Non-Photic Mechanisms of Entrainment in BMAL1 Deficient Conditions

by

Jamie Pham Tran, B.A.

APPROVED:

_____
Dr. Kristin Eckel-Mahan, Ph.D.
Advisory Professor

_____

Dr. Zheng Chen, Ph.D.

_____

Dr. Seung-Hee Yoo, Ph.D.

_____

Dr. Nicholas Justice, Ph.D.

_____

Dr. Joya Chandra, Ph.D.

_____

Dr. J. Leigh Leasure, Ph.D.

_____

APPROVED:

_____
Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

Non-Photic Mechanisms of Entrainment in BMAL1 Deficient Conditions

Thesis

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

by

Jamie Pham Tran, B.A.

Houston, Texas

August 2022

This work is dedicated to Abigail, Jonathan Altamirano, Stephanie Canelas, and Taryn Gray. For without you, I would not have had the strength to go on; and with you, there is nothing I cannot accomplish.

Acknowledgements

First and foremost, this master's thesis would not have been possible without the love, encouragement, and guidance of those who have supported my dreams all of these years. There is no thank you that is enough to express the depth and breadth of my gratitude for you.

To Jonathan & Stephanie – there's nothing I could have done without the both of you cheering me on, pushing me, and being my shelter and comfort. You've given me your unconditional love, your loyalty, and unfailing support. And for that, I was able to give it my all and find the strength to step out of the shadows and fight for what I believe in. Win or lose, you guys are everything to me.

To Taryn – who has no idea what I'm doing 95% of the time, but will support me no matter where I end up in life.

To the late and great Stephanie Bosch – who began this journey with me and was unable to see it through. I made it. I hope I did you proud.

To Katharine Nelson – who has grown up in the scientific community with me and continues to be my loving cheerleader and a shoulder to cry on when I start to doubt my scientific abilities.

To the friends I have made here at GSBS – each and every one of you are brilliant scientists and even better people; even if you don't think so at times. I believe in you.

To the GSBS admins, program managers, directors, and overall community – Thank you for taking a chance on me and working with me through all my troubles. In particular, Drs. Kelly Moore, Natalie Sirisaengtaksin, and Raquel Salinas for being the best writing coaches and

cheerleaders I could ever ask for and giving me the gentle push I needed, even if I cried while doing what needed to be done.

To my advisory committee – who graciously gave their time, support, and wisdom to help shape me into the scientist I wish to become. I feel incredibly fortunate to have been able to work with each and every one of you. Thank you for challenging me in the best way possible.

To my lab mates – who have displayed unending patience with my million and one questions and put up with my presentation anxiety on multiple occasions – may all of your academic and scientific dreams come true. Thank you for taking such good care of me.

To my mentor and advisor, Dr. Kristin Eckel-Mahan – whose gentle encouragement and guidance enabled me to thrive as an upcoming researcher. I have nothing but the deepest appreciation and admiration for the courage and strength it took for you to take me in and go on this journey with me. You are not only one of my greatest role models in the world of academia, but in life itself. Thank you for showing me there is undoubtedly life beyond the lab, that it's worth celebrating all victories big or small, and most importantly, for showing me what it truly means to mentor and support someone both academically and emotionally. I hope to continue your legacy of kindness, compassion, wisdom, and intelligence as I grow to become the researcher I know that I can be.

To Winnifred – my loving pupper noodle whose love for me does not depend upon my social standing, academic achievements, or anything of that sort.

Lastly, to my Abigail – who I love more than life itself, even after death.

Non-Photic Mechanisms of Entrainment in BMAL1 Deficient Conditions

Jamie Tran, B.A.

Advisory Professor: Dr. Kristin Eckel-Mahan, Ph.D.

Maintaining our internal circadian (i.e. 24-hour) clock is imperative to our daily biological and mental well-being. Large epidemiological studies have shown that disruptions of our circadian rhythms can lead to poor mental health, metabolic diseases, and various types of cancer. Various external cues that have become a part of the modern times such as electricity, shift-work, rapid travel across various time zones, easier access to nutritionally unbalanced food items, and various rigid social demands have deleterious effects on our internal clock, and generally reduce robustness of the circadian clock.

The two following projects aim to examine two fundamental aspects of the circadian clock mechanism in health and disease: 1) exercise as an entrainment mechanism for the clock; and 2) targeting of clock proteins to reduce proliferation and survival in acute myeloid leukemia. Both projects center on the important role of the circadian clock protein brain and muscle ARNT-like factor (BMAL1), which is a transcription factor critical for maintaining cellular rhythmicity. Loss of the protein in the PVN of the hypothalamus can lead to arrhythmicity, but can be rescued by exercise. In the case of acute myeloid leukemia, this protein can be targeted to slow the proliferation of transformed cells.

Together, these data suggest that the exploitation of circadian mechanisms of entrainment and molecular targeting can be used to rescue the damage inflicted by circadian disruption.

Table of Contents

Approval Page ..... ***i***

Title Page ..... ***ii***

Dedication ..... ***iii***

Acknowledgements ..... ***iv***

Abstract ..... ***vi***

Table of Contents ..... ***viii***

List of Illustrations ..... ***x***

List of Tables ..... ***xii***

List of Abbreviations ..... ***xiii***

Introduction ***1***

Results ***11***

Exercise as a Potential Therapeutic Strategy for Circadian Disruption in the Paraventricular Nucleus **11**

Characterizing PVN specific, BMAL1-depleted Knockout Mice 11

Restoration of Circadian Locomotor Activity 18

The Synergistic Effects of Nobiletin and Daunorubicin in AML Viability **22**

Characterizing the Molecular Clock in Acute Myeloid Leukemia 23

Optimizing Treatments 25

Discussion ***33***

Exercise as a Potential Therapeutic Strategy for Circadian Disruption in the Paraventricular Nucleus **34**

The Synergetic Effects of Nobiletin and Daunorubicin to Induce Apoptosis in Acute Myeloid Leukemia **40**

Concluding Remarks 45
Methods and Materials 46

Exercise as a Potential Therapeutic Strategy for Circadian Disruption in the Paraventricular Nucleus 46
Animal Model 46
Data Collection 46
Tissue Preparation and Preservation 47
Imaging 47

The Synergetic Effects of Nobiletin and Daunorubicin in AML Viability 47
Cell Culture 47
Cell Treatment 48
Cell Metabolic Assessment 48
Protein Expression Analysis 49

References 52

Background 52
Exercise as a Potential Therapeutic Strategy for Circadian Disruption in the Paraventricular Nucleus 55
The Synergetic Effects of Nobiletin and Daunorubicin in AML Viability 58
Vita 62

List of Illustrations

Figure 1. General representation of how transcription factors function (pg. 4)

Figure 2. Molecular clock, the core loop (pg. 4)

Figure 3. Animal model of a PVN specific, BMAL1 knockout mouse (pg. 11)

Figure 4. Representative Actograms and Periodograms of a GFP-tagged control animal (pgs. 12-13)

Figure 5. Representative actogram and periodogram of a PVN-specific, BMAL1 knockout animal characterizing circadian behavioral phenotype before exercise. (pg. 14)

Figure 6. Representative actograms and periodograms of a PVN-specific, BMAL1 knockout animal before, during, and after exercise. (pgs. 14-15)

Figure 7. Actogram and periodogram of a PVN specific, BMAL1 knockout animal with an alternative behavioral and metabolic phenotype before exercise (pg. 16)

Figure 8. Actograms and periodograms of a PVN specific, BMAL1 knockout animal with an alternative behavioral and metabolic phenotype before, during, and after exercise. (pg. 17)

Figure 9. Contrasting weight gain between two PVN specific, BMAL1 knockout animals (pg. 18)

Figure 10. Overall activity levels of all animals (pg. 19)

Figure 11. Percentage of daily activity for all animals, split into night and day for all conditions (pg. 19)

Figure 12. Weight progression, all animals (pg. 20)

Figure 13. Endogenous BMAL1 protein levels in various AML cell lines (pg. 24)

Figure 14. Metabolic mitochondrial health of AML cells lines and their various responses to 25μM of NOB and 10nM of DNR (pg. 24)

Figure 15. MOLM13, IC50 curves for singular doses of NOB and DNR (pg. 25)

Figure 16. MOLM13, singular and combination doses of NOB and DNR for optimization (pg. 25)

Figure 17. Assessment of cellular growth and proliferation via Trypan Blue with live cells (pg. 26)

Figure 18. Morphological changes of MOLM13 in response to time and treatment conditions (pg. 27)

Figure 19. MOLM13, quantified protein levels of BMAL1 (pg. 28)

Figure 20. MV411, IC50 curves for singular doses of NOB and DNR (pg. 29)

Figure 21. MV411, quantified protein levels of BMAL1 (pg. 30)

Figure 22. MOLM13, quantified protein levels of RORα (pg. 31)

Figure 23. MV411, Quantified protein levels of RORα (pg. 31)

Figure 24. Unpublished actograms and periodograms of time restricted feeding experiments (pg. 36)

Figure 25. MOLM13 preliminary data, western blots of cleaved caspase 3 and p53 (pg. 43)

List of Tables

Table 1. Cell lines, characteristics, and maintenance (pg. 48)

Table 2. Cell lines and singular treatment doses for IC50s (pg. 48)

Table 3. Materials (pg. 51)

Abbreviations

AML – Acute Myeloid Leukemia

BMAL1 – Brain and muscle ARNT-like factor

CCG – Clock-controlled genes

CLOCK – Circadian locomotor output cycles kaput

COM – Combination of nobiletin and daunorubicin

CRY – Cryptochrome

DMH – Dorsal medial hypothalamus

DNR – Daunorubicin

DOX – Doxorubicin

E-Boxes – Enhancer box DNA (CACGTG)

FLT-3 – Fms-like tyrosine kinase receptor 3

NOB – Nobiletin

NT – Non-treated

PER – Period

PVN – Paraventricular Nucleus

PVT – Paraventricular thalamus

RORα - RAR related orphan receptor A

SCN - Suprachiasmatic nucleus

SPZ – Subparaventricular zone

TRF – time-restricted feeding

VIP – Vasoactive intestinal peptide

VPAC2 – Vasoactive intestinal peptide receptor 2

Introduction

Mounting epidemiological evidence this past decade has shown that ill-timed or blunted biological rhythms can have deleterious effects on our physical and mental well-being on a systemic level. For example, according to the US Bureau of Labor Statistics in 2019, approximately 2.3 million people out of our nation's 14.4 million worked on night, early morning, or rotating shift schedules (United States Bureau of Labor Statistics, 2019) placing them at high risk for both long and short-term consequences of constant circadian misalignment and disruptions resulting in systemic consequences such as increased disruptions to metabolic health, propensity towards certain cancers, negative impacts on mental well-being and heart problems compared to their non-shift work counterparts (James, 2017). These negative effects on our overall health are well beyond the scope of what is given in this thesis and we have yet to gain a full understanding of the depth and breadth of the consequences we now face. Given that a large percentage of our population is at risk for such wide-spread effects of circadian disruption, it is of utmost importance to find ways for us to biologically adapt to our ever-changing modern environment.

Thus, finding new and innovative ways to maintain our internal clock is imperative to our daily biological and mental well-being. As our society manages to advance technologically, our bodies become ill equipped to adjust its internal time due to various and constant external cues and disruptions that have become a part of the modern times such as electricity, shift-work, rapid travel across various time zones, easier access to nutritionally unbalanced food items, and various rigid social demands that further damage our own

rhythmicity. Therefore, it is imperative that alternate, non-photic means of adjusting and strengthening our internal clocks are of utmost importance to study.

Circadian rhythms are highly conserved molecular mechanisms which are present throughout the majority of living organisms including single celled bacteria to complex multicellular organisms. This mechanism serves an important evolutionary role in survival by allowing organisms to adapt to the 24-hr. environment using environmental cues (also known as "*zeitgebers*"). This "entrainment" to the 24-hr. environment allows alignment between an internal biological 24-hr. cellular clock and coordinate corresponding behavioral output for optimal success in a variety of behavioral functions. Such adaptation is necessary for a variety of physiological functions, including: 1) finding or conserving energy resources; 2) predatory detection and avoidance; 3) reproduction; and 4) sleep, among others. In order for a biological rhythm to be established as circadian, it must fulfill three requirements: A) have an inherent, near 24-hour periodicity, B) be robust in its oscillation in the face of inherent biological changes such as temperature (known as "temperature compensation"), and C) possess the ability to be tuned to a 24-hour period through environmental cues (otherwise known as entrainment) (Paranjpe & Sharma, 2005).

For most organisms, including humans, the primary means of entrainment to the 24-hr. the environment is light. This entrainment pathway begins when environmental input is received by way of sunlight which enters the eyes and hits the photosensitive retinal ganglion cells which then convert that light into electrical signals that travel down the retinal hypothalamic tract to the suprachiasmatic nucleus (SCN) located in the anterior portion of the hypothalamus (Bass & Takahashi, 2010). The SCN, initially referred to as the "master

pacemaker", then relays this information to other brain areas and peripheral nervous system to synchronize all of our internal clocks to the light signal. Lesions of the SCN in rodents have demonstrated the importance of this region in circadian time-keeping; loss of SCN function results in arrhythmic energy intake, drinking, melatonin secretion, and the sleep/wake cycle (Bass & Takahashi, 2010).

From a micro perspective, the circadian molecular clock is ultimately functional at the cellular level (Patke, 2020), where it is composed of specific genetic components involving a translational-transcriptional feedback loop with the core loop being composed of core clock genes *Bmal1*(Brain and Muscle ARNT-Like 1), *Clock 1/2* (Circadian Locomotor Output Cycles Kaput), *Period 1/2/3*, *Cry 1/2* (Cryptochrome), and their respective protein products. The core loop involves the heterodimerization of two transcription factors, BMAL1 and CLOCK, which together bind to DNA consensus elements known as "E boxes" (CACGTG) to promote the transcription of other core clock genes, notably period (*Per*) and cryptochrome (*Cry*) in respect to the core loop. PER and CRY will accumulate and ultimately interact directly with CLOCK: BMAL1 to and inhibit the further transcriptional activity of the BMAL1: CLOCK transcriptional complex. The PER/CRY proteins will eventually degrade over time allowing the BMAL1 and CLOCK proteins to build up again, allowing further

heterodimerization and transcriptional activation at target genes.

Fig. 1

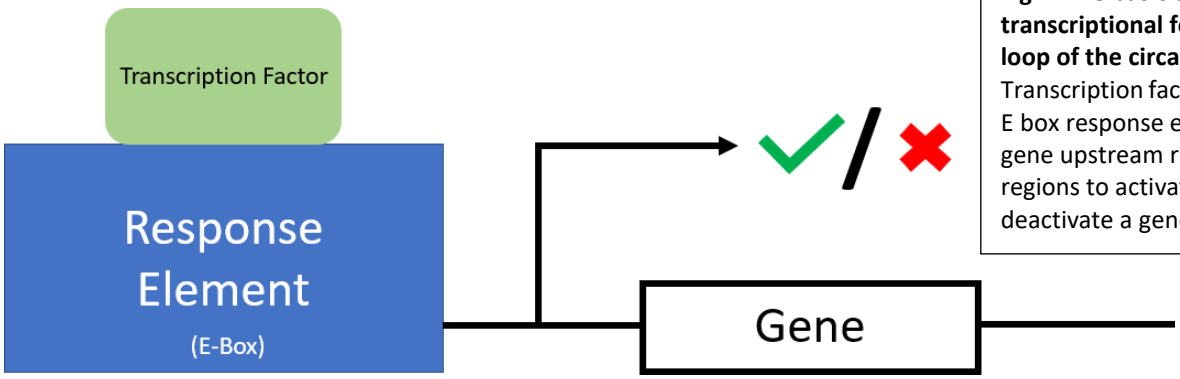
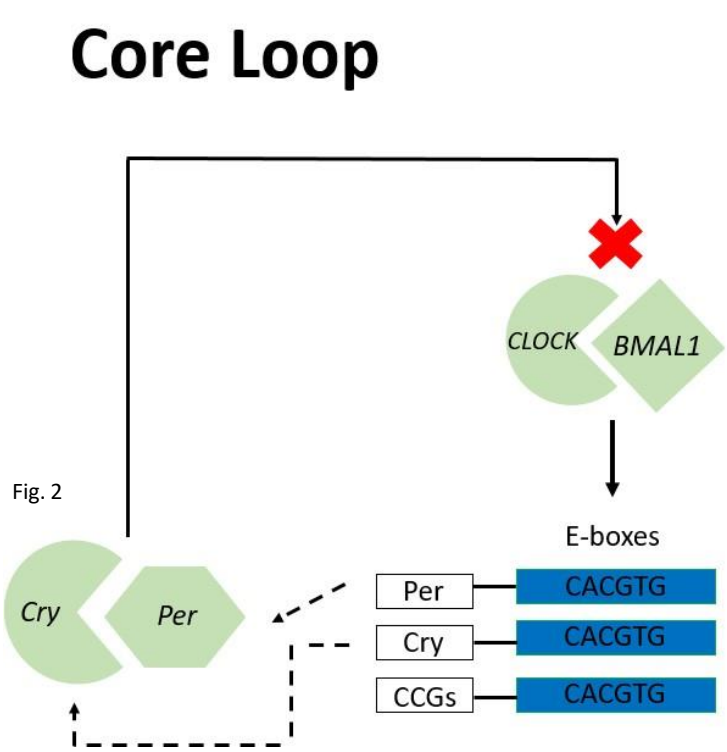


Fig. 1 The basic core transcriptional feedback loop of the circadian clock. Transcription factors bind to E box response elements in gene upstream regulatory regions to activate or deactivate a gene.

Fig. 2



CLOCK = Circadian Locomotor Output Cycles Kaput
BMAL1 = Brain and Muscle ARNT – Like 1
Per = Period (1/2/3)
Cry = Cryptochrome (1/2)
CCG = Clock-controlled genes
E-boxes = Enhancer Boxes

Fig. 2 Proteins involved in the core clock loop. is a simplified diagram of the core loop of the molecular clock depicting the rise of activity in the transcription factors CLOCK: BMAL1and the fall of their activity by their transcriptional targets, the CRY: PER proteins.

In addition to core clock genes, this heterodimer regulates the rhythmic expression of hundreds of genes in the genome across mammalian organisms. This basic transcriptional/translational feedback loop is approximately 24 hours and allows for our bodies to keep their internal time independent of light. Having an ingrained and robust molecular clock allows cells to function and keep biological time stable within the organism

and works as an advantage to the benefit of each organism. It achieves this through signaling the optimal times for each biological system or tissue to function and respond according to its needs.

Although light may be the primary *zeitgeber* for the majority of living species on earth, there are other mechanisms of non-photic entrainment, which have been more recently appreciated and studied. There are *zeitgebers* that can overcome the light-driven time-keeping system of the SCN. For example, even in the absence of light, robust internal circadian rhythms can still persist. Examples of such extreme conditions and circumstances in which traditional light signals are not applicable are extremophiles such as cave fish (Cavallari, 2011), reindeer (Arnold, 2018) and other animals that live near the poles, organisms that hibernate, and people who have lost their ability to detect light due to degradation or injury to retinas (Quera Salva, 2017). Although some of these organisms and situations do not utilize light signals in a traditional manner when it comes to circadian synchronization, they do have other mechanisms and ways in which to process and express these signals such as seasonal clock oscillations within reindeer (Arnold, 2018) and dermal opsins to maintain skin health (Suh, 2020). These organisms and special conditions demonstrate the importance and mystery of other *zeitgebers* in which it is necessary to rely upon an alternate means of entrainment.

Instead of being reliant upon the sun and the resources that wax and wane due to our earth's daily and seasonal cycles, modern day technology has enabled us to achieve extraordinary things such as the invention of electricity and its accompanying technological gadgets, neigh limitless access to various foods of varying nutritional value, and with that,

our society's fast paced expectations due to these modern-day inventions. Being exposed to light at inappropriate times in the day, having the ability to eat anything at any given time, rapid travel across time zones, and lack of social boundaries (such as working night shift or odd hours, constant access to instant communication, etc.) that often accompanies artificial light exposure creates many opportunities for our circadian rhythms to become more atypical or blunted easily and for a longer period of time impacting or leading to various disease states such as disrupted sleep, affective disorders, metabolic disorders, and even certain forms of cancer.

Energy intake in particular has gained research momentum in the last decade as an alternate means of entrainment. Specifically, altered energy intake patterns or nutrient challenge with or without changes in the temporal aspects of energy intake can dramatically alter the circadian clock in a tissue-specific way (Dyar, 2018). One such example is the liver clock. A high fat diet easily results in fatty liver, and results in a dramatic circadian reprogramming of gene expression, to drive fatty acid storage in the liver (Eckel-Mahan, 2013). Fatty liver is now known to be a risk factor for hepatocellular carcinoma of the liver (Fekry, 2019).

To better understand the protective role of the circadian clock in the body, I have undertaken two projects during my master's thesis, both of which rely on a better understanding of the transcription factor BMAL1 in cells and tissues of the body. While both projects center on the important role of the clock through BMAL1, both projects also focus on circadian rhythm restoration through non-photic means in different cell types.

The first project focuses on the loss of BMAL1 in the paraventricular nucleus (PVN), its metabolic and behavioral effects following that loss, the ability of exercise to restore rhythmic behavior, and the magnitude of its effect. This project will address two issues: a) that circadian behavioral output does not solely depend upon the suprachiasmatic nucleus (SCN) and b) that exercise can overcome arrhythmic disruption in the PVN. I hypothesized that loss of rhythmicity by BMAL1 deletion in the PVN could be restored using exercise as a *Zeitgeber*.

The PVN is an area of the brain that is of particular interest to energy balance due to its ability to process information regarding energy intake and expenditure. Interestingly, recent studies (Kim, 2020) demonstrate that BMAL1 expression in the PVN is necessary for driving dramatic changes in circadian behavior. Specifically, previous data has shown that loss of BMAL1 in this area leads to inappropriate energy intake during various hours of the day resulting in obesity (Kim, 2020). This study gave insight into the novel idea of the PVN having an independent influence on circadian behavioral output and suggests that the SCN is not the only area to influence rhythmic behavioral outcomes.

Though light and energy intake are potent *zeitgebers*, in recent years, both modifications to energy intake and expenditure have been proven to drive dramatic changes in rhythmicity across many peripheral tissues. This particular pathway to entrainment differs from the conventional light entrainment pathway in the fact that it uses humoral information (such as nutrient signals) garnered from the paraventricular nucleus (PVN) to modulate the body's circadian rhythm instead of the light information gained from the retinohypothalamic tract (RHT). Although it has been known for quite some time that

environmental cues other than light are able to entrain and regulate circadian rhythms, the strength and permanence of these entrainment methods and the mechanisms in which it is able to do so are not well understood. In particular, exercise as a means to sync and restore circadian rhythmicity has been gaining momentum as a potential therapeutic strategy in the fields of mental illness and metabolic diseases. For example, the Pendergast lab has reported that exercise can induce a phase shift in humans according to their chronotype (Thomas, 2020) providing insight as to how exercise can entrain people of varying circadian rhythms. On a molecular level, the Colwell Lab has found that exercise has the ability alter circadian rhythms on a genetic level in wild type and vasoactive intestinal peptide deficient mice (Schroeder, 2012). Even though these studies have provided great insight into rhythmicity restoration, their studies still have the PVN intact in their models which is in contrast to the Tong Lab's results of aberrant circadian locomotor patterns via BMAL1 depletion in the PVN with an intact SCN (Kim, 2020).

To address this discrepancy, we intend to use exercise as a therapeutic strategy to restore circadian rhythmicity using PVN damaged mice. Two different mice models will be used to assess circadian behavior using actograms to reflect sleep/wake cycles with and without the inclusion of the exercise wheel. These wheels will then be taken away to assess the permanence and magnitude of rhythmicity restoration. This objective will address two issues: a) that circadian behavioral output does not solely depend upon the SCN and b) that exercise can overcome arrhythmic disruption in the PVN.

My second project centers on understanding whether pharmacologically modulating BMAL1 expression via a natural flavonoid, "nobiletin" (NOB) in low BMAL1-expressing acute

myeloid leukemia (AML) cells is a tractable mechanism with or without existing chemotherapeutic agents for slowing the growth of AML. AML is one of the most difficult cancers to treat due to it being an integral part of the circulatory system, its heterogeneity, and aggressive nature. One of the most common treatments for AML is daunorubicin (DNR). It works by interfering with DNA base pairs and causing the helix to uncoil, thus inhibiting DNA replication in the S phase of the cell cycle. However, this effect is not cell specific and can also damage healthy cells as well, causing complications in treating patients (Alves, 2017). Because of these detrimental off-target effects, there is a dire need for a safer administration of DNR in order to treat patients while limiting harm. With this idea in mind, this project seeks to use nobiletin as a pharmacological additive to use in conjunction with traditional chemotherapy. It operates by directly agonizing Retinoic Acid Receptor-Related Orphan Receptor Alpha (RORα), a transcription factor which activates the circadian protein BMAL1 (Chen, 2018). This in turn, activates apoptotic signals downstream to kill cancer cells while leaving healthy cells unharmed (Chen, 2016; Lellupitiyage, 2020). The overall goal of this project is to use NOB in conjunction with traditional daunorubicin to lower the amount of DNR being used to decrease toxic off target effects whilst maintaining apoptotic efficacy in AML. Based on previous data using liver cancer and glioblastoma cells using NOB as a therapeutic reagent (Mahan Lab, unpublished data), I hypothesized that modulating BMAL1 in AML might further slow the growth of AML in combination with standard chemotherapeutic agents.

Ultimately, both projects will hopefully help lay a foundation for future studies, including clinical potential in chronobiology and both are worth exploring as an alternate

means of restoring or strengthening circadian rhythmicity. While pharmacological modulation of the clock is an area of active study, behavioral mechanisms (such as time-restricted feeding) are additional mechanisms by which the circadian clock can be fortified in the body. This is also a feasible clinical approach, and there remains hope that targeting the clock may be an important therapeutic strategy for the future in the context of a number of disease states.

Results

Exercise as a Potential Therapeutic Strategy for Circadian Disruption in the Paraventricular Nucleus

Characterizing PVN specific, BMAL1-depleted Knockout Mice

In addition to the initial metabolic characterization of the PVN specific, BMAL1-deficiency reinforces that circadian behavioral output is not entirely dependent on the SCN. Previous data from the Tong lab suggested that loss of PVN rhythms may drive arrhythmicity independent of the SCN (Kim, 2020), a property that was thought to be driven predominantly by arrhythmicity in energy intake (Izumo, 2014). In further characterizing these mice it was determined whether or not SCN rhythms were still intact in mice in which BMAL1 had been disrupted.

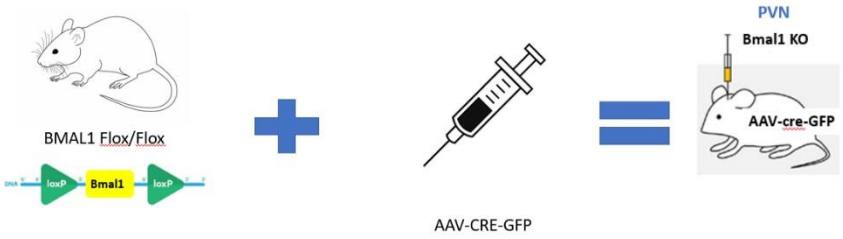


Fig.3

Fig. 3 Mouse model of BMAL1 deletion in the PVN. PVN-specific knockout of BMAL1 is achieved through the viral injection of AAV-CRE-GFP into BMAL1 mice having loxP sites surrounding the Bmal1 gene. Control animals are injected with AAV-GFP.

The circadian molecular clock in the PVN was depleted by using BMAL1 fl/fl mice (Jackson Laboratories, #007668) generated by the CRE-Lox method to create a conditional knockout model. At 8 weeks old, these mice were subject to bilateral intracranial injections of either an AAV-CRE-GFP virus to knock out BMAL1 in the PVN or AAV-GFP for control groups (Kim, 2020). These mice were then put under surgical care observation for the first three days in 12-hour light/12-hour dark entrainment conditions to ensure proper healing

and then further monitored by infrared sensors (Starr Life Sciences) detecting movement for the duration of two weeks in order to define behavioral phenotype of rhythmic, arrhythmic or weakly rhythmic circadian locomotion.

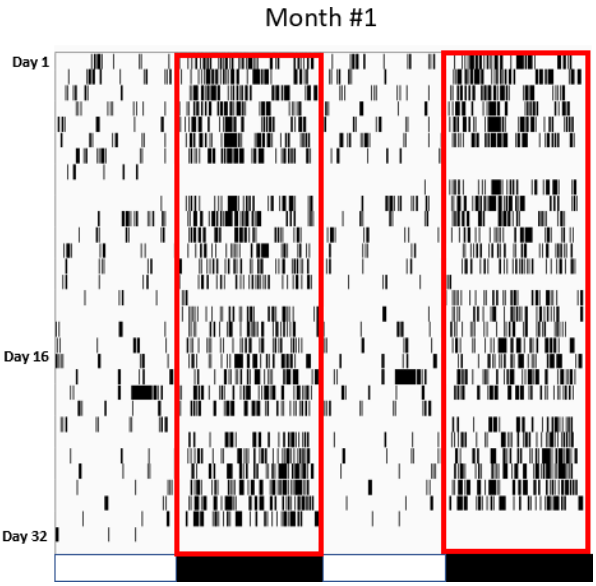


Fig. 4a

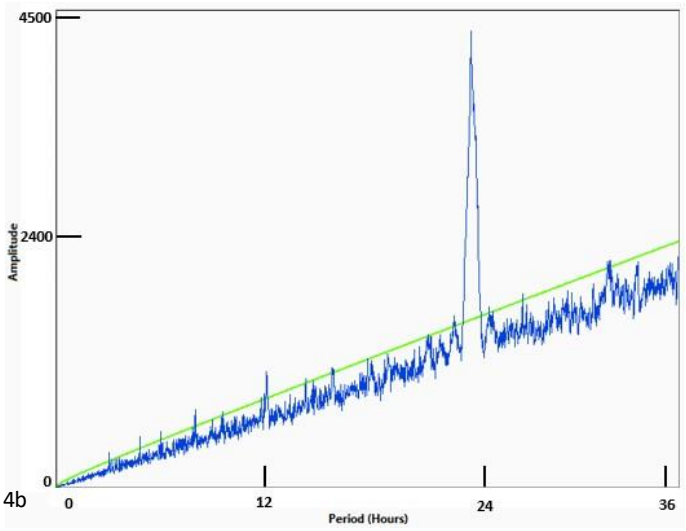


Fig. 4b

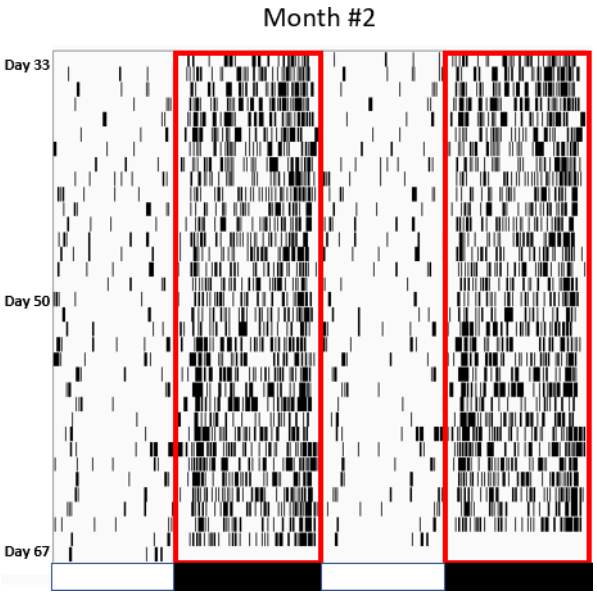


Fig. 4c

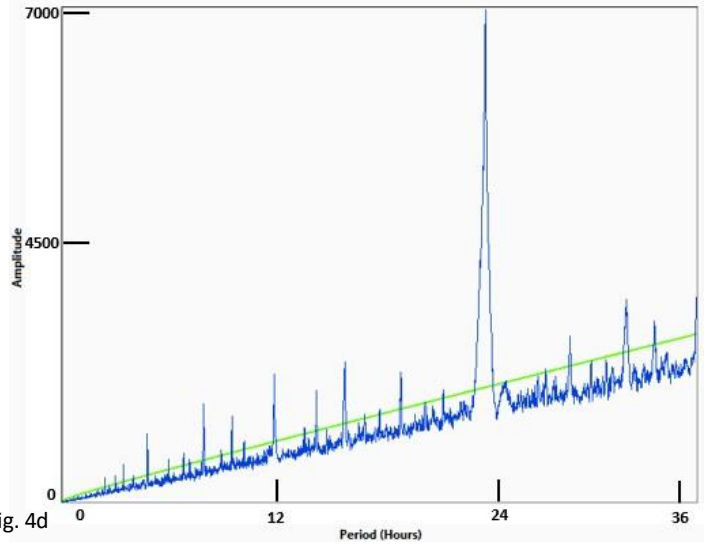


Fig. 4d

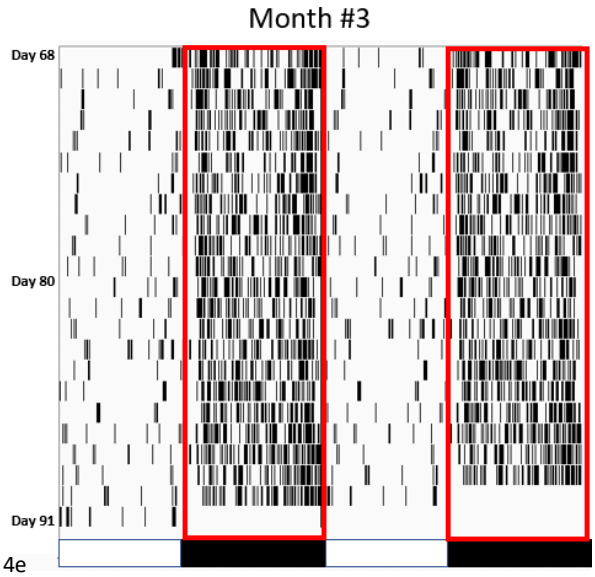


Fig. 4e

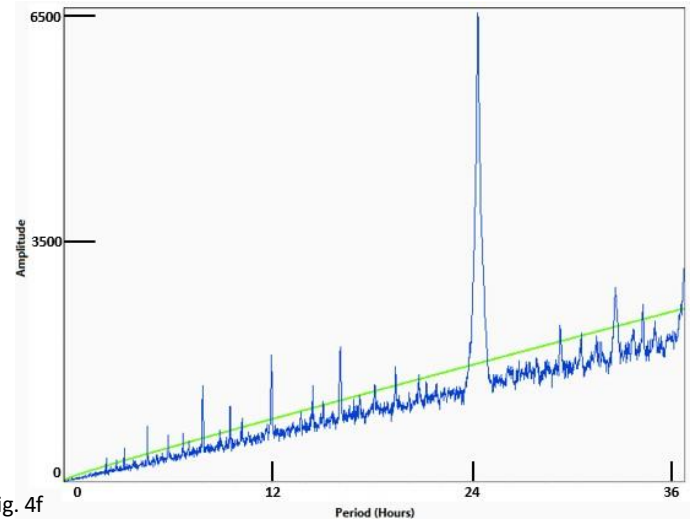


Fig. 4f

Fig. 4 Representative AAV-GFP (control) animal. A side-by-side comparison of actograms and their representative periodograms of an AAV-GFP injected animal over a period of three months. On the left (Fig. 4a, 4c, 4e) are its actograms recorded via infrared sensor with black ticks indicating activity or movement. On the right (4b, 4d, 4f) are their respective periodograms indicating the average number of hours it takes for the animal's period to cycle through and the strength of its period.

Activity was recorded using the Vital View data collection system and analyzed with Clock Labs (Actimetrics, version 6) in order to generate actograms (a visual representation of locomotor activity indicated by a dark tally for movement) and periodograms (used to depict the length and strength of an organism's underlying biological rhythm) to analyze the duration and level of activity. Represented above in Fig. 4a-4f is a representative actogram of a control mouse (n=2) demonstrating a 24-hour periodicity and heightened activity during the 12-hour night dark phase and decreased activity levels during the 12-hour day phase with a clear distinction between both day and night.

Fig. 5a

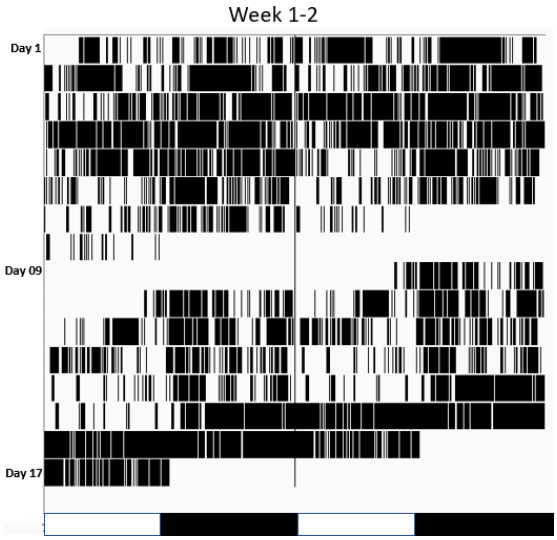


Fig. 5b

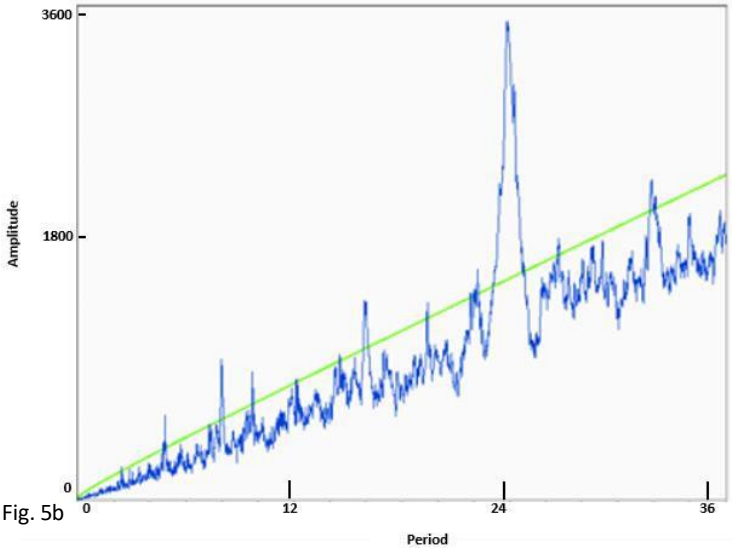


Fig. 5 Representative AAV-CRE (PVN BMAL1 KO) animal, pre-exercise. The figures above represent the PVN-BMAL1 KO animal's actogram (5a) and periodogram (5b) before starting exercise.

While control mice (GFP only) show strong rhythmicity similar to a WT mouse without virus injection, CRE-injected mice show a drastically different phenotype. The BMAL1-deficient PVN mouse displayed elevated erratic locomotor activity during its first two-week post-injection, with no distinct patterns of rest between night and day (Fig. 5a-5b). Though the periodogram suggests some rhythmicity in the CRE-injected mice, notice the scale bar is vastly different than the control, suggesting that the mouse is weakly rhythmic compared to the GFP-only injected mouse.

Fig. 6a

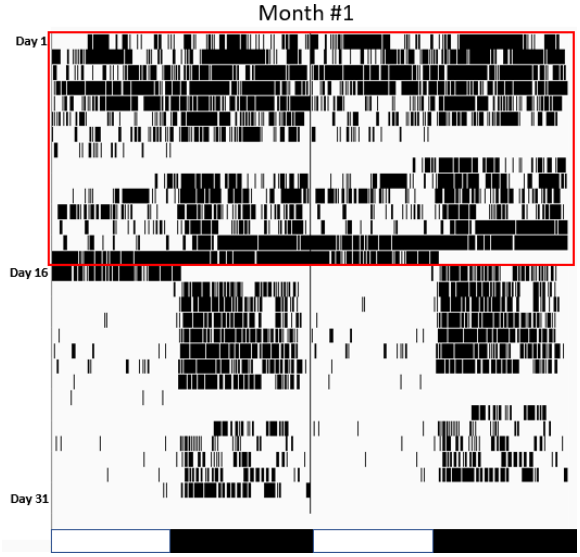


Fig. 6b

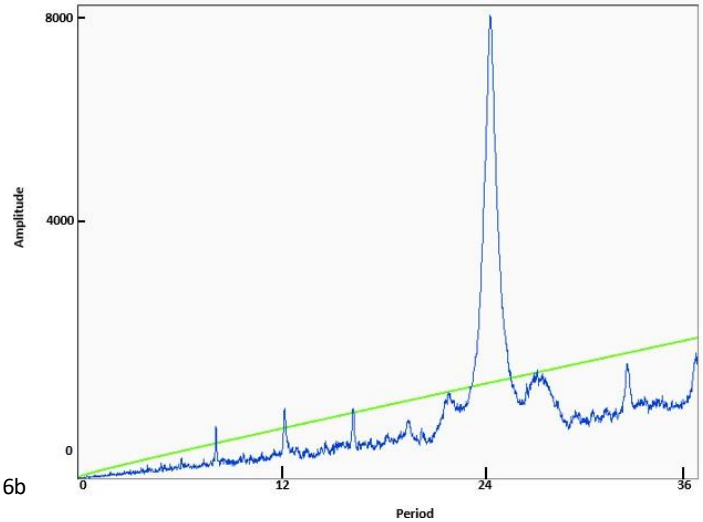


Fig. 6c

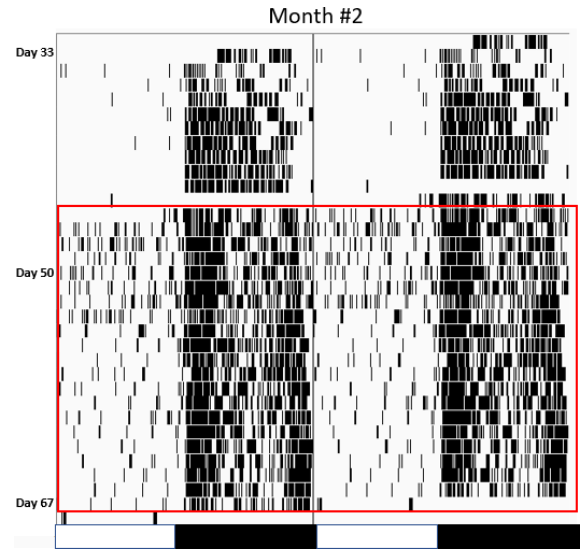


Fig. 6d

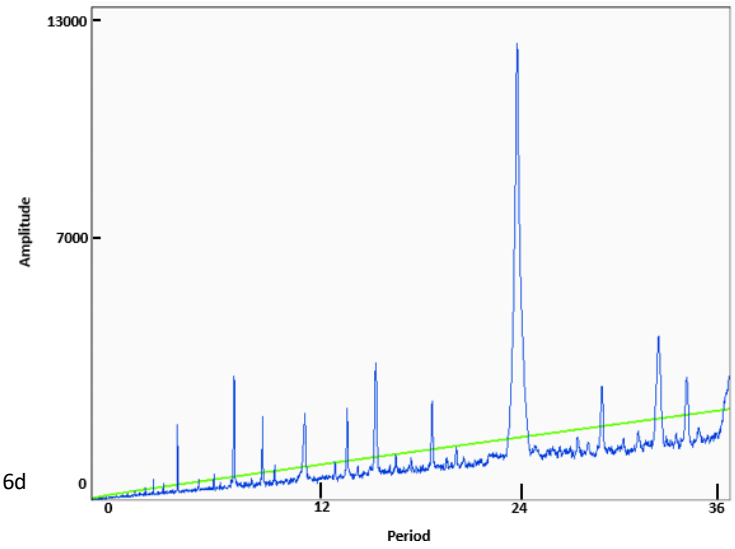


Fig. 6e

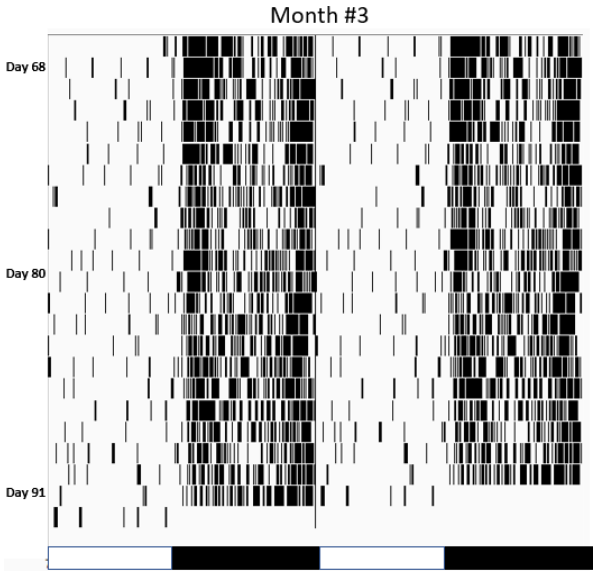


Fig. 6f

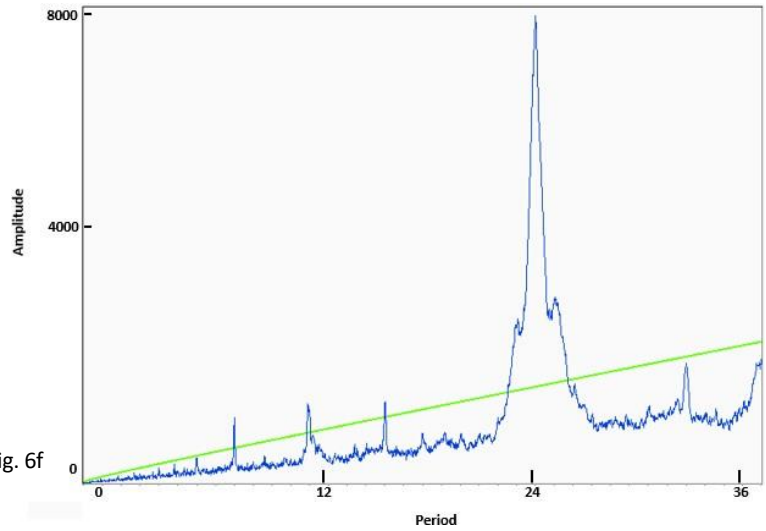


Fig. 6 Actograms and periodicity of a PVN BMAL1-deficient animal (M8) recorded over a duration of three months. Weeks 1-2 were pre-exercise in order to fully characterize the animal as having an arrhythmic behavioral phenotype. Weeks 3-6 were when the animal was given free access to the running wheel. After week 7, the running wheels were taken away to assess the permanence of their circadian rhythmicity. On the left are the actograms measured via infrared sensor (Fig. 6a, 6c, 6e) and their respective periodograms (Fig. 6b, 6d, 6f).

However, when presented with the opportunity to exercise via wheel running, the knockout mice chose to run rhythmically, and their rhythmicity was retained even after the wheel was taken away (Fig. 6a-6f). This information suggests that effects of entrainment are rapid with the animal choosing to run rhythmically on the same day that the wheel was

presented, that entrainment persists with the wheel, and that the effects of entrainment are permanent after the wheel is taken away.

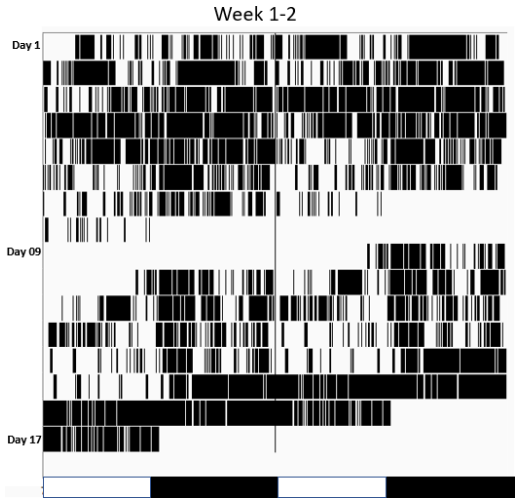


Fig. 7a

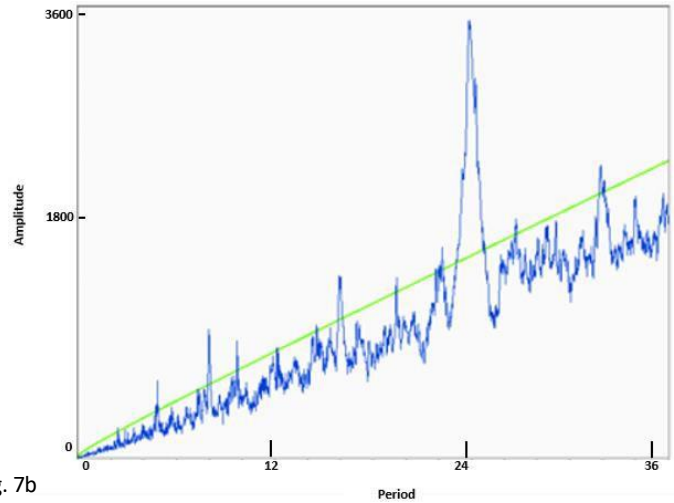


Fig. 7b

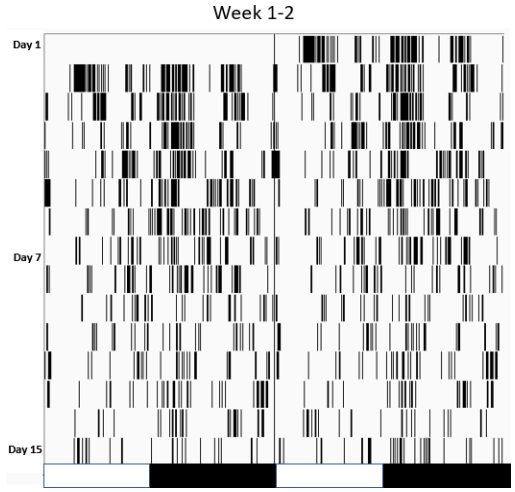


Fig. 7c

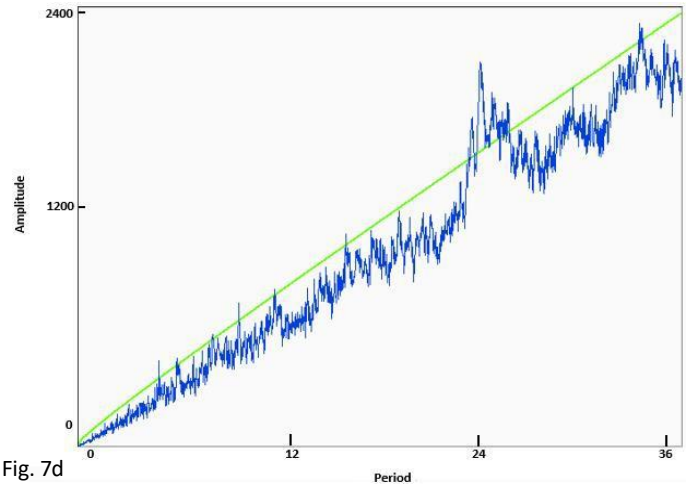


Fig. 7d

Fig. 7 Actograms and periodicity of PVN-deficient BMAL1 knockout mice. Activity (A) and period length (b) of a PVN, BMAL1-deficient mouse (M8) and activity (c) and periodicity (D) of another PVN BMAL1-deficient (M12). Both animals were initially recorded for two weeks to establish arrhythmicity.

In observing their circadian profiles, actograms were generated between the two knockout mice. The M12 actogram reveals that circadian activity is not completely arrhythmic but can be more specifically defined as weakly rhythmic with a blunted periodicity of 24 hours. However, based on the periodograms, it appears to be improved by

wheel running, an improvement which persists even when measured by infrared sensor (Fig. 8a-8c).

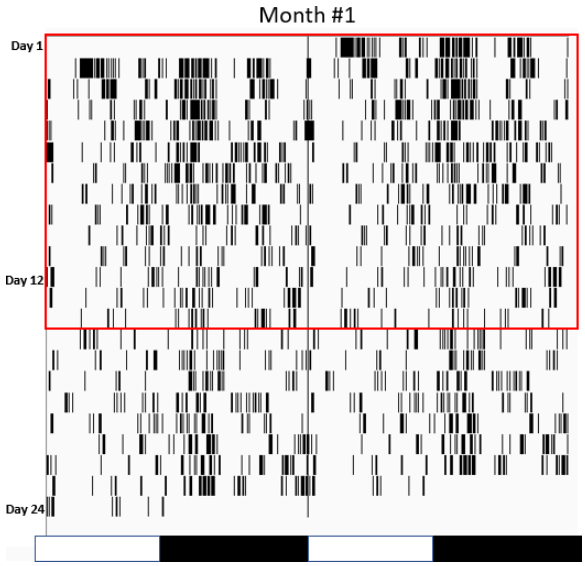


Fig. 8a

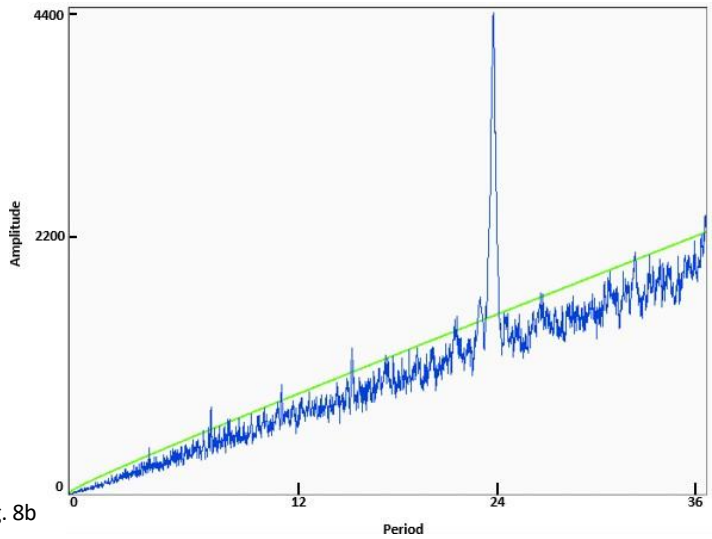


Fig. 8b

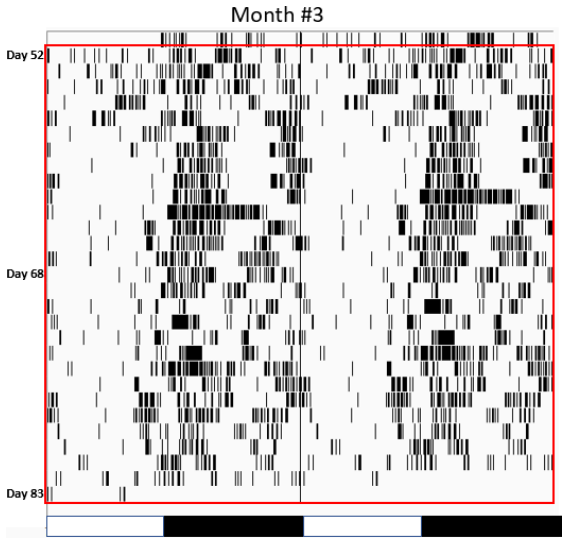


Fig. 8c

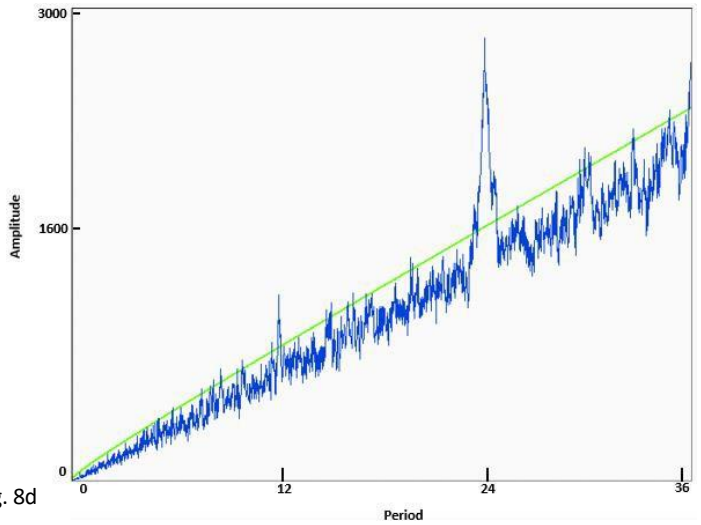


Fig. 8d

Fig. 8 Actograms and periodicity of a PVN BMAL1-deficient animal (M12) recorded over a duration of three months. Weeks 1-2 were pre-exercise in order to fully characterize the animal as having an arrhythmic behavioral phenotype. Weeks 3-6 were when the animal was given free access to the running wheel. After week 7, the running wheels were taken away to assess the permeance of their circadian rhythmicity. On the left are the actograms measured via infrared sensor (Fig. 8a, 8c, 8e) and their respective periodograms (Fig. 8b, 8d, 8f).

In contrast, the activity of M8, is completely arrhythmic, with high activity at all times with no distinct circadian patterns of rest and activity between light and dark. There is

clear rescue by the wheel, which is also maintained when the wheel is removed. Likewise, the relationship between arrhythmicity and body weight regulation is not yet clear (See Fig. 9). Though M8 was initially more arrhythmic, its body weight did not increase to the extent that M12 did. Analysis of more mice will reveal the extent to which BMAL1 loss in the PVN accelerates obesity independent of rhythmicity. Though previous reports demonstrate that loss of BMAL1 in the PVN does result in obesity, these experiments were not done with wheel runners. The enhanced energy expenditure induced by the wheel runners may alter energy balance such that the obesity phenotype is more variable from mouse to mouse depending on the energy expenditure for each mouse.

Fig. 9

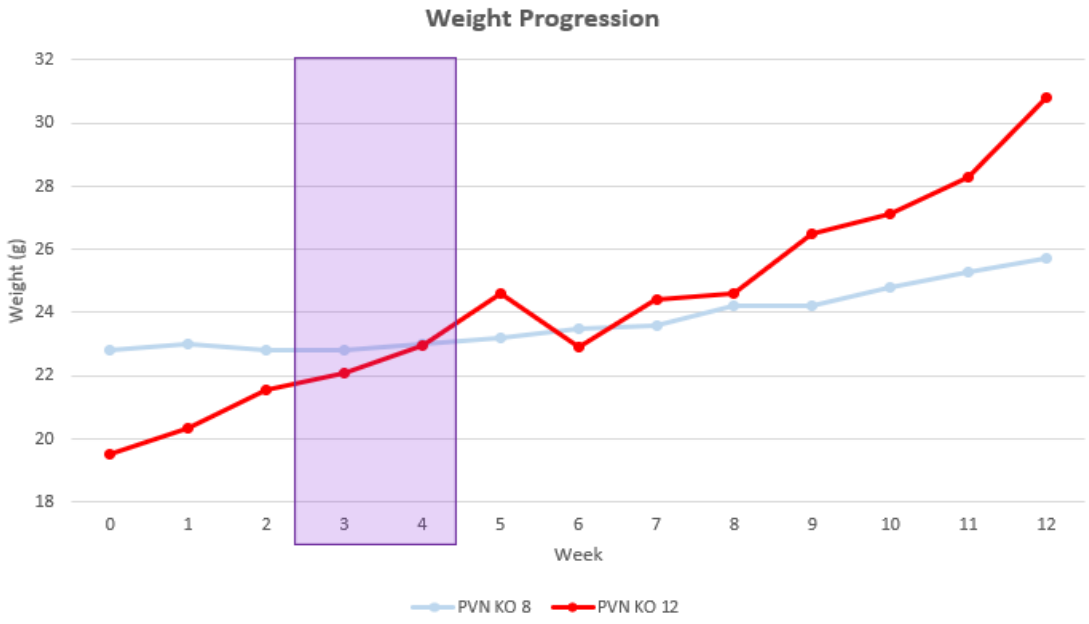


Fig. 9 Body weight progression of PVN BMAL1-deficient mice. Weight progression of two different PVN BMAL1-deficient mice at progressive weeks following BMAL1 loss.

Restoration of Circadian Locomotor Activity

Once an animal was experimentally defined as arrhythmic or weakly rhythmic, animals were voluntarily placed on exercise wheels in 12hr light/12hr dark conditions for an

additional two weeks to determine whether rhythmicity would be able to be restored or strengthened by exercise. The wheels were then removed from the cage and animals were monitored on IR for an additional month to assess the permanence of rhythmicity restoration or strengthening.

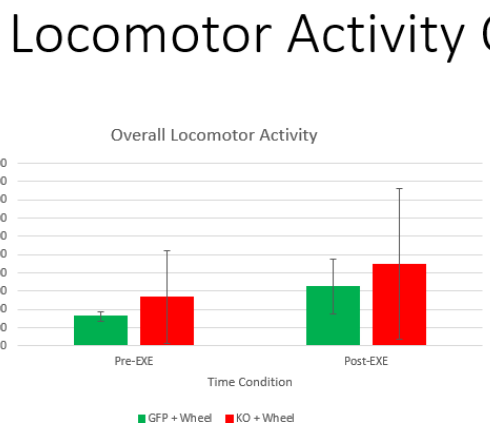


Fig. 10a

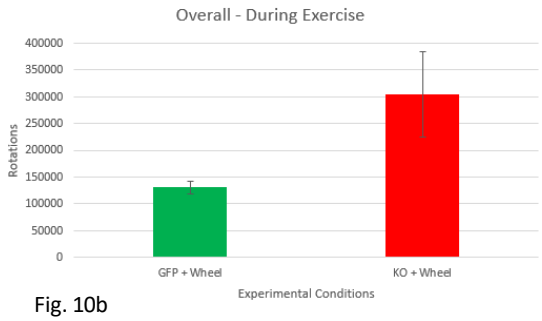


Fig. 10b

Fig. 10 Activity levels of control and PVN BMAL1-deficient mice. Overall activity of mice injected with GFP virus or GFP-CRE virus (a). Exercise-only quantified in control vs. PVN BMAL1-deficient mice (b).

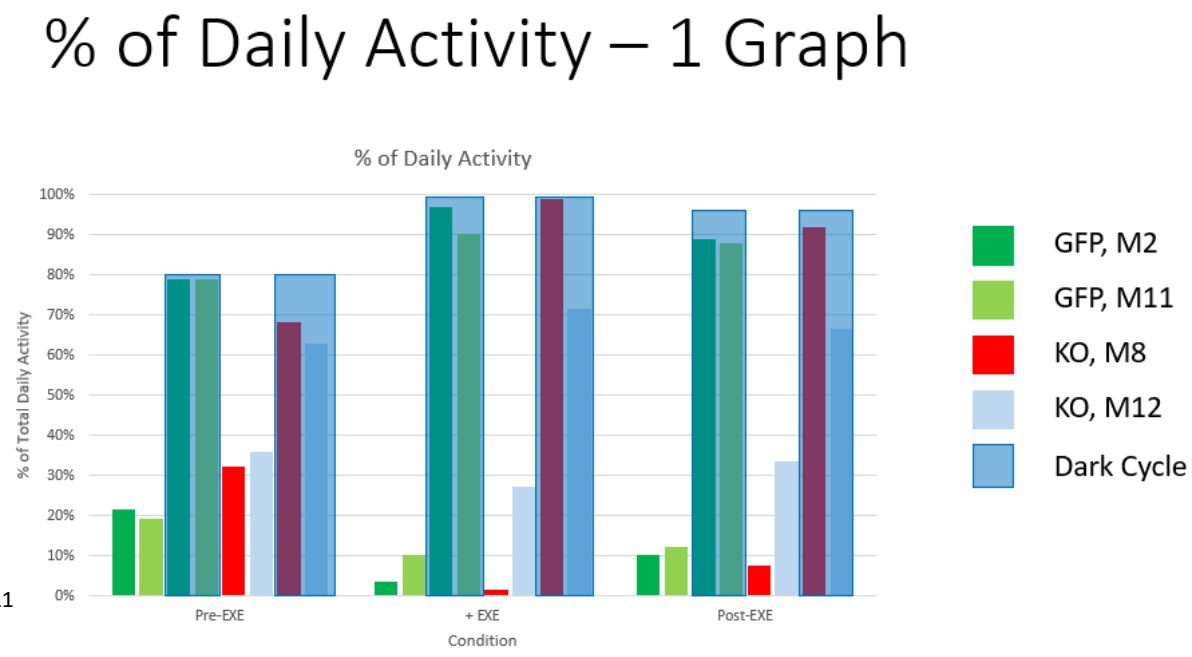


Fig. 11

Fig. 11 Percent of total daily activity for control and PVN BMAL1-deficient mice in each behavioral condition.

As shown, control animals tend to be closely matched together in all three conditions and display very little variation between each other and throughout the different conditions. However, for both knockout animals, it was only M8 that displayed the most significant amount of decreased activity during its rest period and was sustained post-exercise. However, with M12, while there was improvement in decrease of movement during their rest phase in the exercise condition as soon as the wheel was taken away its activity levels during the day and night returned to that of the pre-exercise condition. An interesting thing to note is that the amount of activity during their rest period was significantly improved during and post exercise for all conditions and heightened during their active period with voluntary wheel running which may indicate that exercise may aid in sustaining sleep. For the post-exercise condition, all animals sustained a consistent level of exercise during their active period and their rest period. These two observations suggest that circadian rhythmicity may be improved during both the active and rest periods via sleep maintenance.

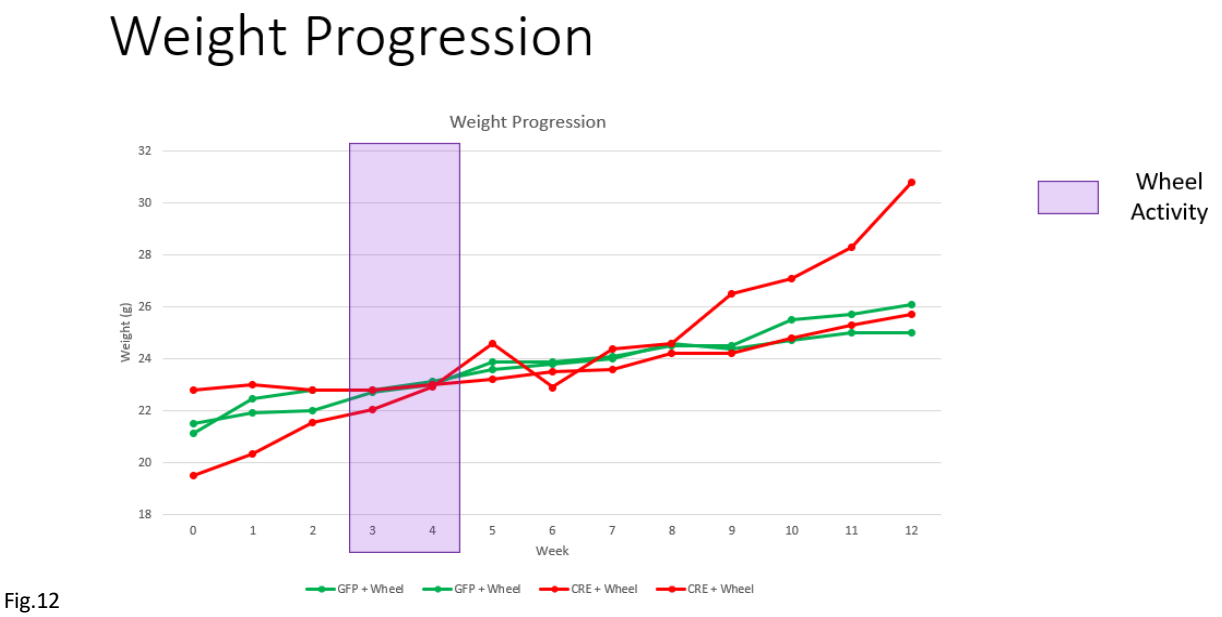


Fig.12

Fig. 12 Body weight progression of both control and PVN BMAL1-deficient mice. Weight progression of all animals over three months following injections, exercise, and post exercise conditions.

Currently we cannot explain these differences in energy expenditure, though it may reflect a difference in overall BMAL1 expression remaining in the PVN. Though target sites of injection were validated by immunohistochemistry, we cannot currently rule out off target effects that are not picked up by protein validation. Nevertheless, this experiment supports the strong role of exercise in clock re-entrainment, which remarkably, can persist even when its *zeitgeber* of exercise is removed. This is the first demonstration of exercise-induced re-entrainment in the BMAL1-deficient PVN model.

The Synergistic Effects of Nobiletin and Daunorubicin in AML Viability

Like metabolic disease, cancer has been linked to circadian disruption in humans (Fekry & Eckel-Mahan, 2022). The molecular basis for this is not entirely clear. However several of the core circadian clock genes have been demonstrated to play a role in cancer prevention (Jiang, 2017; Fu, 2002; Zhang, 2020). For example, circadian gene period 2 is heavily implicated in tumor suppression and is critical for cell cycle control and homeostasis (Fu, 2002; Wang, 2020; Zhang, 2020). Loss of PER2 increases irradiation-induced mutagenesis (Fu, 2002). When absent, thymocytes are prone to unregulated cell growth and proliferation and a decreased sensitivity to p53 apoptotic signaling (Fu, 2002).

Interestingly, our laboratory has shown that in the context of some cancers, BMAL1 expression is quite heterogeneous. In liver cancer, for example, BMAL1 gets completely lost in some tumors (repressed by a fetal isoform of hepatocyte nuclear factor four alpha), while in some tumors BMAL1 is very highly expressed and hepatocyte nuclear factor four alpha expression is lost altogether (Fekry, 2018). Treatment of BMAL1-deficient tumors with the compound nobiletin can cause cell death and apoptosis (Lellupitiyage, 2020). NOB is a compound that targets the circadian molecular clock by directly agonizing Retinoic Acid Receptor-Related Orphan Receptor Alpha (RORα), a transcription factor which activates the circadian protein BMAL1 (He, 2016) We have observed similar heterogeneity in BMAL1 expression in Acute Myeloid Leukemia (AML) cell lines and have been employing a similar strategy of NOB treatment to better understand whether pharmacological targeting of NOB can reduce proliferation and viability in AML. Preliminary data suggests that this natural flavonoid and activator of the circadian system, might be an ideal reagent to use in

combination with DNR to improve AML response. Because standard chemotherapy for AML involves cardiotoxicity (Alves, 2017), we have been using NOB alone and in combination with daunorubicin, to see if there might be additive or synergistic effects of these compounds which could potentially allow for daunorubicin to be used at a lower dosage so as to limit toxicity. Though NOB appears to induce apoptosis and cellular arrest in AML cells, it has minimal to no toxic side effects in conjunction with DNR in non-cancerous cells. Similar findings have been found in metabolic studies, where NOB administration to wild-type mice shows negligible side effects, and even combats metabolic disease in the context of diet-induced obesity (Lee, 2013). In fact, NOB can enhance healthy aging, though not extending the lifespan of laboratory mice (Kazunari, 2019).

For this project, in vitro approaches using several AML cell lines have been used to assess the synergistic strength of NOB and DNR in eliminating AML cells whilst reducing the cardiotoxic off-target effects of DNR required for AML cell death. In doing so, we will determine the minimum amount of DNR to be used in conjunction with NOB to achieve successful elimination of these cells as well as determine the effectiveness of modulating the clock for viability and proliferation of several AML cell lines.

Characterizing the Molecular Clock in Acute Myeloid Leukemia

Due to the heterogeneity of AML, several cell lines were selected to represent the different cell states of AML. Represented below (Fig. 13) are cell lines THP-1 (ATCC, Catalog #: TIB-202), Kasumi-1 (ATCC, Catalog #: CRL-2724), MOLM13 (DSMZ, Catalog #: ACC 554), and MV411 (ATCC, Catalog #: CRL-9591). Each of these lines were probed for inherent levels

of BMAL1. Treatment of 25nM of DNR, 10µM of NOB, and a combination of both reagents was then applied to low BMAL1 expressing lines MOLM13 and MV411 with varying levels of success in killing cells (Fig. 14).

Fig. 13

Fig. 13 Endogenous protein levels of representative AML cell lines THP-1, MV411, Kasumi-1, and MOLM13. Actin was used as a loading control for each cell line.

Fig. 14

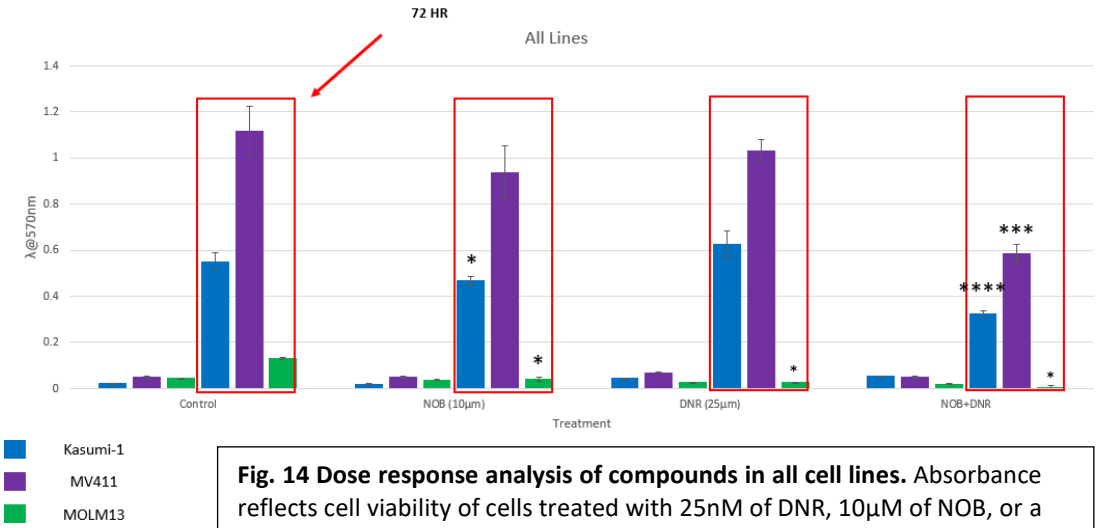


Fig. 14 Dose response analysis of compounds in all cell lines. Absorbance reflects cell viability of cells treated with 25nM of DNR, 10µM of NOB, or a combination of both reagents.

Optimizing Treatments

With the varying responses to the combined treatment of DNR and NOB, it was decided that an IC50 approach was necessary for optimization of the treatment for each cell line. I performed IC50s for both NOB and DNR, and specific doses were chosen for each cell line to ensure the minimal amount of DNR to assess the synergetic effect of NOB in killing cells. After the combined doses were chosen and evaluated via MTT assay, western blots were used to investigate BMAL1 and Cleaved Caspase 3 protein levels to assess the level of NOB influence on downstream apoptotic signaling. These blots were then quantified using ImageJ and graphed via Microsoft Excel.

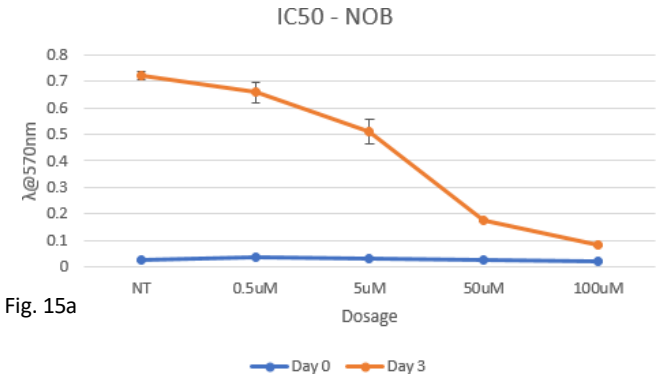


Fig. 15a

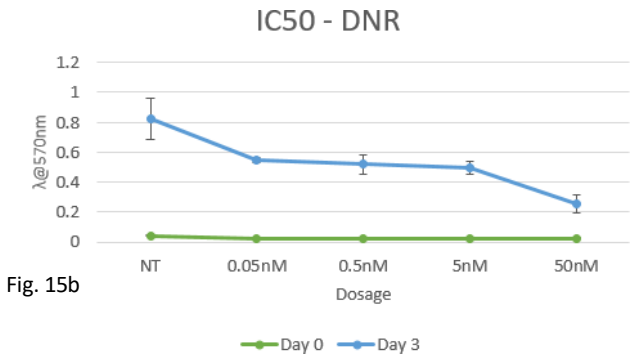


Fig. 15b

Fig 15 Dose response analysis of compounds in MOLM13 cells. Absorbance reflects cell viability of MOLM13 cells treated with varying doses of NOB (a) and DNR (b) individually.

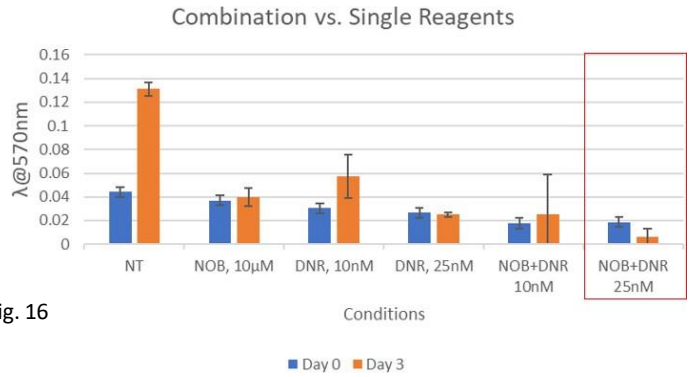


Fig. 16

Fig 16 Dose response analysis of various combinations of NOB and DNR in MOLM13. Various combinations of both NOB and DNR used in order to evaluate the combination most effective to produce a synergetic effect.

Depicted in Fig. 16 and 17 above are the IC50 results chosen for the cell line MOLM13 based on minimal amount of DNR and maximal amount of NOB necessary for killing cells effectively. Ultimately, the dose of 10µM of NOB and 25nM of DNR was deemed the most optimal for observing the synergistic effects of NOB and DNR with cell death in MOLM13 cells.

Fig. 17

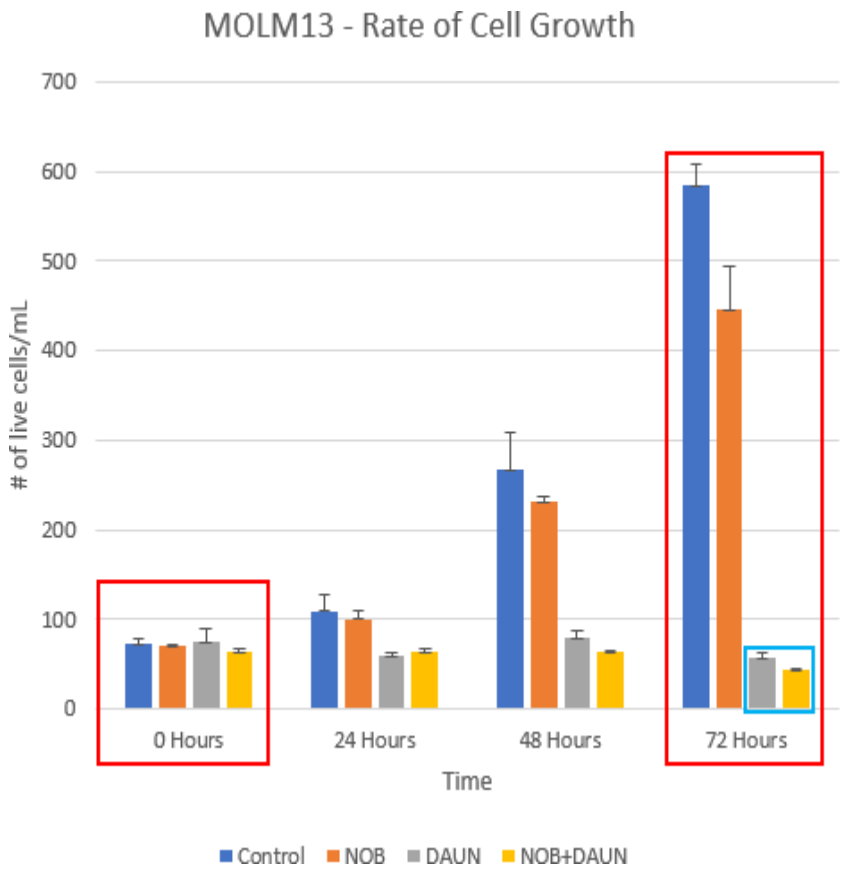


Fig. 17 Cell viability of MOLM13 cells treated with NOB, DAUN, or both in combination at various time points after treatment. Live cell viability was measured via Trypan Blue in cells treated for 24, 48, or 72 hours with vehicle or selected reagents.

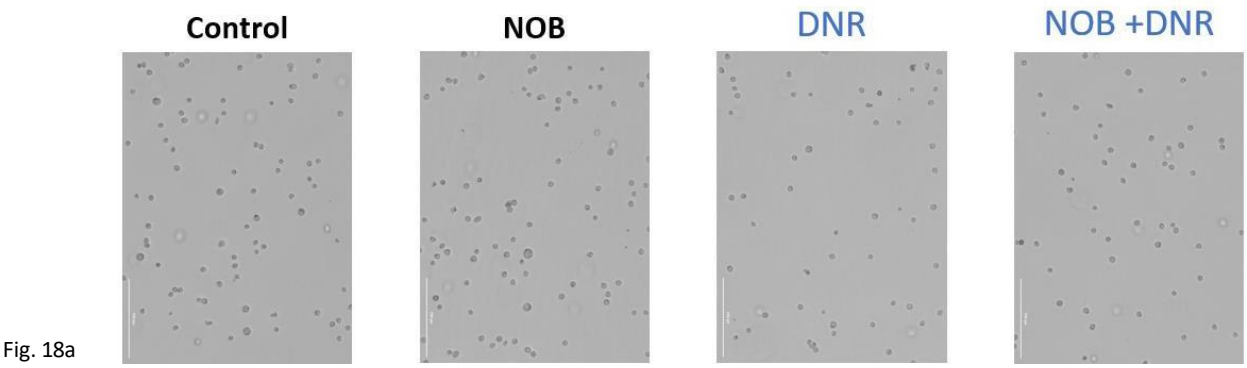


Fig. 18a

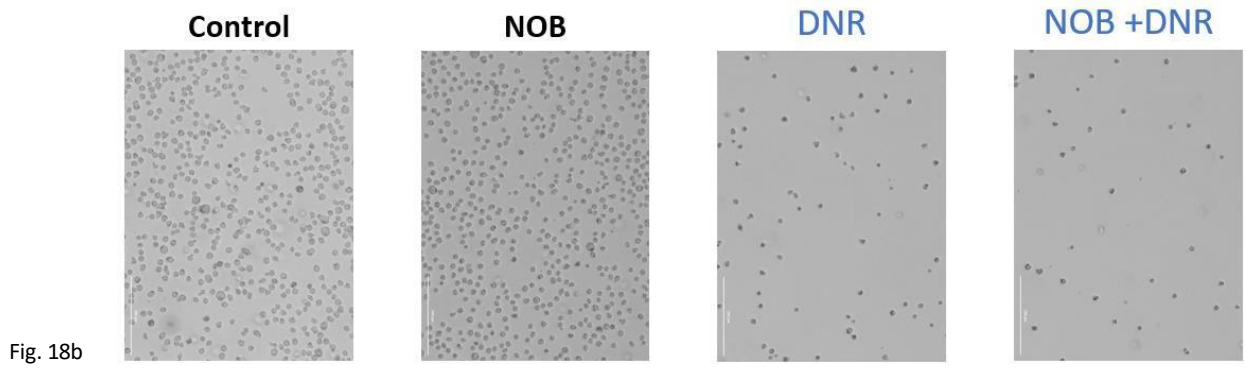


Fig. 18b

Fig. 18 Changes in morphology and proliferation of MOLM 13 cells over time and treatment. Cell morphology and density of cells plated at day 0 (a). Cell morphology and density of MOLM13 cells after three days of treatment with no treatment, NOB, DNR, or NOB+DNR (b).

MOLM13 live cells were assessed and quantified through the use of Trypan Blue (1:100) and BioTek's Cytation 5 machine on 4x over the span of 72 hours. All cells were plated at a density of $1x10^5$ cells per well with no treatment (NT), 10µM of NOB, 25nM of DNR. At zero hours, all cells are at relatively the same health and number per well. However,

the greatest impact can be seen at 72 hours with the NT displaying the highest proliferation and health rates and NOB condition having reduced proliferation. DNR-treated cells had no with additional cell death, and the combined treatment having the most cell death occurs.

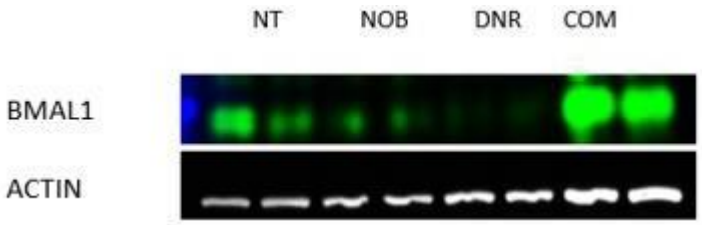


Fig. 19a

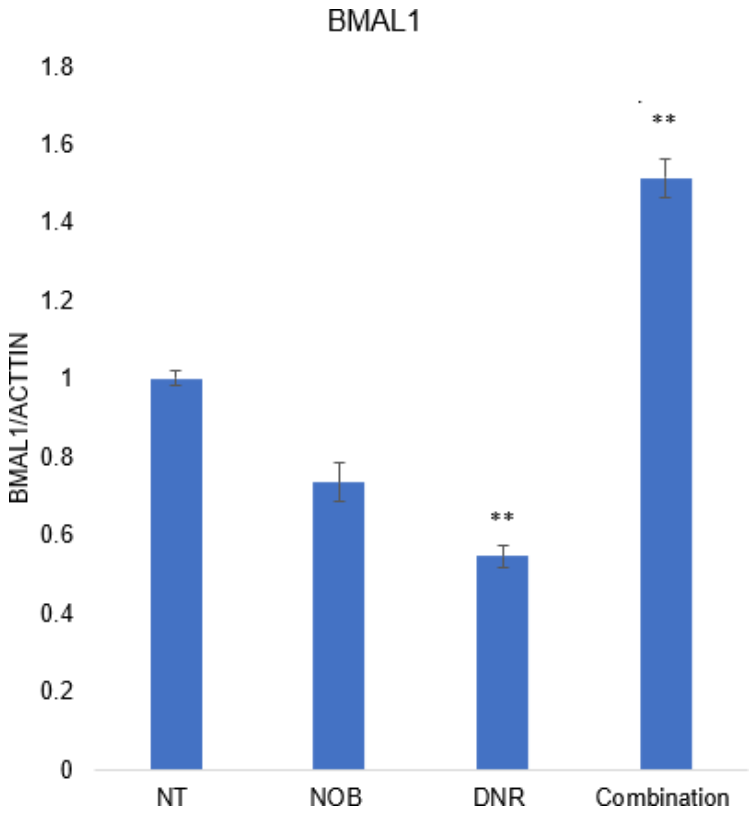


Fig. 19b

Fig. 19 BMAL1 expression in MOLM cells treated with different compounds. BMAL1 levels of MOLM13 in four conditions: NT (Non-treated), 10µM NOB (Nobiletin), 25nM DNR (Daunorubicin), and COM (Combination of NOB and DNR) with n = 4 for all conditions (19a). (19b) Overall quantification of BMAL1 in MOLM13 normalized to Actin protein for each condition.

After optimizing treatment levels for MOLM13, cells were grown at $5x10^6$ per petri dish for 72 hours, cells then treated with either no treatment (NT), 10µM of NOB, 25nM of DNR, or a combination of both NOB and DNR, harvested, and flash frozen via liquid nitrogen to prepare for protein sample analysis via Western Blot. Depicted above in Fig. 19a-19b are

the protein levels of BMAL1 that were quantified by ImageJ (n = 4, all conditions). BMAL1 was chosen as an indirect indicator of downstream ROR-alpha activity. The figure depicts the highest amount of BMAL1 with the combined treatment with the least amount of BMAL1 in the DNR condition.

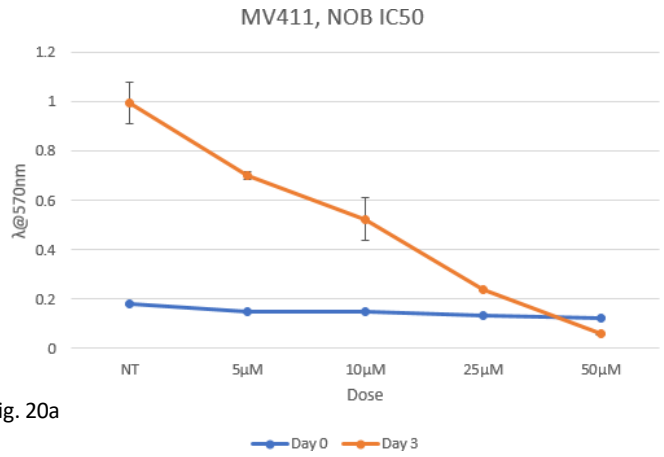


Fig. 20a

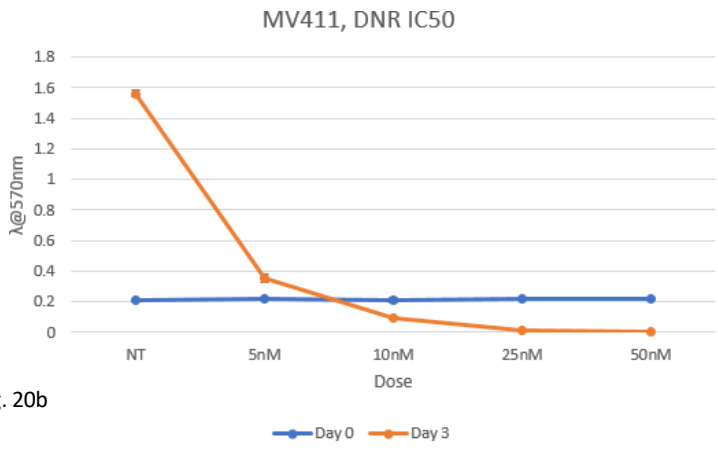


Fig. 20b

Fig. 20 Dose response analysis of compounds in MV411 cells. Absorbance reflects cell viability of MV411 cells treated with varying doses of NOB (Fig. 20a) and DNR (Fig. 20b) individually.

After optimizing treatment levels for MV411 (Fig. 20), cells were grown at $5x10^6$ per petri dish for 72 hours, cells then treated with either no treatment (NT), 25μM of NOB, 10nM of DNR, or a combination of both NOB and DNR harvested, and flash frozen by liquid nitrogen to prepare for protein sample analysis via Western Blot. Depicted below in Fig. 21a-21b are the protein levels of BMAL1 that were quantified by Image J. With this cell line, it appears that it is more sensitive and gives forth a greater BMAL1 output in both the NOB and combined conditions. The discrepancy of BMAL1 output in both cell lines could be attributed to the number of ROR-alpha receptors that are average for each cell line. Other

factors could include the number of cell passages which could negatively influence BMAL1 and or ROR-alpha expression.

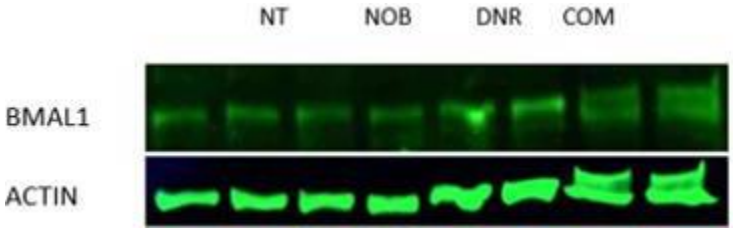


Fig. 21a

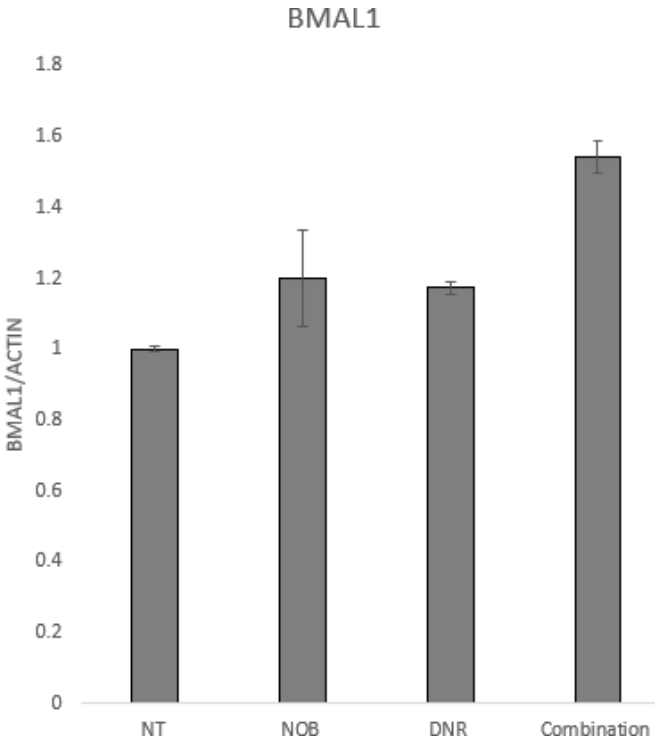


Fig. 21b

Fig. 21 BMAL1 expression in MV411 cells treated with different compounds. BMAL levels of MV411 in four conditions: NT (Non-treated), NOB (Nobiletin), DNR (Daunorubicin), and COM (Combination of NOB and DNR) with n = 4 for all conditions (Fig. 21a). (Fig. 21b) Overall quantification of BMAL1 in MV411 normalized to Actin protein for each condition.

Due to the variable amounts of BMAL1 expression per cell line, I decided to take a closer look at RORα levels of each one to see a) their basic expression levels without modification and b) to see if NOB would modify their expression levels.

Fig. 22

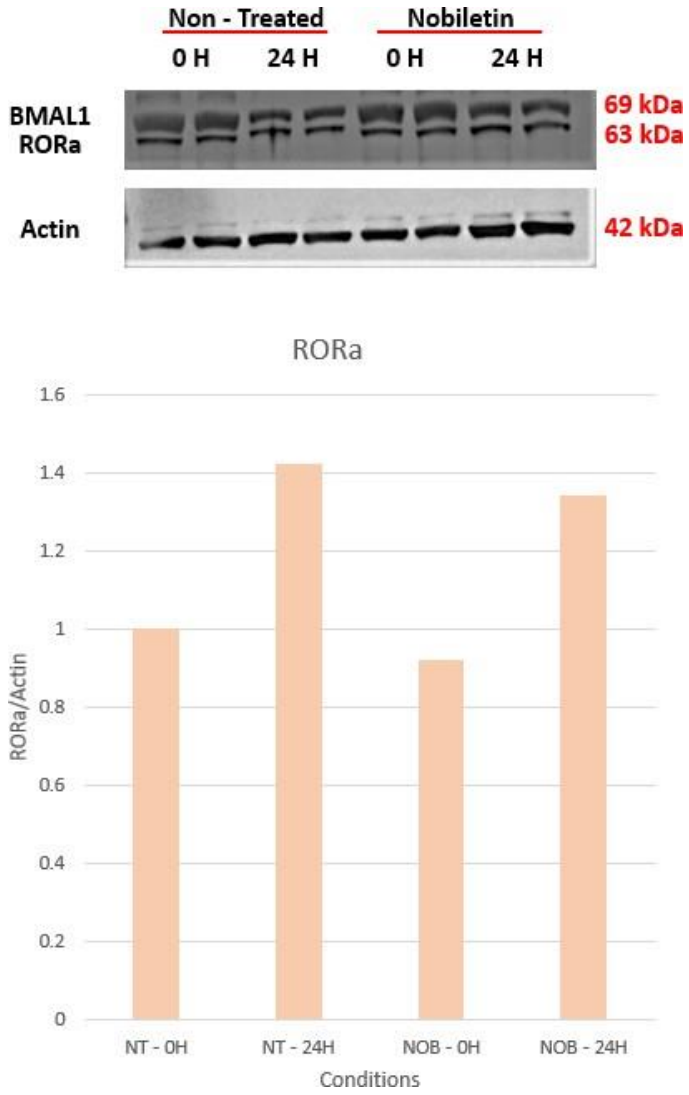


Fig. 22 BMAL1 and RORα expression in MOLM13 cells. Western blot showing BMAL1 and RORα expression (top) and quantification of expression normalized to ACTIN in conditions NT – non-treated and NOB – nobiletin at time points of 0 hours and 24 hours. RORα expression is consistent across both time and treatment.

Fig. 23

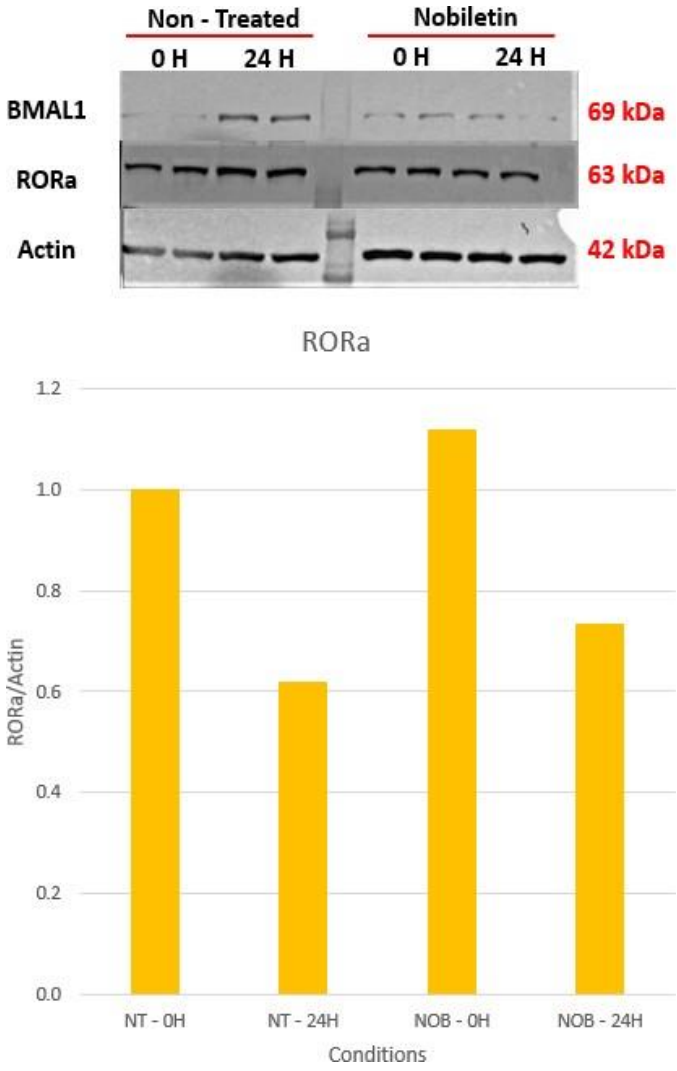


Fig. 23 BMAL1 and RORα expression in MV411 cells. Western blot showing BMAL1 and RORα expression (top) and quantification of expression normalized to ACTIN in conditions NT – non-treated and NOB – nobiletin at time points of 0 hours and 24 hours. RORα expression is consistent across both time and treatment.

To our surprise, both MOLM13 and MV411, RORα and BMAL1 expression remains unchanged regardless of time and treatment within each cell line itself (Fig. 23 and 24). However, in comparing the two cell lines, it appears that they have complementary expressions compared to one another. While the RORα expression in MOLM13 seems to

elevate after 24 hours, the RORα expression in MV411 shows the exact opposite trend by decreasing RORα expressions after 24hours. From these data, it can be concluded that RORα protein expression remains steady within each cell line regardless of treatment with NOB and time but can vary across various cell lines in RORα and BMAL1 upregulation expression and time for reasons that are potentially independent of RORα expression.

Discussion

Based on the increasing number of studies linking circadian disruption to metabolic disease, understanding mechanisms by which to target the clock to delay disease onset or progression is pressing. Collectively, my studies have demonstrated the importance and strength of specific entrainment mechanisms to bolster circadian rhythms in instances where light entrainment is not sufficient or applicable. In the case of circadian dampening or disruption in the hypothalamus, exercise might be one mechanism by which to restore normal rhythms in vivo. On the other hand, exploiting the clock by altering protein expression to treat cancer cell growth and progression may be a tractable solution to limiting toxicity and improving efficacy of specific cancer treatments.

Modern day technology has made us less reliant upon solar cycles by providing access to light at night. This unregulated accessibility to light can be something as small as flipping on a cellphone at night to having the ability to expose entire task forces to working at unconventional hours when our bodies need to be resting. The latter is commonly referred to as “nightshift work” or “rotating shift work” depending on the specific work schedule and is a common form of circadian disruption and misalignment. This growing phenomenon of circadian disruption also extends to future generations and has already been shown to negatively affect children, putting them at risk for future systemic diseases that stem from circadian dysfunction (Crowley, S. 2015). Even though we cannot alter society’s expectations and functions according to what is biologically best for human health, perhaps a more comprehensive knowledge about the beneficial effects of a stricter

circadian cycle will enable individuals to understand the value of the clock in their physiology and health.

Exercise as a Potential Therapeutic Strategy for Circadian Disruption in the Paraventricular Nucleus

In the context of PVN clock disruption, my data suggests that exercise provides a much faster and stronger circadian behavioral restoration as compared to their time-restricted feeding cohorts based on both the actograms and periodograms. Although my number of animals is limiting, both animals demonstrate a strong, exercise-induced behavioral restoration that persists when the wheels have been taken away. Importantly, these results imply separate neural circuits and functions for circadian rhythmicity restoration and metabolism. Interestingly, though both mice gained rhythmicity, one clearly lost weight and had an improved metabolism while maintaining high activity levels, while the other continued to gain weight and remained at activity levels closer to the control. Obviously, more animals are needed, but these results suggest that exercise may be a tractable solution for promoting more robust rhythmicity in the context of aging or chronic jet lab, two situations where the clock is thought to be dampened (Leise, 2013; Oneda, 2022).

In the PVN knockout mice, our data demonstrate that exercise has not only powerful restorative effects on behavioral circadian rhythmicity, but these effects can persist even when the *zeitgeber* is removed. These data suggest a major influence of exercise on circadian locomotion. These studies are also important because they underscore that other

areas besides the SCN are able to influence locomotor rhythmicity Though published recently in the context of energy intake (Kim, 2020), my studies demonstrate for the first time that loss of rhythmicity by PVN deletion of BMAL1 is recoverable, and that exercise is a stronger *zeitgeber* for the central clock in this context than light input to the SCN. Prior to 1990, the primary focus for analyzing circadian behavioral output was the SCN (Saper, 2013). However, in recent times the lens in which we perceive circadian output has been widened and studied in other contexts besides entire lesions such as cell-specific lesions that involve eliminating SCN transmission via various neurotransmitters, deletion of several core clock genes within the SCN itself, or other *zeitgebers* independent of light that are not dependent on the SCN at all. Thus, these studies in conjunction with prior results (Kim, 2020) demonstrate that other areas of the brain such as the PVN may be able to influence overall circadian locomotion independently of the SCN and give vital information as to how non-photic circadian entrainment can influence circadian behavior.

As a behavioral phenotype, we saw rapid weight gain within the first two weeks of injection, arrhythmic eating patterns, and overactive locomotor activity regardless of time of day. Initially, our lab sought to regain rhythmicity through modifying energy intake via time restricted feeding (Mahan Lab, unpublished). Although TRF did manage to restore rhythmicity somewhat, locomotor output was still somewhat fragmented over the 24-hr. cycle, resulting in a weaker period overall. In addition, TRF mice showed slow entrainment as seen below, and not until the second week of TRF did animals reveal rhythmicity that was more similar to the control condition (Fig. 24a-24b).

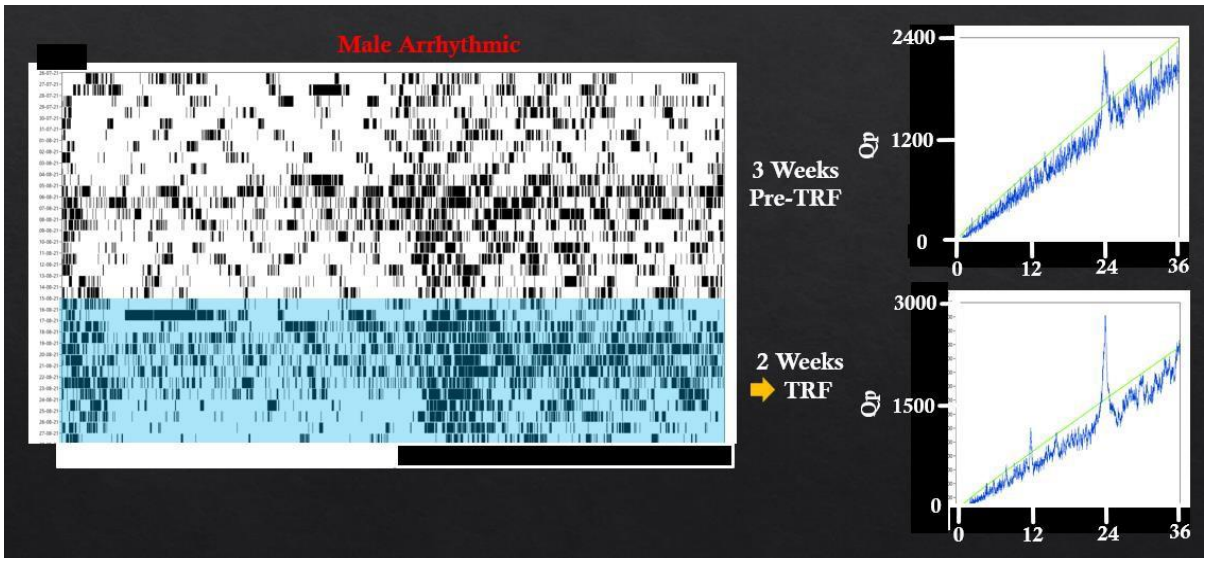


Fig. 24a

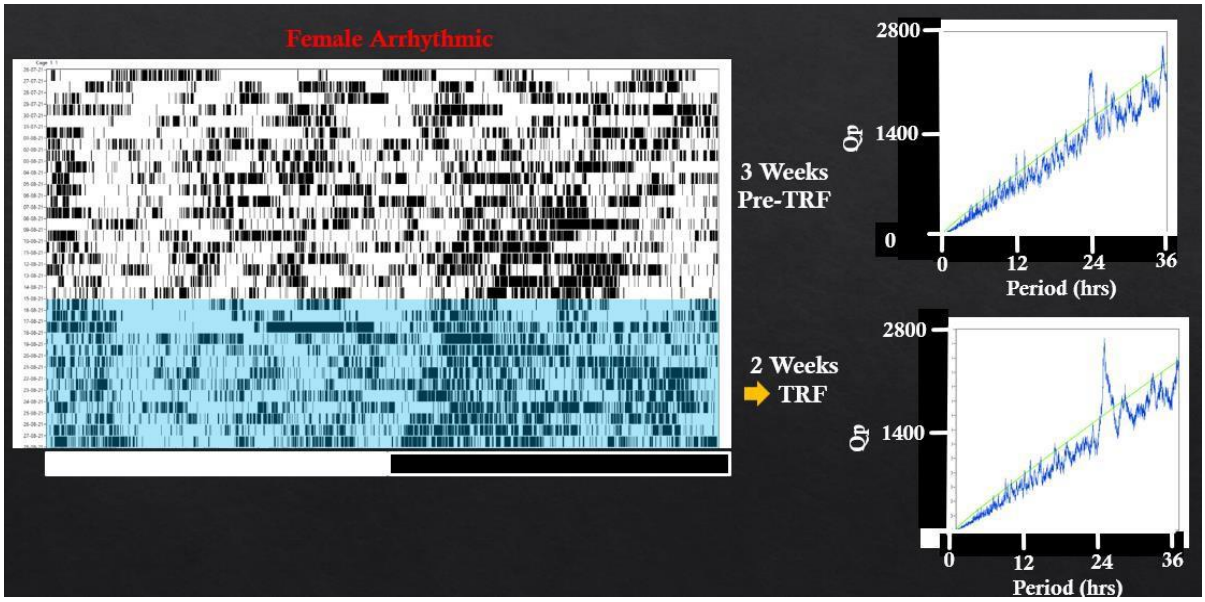


Fig. 24b

Fig. 24 Time Restricted Feeding (TRF) restoration actograms and periodograms. Above (Fig. 24a) depicts an actogram (left) and its respective periodogram of a male arrhythmic animal three weeks before starting TRF and 2 weeks during TRF. Below (Fig. 24b) is the actogram and periodogram of the corresponding female animal on the same paradigm.

With this phenotype and previous experiments in mind, I sought to modulate their arrhythmic behavior by using a counter metabolic behavior in charge of energy expenditure, which is exercise. In my experiments, the PVN BMAL1 KO animal model was behaviorally consistent with previous literature (Kim, 2020), in showing a pronounced effect on

rhythmicity and whole-body energy balance (Kim, 2020). Exercise as a means of circadian restoration may have been possible through an intact SCN VIP and VPAC2 signaling through this loss of BMAL1 since our SCN was intact, but how it connects or if it has the ability to re-entrain the PVN still remains a mystery and that connection may be able to be investigated through observing the neuronal connections and signaling studies between the SCN and PVN as demonstrated in Dr. Herzog's lab (Jones, 2021). Although their primary purpose was to look at how the loss of BMAL1 in CRH neurons affects CORT signaling between these two regions in the hypothalamus, the data suggests important signaling occurs between the two regions. According to their findings, there was no neuronal or synaptic strengthening between these regions when BMAL1 was eliminated, but what was found was an increase in VPAC2 receptors in the PVN as well as the SCN. These data imply that there were more receptors of VPAC2 to volumetrically capture VIP signals coming from the SCN, and thus affecting circadian entrainment. This creates a novel link since literature on exercise and circadian behavioral output was measured primarily in regards to the SCN and its connections to peripheral tissue, bypassing the PVN altogether. By interrupting this "in-between" step, the PVN has its own critical influence on circadian behavior. However, the precise mechanism and molecular pathway and signaling pathways that mediate this effect is elusive. There are several ways in which we can investigate the following further.

From a molecular standpoint, it would be insightful to look at a variety of different molecular inputs and outputs associated with exercise and circadian rhythmicity such as CORT and VIP which are critical in modulating arousal and can be seen as a possible output of circadian rhythmicity from the PVN. By comparing them to base levels with and without

exercise as well as the SCN baselines of VIP and VPAC2 between the two brain regions before and after exercise one could study how these neuronal populations are affected by exercise by their molecular expression. Another route of consideration would be to look at this from the opposite end of the spectrum to see if skeletal muscle could be giving biochemical feedback to the brain in order to influence or correct arrhythmic behavioral patterns. Peripheral clocks are known to feed back to the brain to control circadian behavior. To investigate this particular train of thought, skeletal muscle, the PVN, and the SCN could be analyzed for changes in metabolite expression after exercise. To address whether muscle may be producing myokines that might affect brain function, serum might be analyzed for changes in metabolite content. Finally, retrograde labeling of synapses might reveal potentially new or changed synaptic connections between the PVN and the SCN.

Lastly, looking at the circadian metabolomics, behavior, and molecular changes within both the SCN and PVN in both exercise during the early active period and late active period would be of potential therapeutic benefit. According to a clinical study done by the Pendergast Lab (Thomas, 2020), exercise in the early morning was able to advance the phase of people with circadian rhythms of a late chronotype. In addition to that study, the Sassone-Corsi lab (Sato, 2022) provides strong insight into the metabolic changes and strength of circadian connections to different systems according to the time that exercise was performed giving potential to new, non-invasive therapeutic strategies that can target certain biological systems that are affected by ill synchronized body tissues. The timing and duration of exercise may be a way to assist individuals either trying to alter their chronotype, or deal with circadian rhythm disorders.

Not to be ignored is how feeding and timed exercise can alter circadian metabolic behaviors because of their hedonic value. For example, methamphetamine has been shown to re-entrain arrhythmic mice through a poorly defined oscillator known as the methamphetamine-sensitive circadian oscillator (Tataroglue et al, 2006). This would be a good area to look at in order to answer how powerful food or exercise would be as a mechanism of entrainment potentially why modifying energy intake or expenditure may be so effective. It may also provide insight as to why the animal may be choosing to run rhythmically during its active period when given free access to the wheel. Other experiments to see if exercise is rewarding and therefore, a stronger, faster, and more permanent way of entrainment is comparing not only timed energy intake and voluntary wheel running, but also to look at forced treadmill vs. voluntary wheel running to see if the reward pathway is altered along with entrainment or if they would be separate. By doing this, we can see the extent of how rewarding it is and if exercise, reward, and entrainment are truly tied together. We could do so by looking at the networks in the SCN, PVN, and the paraventricular thalamus (PVT) which is involved in reward. Other areas of interest that could be strengthened to the ties of circadian rhythmicity, metabolism, and reward could be the subparaventricular zone (SPZ) and dorsal medial hypothalamus (DMH) which are directly linked to locomotor output via SCN VIP neurons and are also heavily involved in both energy regulation and circadian rhythms.

And finally, from a systemic point of view careful consideration should be given to sex differences in core clock gene manipulations and exercise due to the fact that both of these factors can have profoundly different effects between the two sexes. For instance, in

the case of BMAL1 depletion within the striatum, the effects of alcoholic preference and drinking behavior differ between the two sexes of mice (de Zavalia, 2021). When knocked out in the striatum of these animals, males exhibited a behavioral phenotype of repressed drinking behavior whilst the females had the exact opposite effect and were more prone to drinking. In the case of exercise, Dr. Asher's lab (Adamovich, 2021) found that circadian rhythms and expressions differed not only according to time dependence, but also between the sexes according to their paradigm. They found that the exercise capacity between the two sexes differed in terms of metabolic output and physiological endurance. With female mice, they were unable to sustain glucose levels for as long as male mice when exercising during their early active period vs. the late active period as well as having a higher respiratory exchange rate and caloric intake. Both of these studies highlight the profound differences between sexes under the same experimental conditions and thus, the absolute need to account for these differences in future studies.

The Synergetic Effects of Nobiletin and Daunorubicin to Induce Apoptosis in Acute Myeloid Leukemia

One important finding of this project is the fact that NOB can help induce apoptosis in low-expressing BMAL1 cancer lines. This finding could have clinical importance because chemotherapy is usually non-discriminant in killing cells and oftentimes causes toxic off-target effects to otherwise healthy cells. However, previous studies have supported the fact that NOB has minimal effects on healthy cells (Huang, 2016; Mahan Lab, unpublished observations). Because NOB acts as an agonist at RORα it may have a unique effect only in

low BMAL1-expressing cancer cells whilst minimally affecting healthy cells with optimal levels of BMAL1 alone. NOB is a natural flavonoid (originating from organic compounds such as citrus peels and is, in general, considered non-toxic to healthy cells). By adding NOB to traditional chemo treatments, cancer cells appear to still be affected, but may be affected at a dose of chemotherapy that minimizes toxic side effects.

As shown in the results above, NOB alone can slow the progression of proliferation, but does not necessarily lead to apoptosis and leaves most of the cells morphologically intact. To further define the effects NOB has on downstream signaling, the use of BRDU and flow analysis would be beneficial in determining what stage of the cell cycle the cells are in before and after treatment of NOB to see if NOB arrests the cell cycle in a particular stage of the cell cycle. In addition, genomic approaches might be used to better understand which ROR-BMAL1-regulated pathways get up- or down-regulated within AML cells.

Treating AML by targeting the clock in vivo could be potentially challenging for several reasons. According to other sources of cancer biology and circadian pharmacological manipulation (Lellupitiyage, 2020) each tissue has a different rhythmicity, but not only that, each cell type within a system may have a different oscillation as well. As of now, there is not much literature on the effects of NOB on AML specifically, but NOB has been known to aid in cancer treatments since the 1990 (Bracke, 1990) and has shown promise in the current treatment of different cancers such as breast cancer as shown in Dr. Seung-Hee Yoo's lab (Kim, 2022). Her lab exploits the findings from Dr. Chen's lab (Chen, 2018) which uses NOB to induce apoptosis to target the molecular clock by activating RORs in order to

induce apoptosis in triple-negative breast cancer. While NOB did display anti-tumorigenic effects in regards to cancer cell growth and migration, it was found that it was not due to the modulation of the circadian clock at all but rather through activating ROR binding to the ROR response elements of the IkBa promoter to inhibit proliferation. It did not restore circadian rhythmicity of these naturally arrhythmic cells with or without NOB treatment, suggesting that NOB may be affecting AML growth partially independent of BMAL1. Studies using serum shock (which synchronizes cells in a dish) may be helpful in determining the rhythmicity of AML cells, and to what extent it is altered in the context of AML treatment. This distinction has been addressed in some other cancers. For example, in Lellupitiyage et al. (Lellupitiyage, 2020) different clock profiles in various cancers and their varying effects in conjunction with NOB were observed. Using a bone osteosarcoma line (U2OS) and two breast cancer lines, MCF7 (known for its weak rhythmicity) and MDA-MB-231 (known for its arrhythmicity) authors found that all three lines reacted differently to NOB and its modulatory effects, suggesting that how a cell responds to NOB treatment may be based upon its unique circadian molecular profile.

In preliminary studies (Mahan Lab, unpublished) our lab initially tried this treatment of NOB combined with chemotherapeutic agent doxorubicin (DOX) with established cell line MOLM13, which was chosen for its deadly fms-like tyrosine kinase receptor 3 (FLT-3) mutation and is generally associated with poor patient survival. It was shown through proliferation and western blot analysis that this cell line did best with both reagents and showed a marked decrease in cell proliferation and an increase in p53 and cleaved caspase three expression, indicating a drastic increase of cell apoptosis in the combined treatment

conditions. However, since AML is heterogeneous in nature, testing other cell lines related to AML progression and disease became imperative to see if a) their circadian molecular signature was the same as that of MOLM13 and b) if the same doses of both NOB and DOX would be as effective in guiding cells towards death (Fig.25).

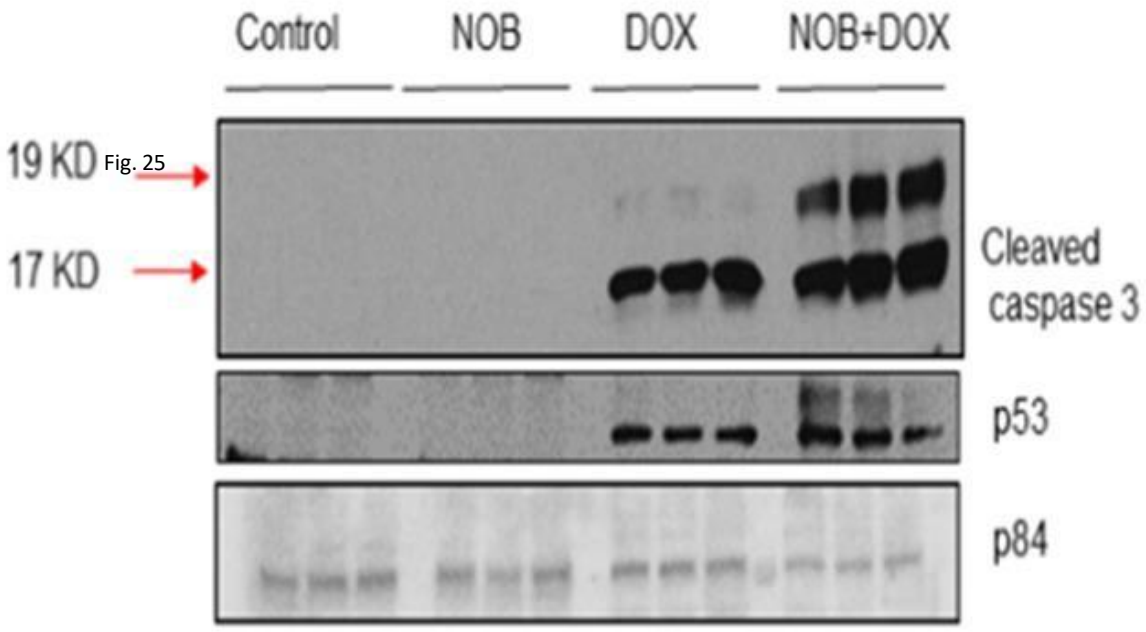


Fig. 25 Cleaved Caspase 3 and p53 expression in MOLM13 cells treated with different compounds. These cells were subject to four conditions: NT (Non-treated), NOB (Nobiletin), DOX (Doxorubicin), and NOB + DOX.

Thus, our lab had chosen the cell lines of MOLM13, MV411, Kasumi-1, and THP-1 to test this treatment and see if the apoptotic and proliferation effects would be the same. To our surprise, there were some cell lines that were more sensitive to treatment than others and one that had no response whatsoever. To investigate this perplexing matter further, a western analysis of each cell line was performed and probed for RORα and BMAL1 as direct and indirect outputs of RORα expression. What we found was varying molecular profiles for

each cell line and that for this particular treatment to work, we had to optimize levels of NOB and DNR to see the synergetic effects of cellular apoptosis within each line.

Although three out of four cell lines were responsive towards treatment and had low BMAL1 expression, the line of THP-1 (which is distinct in its endothelial origin compared to the other AML lines) did not respond to NOB, potentially due to the fact that it displayed an already high level of BMAL1 expression. We have previously observed that liver cancer lines with high BMAL1 expression were also immune to the inhibitor effects of NOB on cell growth (unpublished data). Though protein levels of RORα does not appear to change across the cell lines, more analysis of ROR activity (i.e. nuclear localization, etc.) will be necessary to fully understand why NOB has its specific effects. Other avenues of exploration regarding ROR activity, the molecular clock, and apoptosis would be to look at alternative ROR expressions such as RORγ levels in conjunction with BMAL1 expressions and apoptotic signaling as well as RORα knockdowns and overexpression to see how they affect BMAL1 output levels with and without NOB. Experiments investigating RORα, its influence on the molecular clock, and links to apoptosis signaling are certainly a highly valuable area for future studies.

Finally, I noticed that when passaging cell lines over a prolonged period of time BMAL1 levels had changed with cell appearing to lose BMAL1 expression. This is an additional variable to consider when testing the cell-specific effect of NOB. Nevertheless, our data is promising, in that some AML lines, NOB in conjunction with DNR appears to provide a beneficial effect over a single treatment.

Concluding Remarks

In summary, my results support the idea that targeting the clock could be a tractable solution for treating disease. In the context of metabolic disease, paradigms such as restricted feeding and rhythmic energy expenditure may be means by which to increase the amplitude of the clock in vivo and potentially to re-align clocks across the body. More studies are required to determine the mechanisms by which exercise re-entrains the clock in vivo, however targeted deletion of the clock in a tissue-specific manner would help determine other organs potentially involved in re-entrainment.

In the context of cancer, targeting the clock may be an added mechanism by which to achieve growth arrest. This is important for several reasons, one of which is reducing the toxicity of existing drugs when used in combination. More work is needed to fully understand the BMAL1-dependent vs. independent effects of NOB, but our preliminary data suggests that this may be a promising solution for future therapy.

Methods and Materials

All materials referenced will be found in Table 3.

Exercise as a Potential Therapeutic Strategy for Circadian Disruption in the Paraventricular Nucleus

Animal Model

100-150 nl of AAV-GFP or AAV-CRE-GFP viral vectors were bilaterally injected with a 0.5ul syringe at a rate of 25-40nl per minute into the paraventricular nucleus (coordinates: anteroposterior (AP): −0.5 to −0.6 mm; mediolateral (ML): ±0.2 mm; dorsoventral (DV): −4.8 mm) of 12 (6 experimental AAV-CRE-GFP, 6 control AAV-GFP) male mice at 8 weeks old and then placed under a two-week observation via infrared sensors to establish an arrhythmic/rhythmic locomotor phenotype. Once established, animals were then exposed to free-running conditions for 2 weeks via running wheel. After two weeks, the running wheel was removed and animals were placed back onto IR for an additional month to assess permeance of locomotor circadian rhythmicity recovery.

Data Collection

Activity bouts and wheel rotations were recorded using hardware and software (Vital View) acquired from Starr Life Sciences and converted into a csv file. Data was then analyzed using Actimetrics' ClockLabs (version 6) Software and Microsoft Excel.

Tissue Preparation and Preservation

Mice were injected with a concentration of 10µl/g of ketamine/xylazine cocktail (10 Ketamine:1 Xylazine) to slow the heart rate of each animal in preparation for the flushing and cardiac profusion of each animal. They were then flushed with 30ml of 0.9% saline and then fixed with 4% PFA. Brains were then extracted from the cranium and then placed in a solution of 4% PFA for 24 hours. After 24 hours, brains were then placed in a solution of 10% sucrose at 4°C for preservation. Coronal sections were then sliced at 30 microns per slice using a freezing microtome and preserved in 0.1% Sodium Azide in PBS at 4°C. Tissue was then mounted, dried, and cover slipped with 100µl of flouromount and sealed with clear nail polish.

Imaging

Images of the PVN were then captured and stitched via Cytation5 at 4x magnification.

The Synergetic Effects of Nobiletin and Daunorubicin in AML Viability

Cell Culture

Kasumi-1, MV411, and THP-1 cell lines were acquired through ATCC. MOLM 13 cells were gifted from the Chandra lab. All cell lines were cultured at 37°C with 5% CO2 in an incubator. Cell lines Kasumi-1, MV411, THP-1, and MOLM13 were chosen to be representative cell lines of AML. These lines were cultured as follows:

Cell Line	Characteristics	Media
MOLM 13	FLT-3 mutation, monocytes	1640 RPMI (Hyclone), 5% Pen/Strep (Gibco), 10% FBS (Gibco)
Kasumi-1	TP53 mutation, myeloblasts	1640 RPMI (ATCC), 5% Pen/Strep (Gibco), 20% FBS (Gibco)
MV411	FLT-3 mutation, macrophages/lymphoblasts	IMDM (ATCC), 5% Pen/Strep (Gibco), 10% FBS (Gibco)
THP-1	Monocytes	RPMI 1640 (Hyclone) 10% FBS BME (0.05mM)

Table 1

Cell Treatment

Cell treatment was optimized using an IC50 approach via single doses of each reagent using the following doses:

Cell Line	NOB Doses (μM)	DNR Doses (nM)
MOLM 13	5, 10, 25, 50	5, 10, 25, 50
Kasumi-1	5, 10, 25, 50	5, 10, 25, 50
MV411	5, 10, 25, 50	5, 10, 25, 50
THP-1	5, 10, 25, 50	5, 10, 25, 50

Table 2

Combination doses of NOB and DNR were chosen for each cell line to assess the synergetic effects of both reagents together.

Cell Metabolic Assessment

An MTT Assay was used to assess metabolic health of cells at a time point of 0 hours, 24 hours, and 72 hours. Raw data was acquired through Cytation5 machine and background subtracted (with background being the absorbance wavelength for vehicle-only treated cells).

Protein Expression Analysis

Harvesting and Quantifying Protein

All cell lines were grown at $5x10^6$ per condition and harvested 72 hours later and washed with 1x PBS. Supernatant was aspirated and the pellets were flash frozen in liquid nitrogen and stored at -80°C for later protein extraction. Protein was extracted by pipetting 100-300µl of RIPA buffer with protein inhibitors and sonicating each sample for 30 secs. Samples were then spun down at 10,000g for 10 minutes at 4°C. Supernatant was then collected and frozen down at -80°C with 10µl-25µl taken for protein quantification via BCA assay and read using the Cytation5 machine. Samples were then prepared with additional RIPA buffer with protein inhibitors and 5x Laemmeli buffer to a concentration of 4µg of protein/µl and a Laemmeli concentration of 1x.

Running and Transferring Western Blot

Thirty µg of protein from each condition and each cell line were boiled at 100°C for 10 minutes and then pipetted into each well of either 8% or 12% acrylamide gel and run at 70mV for 1.5-2 hours. The gel was then transferred over with 0.45um nitrocellulose and run at 4°C for 1.5 hours at 100mV. After transferring, blot was immediately removed and stained with Ponceau S to ensure correct protein transfer and then blocked with 5% non-fat milk and 1x TBST for 1 hour at room temperature. Blot was then washed with 1x TBST for five minutes in three rounds.

Protein Detection

After washing, the blot was placed and sealed within a bag containing the primary antibody of choice (BMAL1, RORα, Cleaved Caspase 3, Actin) at a dilution of 1:1,000 with 1x TBST and then incubated overnight at 4°C. The blot was then removed and washed an additional three times with 1x TBST for 5 minutes each and then blocked with the corresponding secondary antibody (Anti-Mouse, Anti-Rabbit) at a dilution of 1:10,000 in 5% blocking buffer for 1 hour at room temperature and then washed using the same procedure mentioned above. One mL of developer was then used to develop the western and finally detected with a Chemidoc Imaging System.

Protein Quantification

Protein was quantified using Image J software and then analyzed using Microsoft Excel with proteins indicating BMAL1, RORα, and Cleaved Caspase 3 normalized to Actin to achieve the final amount of protein per well.

BMAL1 PVN KO Project	*AML Project*
Animal Model	**Cell Lines**
BMAL1 fl/fl animals (Jackson Laboratories, Strain# 007668)	Kasumi-1 (ATCC, Catalog #: CRL-2724)
AAV-CRE-GFP Virus (Core Facility, University of Pennsylvania)	MOLM13 (ATCC, Catalog #: ACC-554)
AAV-GFP Virus (Core Facility, University of Pennsylvania)	MV411 (ATCC, Catalog #: CRL-9591)
Stereotaxic Injections via 0.5 µL syringe (Neuros Model 7000.5 KH, point style 3)	THP-1 (ATCC, Catalog #: TIB-202)
Motorized Stereotaxic Injector (Quintessential Stereotaxic Injector; Stoelting, Wood Dale, IL, USA)	**Cell Culture**
Behavioral Recording and Analysis	UV Water Incubator, 37C, 5% CO2 (Panasonic)
Home Cage Infrared Sensors (Starr Life Sciences)	RPMI 1640 (ATCC, Catalog #: 30-2001)
Home Cage Running Wheel Rod Sensors (Starr Life Sciences)	IMDM (ATCC, Catalog #: 30-2005)
Home Cage Running Wheels (Starr Life Sciences)	RPMI 1640 (Hyclone, Catalog #: SH30027.FS)
Vital View Software (Starr Life Sciences)	Fetal Bovine Serum (Gibco, Catalog #: 10082147)
Clock Labs (Actimetrics, version 6)	**Assessment of Cellular Metabolic Health**
Imaging	CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) (Promega, Catalog # G4000)
Cytation5 Machine (Biotek)	Trypan Blue (Sigma, Catalog #: T8154)
Tissue Preparation and Preservation	**Treatments**
4% Paraformaldehyde (Fisher, Catalog #: J19943K2)	Nobiletin (Cayman Chemicals, Catalog #: 15421)
Freezing Microtome (Leica SM 2010R)	Daunorubicin (MD Anderson Cancer Center Pharmacy)
	Doxorubicin (MD Anderson Cancer Center Pharmacy)
	Western Blot Detection
	Clarity Western ECL Substrate (Bio-Rad, Catalog #: 1705061)
	Chemidoc Imaging System (Bio-rad)
	Antibodies
	BMAL1 (Abcam, Catalog #: ab93806)
	RORα (Abcam, Catalog #: ab70061)
	Actin (Abcam, Catalog #: ab8227)
	Cleaved Caspase 3 (Abcam, Catalog #: ab2302)

Table 3

References

Background

Arnold, W., Ruf, T., Loe, L. E., Irvine, R. J., Ropstad, E., Veiberg, V., & Albon, S. D. (2018). Circadian rhythmicity persists through the Polar night and midnight sun in Svalbard reindeer. Scientific reports, 8(1), 14466. https://doi.org/10.1038/s41598-018-327784

Bass, J., & Takahashi, J. S. (2010). Circadian integration of metabolism and energetics. Science (New York, N.Y.), 330(6009), 1349–1354. https://doi.org/10.1126/science.1195027

Cavallari, N., Frigato, E., Vallone, D., Fröhlich, N., Lopez-Olmeda, J. F., Foà, A., Berti, R., Sánchez-Vázquez, F. J., Bertolucci, C., & Foulkes, N. S. (2011). A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception. PLoS biology, 9(9), e1001142. https://doi.org/10.1371/journal.pbio.1001142

Crowley, S. J., Cain, S. W., Burns, A. C., Acebo, C., & Carskadon, M. A. (2015). Increased Sensitivity of the Circadian System to Light in Early/Mid-Puberty. The Journal of clinical endocrinology and metabolism, 100(11), 4067–4073. https://doi.org/10.1210/jc.2015-2775

Dyar, K. A., Lutter, D., Artati, A., Ceglia, N. J., Liu, Y., Armenta, D., Jastroch, M., Schneider, S., de Mateo, S., Cervantes, M., Abbondante, S., Tognini, P., Orozco-Solis, R., Kinouchi, K., Wang, C., Swerdloff, R., Nadeef, S., Masri, S., Magistretti, P., Orlando, V., … Sassone-Corsi, P. (2018). Atlas of Circadian Metabolism Reveals System-wide Coordination and Communication between Clocks. Cell, 174(6), 1571–1585.e11. https://doi.org/10.1016/j.cell.2018.08.042

Fekry, B., Ribas-Latre, A., Baumgartner, C., Mohamed, A., Kolonin, M. G., Sladek, F. M., Younes, M., & Eckel-Mahan, K. L. (2019). HNF4α-Deficient Fatty Liver Provides a Permissive Environment for Sex-Independent Hepatocellular Carcinoma. Cancer research, 79(22), 5860–5873. https://doi.org/10.1158/0008-5472.CAN-19-1277

Gangwisch J. E. (2009). Epidemiological evidence for the links between sleep, circadian rhythms and metabolism. Obesity reviews: an official journal of the International Association for the Study of Obesity, 10 Suppl 2(0 2), 37–45. https://doi.org/10.1111/j.1467789X.2009.00663.x

James, S. M., Honn, K. A., Gaddameedhi, S., & Van Dongen, H. (2017). Shift Work: Disrupted Circadian Rhythms and Sleep-Implications for Health and Well-Being. Current sleep medicine reports, 3(2), 104–112. https://doi.org/10.1007/s40675-017-0071-6

Masri, S., & Sassone-Corsi, P. (2018). The emerging link between cancer, metabolism, and circadian rhythms. Nature medicine, 24(12), 1795–1803. https://doi.org/10.1038/s41591-018-0271-8

Paranjpe, D.A. and Sharma, V.K., 2005. Evolution of temporal order in living organisms. Journal of Circadian Rhythms, 3, p.Art. 7. DOI: http://doi.org/10.1186/1740-3391-3-7

Patke, A., Young, M.W. & Axelrod, S. Molecular mechanisms and physiological importance of circadian rhythms. Nat Rev Mol Cell Biol 21, 67–84 (2020). https://doi.org/10.1038/s41580-019-0179-2

Quera Salva, M. A., Hartley, S., Léger, D., & Dauvilliers, Y. A. (2017). Non-24-Hour Sleep Wake Rhythm Disorder in the Totally Blind: Diagnosis and Management. Frontiers in neurology, 8, 686. https://doi.org/10.3389/fneur.2017.00686

Saper C. B. (2013). The central circadian timing system. Current opinion in neurobiology, 23(5), 747–751. https://doi.org/10.1016/j.conb.2013.04.004

Scott J. (2011). Clinical parameters of circadian rhythms in affective disorders. European neuropsychopharmacology: the journal of the European College of Neuropsychopharmacology, 21 Suppl 4, S671–S675. https://doi.org/10.1016/j.euroneuro.2011.07.006

Suh, S., Choi, E. H., & Atanaskova Mesinkovska, N. (2020). The expression of opsins in the human skin and its implications for photobiomodulation: A Systematic Review. Photodermatology, photoimmunology & photomedicine, 36(5), 329–338. https://doi.org/10.1111/phpp.12578

United States Bureau of Labor Statistics. [Accessed 29 July 2022]; Job Flexibilities and Work Schedules Summary. 2019. https://www.bls.gov/news.release/flex2.nr0.htm

Vosko, A. M., Colwell, C. S., & Avidan, A. Y. (2010). Jet lag syndrome: circadian organization, pathophysiology, and management strategies. Nature and science of sleep, 2, 187 198. https://doi.org/10.2147/NSS.S6683

Wahl, S., Engelhardt, M., Schaupp, P., Lappe, C., & Ivanov, I. V. (2019). The inner clock-Blue light sets the human rhythm. Journal of biophotonics, 12(12), e201900102. https://doi.org/10.1002/jbio.201900102

Adamovich, Y., Dandavate, V., Ezagouri, S., Manella, G., Zwighaft, Z., Sobel, J., Kuperman, Y., Golik, M., Auerbach, A., Itkin, M., Malitsky, S., & Asher, G. (2021). Clock proteins and training modify exercise capacity in a daytime-dependent manner. Proceedings of the National Academy of Sciences of the United States of America, 118(35), e2101115118. https://doi.org/10.1073/pnas.2101115118

Ferguson, A. V., Latchford, K. J., & Samson, W. K. (2008). The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction. Expert opinion on therapeutic targets, 12(6), 717–727. https://doi.org/10.1517/14728222.12.6.717

Hughes, A., Samuels, R. E., Baño-Otálora, B., Belle, M., Wegner, S., Guilding, C., Northeast, R. C., Loudon, A., Gigg, J., & Piggins, H. D. (2021). Timed daily exercise remodels circadian rhythms in mice. Communications biology, 4(1), 761. https://doi.org/10.1038/s42003-021-02239-2

Izumo, M., Pejchal, M., Schook, A. C., Lange, R. P., Walisser, J. A., Sato, T. R., Wang, X., Bradfield, C. A., & Takahashi, J. S. (2014). Differential effects of light and feeding on circadian organization of peripheral clocks in a forebrain Bmal1 mutant. eLife, 3, e04617. https://doi.org/10.7554/eLife.04617

Jones, J. R., Chaturvedi, S., Granados-Fuentes, D., & Herzog, E. D. (2021). Circadian neurons in the paraventricular nucleus entrain and sustain daily rhythms in glucocorticoids. Nature communications, 12(1), 5763. https://doi.org/10.1038/s41467-021-25959-9

Kim, E. R., Xu, Y., Cassidy, R. M., Lu, Y., Yang, Y., Tian, J., Li, D. P., Van Drunen, R., Ribas-Latre, A., Cai, Z. L., Xue, M., Arenkiel, B. R., Eckel-Mahan, K., Xu, Y., & Tong, Q. (2020). Paraventricular hypothalamus mediates diurnal rhythm of metabolism. Nature communications, 11(1), 3794. https://doi.org/10.1038/s41467-020-17578-7

Kim, S. E., Ko, I. G., Ji, E. S., Jin, J. J., Hwang, L., Kim, S. H., Cho, S., Kim, C. J., Kim, K., & Kim, K. H. (2019). Treadmill Exercise Alleviates Circadian Rhythm Disruption-Induced Memory Deficits by Activation of Glucocorticoid Receptor and Brain-Derived Neurotrophic Factor-Dependent Pathway. International neurourology journal, 23(Suppl 1), S40–S49. https://doi.org/10.5213/inj.1938048.024

Leise, T. L., Harrington, M. E., Molyneux, P. C., Song, I., Queenan, H., Zimmerman, E., Lall, G. S., & Biello, S. M. (2013). Voluntary exercise can strengthen the circadian system in aged mice. Age (Dordrecht, Netherlands), 35(6), 2137–2152. https://doi.org/10.1007/s11357-012-9502-y

Oneda S., Cao S., Haraguchi A., Sasaki H. and Shibata S. (2022) Wheel-Running Facilitates Phase Advances in Locomotor and Peripheral Circadian Rhythm in Social Jet Lag Model Mice. Front. Physiol. 13:821199. doi: 10.3389/fphys.2022.821199

Pastore, S., & Hood, D. A. (2013). Endurance training ameliorates the metabolic and performance characteristics of circadian Clock mutant mice. Journal of applied physiology (Bethesda, Md.: 1985), 114(8), 1076–1084. https://doi.org/10.1152/japplphysiol.01505.2012

Sato, S., Dyar, K. A., Treebak, J. T., Jepsen, S. L., Ehrlich, A. M., Ashcroft, S. P., Trost, K., Kunzke, T., Prade, V. M., Small, L., Basse, A. L., Schönke, M., Chen, S., Samad, M.,

Baldi, P., Barrès, R., Walch, A., Moritz, T., Holst, J. J., Lutter, D., … Sassone-Corsi, P. (2022). Atlas of exercise metabolism reveals time-dependent signatures of metabolic homeostasis. Cell metabolism, 34(2), 329–345.e8. https://doi.org/10.1016/j.cmet.2021.12.016

Schroeder, A. M., Truong, D., Loh, D. H., Jordan, M. C., Roos, K. P., & Colwell, C. S. (2012). Voluntary scheduled exercise alters diurnal rhythms of behaviour, physiology and gene expression in wild-type and vasoactive intestinal peptide-deficient mice. The Journal of physiology, 590(23), 6213–6226. https://doi.org/10.1113/jphysiol.2012.233676

Tataroglu, O., Davidson, A. J., Benvenuto, L. J., & Menaker, M. (2006). The methamphetamine-sensitive circadian oscillator (MASCO) in mice. Journal of biological rhythms, 21(3), 185–194. https://doi.org/10.1177/0748730406287529

Thomas, J. M., Kern, P. A., Bush, H. M., McQuerry, K. J., Black, W. S., Clasey, J. L., & Pendergast, J. S. (2020). Circadian rhythm phase shifts caused by timed exercise vary with chronotype. JCI insight, 5(3), e134270. https://doi.org/10.1172/jci.insight.134270

de Zavalia, N., Schoettner, K., Goldsmith, J. A., Solis, P., Ferraro, S., Parent, G., & Amir, S. (2021). Bmal1 in the striatum influences alcohol intake in a sexually dimorphic manner. Communications biology, 4(1), 1227. https://doi.org/10.1038/s42003-02102715-9

Alves, A. C., Nunes, C., Lima, J., & Reis, S. (2017). Daunorubicin and doxorubicin molecular interplay with 2D membrane models. Colloids and surfaces. B, Biointerfaces, 160, 610–618. https://doi.org/10.1016/j.colsurfb.2017.09.058

Bracke, M., Vyncke, B., Opdenakker, G., Foidart, J. M., De Pestel, G., & Mareel, M. (1991). Effect of catechins and citrus flavonoids on invasion in vitro. Clinical & experimental metastasis, 9(1), 13–25. https://doi.org/10.1007/BF01831706

Eckel-Mahan, K. L., Patel, V. R., de Mateo, S., Orozco-Solis, R., Ceglia, N. J., Sahar, S., Dilag Penilla, S. A., Dyar, K. A., Baldi, P., & Sassone-Corsi, P. (2013). Reprogramming of the circadian clock by nutritional challenge. Cell, 155(7), 1464–1478. https://doi.org/10.1016/j.cell.2013.11.034

Fekry, B., & Eckel-Mahan, K. (2022). The circadian clock and cancer: links between circadian disruption and disease Pathology. Journal of biochemistry, 171(5), 477–486. https://doi.org/10.1093/jb/mvac017

Fu L, Pelicano H, Liu J, Huang P, Lee C. The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo [published correction appears in Cell 2002 Dec 27;111(7):1055]. Cell. 2002;111(1):41-50. doi:10.1016/s0092 8674(02)00961-3

He, B., Nohara, K., Park, N., Park, Y. S., Guillory, B., Zhao, Z., Garcia, J. M., Koike, N., Lee, C. C., Takahashi, J. S., Yoo, S. H., & Chen, Z. (2016). The Small Molecule Nobiletin Targets the Molecular Oscillator to Enhance Circadian Rhythms and Protect against

Metabolic Syndrome. Cell metabolism, 23(4), 610–621. https://doi.org/10.1016/j.cmet.2016.03.007

Huang, H., Li, L., Shi, W., Liu, H., Yang, J., Yuan, X., & Wu, L. (2016). The Multifunctional Effects of Nobiletin and Its Metabolites In Vivo and In Vitro. Evidence-based complementary and alternative medicine: eCAM, 2016, 2918796. https://doi.org/10.1155/2016/2918796

Jiang, W., Zhao, S., Jiang, X., Zhang, E., Hu, G., Hu, B., Zheng, P., Xiao, J., Lu, Z., Lu, Y., Ni, J., Chen, C., Wang, X., Yang, L., & Wan, R. (2016). The circadian clock gene Bmal1 acts as a potential anti-oncogene in pancreatic cancer by activating the p53 tumor suppressor pathway. Cancer letters, 371(2), 314–325. https://doi.org/10.1016/j.canlet.2015.12.002

Kim, E., Kim, Y. J., Ji, Z., Kang, J. M., Wirianto, M., Paudel, K. R., Smith, J. A., Ono, K., Kim, J. A., Eckel-Mahan, K., Zhou, X., Lee, H. K., Yoo, J. Y., Yoo, S. H., & Chen, Z. (2022). ROR activation by Nobiletin enhances antitumor efficacy via suppression of IκB/NF-κB signaling in triple-negative breast cancer. Cell death & disease, 13(4), 374. https://doi.org/10.1038/s41419-022-04826-5

Lee, Y. S., Cha, B. Y., Choi, S. S., Choi, B. K., Yonezawa, T., Teruya, T., Nagai, K., & Woo, J. T. (2013). Nobiletin improves obesity and insulin resistance in high-fat diet-induced obese mice. The Journal of nutritional biochemistry, 24(1), 156–162. https://doi.org/10.1016/j.jnutbio.2012.03.014

Lellupitiyage Don, S. S., Robertson, K. L., Lin, H. H., Labriola, C., Harrington, M. E., Taylor, S. R., & Farkas, M. E. (2020). Nobiletin affects circadian rhythms and oncogenic

characteristics in a cell-dependent manner. PloS one, 15(7), e0236315. https://doi.org/10.1371/journal.pone.0236315

Li H. X. (2019). The role of circadian clock genes in tumors. OncoTargets and therapy, 12, 3645–3660. https://doi.org/10.2147/OTT.S203144

Nohara, K., Mallampalli, V., Nemkov, T., Wirianto, M., Yang, J., Ye, Y., Sun, Y., Han, L., Esser, K. A., Mileykovskaya, E., D'Alessandro, A., Green, C. B., Takahashi, J. S., Dowhan, W., Yoo, S. H., & Chen, Z. (2019). Nobiletin fortifies mitochondrial respiration in skeletal muscle to promote healthy aging against metabolic challenge. Nature communications, 10(1), 3923. https://doi.org/10.1038/s41467-019-11926-y

Numata, M., Hirano, A., Yamamoto, Y., Yasuda, M., Miura, N., Sayama, K., Shibata, M. A., Asai, T., Oku, N., Miyoshi, N., & Shimoi, K. (2021). Metastasis of Breast Cancer Promoted by Circadian Rhythm Disruption due to Light/Dark Shift and its Prevention by Dietary Quercetin in Mice. Journal of circadian rhythms, 19, 2. https://doi.org/10.5334/jcr.203

PDQ® Adult Treatment Editorial Board. PDQ Adult Acute Myeloid Leukemia Treatment. Bethesda, MD: National Cancer Institute. Updated <03/06/2020>. Available at: https://www.cancer.gov/types/leukemia/patient/adult-aml-treatment-pdq. Accessed <10/15/2020>. [PMID: 26389377]

Puram, R. V., Kowalczyk, M. S., de Boer, C. G., Schneider, R. K., Miller, P. G., McConkey, M., Tothova, Z., Tejero, H., Heckl, D., Järås, M., Chen, M. C., Li, H., Tamayo, A., Cowley, G. S., Rozenblatt-Rosen, O., Al-Shahrour, F., Regev, A., & Ebert, B. L. (2016). Core

Circadian Clock Genes Regulate Leukemia Stem Cells in AML. Cell, 165(2), 303–316. https://doi.org/10.1016/j.cell.2016.03.015

Sak, K., & Everaus, H. (2017). Established Human Cell Lines as Models to Study Anti-leukemic Effects of Flavonoids. Current genomics, 18(1), 3–26. https://doi.org/10.2174/1389202917666160803165447

Thorn, C. F., Oshiro, C., Marsh, S., Hernandez-Boussard, T., McLeod, H., Klein, T. E., & Altman, R. B. (2011). Doxorubicin pathways: pharmacodynamics and adverse effects. Pharmacogenetics and genomics, 21(7), 440–446. https://doi.org/10.1097/FPC.0b013e32833ffb56

Wang, N., Mi, M., Wei, X., & Sun, C. (2020). Circadian clock gene Period2 suppresses human chronic myeloid leukemia cell proliferation. Experimental and therapeutic medicine, 20(6), 147. https://doi.org/10.3892/etm.2020.9276

Zhang, J., Lv, H., Ji, M., Wang, Z., and Wu, W. (2020) Low circadian clock genes expression in cancers: a meta-analysis of its association with clinicopathological features and prognosis. PLoS One 15, e0233508

Vita

Jamie Pham Tran is the child of Jeanie Tran and John Pham. After completing their work at Westchester Academy for International Studies, Houston, Texas in 2008, they entered the University of Houston, Houston, Texas. They received the degree of Bachelor of Arts with a major in psychology from University of Houston in May 2013. For the next six years, they worked as a research assistant in the Department of Stem Cell Transplantation & Cellular Therapy at The University of Texas MD Anderson Cancer Center. In August of 2019 they entered The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences.

Permanent address:

4315 Wildacres Drive

Houston, Texas 77072