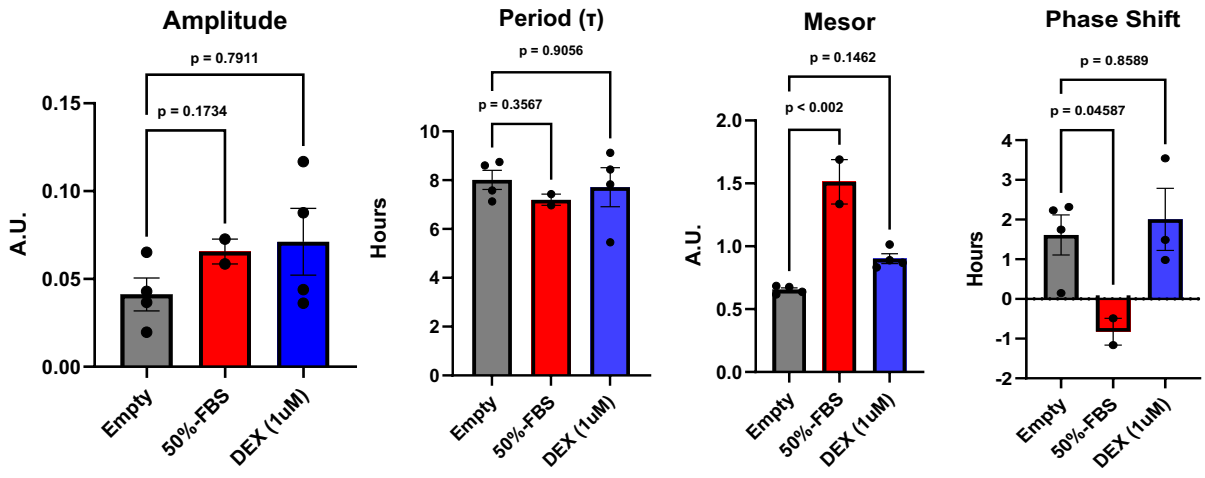


Figure 7: Comparison of Cosinor Parameters of Untreated (*gray, n=4*), 50%-FBS-treated (*red, n=2*), and Dex-treated (*blue, n=4*) cells. Error bars represent *SEM*. One-Way ANOVA was used to determine significance. Only FBS-treated cells exhibited either significant or trending toward significant changes in Cosinor parameters.

untreated cells. The only Cosinor parameter that trended towards significance was mesor, although the magnitude of difference was not very notable. This is unexpected, as DEX is an established inducer of circadian rhythms. This lack of impact could be explained by several factors. First, all experiments were conducted with a polyclonal population of reporter AML12 cells. Because the insertion of transgenic genes is biased towards transcriptionally active regions under lentiviral transfection, chromatin structure has large impact on the sensitivity of the promoter region [103]. This imparts variance in sensitivity of the reporter in our cell population. It is possible that surviving population of cells treated with DEX had lower sensitivity due to its position in the insertion locus, where it competed with other existing promoter regions. Another factor is that transfection of the reporter was performed on AML12 cells that retained the native PER2 gene. It is possible that DEX-treatment did induce the formation of the CLOCK/BMAL1 heterodimer but favored binding to the native PER2 promoter. Again, chromatin structure would play a major role if this were to be true, as the transgene may have inserted in less accessible region. In summary, the DEX-treatment failed to elicit the expected effect on circadian parameters.

2.4: Discussion, Conclusions, and Limitations

In chapter 1, we described the justification for developing the circadian reporter. To summarize, we discussed emerging relationship between circadian rhythm and metabolism. Because of the inherent labor-intensive nature of circadian research, there is limited potential for knowledge generation. Current methods for circadian research

address these limitations, but many have inherent complications. Thus, the field would benefit from the development of a circadian reporter that is easy to use, while limiting labor and complications. In Chapter 2, we discussed the logic behind the design of our novel circadian reporter and tested its efficacy. When treated with known *in vitro* circadian inductors, our results demonstrated that reporter is sensitive to the 50%-FBS treatment but failed to respond to the DEX treatment. In section 2.3, we discussed the possible reasons for the disparity in sensitivity. As it stands, the reporter requires more optimization to improve function and efficacy. Here, we discuss suggestions for improving the system.

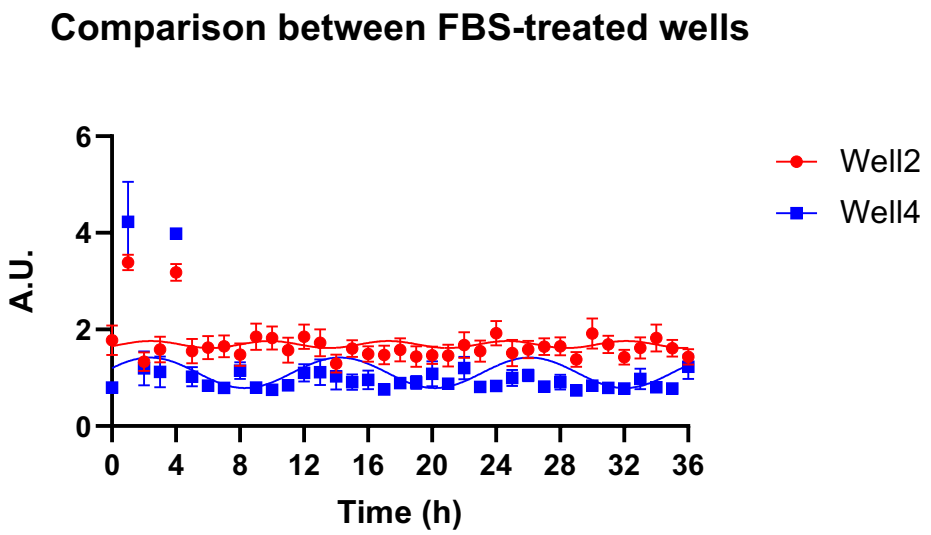
Firstly, due to the mechanism of lentiviral transfection, there is little control over where the transgene would insert into the genome. It is random and thus each cell would behave slightly differently under circadian induction. Instead of transfecting wildtype AML12 cells, we suggest transfecting $PER2^{-/-}$ cells. This addresses the stoichiometric limitations of CLOCK/BMAL1 heterodimer. In other words, the presence of more than one PER2 promoter region in the genome creates competition for CLOCK/BMAL1 binding, which would reduce the overall sensitivity of the reporter. Cheon et al. improved the sensitivity of the PER2:LUC construct with this approach [104]. Another approach is to isolate individual cells with higher baseline fluorescence expression from the polyclonal population using fluorescence-activated cell sorting (FACS). FACS is specialized type of flow cytometry and provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific fluorescent characteristics of each cell. The approach taken by Cheon et al. in combination with FACS

would generate a pool of highly sensitive cells and improve efficacy in subsequent experiments.

Furthermore, there are some issues that could arise from the design of the reporter itself. Here, we designed the lentiviral plasmid to position the CMV-driven D2mCherry construct upstream of the PER2-driven D2eGFP. CMV is highly-constitutively active and there is the possibility that CMV could mask PER2-driven transcription. We initially considered this potential limitation but at the time and as of now, there was no substantial evidence in the literature that the presence two independent promoter regions would affect the other. However, there are existing solutions that would address this if it were so. The first step is to place each reporter construct in separate plasmids with different selection markers. Then, transfect one of individual plasmids into a cell population while administering the respective selective antibiotic. After selection, surviving cells would be subjected to transfection with the second plasmid and undergo another round of selection with the secondary antibiotic.

The disparity between the FBS and DEX treatments was unexpected. The traditional method for circadian induction performed in these experiments were chosen based on the substantial empirical evidence of their effectiveness present in the cited sources. Optimization of the circadian induction method may enhance reporter sensitivity. Of the tested methods, only the mechanism behind the DEX-treatment is more understood. Despite its widespread use in cell culture, the components of FBS are not completely defined and not standardized. Blood serums contain a myriad of potential inducing agents such as steroids (primarily glucocorticoids), insulin, and high nutrient

abundance. However, these components' identities and their relative contributions are not well characterized. As such, the higher sensitivity to FBS could be attributed to the higher abundance and diversity of circadian inducers compared to singular presence of dexamethasone. This, however, has not been robustly tested in the literature as far as we know. Interestingly, this may also explain the disparity we observed between some of FBS-treated biological replicates. Decomposition the Cosinor curves fitted to the FBS-treated data revealed that the overall rhythm was mostly driven by cell populations from one well despite treated with the same batch of FBS. Figure 8. compares Cosinor parameters of each FBS-treated well. Notably, the fourth well (n=3) had higher parameters across the board. The issue of the inconsistency with FBS is difficult to address. However, the previous stated suggestion for optimization will reduce the inherent variance.



	Well2	Well4
Amplitude	0.07272	0.3159
Wavelength	7.426	11.87
PhaseShift	-0.4861	0.3135
Baseline	1.689	1.104

Figure 8: Comparison of FBS-treated cell populations. Error bars represent *SEM.* Statistical significance could not be determined (each well is treated as n=1). Also shown are tabulated values of each Cosinor parameter. Note that cells in well 4 exhibit higher sensitivity to the FBS treatment.

Finally, there are some factors in the experimental pipeline that would benefit from further optimization. Cell cultures are typically grown in synthetic culture mediums like Minimal Essential Medium (MEM) and other derivatives. The mediums are high in phenolic compounds such as phenol red, which is a standard pH indicator and provides a quick check for the health of the culture. Because phenol-red is auto-fluorescent, there is potential for emission interreference when images are collected by the Incucyte microscope. Indeed, we observed some interference in the collected images (not shown). However, the Cell-by-Cell module allows for the adjustment of parameters that reduce

the interference, which we optimized prior to data collection. Fortunately, there are phenol-free cell culture mediums offered by Thermo-Fischer. Another method is to reduce auto- fluorescence naturally given off by cells. Auto-fluorescence is in part the result of accumulation of oxidized proteins and fatty acids granules in cells called lipofuscin, which is caused by oxidative stress [105]. There are commercial quenchers available that removes lipofuscin auto-fluorescence. (TrueBlack© - Biotum). In summation, we are confident that these suggested optimizations will improve reporter efficacy.

Future Directions:

In summation, the novel circadian reporter provides promising method for analyzing circadian rhythms but requires more optimization in both experimental and analytical parameters. Future work requires investigating other potential reporter designs and generating the more sensitive stable cell lines. After these suggested optimizations are applied and improve efficacy, we will apply the circadian reporter to investigate the effects of dietary fats on circadian expression and corroborate previous findings regarding the role of fats in regulating circadian rhythms. We will also utilize the reporter system in investing the contribution of ATGL signaling in the SIRT1-PER2 axis. Due to the high through-put nature of our reporter, we aim to use mutagenic screenings in reporter cells to identify novel circadian regulators with our experimental pipeline. Lastly, we aim to distribute our reporter system with collaborators to assist with hypothesis/knowledge generation in other circadian related fields.

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