Interferon Induced Transmembrane Protein 1

as a

Candidate Clock Regulator Gene

by

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to my family and friends…

ABSTRACT

Daily rhythms, known as the circadian rhythm, are ever-present in the living world, driving the sleep–wake cycle and many other physiological changes. Most of the physiological pathways are controlled by the interaction between different signal transduction pathway and core clock mechanism. However, many aspects of circadian molecular physiology remain unexplained. In the last two decades, several groups have identified "clock genes" that interact to generate underlying molecular oscillations and, in turn, regulate the different physiological variables. At the molecular level the CLOCK/BMAL1 transcriptional complex lies at the core of the molecular clock. These proteins bind E-box elements in the promoters of target genes. The *Period* and *Cryptochrome* gene families are prominent among these targets, and their products ultimately repress CLOCK/BMAL1 activity and their own transcription.

A second loop regulates *Bmal1* expression through the opposing actions of the REV–ERB and ROR nuclear receptor protein families. A previous study used a simple "machine learning" approach to identify new clock genes by searching the genome for candidate genes that share clock-like features such as cycling, broad-based tissue RNA expression, in vitro circadian activity, genetic interactions, and homology across species. This study resulted in identification of a gene, Interferon Induced Transmembrane Protein 1 (IFITM1), may also act as in a clock function.

In this thesis work, I showed that IFITM1 protein specially interacts with CRY1 proteins using mammalian two-hybrid and co-immunoprecipitation. Further studies showed last 20 amino acids in CRY1 C-termini are responsible for the interaction with IFITM1. To evaluate the functional consequences of these physical interactions, I monitored *Per1::luciferase* activity in unsynchronized HEK 293T cells transiently transfected with *Clock/Bmal1*, *Cry1* and *Ifitm1*. It is known that *Per1::luc* reporter activity is enhanced by CLOCK/BMAL1 transfection but repressed by the overexpression of *Cry1*. When all of the constructs were transfected in HEK 293T cells, we observed *Ifitm1* inhibits repressor activity of the CRY1. In this study for the first time, I provided evidence that IFTIM1 interacts with CRY1 and such interaction

inhibits the activity of CRY1. This study improves our understanding on how clock works and will enable us to develop new drugs curing clock-related diseases such as insomnia, diabetes, obesity, jetlag and psychological disorders.

ÖZET

Canlılar dünyasındaki uyku-uyanıklık ve daha birçok fizyolojik değişim sirkadiyen ritim olarak da bilinen günlük ritimler ile kontrol edilir. Fizyolojik yolaklar, farklı sinyal iletim yolakları ve saat mekanizmalarıyla kontrol edilebilirler. Buna rağmen, sirkadiyen moleküler fizyolojisinin birçok özelliği hala açıklanamamıştır. Geçtiğimiz yirmi yıl içerisinde, birçok grup moleküler salınım gösteren ve fizyolojik değişkenleri kontrol eden birçok "saat geni" tanımlamışlardır. CLOCK/BMAL1 anlatım kompleksi moleküler saat mekanizmasının temelini oluşturmaktadır. Bu proteinler, hedef genlerin E-Box elemanları bulunduran promotor bölgelerine bağlanırlar. *Period* ve *Cryptochrome* gen aileleri bu bilinen hedef genler arasındadır ve bu genlerin anlatımları CLOCK/BMAL1 aktivitesini ve kendi gen anlatımlarını baskılamaktadır. İkinci bir döngü de *Bmal1* anlatımını kontrol eden, nükleer almaç protein ailesine ait birbiriyle ters etkili biçimde çalışan REV-ERBα ve ROR proteinleriyle düzenlenir. Geçmiş bir çalışmada kullanılan basit "makine öğrenmeli (machine learning)" yaklaşımını kullanarak genomdaki saat benzeri özellikler taşıyan aday saat genler belirlenmiştir. Bu belirlemede genlerin saat döngüsüne sahip olup olmadığı, doku düzeyinde mRNA anlatımına etkisi olması, in vitro saat aktivitesi gösterip göstermediği, genetik etkileşimleri ve türler arası genetik kod korunumları dikkate alınmıştır. Bu çalışma sonucunda interferon ile indüklenmiş transmembran protein 1 (IFITM1) proteininin aday saat genlerinden biri olabileceği bulunmuştur.

Bu tez çalışmasında, IFITM1 proteininin spesifik olarak CRY1 proteini ile etkileştiğini memeli-ikili melez yöntemi ve antikor yardımıyla eş zamanlı çöktürme yöntemleriyle gösterdim. İleri çalışmalar CRY1 proteininin karboksil son 20 aminoasitin IFITM1 proteini ile etkileştiğini gösterdi. Bu etkileşimin fizyolojik sonucunu bulabilmek için, geçici olarak *Clock/Bmal1, Cry1* ve *Ifitm1* ile transfekte edilmiş, aseknronize HEK293T hücrelerindeki *Per1:lusiferaz* aktivitesi görüntülenmiştir. *Per1:lusiferaz* aktivitesinin CLOCK/BMAL1 transfeksiyonu ile arttığı, CRY1 yüksek anlatımı ile baskılandığı bilinmektedir. Bütün bu genlerin transfekte edildiği HEK293T hücrelerinde, IFITM1'in CRY1 proteinini baskılayıcı etkisini inhibe ettiği bulunmuştur. Bu çalışmada ilk kez IFITM1'in CRY1 ile bağlandığı ve bu bağlanmanın CRY1'in baskılayıcı etkisini

engellediği bulunmuştur. Bu çalışma bize, saat mekanizmasının nasıl çalıştığı hakkında bilgi vermekle birlikte, saate-dayalı uykusuzluk, diyabet, obezite, jetlag ve diğer fizyolojik hastalıkların tedavisinde kullanacak yeni ilaç tasarımlarının yapılması için izin vermektedir.

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Eylem KÜLKÖYLÜOĞLU

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NOMENCLATURE

CDT	C-terminal domain
Co-IP	Co-immunoprecipitation
CML	Chronic Myeloid Leukemia
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
IFITM1	Interferon induced transmembrane protein 1
IFITM3	Interferon induced transmembrane protein 3
GR	Growth receptor
GREs	Growth receptor elements
GWAS	Genome-wide association scans
HPA	Hypothalamic–pituitary–adrenal axis
HRP	Horseradish peroxidase
LB	Luria Bertani
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonylfluoride
pRGCs	Photosensitive retinal ganglion cells
PTR	Post-translational regulation loop
RNA	Ribonucleic acid
ROREs	Retinoic acid-related orphan nuclear receptor response elements
SCN	Suprachiasmatic nuclei

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
WT	Wild type
TTR	Translational feedback loop
GSK-3	Glycogen synthase kinase-3
CK1ε	Casein kinase 1 epsilon
CK1δ	Casein kinase 1 delta
ARNTL-1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
FBXL3	F-Box and leucine-rich repeat protein 3
β-TrCP	Beta-transducin repeat-containing protein

CHAPTER 1

INTRODUCTION

The circadian clock is a biological timing system that oscillates with a period of 24 hours and is coordinated with the day-night cycle. Therefore, understanding the molecular clockwork helps us to develop treatments against various disease and disorders such as diabetes, cardiovascular diseases, obesity, jetlag, insomnia, and psychological disorders. The mammalian circadian clock is composed of transcriptional-translational feedback loop which allows the generation of daily rhythms. Core clock genes that have been identified so far that constitute the molecular feedback loop are: *Bmal1, Clock, Npas2, Period1, 2, 3, Cryptochrome 1, 2*. Besides known core clock components, more gene loci have been discovered that change the wild-type oscillation pattern of core clock genes.

The circadian rhythm is one of the hottest topics in molecular biology and according to deep high-throughput sequencing studies, almost 40 % of human genome works under the control of core clock components. The circadian clock system is controlled by multiple functional positive (BMAL1 and CLOCK) and negative regulators (PER and CRY). The molecular mechanism is under the control of transcriptional-translational feedback loops. The CLOCK-BMAL1 heterodimer activates the transcription of the *Period (Per)* and *Cryptochrome (Cry)* genes and PER and CRY form a complex and suppress their own gene expression by inhibiting the CLOCK-BMAL1 heterodimer-centered transcription. This kind of far-reaching system has more key players involved in the regulation of this system. Therefore, many candidate core clock proteins have

been identified by GWAS studies. One of these studies was done by Anafi et al. (2014) and their deep sequencing and microarray results identified almost 20 candidate core clock components. To our knowledge, recent studies have determined the functions and effects of candidate proteins on core clock components.

Therefore, I examined the role of interferon-induced transmembrane protein-1 (IFITM1) protein in circadian clock mechanism to determine a new core clock component. IFITM1 is a 17 kDa transmembrane protein that transduces anti-proliferative and homotypic adhesion signals in lymphocytes. It is also responsible for mouse primordial germ cell development. The protein is also known as 9-27, CD225 and Leu13 in the literature. Not only does affect the immune system, it can also act as a key player in different mechanisms such as circadian rhythm and cancer. As a transmembrane protein, IFITM1 can interact with both transmembrane and cytosolic proteins in different ways. Hence, finding its binding partners will illuminate its function in the cell. Besides its anti-proliferative role, it has been shown to promote cancer progression by enhancing cell migration and invasion in gastric, head and neck, glioblastoma, Chronic Myeloid Leukemia (CML) and ovarian cancer. These broad spectrum of functions brought IFITM into the limelight of molecular biology studies. To identify the importance of IFITM1 for circadian rhythmicity, I conducted this project.

In chapter 2, we give detailed information about molecular mechanism of circadian clock, different molecular clockwork examples in different model organisms and the relationship between circadian clock and immune system.

Chapter 3 explains all experimental procedures and used materials. Also, construct generation, co-immunoprecipitation, mammalian-two hybrid assay, siRNA knockdown, RNA isolation, qPCR and cellular imaging methods are included in this chapter.

Chapter 4 presents the results. The thesis is finalized in Chapter 5 by giving concluding remarks and discussion part.

CHAPTER 2

LITERATURE REVIEW

2.1. Overview of Biological Timing System from Single Cells to Humans

From single cells to humans, all living organisms have developed innate biological timing systems called “biological clocks”. These systems are subject to universal rhythms. The scientific area that studies adaptations evolved by living organisms to cope with regular geopyhsical cycles in the environment is chronobiology [1]. Numerous different oscillatory clocks comprise circatidal (12,4 hours), circalunidian (29,5 days), circadian (24 hours) and circaannual (365 days) oscillations which keep time with environmental periodicities such as ovarian cycles and oscillations in development and in the brain keep time with biological timescales [2]. Most of the human behaviours such as feeding, reproduction, seasonal migration and hibernation are regulated according to these geophysical oscillations. Recent evidence shows that these rhythms may be important for human health. DisturbanceS of circadian rhythms by night shift work or jet lag is very commonly seen in the population. There is now increasing evidence that disturbance of biological rhtytms and their desyncronization results in loss of health. Accumulating epidemiological and genetic evidence indicates that distruption of circadian rhythms can be directly linked to many pathological conditions, including sleep disorders, depression, metabolic syndrome and cancer [3]. In order to illuminate the relationship between circadian rhythm and metabolic disorders, huge amount of research has been focused on this area.

The occurance of biological rhythms in living organisms is adventageous for several ways. Firstly, it provides metabolic stability. Control of heat-rate is achieved by hormonal oscillation which is controlled by external stimuli. Other advantage is tuning and sycnronization of rhythms saves energy. For example, when we relax or sleep, our energy consumption is relatively lower then when we run or walk. Lastly, temporal compartmentalization allows seasonal events to occur in the same space unit. These situations are balanced by means of circadian oscillations [4]. In response to external influences all of metabolic cycles have been internalized into organisms by complicated neuronal integrations and genetic control [5]. For example, children exposed to a light cycle program displayed improved growth compared to ones that exposed continous dim or bright light. Additionally, the period of lunar cycle is possibly found today in the female menstural cycle, but some studies reported significant connection between metabolic regulations and circadian timimg. This is probably due to the light pollution present in cities, obscuring the lunar influence [6]. As we have seen from the examples, biological timing system works and syncronized by geophysical effects and it gives us the motivation to find out the mechanism of this co-operativity.

2.2. The Pervasive Effects of Light

The Earth completes a full turn on its axis almost 24 hours. The 24-hour light-dark cycle is a fundamental characteristic of Earth's environment and it has significant effects on behavior and physiology of living organisms. Especially, the effect of the light on animal and human physiology can be clearly seen. Light coordinates the changing behavior and physiology by sending inputs to specific locations in the brain that is called superchiasmatic nucleus (SCN) and these signals are mediated either by chemicals (melanopsin) or physical impulses (electrical impulses in retina) [7].

In humans and other diurnal mammals, most daily activities occur during the day, whereas nocturnal animals a reactive in dark conditions. More than 35 years ago, it was found that circadian rhythm in mammals is regulated by specific centers called

"pacemakers" in the brain. This region consists of thousands of neurons and called the superchiasmatic nucleus (SCN) in the anterior hypothalamus [8].

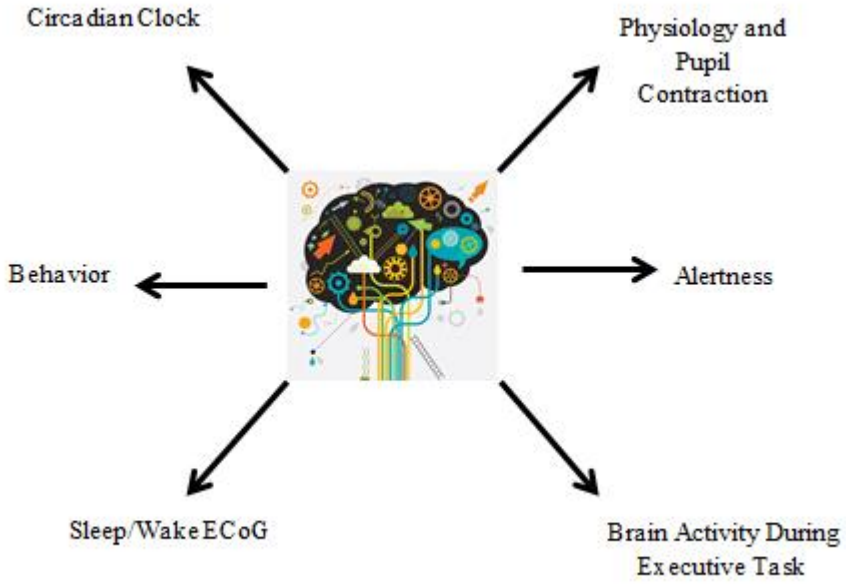


Figure 2.1.Summary of pervasive effects of light [*Adapted from 7*]

Either light exposure in dawn or light exposure late in the night will cause phase-shifting in diurnal animals. The dark condition also creates the same situation in nocturnal animals. This phase-shifting occurs as a result of the non-image forming effect of the light, which depends on circadian phase and plays important role in the temporal organization of behavior in animals [9]. One of the most important behavioral effects of light can be clearly seen in sleep-awake activity in animals including humans. Sleep is regulated by an interaction of homeostatic and circadian process. The homeostatic mechanism keeps track of how long we have been awake and asleep, and circadian process determines the optimal time for sleep. The SCN generates signals that influence sleepiness, sleep duration and sleep structure. There is now large body of evidence shows that, any lesions on SCN cause light dependent phase-shifting and this phase-shifting causes sleep disorders [10]. Besides SCN, there are other important components that lie under light-dependent circadian rhythm such as melanopsin, rods

and cones. The photo pigment melanopsin is expressed in the inner retina of humans [11] and in particular, in a subclass of photosensitive retinal ganglion cells (pRGCs). Recent studies indicate that, mice lacking melanopsin coding gene show abnormal pupillary constriction and create changes in circadian oscillations [12].

2.3. What is Circadian Clock?

The circadian clock is the biological timing mechanism to adapt 24 hour environmental light/dark cycle and adapt to external conditions that is based on Earth's daily rotation around its axis. According to the definition in *Overview of Circadian Rhythms*, "In its broadest sense, chronobiology encompasses all research areas focusing on biological timing, including high-frequency cycles (e.g., hormone secretion occurring in distinct pulses throughout the day), daily cycles (e.g., activity and rest cycles), and monthly or annual cycles (e.g., reproductive cycles in some species). Among these interrelated areas of chronobiology, this article focuses on one frequency domain-the daily cycles known as circadian rhythms (The term "circadian" derives from the Latin phrase "circa diem," which means "about a day.") [13].

The physiology and behavior of living systems works in a circadian manner. Therefore, organisms have a precise internal biological clock that organizes daily incidents and synchronizes physiological rhythmic alterations within the day [14] .More than 35 years ago, it was discovered that circadian rhythm in mammals are driven by a specific part of brain which is called superchiasmatic nucleus (SCN) [15]. This specific region is located in the anterior hypothalamus and it receives environmental signals from different receptor organs in the body. In the central superchiasmatic nucleus (SCN) of the hypothalamus, a coupled population of neuronal circadian oscillators acts as a master pacemaker for the organism to drive rhythms in activity and rest, feeding, body temperature, and hormones. Coupling within the SCN network confers robustness to the SCN pacemaker, which in turn provides stability to the overall temporal architecture of the organism [16][17]. Almost all living organisms from single cell to humans use this mechanism to adapt to their environment [16]. Environmental signals are received by

receptor organs and these signals are processed in SCN [18]. Depending on the characteristic of the signal, different metabolic output signals are given. SCN has ability to synchronize and control the peripheral organs. This control can be provided by hormone secretion and physical signal transmission.

here main characteristics define circadian clock oscillations: i) periodicity ii) robustness iii) entrainment. These main attributes of circadian clock were established in light of Pittendrigh and Aschoff's researches in the second half of the 20^{th} century [13].

2.3.1. Periodicity

Periodicity refers the reoccurrence of the same conditions in the clock oscillation repeatedly. As a basic rule of circadian clock, period length is persistent and sustained under constant conditions [13]. Although there is an intrinsic circadian rhythm mechanism which exists without any sensory input from the environment, circadian clock characteristics like period length and phase can be reset by equivalent external inputs. However, oscillations are resistant to broad range of environmental variations [19].

2.3.2. Robustness

Robustness is the other important characteristic of circadian clock oscillations. It means resilience of an oscillator to both intrinsic and extrinsic perturbations [20]. Here, robustness is defined as the ability to sustain daily oscillations with an accurate period length day after day and robustness of the system is determined by intercellular and intracellular processes [21]. T external stimuli are important because of their effects on intercellular and intracellular processes inside the cell. Entrainment by light/dark cycles and cooperativity in repression enhance the robustness of circadian oscillations with respect to molecular noise [22].

2.3.3. Entrainment

A third characteristic of circadian rhythm is entrainability which means being synchronized according to external stimuli. Despite existing without any external stimuli, there are strong evidences that oscillation patterns are affected by

environmental conditions. Accordingly, if a shift in external cues occurs because of time-zone changes, the rhythms will be aligned to the new cues. This alignment is called entrainment [13][23].

There are three main ways to entrain a circadian system which are called pacemakers. Although the major potent factor is light, other factors-such social interactions, activity, exercise and temperature can also modulate the circadian mechanism of a living system [24] [25] [13].

External stimuli are taken up by receptor organs in the system and they are processed in the master clock (SCN). Then environmental signals and internal body signals are synchronized and a complex response is established and integrated into the system as an output signal [26].

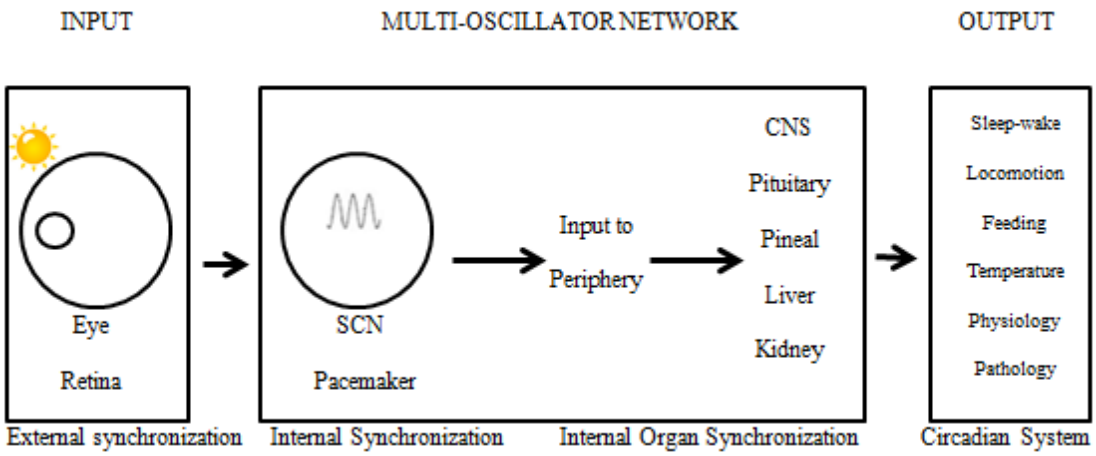


Figure 2.2. The mammalian circadian timekeeping system [*Adapted from 27*]

2.4. Biological Importance of the Circadian Rhythm

For living systems, the basic function of having a circadian rhythm is providing a prediction mechanism for environmental alterations. From that perspective, it is very clear that having a circadian rhythm is a “must” for almost all organisms on the Earth [28] [29]. The circadian oscillators provide ability to respond complex metabolic incidents like sleeping and feeding. This system regulates almost all cyclic behavior in living systems such as gastrointestinal tract motility, cardiovascular activity, endocrine secretion, body temperature regulation, renal activity, osmotic pressure control, locomotor activity, sleep-wake order, cell division and leaf movement [30].

Since there is a direct connection between circadian clock and metabolism, the reason behind most of the health problems is disruption of this internal rhythm [17]. The control of different biological processes by circadian clock has been demonstrated by occurrences of several health problems including asthma, allergy attacks, metabolic and psychological disorders, myocardial infarction, fatigue, disorientation, insomnia and distorted hormone profiles are seen in jet-lagged travelers and shift workers [31] [32].

This connection between two clinical situations is the reason of generating medical treatment strategies during the last decade [33].On this purpose, people have focused on studies that addresses discovery of the molecular clockwork and developed specific drugs against circadian clock disorders [34]. Especially, small molecule design strategy have been successful and drug development studies gave positive results for several treatments [35] [36].

2.5. Historical Background of Chronobiology

The research field now known as chronobiology deals with the body's biological rhythms. Greek natural historians had noted on the rhythms of birdsong or leaf movements, none had considered that the response to environmental cycles was more than a passive exogenous reaction to external stimuli [23]. Although many observations have been shared during ancient times to 18th century, the story began with the discovery of connection between geophysical and circadian cycles in 1729 by the French astronomer Jean-Jacques d'Ortous DeMarian. He took plants that displayed leaf movements and put them in the dark for several days. He noted that the leaves of the plants continued to open during the day and close at night, despite the absence of sunlight. Based on this observation, he concluded that the rhythm was not passively driven by cyclic environment but was an innate reaction of the plant [37] .After DeMarian's discovery, new information was added in the next century. The Swiss botanist Alphonse de Candolle demonstrated that the *Mimosa* daily leaf movements persisted in darkness, too [38]. Although several later observers noted behavioral patterns of internal timing, a major obstacle to further progress was the wrong choice of words. The internal timing of living organisms were defined in 1929 with the German Word "Zeitgedächtnis" which means time memory [39].

In the beginning of 20^{th} century, studies in chronobiology accelerated. In 1939, Maynard S.Johnson made an experiment to measure internal free-running activity of white-footed mice in wheel cages. Johnson showed that the internal rate of mice was dependent on the intensity of the continuous light regime. This experiment was lost in the shuffle, but it was the first attempt to measure the internal rhythm of a mammalian organism [30]. The other Pioneer of plant physiology was the German scientist Erwin Bünning. He started research on circadian rhythms in 1928 and proposed his hypothesis that circadian rhythms have adaptive value for organisms. When he revised his book The Physiological Clock in 1973, he wrote that "As recently as 15-20 years ago, the existence of an endogenous circadian rhythm was accepted as a mystical notion." His discoveries are credited today with the beginning of plant photoperiodism [38]. In the

late 1940s, the German scientist Gustav Kramer was studying time-compensated celestial navigation in bird migration. He had shown that birds require a sun compass for flying North in the spring and he wrote that to use such a continuously moving reference point for flying in a fixed direction, the bird should have a biological clock [40].

Previous to these milestone, there were two important names in chronobiology. They were Colin S. Pittendrigh and Jürgen Aschoff. Pittendrigh had studied mosquito behavior as a malaria biologist during 2nd World War. He then decided to study daily organization of *Drosophila melanogaster*. He is known for his careful descriptions of the properties of the circadian clock in Drosophila and other species, and providing the first formal models of how circadian rhythms entrain (synchronize) to local light-dark cycles [41]. Jürgen Aschoff was the other important collaborator of Pittendrigh. He also worked on properties of circadian clock, especially entrainabiliy of the system [42].

After Kramer, Pittendrigh and Aschoff's studies', many laboratories around the world turned their attention to chronobiology. At Northwestern University, a young professor Woody Hastings and his collaborator Beatrice Sweeney noticed rhythms of bioluminescent flashing and glowing single cell *Gonyaulax* [43]. At University of Wisconsin, Kenneth Rawson noted highly precise circadian rhythm in nocturnal deer mice and flying squirrels by measuring free-running activity on wheel cage [44]. At that time, a young biologist Michael Menaker at University of Texas worked on circadian rhythm of birds, fish and reptiles [1].

The molecular mechanism behind circadian rhythm was a question mark before 1971. Ron Konopka and Seymour Benzer discovered one of the core genetic components of the circadian clock called period (*Per*) in *Drosophila melanogaster.* They demonstrated all possible mutant phenotypes such as long-period, short-period, arrhythmic mutant phenotypes in *Drosophila* as a model organism[45].

In 1994, another core clock component, the "circadian locomotor output cycles kaput" (*Clock*) was discovered by Takahashi group. The group described a period-lengthening mutation in a mice and characterized the gene [46]. The discovery of *Period* and *Clock*

genes were milestone studies for identification of molecular mechanism of circadian clock. The last core component Brain and muscle Arntl-1 like protein (*Bmal1*) was discovered as a core component [47]. After understanding the regulatory role of molecular clock mechanism on metabolism, scientists turned their attention to finding novel core components. To do this, genome-wide studies are done by high-throughput tools.

2.6. The Molecular Circadian Clock in Prokaryotes, Fungi, Plants, and Insects

It has been found that from cyanobacteria to mammals in almost all living organisms, there is a circadian clock system. Although the basic mechanism is generally similar, components in these diverse systems differ. It has been known that master clock mechanism is conserved among kingdoms [48]. Interestingly, clock components that are conserved between species can be used in different ways. The different usage of components may be the reason of different environmental adaptations to the system.

As it was described before, circadian clock system consists of three basic components: a central oscillator that generates rhythmicity; input pathways that receive and relay environmental cues that entrain the oscillator; and output components that create changes in rhythms. But this expression is an oversimplification. In reality, the system is complex because it contains positive and negative feedback loops [48]. In almost all organisms except cyanobacteria, the system is supported at least one self-governing internal oscillator that consists of auto regulatory negative and positive feedback loops [49].

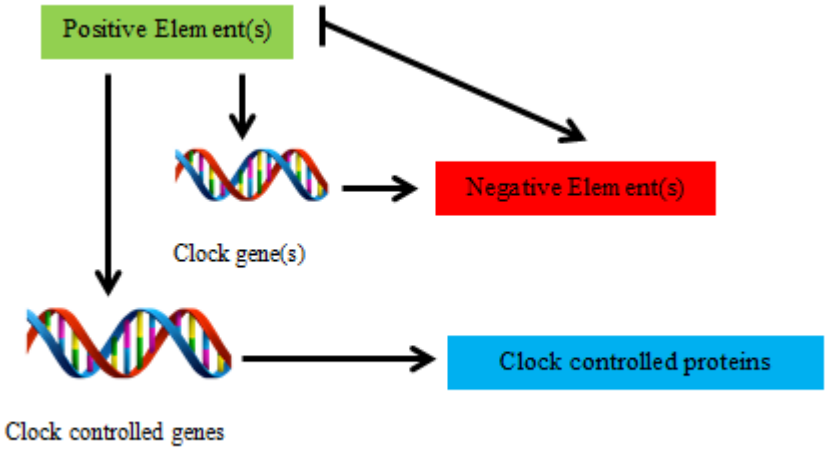


Figure 2.3. Common elements in the circadian feedback loops [*Adapted from 46*]

2.6.1. Circadian Clock in Cyanobacteria: *Synechococcus elongatus*

Before 1990s, it was assumed that prokaryotes did have circadian rhythm. In 1997, circadian clock mechanism in *Synechococcus elongatus* was characterized [50]. The cyanobacterial circadian clock is responsible for controlling cell division, amino acid uptake, nitrogen fixation, photosynthesis and respiration [51]. There are three proteins that regulates cyanobacterial circadian rhythm: KaiA, KaiB and KaiC [52] .KaiC functions as autokinase, autophosphatase and ATPase. KaiA stimulates the autophosphorylation of T432 residue of KaiC. Phosphorylated KaiC subsequently encounters spontaneous phosphorylation at T432 and binds to the KaiA [53]. *KaiB* and *KaiC* are transcribed together from the same promotor and overexpression of *KaiC* represses the expression of both *KaiA* and *KaiB*, but overexpression of *KaiA* enhances the transcription of *KaiB* and *KaiC*.

As it was indicated in Figure 2.4., there are two feedback loops in the cyanobacterial circadian clock. One of them is post-translational regulation loop (PTR) and transcriptional and translational feedback loop (TTR). When PTR is responsible for rhythmic oscillation of *KaiC*, TTR is responsible involved in the control of *kaiBC* promotor [54].

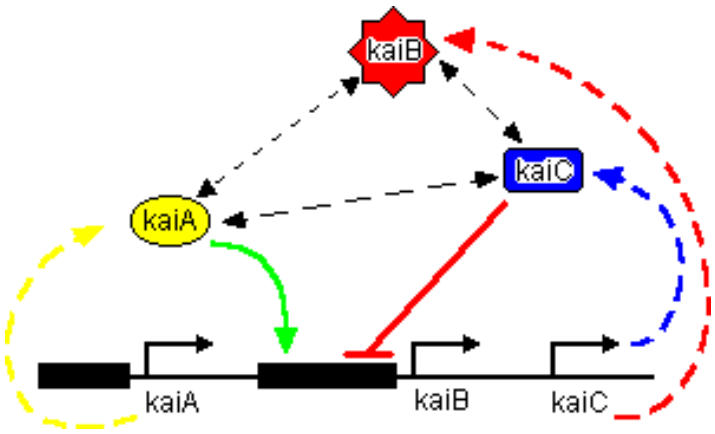


Figure 2.4. Molecular mechanism of cyanobacteriai circadian rhythm [*Adapted from 55*]

2.6.2. Circadian Clock in *Neurospora crassa*

The circadian clock of fungus *Neurospora crassa* was first identified in 1970s. People found that mutations in three genes *frq, wc-1,wc-2* cause period length defections [56]. FRQ is known as a transcription factor and this protein negatively regulates *frq* transcription. *frq* expression is provided by WC-1 and WC-2 proteins which have PAS domain and zinc-finger DNA-binding domains [57] [56]. Basically WC-1 and WC-2 heterodimerize in vivo for the induction of gene expression in response to blue light [58].

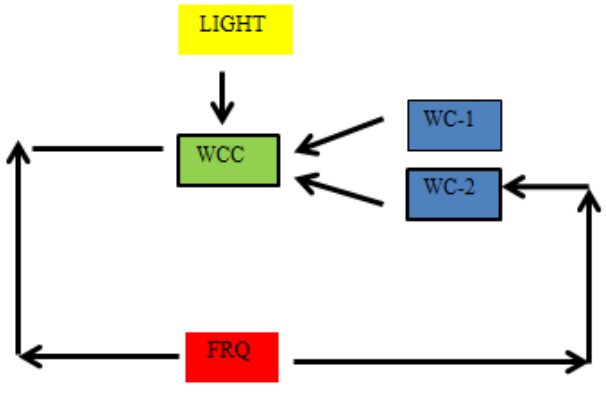


Figure 2.5. Feedback loops within the Neurospora circadian system [*Adapted from 59*]

2.6.3. Circadian Clock in *Arabidopsis thaliana*

The plant circadian clock is different from other organisms. There are two basic loops that are formed by the interaction of transcription factors acting as repressors. Overexpression of two transcription factors CCA1 and LHY causes arhythmicity [18]. Two-morning-phased transcription of CCA1 and LHY repress the expression of an evening-based pseudo-response regulator, timing of CAB expression 1 protein (TOC1) by binding cis-regulatory element named the evening element (EE) found in the promotor regions of most evening-phased clock genes [60]. In contrast, TOC1 is a repressor for CCA1 and LHY during the night. [61] The photosynthesis reactions work according to circadian clock. From dawn, light activates *PRR7* drives photosynthesis. High endogenous sugar concentrations lead to suppression of the *PRR7* promoter, contributing to the phase of *PRR7* rhythms. PRR7 is a transcriptional repressor of the circadian clock component CCA1. The metabolic gradient lags behind that of light and contributes to the setting of the circadian clock [61].

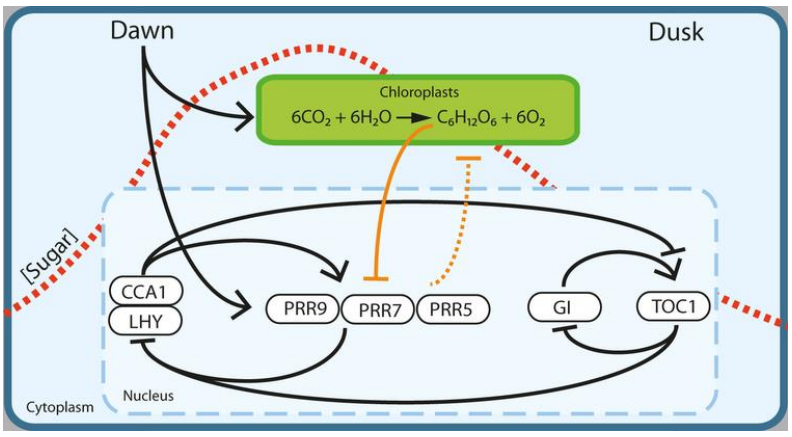


Figure 2.6. A model for entrainment of the A. thaliana circadian clock by photosynthetically derived sugars *[Adapted from 61]*

2.6.4. Circadian Clock of *Drosophila melanogaster*

The molecular genetic studies had started in *Drosophila melanogaster* with identification of the period (*dper*) gene [45]. Later the, timeless (*dtim*) gene was found based on its ability to bind the period gene [62]. The circadian clock of *Drosophila melanogaster* has only one transcriptional-translational feedback loop and the transcription factors of this system are clock (CLK) and cycle (CYC) which both have DNA-binding domains and PAS domains. dCLK/CYC heterodimers binds directly to E-box elements found in the *dper* and *dtim* promotors and enhance expression of reporter genes driven by these E-Box elements [63][64]. This induction on the system is negatively regulated by dPER AND dTIM elements [65]. Different kinases DOUBLETIME, casein kinase2 (CK2), glycogen synthase kinase-3 homolog (GSK-3) and SHAGGY, gradually hyper phosphorylate dPER and dTIM during the night [65] [66]. Hyper phosphorylated proteins inhibit the activity of the CLK/CYC complex by interaction. CLK/CYC complex is relieved in the morning when dPER and dTIM start to be degraded [67].

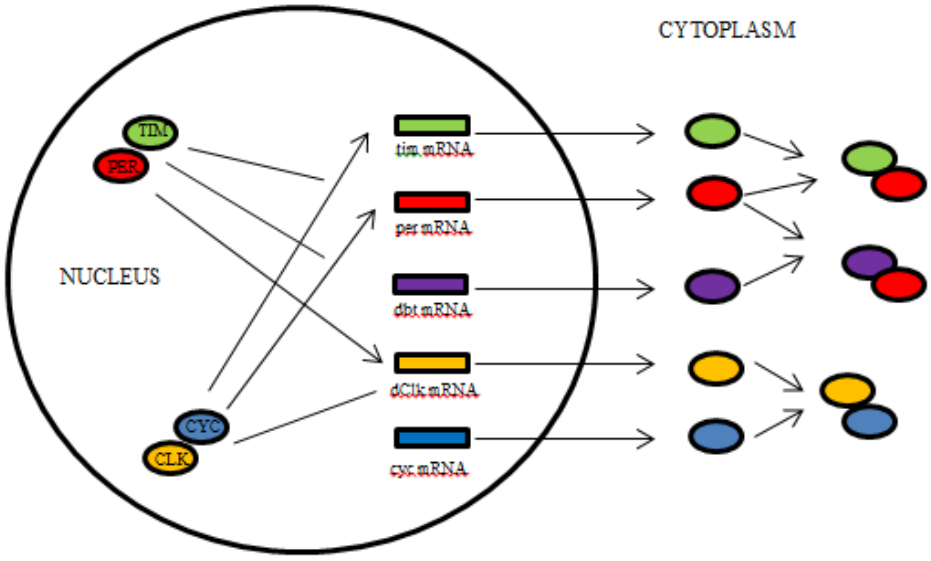


Figure 2.7. Schematic illustration of circadian clock in *Drosophila [Adapted from 68*]

2.7. The Molecular Circadian Clock in Mammals

Similar to other organisms, mammalian circadian clock is also responsible for regulating a wide variety of physiological and behavioral rhythms [26]. A core set of circadian clock genes common to most cells throughout the body code for proteins that feed back to regulate not only their own expression, but also that of clock output genes and pathways throughout the genome [69]. The mammalian circadian clock is organized in a hierarchical way. The master clock, superchiasmatic nuclei is found at the top of this hierarchy. SCN is found in the anterior hypothalamic region of the brain and it is responsible for coordinating independent peripheral oscillators and external stimuli which are coming from the environment [30]. By means of recent studies in the last decade, molecular mechanisms of circadian clock were discovered and new models of core mechanism were built [70][30].

According to the current model, core components form a transcriptional-translation feedback loop. The first feedback loop elements are the basic helix-loop-helix transcription factors BMAL1 (brain and muscle ARNTL-like protein-1 (ARNTL), an orthologous of the *Drosophila cyc* gene) and its heterodimeric partner CLOCK. They interact with each other and bind to E-Box *cis* elements which are found in the promotor regions of target genes [71].

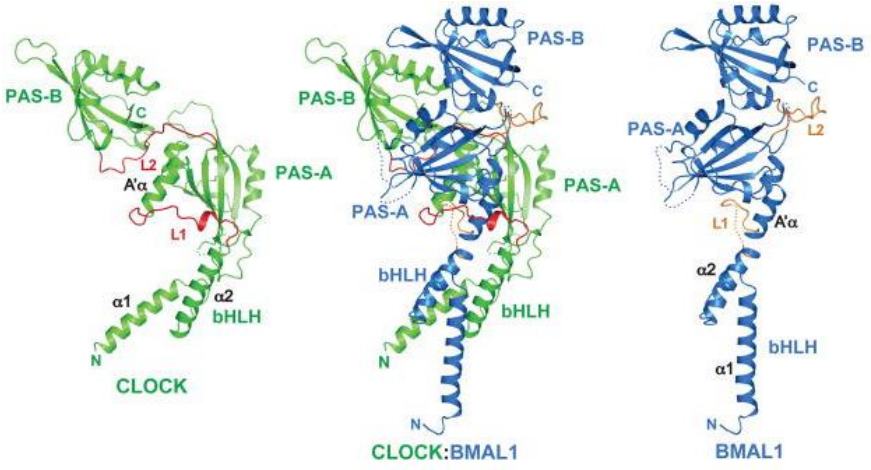


Figure 2.8. Overall structure of mouse CLOCK:BMAL1[*72*]

period 1, period 2 , period 3 and cryptochrome 1, cryptochrome 2 genes are negative regulators of the system and BMAL1-CLOCK complex binds to their E-Box elements in their promotor region [73]. The repressor proteins PERs and CRYs interact in a protein complex that translocate from the cytoplasm to the nucleus. PER-CRY complex in the nucleus physically interacts with BMAL1-CLOCK heterodimer and restrain this activator complex from binding to their own promotors [74]. In summary, when the BMAL1-CLOCK complex forms the positive feedback loop of the system, PER-CRY heterodimer is the negative feedback loop of the molecular mechanism of the circadian clock [75].

Another regulatory feedback loop is composed of the nuclear receptors called retinoic acid-related orphan nuclear receptor *REV-ERBα* and RAR-related orphan receptor A *RORα* [76] [77]. These two transcription factors compete to bind retinoic acid-related orphan nuclear receptor response elements (ROREs) present in *Bmal1* promotor and regulates the transcription [78]. RORs activate the transcription of *Bmal1*, whereas REV-ERBα represses *Bmal1* transcription [79]. In addition to these roles, interaction between REV-ERBα and PER2 fine-tunes *Bmal1* expression [80]. Besides controlling *Bmal1* and *Clock* gene expressions, these two nuclear receptor structures are activators for their own transcription [77]. Lastly, REV-ERBα represses the transcription of *Clock* by binding to REV-ERBα response elements [81].

Post-translational modifications of the core component proteins are important for regulation of circadian oscillations. *Casein kinase 1 epsilon* (*CK1ε*) and *Casein kinase 1 delta* (*CK1δ*) are important genes for the protein turnover of core clock components [82][83]. The basic role of these proteins is to phosphorylate core components leading their degradation. Phosphorylation causes ubiquitin ligases to be recruited eventually leading to proteosomal degradation of core components. Any mutations in either *CK1ε or CK1δ* results in period-lengthening of the oscillation [84]. Each core component degradation is achieved by different mechanisms. For example, PERs are phosphorylated by CK1ε and acquire binding sites for E3 ubiquitin ligase, beta-transducin repeat-containing protein(β-TrCP) [85]. On the other hand, CRYs are

phosphorylated by AMP-activated protein kinase (AMPK) and then ubiquitinylated by F-box leucine-rich protein 3 (FBXL3). [86] BMAL1 phosphorylation is also achieved by casein kinase 2 alpha (CK2α).

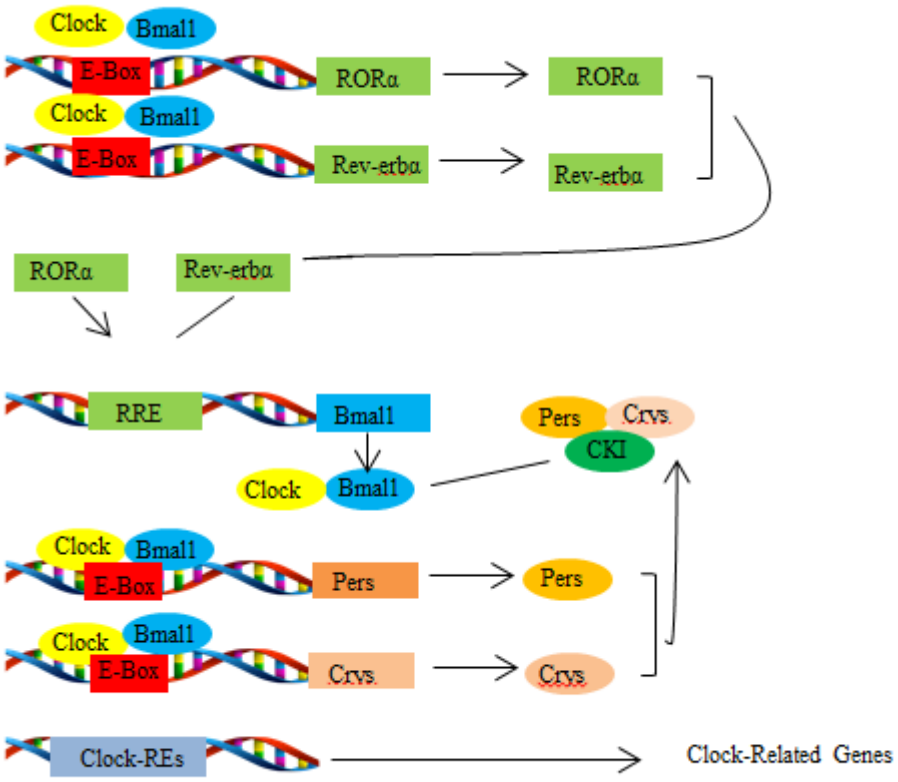


Figure 2.9. The molecular mechanism of mammalian circadian clock *[Adapted from 87]*

2.8. The Connection between Immune System and Circadian Clock

Most of the physiological and behavioral systems are regulated by effects of light and dark which can also be referred to 24 hour cycling. Especially mammals receive light by their retina and process the light stimulus by their Suprachiasmic Nucleus (SCN) which is situated close to hypothalamic part of the brain [88]. This part of the brain is known as the "master clock" and controls the rhythm in cooperation with a neuroendocrine system like the HPA axis. The peripheral organs, which are far from the brain, are also regulated by the master clock [89].

Basically, the system starts when the light reaches to the SCN. BMAL1 and CLOCK expressions start to increase and because of the acetyltransferase activity of CLOCK protein, glucocorticoid receptors (GR) are acetylated by CLOCK [90]. This acetylation inhibits GRs to bind to glucocorticoid-responsive-elements (GREs) of some genes. Conversely, BMAL1 and CLOCK expression is not high during daytime, so GRs cannot be acetylated by CLOCK and they can bind to GREs. Due to this binding, glucocorticoid responsive gene transcription starts [88].

When the inner mechanism of *Clock* acetylation is examined, it acetylates GRs at their multiple lysine clusters in the hinge region, which includes lysine amino acids at 494-495 located in the KXKK motif and represses GR-dependent transcription of several glucocorticoid responsive genes [88] [91] [92].

In conclusion, BMAL1/CLOCK expression oscillates oppositely to glucocorticoid synthesis by means of the HPA axis. The regulation which starts with circadian clock continues with negative-feedback loop by HPA axis and at the end HPA axis controls metabolic clock by secreting cortisol from the adrenal glands.

2.8.1. Mechanism of Hypothalamic-Pituitary-Adrenal (HPA) Axis during stress conditions

Organisms regulate their inner balance (homeostasis) according to external stimuli. These long or short-termed environmental stimuli are called “stressors”, which can be caused by external factors such as environmental temperature, food availability, sunlight and infections [88]. Their behavioral and physical adaptations to different factors are provided by both hormonal and neural cascades. One of the most important physiological mechanisms is hypothalamic-pituitary-adrenal axis to deal with the stressors.

When a stress stimulus comes to the Hypothalamic Paraventricular Nucleus (PVN) region in the brain, it releases Corticotrophin-Releasing-Hormone (CRH) and Arginine Vasopressin (AVP) hormones to stimulate posterior part of the pituitary gland. As soon as the hormones reach the pituitary gland, it releases another hormone which is called ACTH to trigger glucocorticoid secretion from adrenal glands which are located at the retroperitoneal part of the human body [91]. Glucocorticoid secretion has extremely important effects the on generation of homeostatic balance. For this reason, it is controlled by physiologic control mechanisms, especially by positive and negative feedback loops.

The time-integrated secretion of glucocorticoids which is regulated by the HPA axis, is tightly controlled by circadian rhythm and as a result, expression of core clock proteins are determined by means of negative feedback mechanism

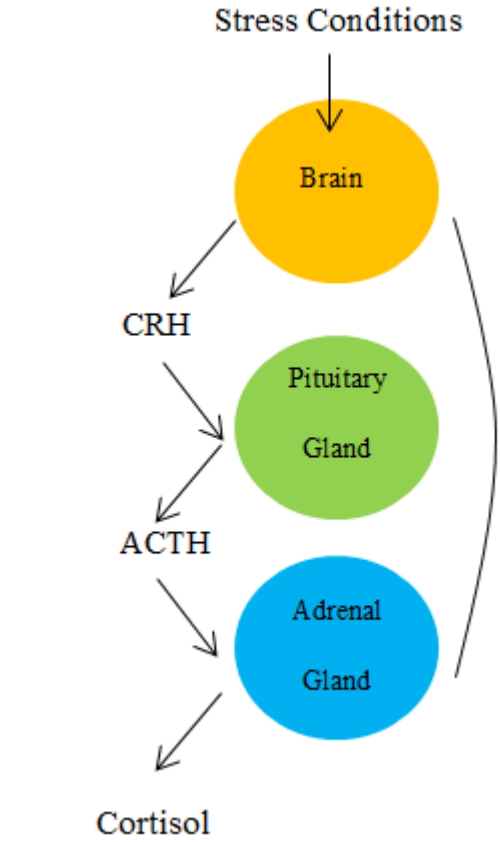


Figure 2.10. Schematic illustration of HPA axis in stress condition [*Adapted from 88*]

2.8.3. HPA axis and Circadian Clock play important role in Immune System

Crosstalk between HPA axis and circadian clock which were mentioned earlier is important for several crucial metabolic mechanisms such as gluconeogenesis stimulation, glycogenolysis, lipolysis, degradation of proteins and amino acids. It is also important for some clinical diseases such as insulin resistance which causes Diabetus mellitus Type II [3] [93] [94] muscle loss and skin thinning in Cushing syndrome [95] and finally cardio metabolic dysregulation because of jetlag. Besides these metabolic problems, researches also show that HPA/Clock crosstalk has an important role on immune system. In both human and rodents, Clock mechanism produces fluctuation of several cytokines such as INF-γ, IL-β1, IL-6 and TNFα and also T and B lymphocytes [88][96]. In clinical medicine, physicians use glucocorticoid-containing creams in

order to suppress local inflammations on skin which occurs because of high-immune response, so there is a triple connection between glucocorticoids (response of HPA axis), immune system and outcome proteins of circadian rhythm. Although this connection has not been explained properly, new potential key proteins are discovered each day.

One of the most important ones is interferon-induced transmembrane (IFITM) protein family. In the last part of this thesis, roles of IFITM-1 protein will be evaluated from different perspectives. IFITM-1 protein is a component of protein complexes involved in homotypic adhesion and the transduction of antiproliferative signals [97] .IFITM-1 expression increases when a viral infection occurs in the cell [98–101].

2.9. Interferon-Induced Transmembrane Protein Family

Interferons (IFN) are a family of proteins secreted by the host cell in response to various pathogens such as viruses, bacteria, fungi or parasites [102]. They are the hallmark of host antiviral immunity. There are three types of interferon and each one has a specific effect on different host immune system [103]. The antiviral IFITM proteins are nearly ubiquitously expressed and upregulated by Type I interferons (IFN) [104]. Interferon-induced transmembrane (IFITM) genes are transcribed in most tissues and all of them are interferon inducible except IFITM5. Most IFITM genes are activated in response to bacterial and viral infections, and the exact host immune defense mechanisms are still unknown [104]. In spite of the unknown parts in the mechanism, it has been known that IFITM proteins inhibit steps leading to formation of a viral infection area by increasing the curvature of cell membranes and their fluidity [105][106].

IFITM1 is a member of this protein family and it is a 17 kDa transmembrane protein that transduces anti-proliferative and homotypic adhesion signals in lymphocytes [97]. It is also responsible for mouse primordial germ cell development. It is named as 9-27, CD225 and Leu13. IFITM proteins may be localized to different cellular compartments in the absence of an infection, with IFITM1 residing primarily at the plasma membrane and IFITM2 and IFITM3 residing in late endosomes and lysosomes [107] [108].

IFITM1can act as a key player of different metabolic mechanisms outside of the immune system such as circadian rhythm, immunologic response and cancer [109–112]. As a transmembrane protein, IFITM1 can interact with both transmembrane and cytosolic proteins in different ways, therefore finding its binding partners will illuminate its function in the cell [97]. Besides its anti-proliferative role, it has been shown to promote cancer progression by enhancing cell migration and invasion in gastric, head and neck, glioblastoma, Chronic Myeloid Leukemia (CML) and ovarian cancer. Although IFITM1 is a multi-pass transmembrane protein, recent studies has shown that it can also be found in the cytosol. It has been proposed that human IFITM1 has a membrane topology in which the N-terminal domain resides in the cytoplasm, and the C-terminal domain is extracellular [113]. Like IFITM1, IFITM3 is in the same family and it is responsible for viral inhibition. IFITM3 partially resides in late endosomal and lysosomal structures, placing it in the path of invading the viruses [114].

2.10. Novel Clock Component Discovery Studies

In the last decade, a great number of clock components were identified by using either forward mutagenesis or reverse genetics methods. In the forward genetics approach, random mutagenesis is applied on an organism and abnormal phenotypes are screened [115]. Most of the clock components such as *Period* and *Clock* genes were discovered by forward genetics approach. Although this method is very useful, high throughput techniques supersede the forward genetics techniques [116]. By means of computer assisted Bayesian integration strategy method, genome-wide data sets were integrated and a list of high probability core clock components was identified. The Bayesian strategy uses these five main attributes to define a candidate gene as a core component:

1. Core clock transcription oscillates within approximately 24 hour.

2. Core clock components interact with other core clock components.

3. When core clock components are knockdown, a cellular and behavioral phenotype change is observed.

4. Core clock components oscillate continuously.

5. Core clock components are conserved through phylogeny.

CHAPTER 3

MATERIAL AND METHODS

3.1. Gene Cloning

To express IFITM1 and IFITM3 genes, they were inserted into pACT and pCMV Sport6 vectors. pACT and pCMV Sport 6 vectors were purified by GeneJet Plasmid Miniprep Kit (Thermo Scientific). Amplified IFITM1 and IFITM3 genes were double digested with EcoRV (Thermo Scientific) and NotI (Thermo Scientific) or FD-EcoRI (Thermo Scientific) and FD-HindIII (Thermo Scientific) restriction enzymes at 37ºC for 2 hours. After digestion, both amplified genes and pACT vector were extracted from 0.8% agarose gel and they were purified by Gel Extraction Kit (QIAQuickQiagen).The pACT, pCMV Sport6 plasmid vectors and insert DNA which have same flanking regions were ligated at room temperature for 20 minutes by using Rapid DNA Ligation Kit (Thermo Scientific). Ligation products were transformed into chemically competent DH5α cells with heat shock transformation method. In order to perform heat shock transformation, chemically competent DH5α cells were added into ligation products and they were incubated on ice for 10 minutes. The cells were kept at 42ºC for 90 seconds and they were taken back onto ice for 1 minute. After, 900µl LB medium addition, transformants were incubated in 37ºC shaker incubator for 45 minutes. At the end of this incubation time, 100 µl products were spread on LB Agar plate supplemented with 100µg/µl ampicillin. They were left to grow at 37ºC incubator overnight. After overnight incubation, several colonies were picked and grown in LB broth including 100µg/µl ampicillin at 37ºC shaker incubator. Newly constructed plasmids were isolated by using GeneJet Plasmid Miniprep Kit (Thermo Scientific).

3.2. Preparation of Chemically Competent DH5α Cells

Escherichia coli (*E.coli*) DH5α cells were streaked on LB Agar plate and incubated overnight at 37ºC incubator. Following day, single colony was picked and grown overnight in 5 ml LB medium at 37ºC shaker incubator. Bacterial culture was added into 500 ml LB medium and it was inoculated until its OD_{600} value reached approximately 0.8-0.9. Culture was kept on ice for cooling at cold room and centrifuged in pre-chilled centrifuge tubes at 4000 rcf, 4ºC for 10 minutes. After discarding the supernatant, the pellet was solved in 250 ml 80 mM $CaCl_2$ solution, centrifuged again at 4000 rcf, 4ºC for 10 minutes. Supernatant was discarded again and the washing step was repeated by using 100 ml, 50 ml and 25 ml $CaCl_2$ solution. After the washing step, all of the supernatant was discarded and the pellet was dissolved in 7 ml 50 mM $MgCl_2$ and 3 ml 80% Glycerol mixture. Aliquots were frozen in liquid nitrogen and they were kept at -80ºC.

3.3. Polymerase Chain Reaction

From the list created by the Bayesian method, two candidate genes IFITM1 and IFITM3 were selected for further studies. Primers were designed as forward and reverse including their specific restriction sites. All the primer sequences are listed in Appendix A. The reaction mixture for PCR was prepared by using 5X High Fidelity Reaction Buffer (Thermo Scientific), 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP, 50 ng template DNA, 0.5 µM from each primer and 1 unit of Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The PCR reaction was performed in conditions: 95ºC for 30 seconds, 58ºC for 30 seconds, and 72 º C for 1 minute per 1 kb, and 32 cycles. Amplified genes were visualized in 1% agarose gel and correct-sized band were extracted by using Gel Extraction Kit (QIAQuickQiagen).

3.4 Cell Culture

HEK 293T, NIH 3T3 and U2OS cells were grown in high-glucose Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10% FBS (Sigma-Aldrich), 100

μg/μl Streptomycin and 100 μg/μl Penicillin (Gibco) and 2 mM L-Glutamine (Gibco). Cells were incubated at 37ºC humidified incubator that has constant 5% CO_2. In order to passage confluent cells on dish, all the media was first aspirated, then cells were washed with 5 ml of 1X PBS. After the washing step, cells were resuspended in 5 ml of culture medium and they were seeded on a new 10 cm plate containing fresh culture medium for further growth.

3.5. Mammalian Two Hybrid System

80-90% confluent HEK 293T cells were splitted on a new 10 cm plate 1 day before transfection. Using 70-80% confluent 10 cm plate is optimal for this assay. DNA mixture to be transfected was prepared as follows: 50 ng PG5-*luc* vector, 50 ng clock gene in pBIND (*Cry1*), 50 ng candidate gene in pACT (*IFITM1, IFITM3*), 1 ng *Renilla* in pGL4 plasmid. For each well, total DNA concentration was determined as 300 ng and total DNA mixture volume was 50 μl. Total DNA amount was equalized by using 50 ng empty pCMV Sport 6 plasmid. 50 ng *Per2* in pACT, 50 ng Cry1-pBIND and 50 ng PG5-*luc* was used as positive control while negative control mixture included only 50 ng PG5-*luc* and 250 ng pCMV Sport 6. Prepared DNA mixtures were diluted in 50 μl serum-free DMEM and 0.9 μl TurboFect transfection reagent (Thermo Scientific). Following dilution, all the mixtures were mixed thoroughly and incubated for 20 minutes at room temperature. In these 20 minutes, the medium of the cells which were seeded 1 day before transfection was aspirated and cells were washed with 1X phosphate buffered saline, then the cells were resuspended with culture medium which includes 20% fetal bovine serum, 200 μg/μl Streptomycin and 200 μg/μl Penicillin (Gibco) and 4 mM L-Glutamine (Gibco). Resuspended cells were counted under microscope by using hemocytometer and $5x10^4$ cells were seeded to 96-well plate in 50 μl total volume. After 20 minutes incubation, 50 μl DNA containing reaction mixture was added in each well and 96-well plate was incubated for 24-36 hours at 37ºC incubator (constant 5% CO_2). After 24 h, activity of both firefly and Renilla luciferase was measured using a luminometer. The firefly luciferase activity was normalized

using Renilla luciferase for transfection efficiency. All experimental results are the average of at least three experiments.

3.6. Repression Assay

Confluent HEK 293T cells were splitted into a new 10 cm plate 1 day before transfection. Using 70-80% confluent 10 cm plate is optimal for this assay. For each well, total DNA concentration was 300 ng and total DNA mixture volume was 50 µl. DNA mixture to be transfected was prepared by using 50 ng *mPer1-luc* vector, 50 ng *Bmal1* gene in pCMV Sport 6, 125 ng *Clock* gene in pCMV Sport6, 1 ng *Renilla* in pGL4 plasmid constructs were diluted in 50 µl serum-free DMEM and 0.9 µl TurboFect transfection reagent (Thermo Scientific) was used for efficient transfection. In order to equalize DNA concentration in each well, 50 ng pCMV Sport6 plasmid was used. To be able to see dose dependent effect of IFITM1 and IFITM3 on *mPer1-luc* promotor, different doses of IFITM1 and IFITM3 (from 100 ng to 25 ng) were pipetted into each reaction tubes. On the other hand, 0.5 ng *Cry1* in pcDNA4/myc-His A plasmid was also added into reaction tubes together with IFITM1 and IFITM3 to observe the effect of this combination on BMAL1-CLOCK heterodimerization. 50 ng *Bmal1* in pCMV Sport6, 125 ng *Clock* in pCMV Sport6, 50 ng *mPer1::luc*, 100 ng empty pCMV Sport6 and 1 ng *Renilla* in pGL4 promotor constructs were mixed as positive control while 50 ng *mPer1-luc* vector and 250 ng empty pCMV Sport6 were mixed as negative control. As an extra control, 50 ng *Bmal1* in pCMV Sport6, 125 ng *Clock* in pCMV Sport6, 50 ng *mPer1-luc*, 100 ng empty pCMV Sport6 and 1 ng *Renilla* in pGL4 promotor and 0.5 ng *Cry1* in pcDNA4/myc-His A plasmid was diluted in 50 µl serum-free DMEM like other control reactions. Following dilution, all the mixtures were mixed thoroughly and incubated for 20 minutes at room temperature. In these 20 minutes, the medium of the cells which were seeded 1 day before transfection was aspirated and cells were washed with 1X phosphate buffered saline, then the cells were resuspended with culture medium which includes 20% fetal bovine serum, 200 µg/µl Streptomycin and 200 µg/µl Penicillin (Gibco) and 4 mM L-Glutamine (Gibco). Resuspended cells were counted under microscope by using hemocytometer and $5x10^4$ cells were seeded to 96-well plate

in 50 µl total volume. After 20 minutes incubation, 50 µl DNA containing reaction mixture was added in each well and 96-well plate was incubated for 24-36 hours at 37ºC incubator (constant 5% CO_2). After 24 h, activity of both firefly and Renilla luciferase was measured using a luminometer. The firefly luciferase activity was normalized using Renilla luciferase for transfection efficiency. All experimental results are the average of at least three experiments.

3.7. Protein Overexpression and Western Blot

Approximately $3x10^4$ HEK 293T cells in 2 ml culture medium were seeded on each well of 6-well plate and grown 1 day before transfection. On the day of transfection, each well should be 70-80% confluent. In each well, 2 µg DNA was diluted in 2 ml serum-free DMEM and 3 µl TurfoFect transfection reagent (Thermo Scientific) was used to increase transfection efficiency. The reaction mixture was incubated for 20 minutes at room temperature, and then they were added on the HEK 293T cells which were seeded 1 day before transfection. Plate was rocked gently and incubated in 37ºC incubator containing constant 5% CO_2. 48 hours after transfection, culture medium was aspirated and cells were collected by scrapping with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) Collected cells in 1X PBS were centrifuged at 1800 g, 4ºC for 5 minutes and supernatant was discarded. Pellet was solved in 200 µl cold 1X PBS and mixed with 40 µl 6X protein loading dye (200 mM Tris-Cl, pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol). Cells were mixed with dye and the mixture was boiled at 95ºC for 7 minutes and then they were kept on ice for 1 minute. In order to get rid of cell debris, samples were centrifuged for 10 minutes at 12000 g, room temperature and the soluble phase was loaded onto SDS-PAGE (15% separating gel and 5% stacking gel). The gel was run at 140 mV for 90 minutes and proteins were transferred to polyvinylidene difluoride membrane (Biotrace PVDF, Pall Corporation, FL, and USA) and blocked with 5% milk solution (2 grams milk powder in 40 ml TBS-0.015%Tween20) for 1 hour. Finally, the membrane was incubated in His-tagged primary antibody (1:5000 diluted in TBS-0.015%Tween20) (Anti-6X His tag® antibody-HRP, Abcam) or Flag-tagged (1:1000

diluted in TBS-0.015%Tween20) (Monoclonal ANTI-FLAG® M2 antibody, Sigma-Aldrich). Proteins are visualized by ECL system.

3.8. $(Ca_3PO_4)_2$ Transfection and Co-Immunoprecipitation (Co-IP)

Co-immunoprecipitation of overexpressed proteins was performed using HEK 293T cells. $2x10^6$ cells were seeded in 10 cm plate 1 day prior to transfection. 5-10 µg DNA was diluted in 50 µl 2,5 M $CaCl_2$ and 500µl double distilled water, then 500 µl 2X HBS medium (50 mM HEPES, 10 mM KCl, 12 mM Dextrose, 280 mM NaCl, 1,5 mM Na_2HPO_4) was given into DNA mixture by aeration. This DNA mixture was incubated for 20 minutes at room temperature and pipetted on HEK 293T cells. Cells were incubated at 37ºC incubator containing constant 5% CO_2. In the 12^{th} hour of the incubation, culture medium was changed and after 48 hours incubation, cells were collected by solving in 5 ml cold 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). Cells were centrifuged at 1800 rcf, 4ºC for 15 minutes. Cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton 100-X, 1:100 Proteinase inhibitor, 1:100 PMSF, 1:100 DTT were freshly added). Extracts were incubated overnight with 0.5 mg/ml of ANTI-FLAG® M1 Agarose Affinity Gel (Sigma-Aldrich). Resulting complexes were washed with 1X TBS (50 mM Tris, 150 mM NaCl, 0.1M $CaCl_2$), denatured and eluted. Protein extracts were visualized on SDS-PAGE gel (15% separating, 5% stacking).

3.9. siRNA Knock Down Experiments

$2x10^5$ U2OS *Bmal1::Luc* cells/well was seeded in 6-well plate 1 day prior to transfection. In the day of transfection, each well should be 80-90% confluent. 6 µl of 2 mM siRNA cocktail (Appendix 6) for *Ifitm1*, *Ifitm3* and *Cry1* genes (Qiagen) were diluted in 250 µl Opti-MEM® I Reduced Serum Medium (Gibco). In the meantime, 5 µl Lipofectamine® RNAiMAX was added in 250 µl Opti-MEM® I Reduced Serum Medium (Gibco) for each reaction. These two batches were incubated separately for 5 minutes at room temperature. In the end of 5 minutes, 250 µl Lipofectamine®

RNAiMAX and Opti-MEM® I Reduced Serum Medium (Gibco) mixture was added in siRNA cocktail and Opti-MEM® I Reduced Serum Medium (Gibco) mixture. 500 µl reaction mixture was incubated for 20 minutes at room temperature. In the end of incubation time, 500 µl reaction mixture was pipetted on the cells which were seeded 1 day before transfection. Cells were incubated for 48 hours at 37ºC incubator containing constant 5% CO_2. After 48 hours incubation, cells were collected by using 1 ml TRIzol reagent (Invitrogen, USA) and they were incubated for 5 minutes at room temperature. 200 µl chloroform (Sigma-Aldrich) was added and incubated for 2 minutes at room temperature, then it was centrifuged at 12000 g, 4ºC for 15 minutes. Aqueous phase was taken in a new centrifuge tube and 500 µl isopropanol (Sigma-Aldrich) was added onto the mixture. After inverting the tubes gently, mixture was incubated at room temperature for 10 minutes and centrifuged at 12000 g, 4ºC for 10 minutes. Pellet was washed with 1 ml 70% ethanol solution (Sigma-Aldrich) and dried for 10 minutes at room temperature. Dried pellet was eluted in 40 µl RNAase-free double distilled water at 55-60 ºC heater. (Thermo Scientific). Eluted RNA was loaded in 1% agarose gel and correct-sized band was observed. After extraction of total RNA using TRIzol reagent (Invitrogen, USA), 1 µg of total RNA was used for first-strand cDNA synthesis using random hexamer oligos. qPCR was performed with SuperReal PreMix Plus (SYBR Green) (Tiangen Biotech, Beijing) on the Light Cycler 1.5 (Roche Diagnostics, Germany). qPCR was performed with gene specific primers. Primer sequences were given in Appendix A. 18S rDNA gene was used as an internal reference gene. The amplification programs were performed according to the protocol: 95 ^{0}C for 10 min; 45 cycles of 95 ^{0}C for 2s, 62 ^{0}C for 10s and 72 ^{0}C for 10s, and followed by a thermal denaturing step to generate the melting curves. All reactions were performed in biological triplicates (each triplicate with 2 technical replicates), and the results were expressed relative to the transcription level of 18S rDNA in each sample by using the $2^{-\Delta\Delta CT}$ method.

3.10. Kinetic Luminescence Assay

$2x10^4$ U2OS *Bmal1::luc* cells were seeded in 35 mm plates 1 day before transfection. siRNA transfection was performed as it was written under 3.9.1. Cells were incubated for 48 hours at 37ºC incubator containing constant 5% CO_2. After 48 hours, culture medium was removed and replaced with 2 ml of Lumicycle medium (without Phenol red, with 10% FBS /NEAA/ 10 mM HEPES, 0.1 mM dexamethasone, 0.1 mm luciferin). Bioluminescence patterns were monitored using a LumiCycle luminometer (Actimetrics). Briefly, after change to fresh explant medium at ambient temperature, culture dishes containing cells or explants were sealed and placed into the luminometer, which was kept inside a standard tissue culture incubator at 36°C. Bioluminescence from each dish was continuously recorded with a photomultiplier tube (PMT) for ~70 sec at intervals of 10 minutes. Raw data (counts/sec) were plotted against time (days) in culture. For analysis of rhythm parameters, we used the LumiCycle Analysis program (Actimetrics). Raw data were baseline fitted, and the baseline-subtracted data were fitted to a sine wave (damped), from which the period was determined.

CHAPTER 4

RESULTS

4.1. Gene Cloning

A previous study showed that *Ifitm1* gene is most likely a clock modifier or acts as core clock component [116]. Study by Anafi et al. showed that, *Ifitm1* interacts with CRY1 protein and siRNA of the *Ifitm1* in U2OS cell line results in long period. We used Circa DB [117] to see mRNA level oscillations of the *Ifitm* gene family and we observed that both *Ifitm1* and *Ifitm3* are oscillating in the SCN. Therefore, we decided to work with these two genes to discover their role in the circadian clock.

These genes were amplified by polymerase chain reaction (PCR) using primer *Iftim1*-Forward, *Iftim1*-Reverse, *Iftim3*-Forward, *and Iftim3*-Reverse (Appendix 3) to clone into mammalian expression vectors. The qualities of PCR product were assessed in 0.8% agarose gel (Figure 4.1.A). As seen in Figure 4.1A, the size of the fragments were around approximately 300 and 450 bp which corresponds to the actual sizes of the open reading frames of 321bp for IFITM1 and 441 bp for IFITM3, respectively. Then, PCR products along with pACT were purified and then digested with NotI and EcoRV restriction enzymes. *Ifitm1* was ligated into both pACT and Flag-tagged pCMV-Sport6 shown in Figure 4.1.

In order to confirm the presence of the inserts in pACT and pCMV-Sport6 plasmids, purified plasmids after sub-cloning were double digested with EcoRI and HindIII. Positive colonies were shown in the Figure 4.1.B (right panel and the left panel) for pACT and pCMV-Sport6.

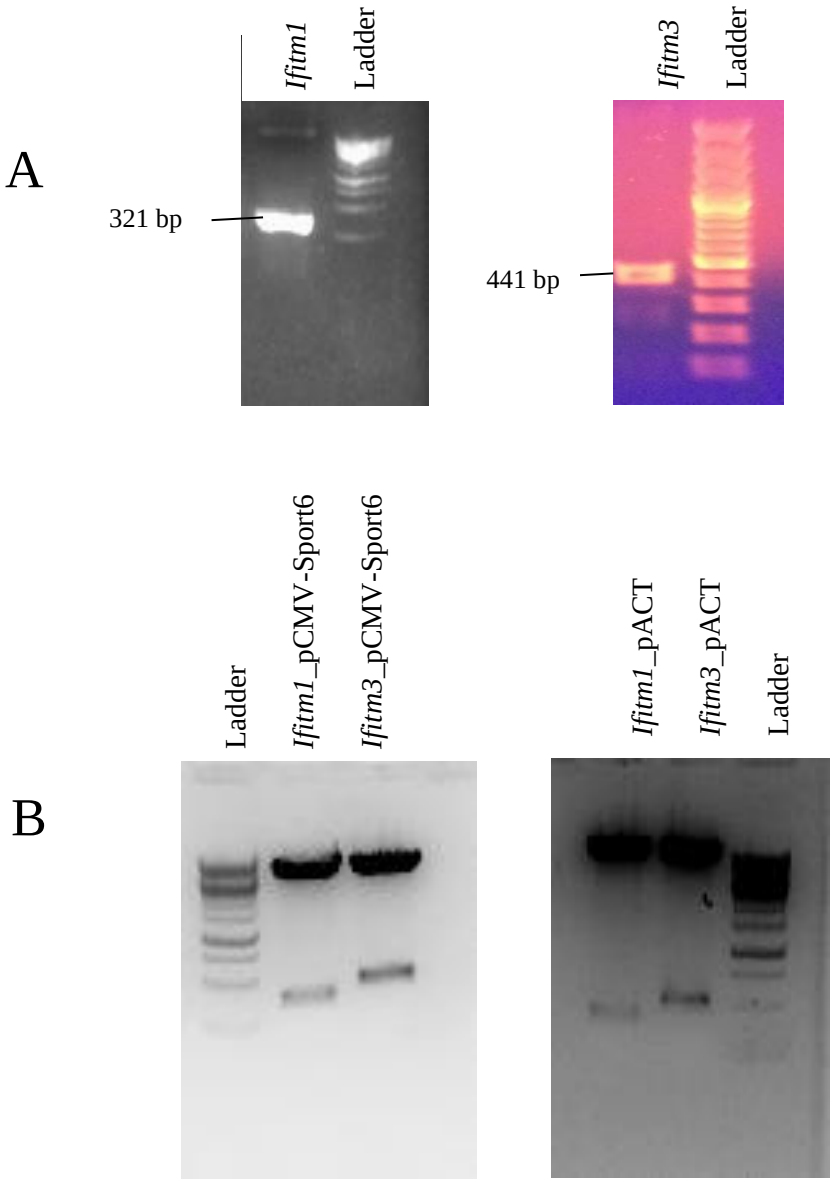


Figure 4.1. Cloning of IFITM1 and IFITM3 (A) PCR product of IFITM1 gave a single band with a size of 321 bp and IFITM3 gave a single band with a size of 414 bp. (B) To confirm the existence of the inserts in the vectors, constructs were double digested and run in 0.8% agarose gel.

4.2. Mammalian Two-Hybrid Assay

Mammalian two-hybrid assay is a powerful method to investigate protein-protein interactions. This is a genetic, in vivo assay based on the reconstitution of the function of a transcriptional activator. In this system, one protein of interest is cloned into a pBIND plasmid which carries GAL4 DNA binding domain, the other possible interacting partner is cloned in a pACT plasmid which carries transcriptional activation domain called VP16. The basis of this method is the interaction between DNA-binding domain (GAL4) and transcriptional activation domain (VP16). Additionally, PG5*luc* plasmid carries firefly luciferase reporter gene fused with of five GAL4 DNA-binding domains. When the protein fused with GAL4 DNA binding domain, which is cloned to pBIND plasmid interacts with the other protein fused with VP16 domain expressed from pACT, they form a complex and it leads to recruitment of RNA polymerase II complex onto the *Gal4* promoter. As an overall result, this binding increases the firefly luciferase reporter gene transcription [118].

The mammalian two-hybrid system can be used as a complementary approach to verify protein-protein interactions. This system is more advantageous than the other methods which test protein-protein interaction *in vivo*. This assay generate results within 48 hours of transfection and protein interactions in mammalian cells can better mimic the actual mammalian cell environment [119].

In order to confirm the interaction between IFITM1 and CRY1, HEK 293T cells were co-transfected with pACT-*Ifitm1-VP16*, pBIND-*GAL4-Cry1* and *pGL5luc* plasmids. In addition, a possible interaction between IFITM3 and CRY1 was investigated by using pACT-*Ifitm3-VP16*, pBIND-*GAL4-Cry1*and *pGL5luc* plasmids.

In all experiments, luciferase activity was normalized to *Renilla* containing pGL4 plasmid to eliminate fluctuations in transfection efficiency between different experiment sets. The luciferase activity between empty pACT and pBIND vectors and their crossed configurations (pACT-*Ifitm1-VP16*_pBIND and pBIND-*GAL4-Cry1*_pACT) gave negative results. For IFITM1 and CRY1 interaction, almost 5-fold difference was confirmed as it was indicated in Figure 4.2.B. As it was indicated in Figure 4.2.A,

expression of pACT-*Ifitm1-VP16* construct is confirmed by WB to make sure that genes are being expressed.

With a similar experiment, the interaction between IFITM3 and CRY1 was also investigated. As seen in Figure 4.3.A., there was no interaction between IFITM3 and CRY1 proteins. To see whether *Ifitm3* is expressed under those conditions, I performed Western Blot analysis. The result indicated that *Ifitm3* was expressed in HEK293T cells (Figure 4.3.B.)

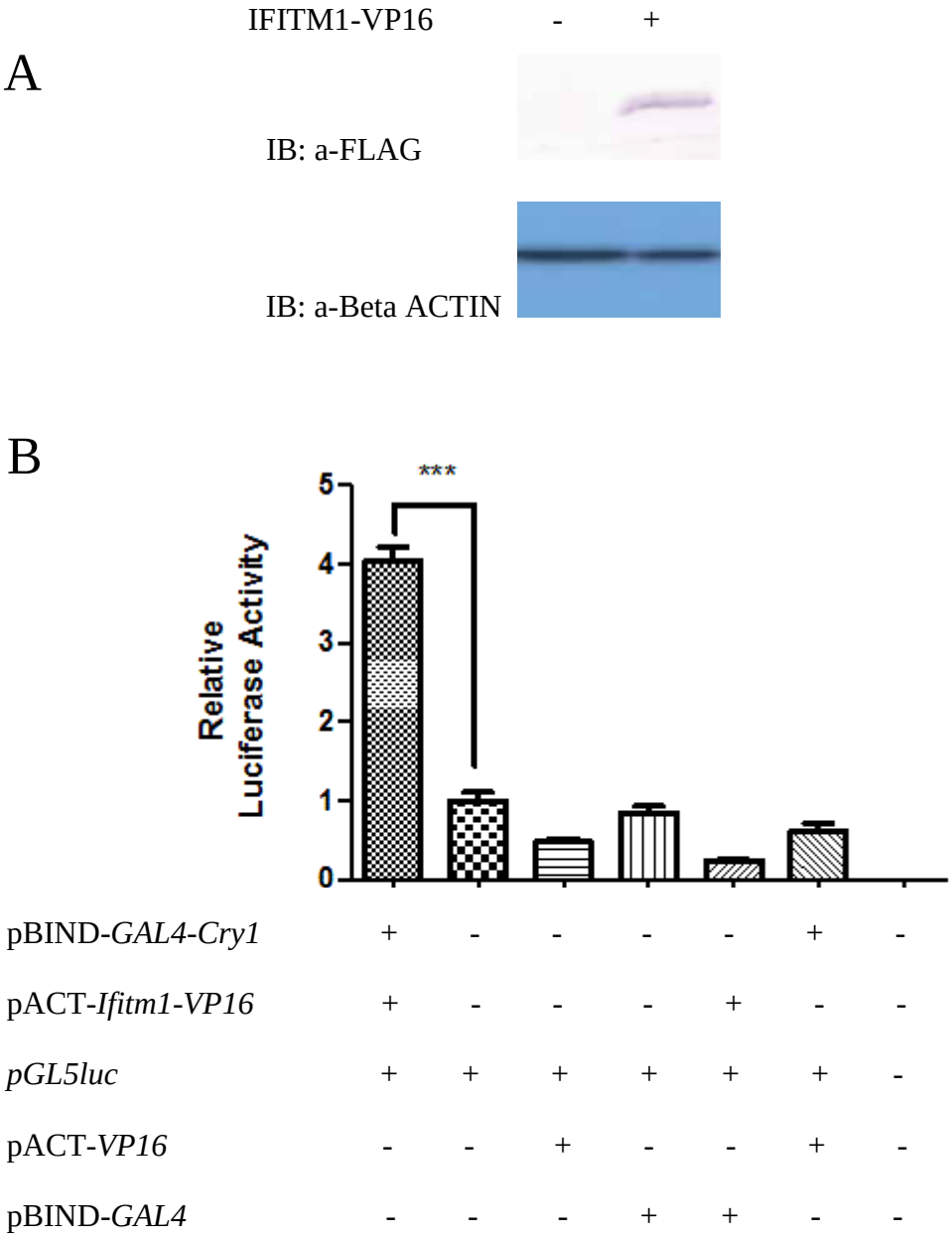


Figure 4.2.. Interaction between IFITM1 and mCRY1 domains (A) Mammalian two-hybrid assay of IFITM1 and mCRY1. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *pGL5luc.* Student's t test shows statistically significant results (***p<0.005). Error bars represent mean ±SEM. (B) Total cell extract (upper panel) of pACT-*Ifitm1-VP16* transfected cells were subjected to Western blotting using an anti-Flag antibody.

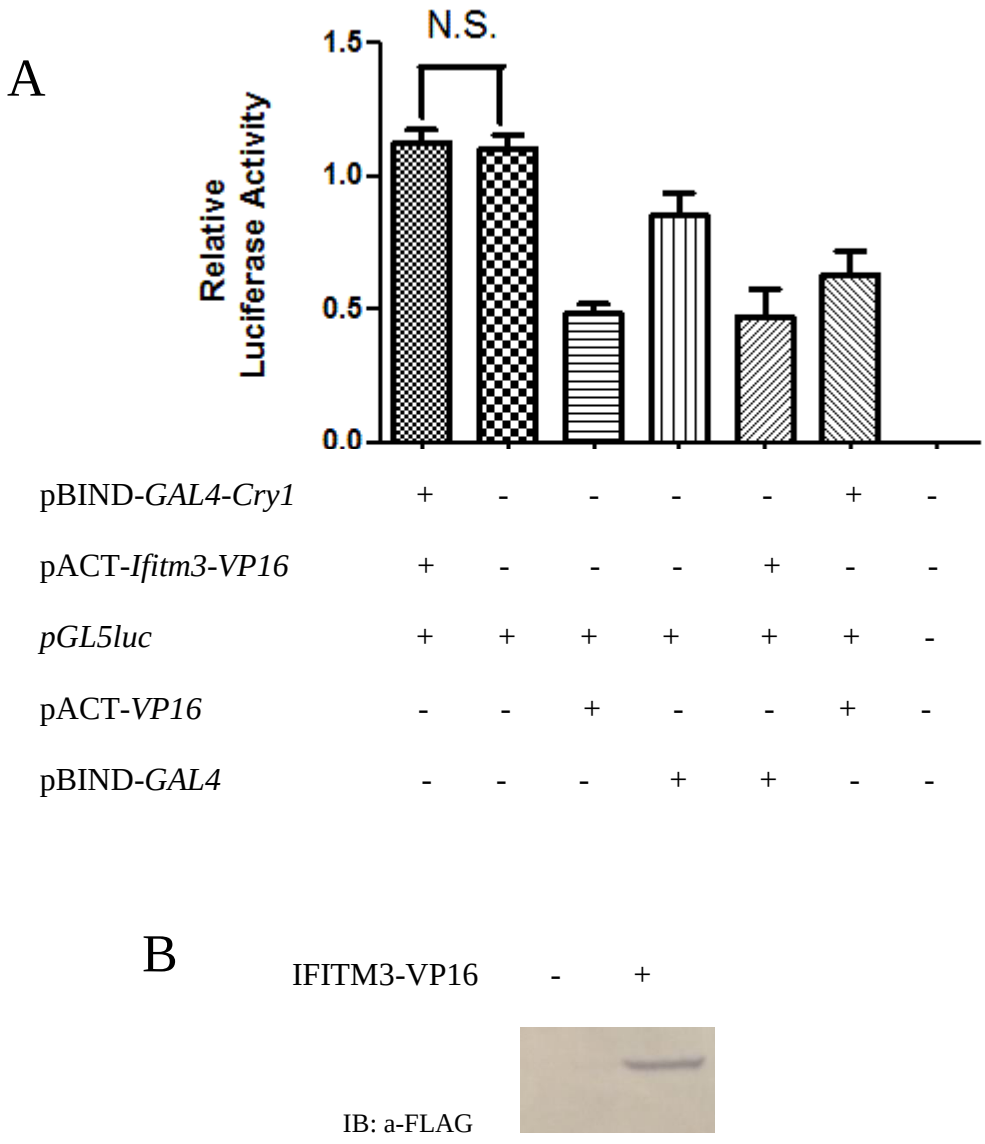


Figure 4.3. IFITM3 does not interact with mCRY1 (A) Mammalian two-hybrid assay of IFITM3 and mCRY1. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *pGL5luc.* Student's t test shows statistically significant results (**p<0.05). Error bars represent mean ±SEM. (B) Total cell extract (upper panel) of pACT-*Ifitm3-VP16* transfected cells were subjected to Western blotting using an anti-Flag antibody.

4.3. The specificity of the interaction between IFITM1 and CRY1

To identify the exact region of CRY1 interacting with IFITM1, M2H assay was performed. There are three distinct regions of CRY1 (Figure 4.4.A.). These domains are α/β domain (D1) and α-helical domain in photolyase homology region (D2), carboxyl-terminal domain (D3). The PHR domain is the evolutionary conserved core domain and it is essential for the protein to be functional, but C-terminal tail domain is not necessary for the repression. However, it has important roles in modulating rhythm amplitude and period length [49]. For example, PER2 [74] and FBXL3 [18] have been previously shown to interact with CTD of CRY1.

Therefore, we used truncated version of *Cry1*, which lacks 20, 40, 60, and 100 amino acids from the C-termini (called mCry1-T1, mCry1-T2, mCry1-T3, mCry1-T4, mCry1-T5, and mCry1-T6 respectively) to investigate the exact interaction region of CRY1. (Figure 4.4.B.)

To find the exact interaction region between IFTIM1 and CRY1, M2H assay was performed. The result showed that last 20 amino acid of CRY1 was important for the interaction with IFITM1 (Figure 4.5.).

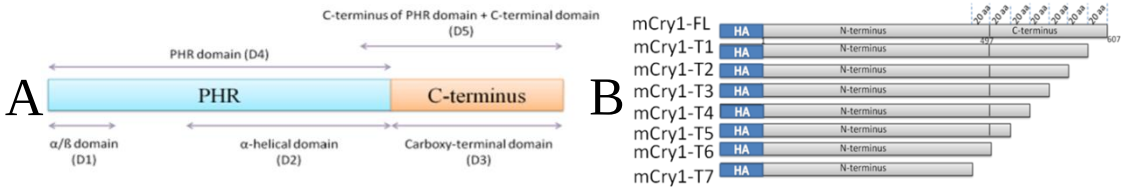
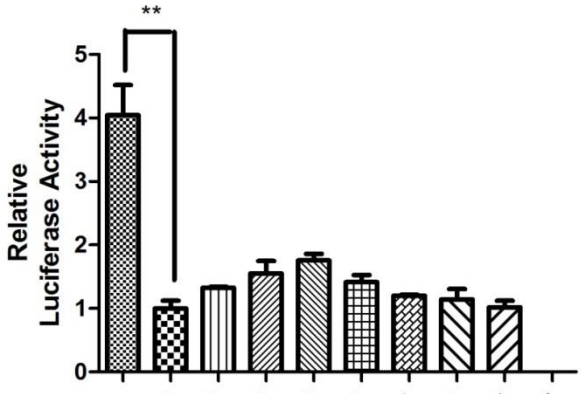


Figure 4.4. Schematic illustration of mCRY1 (A) Schematic illustration of mCRY1. (B) Schematic illustration of truncated structures of mCRY1



pACT-*Ifitm1-VP16*	+	-	+	+	+	+	+	+	+	-
pBIND-*GAL4-Cry1*	+	-	-	-	-	-	-	-	-	-
pBIND-*GAL4-Cry1*T1	-	-	+	-	-	-	-	-	-	-
pBIND-*GAL4-Cry1*T2	-	-	-	+	-	-	-	-	-	-
pBIND-*GAL4-Cry1*T3	-	-	-	-	+	-	-	-	-	-
pBIND-*GAL4-Cry1*T4	-	-	-	-	-	+	-	-	-	-
pBIND-*GAL4-Cry1*T5	-	-	-	-	-	-	+	-	-	-
pBIND-*GAL4-Cry1*T6	-	-	-	-	-	-	-	+	-	-
pBIND-*GAL4-Cry1*T7	-	-	-	-	-	-	-	-	+	-
pGL5luc	+	+	+	+	+	+	+	+	+	-

Figure 4.5. IFITM1and CRY1 interaction requires an intact CTD domain of CRY1. M2H assay of IFITM1 and CRY1 domains.

4.4. Identification of the amino acids in CRY1 interact with IFTIM1

To map the amino acids in CRY1 which mediate interaction with IFITM1, PCR based approach was used in the presence of degenerate primers that target the last 20 amino acids. PCR product of CRY1 gene was cloned into pBIND vector and 15 colonies were picked up. Mutation carrying plasmids of *Cry1* were prepared by Dr. Asimgil and Hasimcan Oner and transfected to HEK293T cell line along with plasmids contains luciferase gene and *Ifitm1* cDNA to identify mutants that do not interact with *Ifitm1*. As seen in Figure 4.6., most of the mutants fail to interact with IFTIM1 except *Cry1* mutants named Deg 5, 9 and 10. To find out the nature of the mutants all *Cry1* mutants were sequenced and last 20 amino acids were compared with wild type amino acid sequence of the CRY1 (Table 4.1). The comparison of wild type and mutant amino acid alignments indicated that whenever Arg replaced with Pro or Leu at the position 602 cause disruption of interaction between those proteins. Over all these study indicated that the last 20 amino acids of the CRY1 are necessary for the interaction with IFITM1. (Figure 4.6.)

Table 4.1. The list of degenerate CRY1 consrtucts with their aminoacid changes.

WT	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	R	Q	S	S	N
DEG1	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	R	Q	I	S	K
DEG2	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	P	Q	S	S	K
DEG3	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	L	Q	I	S	N
DEG4	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	P	Q	S	S	K
DEG5	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	R	Q	S	S	K
DEG7	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	P	Q	S	S	K
DEG8	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	P	Q	S	S	K
DEG9	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	R	Q	S	S	K
DEG10	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	R	Q	S	S	K
DEG11	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	L	Q	S	S	K
DEG12	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	P	Q	S	S	K
DEG13	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	P	Q	I	S	K
DEG14	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	P	Q	S	S	K
DEG15	P	S	Q	E	E	D	A	Q	S	V	G	P	K	V	Q	P	Q	S	S	K

pACT-*Ifitm1-VP16*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	
pBIND-*GAL4-Cry1*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG2	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG3	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG4	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG5	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG7	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG8	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG9	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG10	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG11	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG12	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	
pBIND-*GAL4-Cry1*DEG13	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	
pBIND-*GAL4-Cry1*DEG14	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	
pBIND-*GAL4-Cry1*DEG15	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	
pGL5luc	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-		

Figure 4.6. IFITM1and degenerate CRY1 interaction requires an intact CTD domain of CRY1. M2H assay of IFITM1 and degenerate CRY1.

4.5. Physical Interaction between IFITM1 and CRY1 by Co-immunoprecipitation

Co-IP is a technique to identify the interacting partners of a protein or protein complex. An antibody against a specific target protein forms an immune complex with the protein in the cell lysate. After the target protein in the cell lysate is captured by specific antibody, non-specific binding partners are washed away by different washing solutions which contain different detergents. The stringency of these washing solutions depends on the salt concentration, pH, and detergent content in the buffers. When all the nonspecific interactions are discarded from the system, target protein which is captured by antibody is loaded in SDS gel. Finally, the target protein and its interacting partners is visualized by Western blot [120].

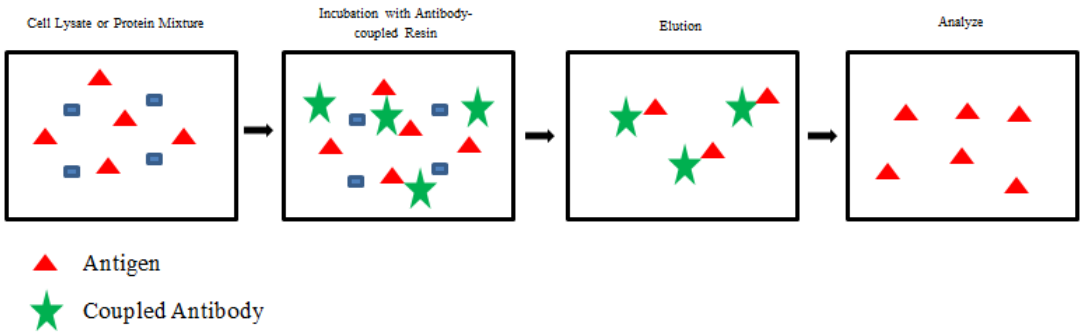


Figure 4.7. Schematic representation of co-immunoprecipitation assay [*Adapted from 120*]

To see a direct physical interaction these CRY1 and IFITM1 between these proteins Co-IP was performed. HEK 293T cells were transiently transfected with Flag tagged pCMV-Sport6-*Ifitm1* and pcDNA4/*myc*-His-*Cry1*plasmids. After 48 hours incubation, cell lysates were collected with cold 1X PBS buffer and boiled until the cell structure is disrupted. Then pcDNA4/*myc*-His-*Cry1* overexpressed cell lysate was incubated with Anti-Flag resin to eliminate nonspecific interactions and pCMV-Sport6-*Ifitm1* containing cell lysate was mixed with pcDNA4/*myc*-His-*Cry1* containing lysate and IFITM1 target proteins were captured by Anti-Flag resin. After overnight incubation,

lysate mixtures were washed with washing buffer and the final results were shown following standard Western blot procedure by using anti-His antibody.

As it is seen in Figure 4.8., there is a physical interaction between IFITM1 and CRY1 proteins. The first band indicates that Flag-tag containing IFITM1 protein was captured by Anti-Flag resin. The second band shows that, CRY1 proteins which interacts with IFITM1 was stained with Anti-His antibody. Additionally, this result also indicates that the interaction between IFITM1 and CRY1 proteins is specific, because there is no band seen in the CRY1 gene which lacks 20 amino acid on its C termini.

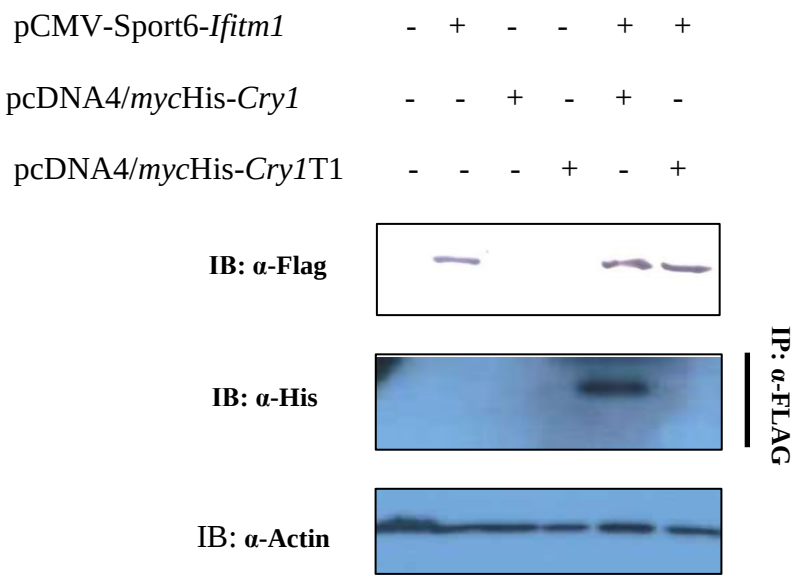


Figure 4.8. IFITM1 physically interacts with mCRY1. HEK 293T cells were transfected with pCMV-Sport6-*Ifitm1*, pcDNA4/*myc*His-*Cry1* and pcDNA4/*myc*His-*Cry1*T1. Total cell extracts or immunoprecipitates using an Anti-Flag antibody were subjected to Western blotting using an antibody against His-tagged mCRY1.Blots and co-immunoprecipitation was repeated twice. IB Immunoblot, IP Immunoprecipitation.

4.6. The Effect of IFITM1 on *mPer1* Promotor

As it was referred in Chapter 2, the mammalian circadian clock operates through an auto-regulatory transcriptional and translational feedback loop composed of four clock components. The positive arm of this loop consists of BMAL1 and CLOCK proteins, which are called transcriptional activators, and the negative arm elements are Periods (PERs) and Cyrptochromes (CRYs) called transcriptional repressors. The positive arm elements bind E-box and initiate the transcription of *Period* and *Cryptochrome* gene expressions along with other clock controlled genes [121]. Then CRYs and PERs accumulate in cytosol and translocate into the nucleus, where they repress BMAL1-CLOCK driven transcription. To see the effect of the IFITM1 on the CRY1 repression activity *mPer1-luc* assay was used.

To do that HEK 293T cells were transiently transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and *mPer1::Luc* vectors along with Cry1 and Ifitm1. As seen in Figure 4.9., in the presence of the IFITM1, CRY1 repressor activity was reduced by approximately 15%. On the contrary, under the same conditions IFITM3 did not have any effect on CRY1 activity. (Figure 4.10.) To see is whether the effect is not E-box-dependent or not, the same experiment was performed in the absence of the *Bmal1* and *Clock*. As it was seen in the Figure 4.12., *luciferase* gene activity did not change for IFITM1 and IFITM3, so either IFITM1 or IFITM3 does not bind directly to the *mPer1::Luc* promotor.

Besides inhibition activity on CRY1 repressor, only IFITM1 enhances the transcription of BMAL1-CLOCK complex. As it is clearly seen in the Figure 4.10, when HEK 293T cells were transiently transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and *mPer1::Luc* vectors along with the *Ifitm1*, BMAL1-CLOCK activity increased by approximately 25%. In contrast, only IFITM3 did not give the same effect on *mPer1::Luc* promotor. (Figure 4.11.) Such observation is may be due to the interaction between endogenous CRY1 and IFTIM1 and this cause release of CRY1 repressor activity.

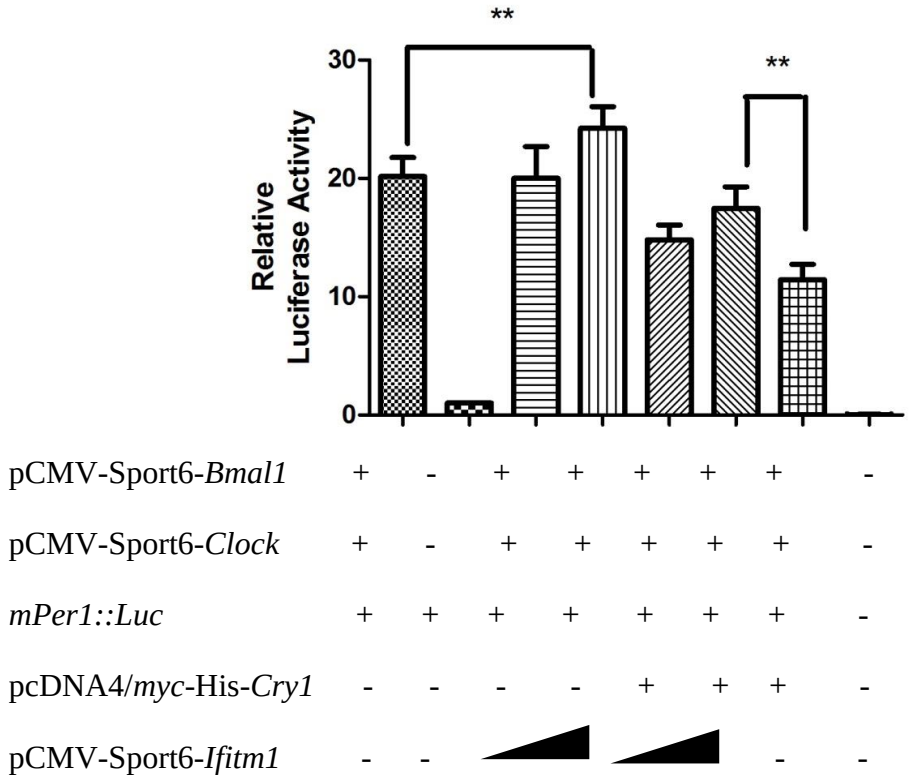


Figure 4.9. Dose dependent IFITM1 has a significant effect on *mPer1::Luc* promotor with BMAL1-CLOCK complex. HEK 293T cells were co-transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and increasing doses of pCMV-Sport6-*Ifitm1* plasmids. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *mPer1::Luc.* Student's t test shows statistically significant results (**$p<0.05$). Error bars represent mean ±SEM.

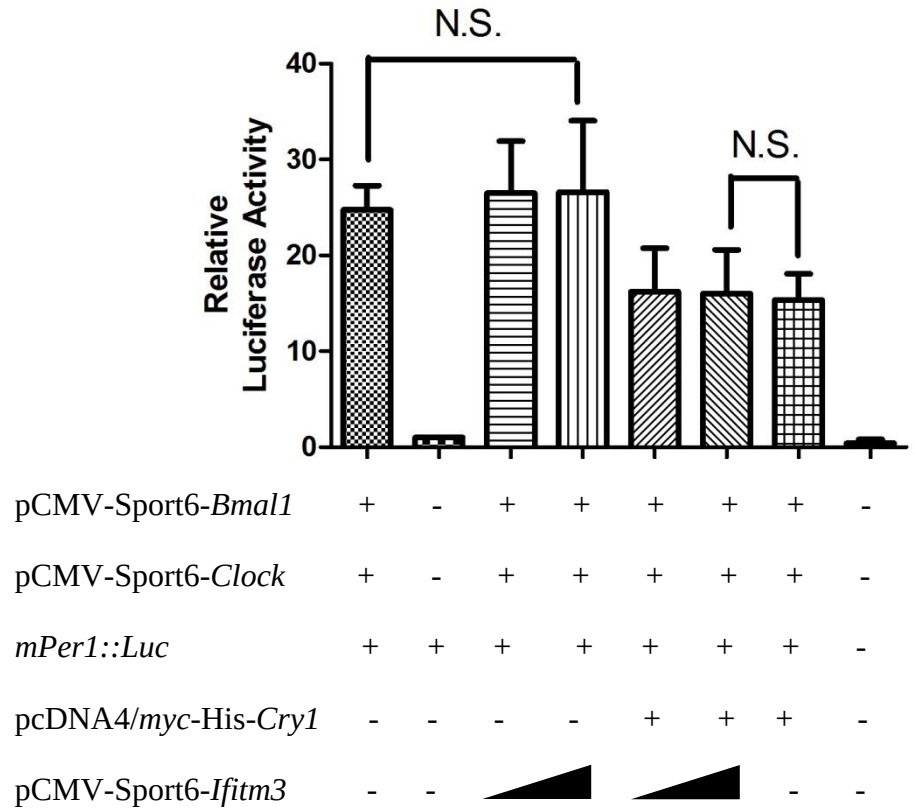


Figure 4.10. IFITM3 does not have a significant effect on *mPer1::Luc* promotor with BMAL1-CLOCK complex. HEK 293T cells were co-transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and increasing doses of pCMV-Sport6-*Ifitm3* plasmids. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *mPer1::Luc*. Student's t test shows statistically significant results (**$p<0.05$). Error bars represent mean ±SEM. N.S. means nonsignificant.

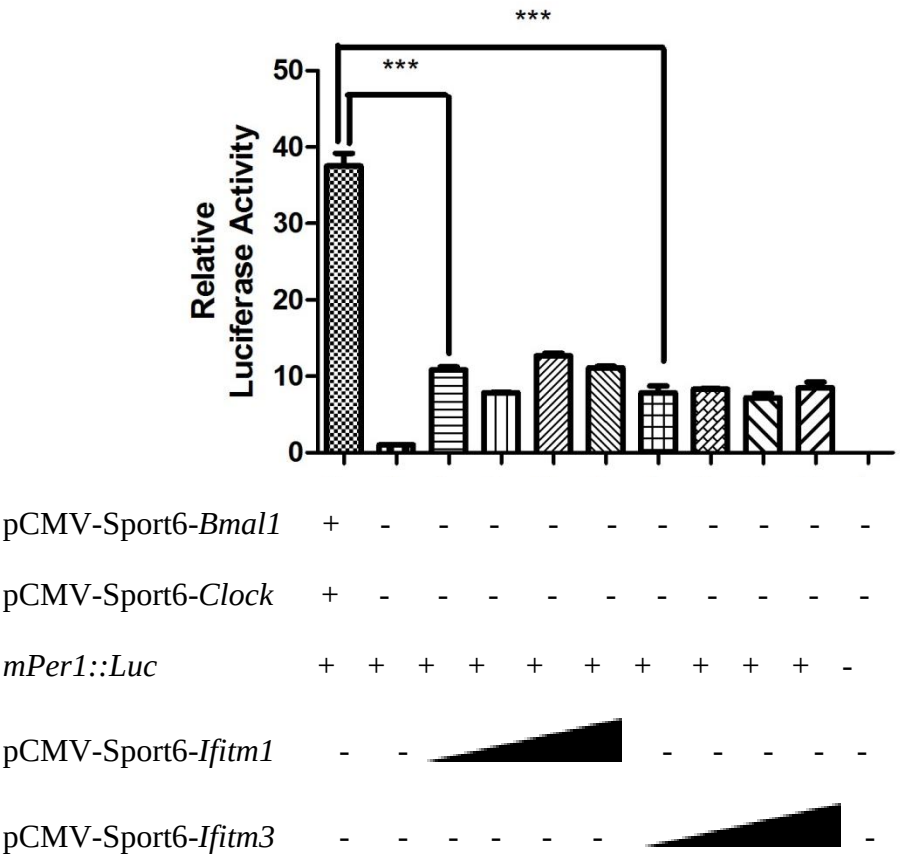
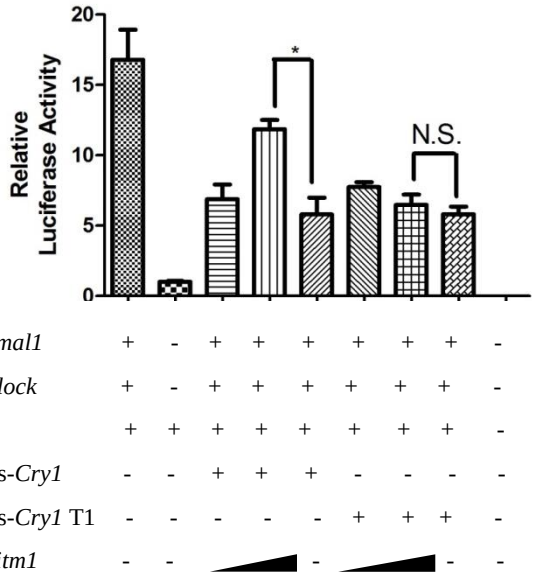


Figure 4.11. Either IFITM1 or IFITM3 does not have a direct effect on *mPer1::Luc* promotor. HEK 293T cells were transfected with increasing doses of pCMV-Sport6-*Ifitm1*and pCMV-Sport6-*Ifitm3* plasmids. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *mPer1::Luc.* Student's t test shows statistically significant results (***p<0.005). Error bars represent mean ±SEM.

After seeing the effect of IFITM1 on mPER1 promotor, we decided to control the specificity of this interaction by doing the same assay with truncated CRY1 protein (T1). It is already known that C-terminal domain is not essential for the protein to be functional [49]. For this reason, *Bmal1-Clock* genes were overexpressed in HEK 293T cells. In addition *Cry1* and truncated *Cry1* (T1) were transfected to see the effects on increasing doses of IFITM1 and IFITM3. The results show that, IFITM1 represses repression activity when it binds to C-termini of the CRY1, but such effect is not observed when truncated CRY1 does not interact with IFITM1. (Figure 4.12.A.) On the other hand, the same constraint effect is not the case for our negative control, IFITM3 (Figure 4.12.B.)

A



B

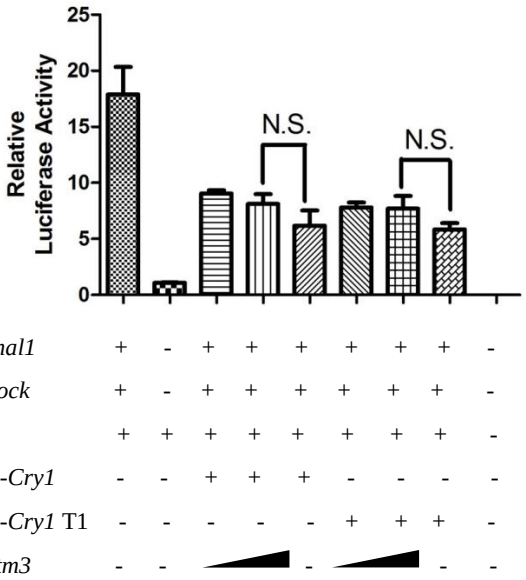


Figure 4.12. IFITM1 represss the repression activity of CRY1, but IFITM3 does not. HEK 293T cells were co-transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock*, pcDNA4/*myc*-His-*Cry1*, pcDNA4/*myc*-His-*Cry1* T1 and increasing doses of pCMV-Sport6-*Ifitm1* and 3 plasmids. For each experiment (n=3), values obtained for cells transfected with the luciferase reporter alone were normalized to *mPer1::Luc*. (A) Student's t test shows statistically significant results (*p<0.5). (B) N.S.means nonsignificant. Error bars represent mean ±SEM.

4.8. The Effect of IFITM1 and IFITM3 on *mCry1* Promotor

To further see the effects of IFITM1 and IFITM3 proteins on different E-Box containing promotor, similar experiment sets were also performed for m*Cry1*promoter. In light of the results which were performed on *mPer1* promoter, BMAL1, CLOCK and *mCry1::luc* promotor carrying plasmids were overexpressed in HEK293T cells. The results were similar with the experiments, which were done on *mPer1*. In Figure 4.13., increasing IFITM1 level elevates the luciferase reporter gene expression which means repressing the repression activity on BMAL1-CLOCK heterodimerization. Moreover, IFITM3 does not have any significant effect on overexpressed *mCry1::luc* promoter (Figure 4.14.)

Similar to my results using the *mPer1::Luc* promoter, when the HEK 293T cells were transiently transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and *mCry1::Luc* vectors along with the *Ifitm1*, BMAL1-CLOCK, transcription increases by 60% relative to the cells transfected pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and *mCry1::Luc* vectors except *Ifitm1*.

To see whether IFITM1 and IFITM3 have a direct effect on *mCry1::luc* promoter, HEK 293T cells were transfected with the increasing doses of both IFITM1 and IFITM3. Figure 4.15. and Figure 4.16. show that IFITM1 has a direct effect on *mCry1::luc* promoter, but IFITM3 does not. All the data support that, IFITM1 has a direct and indirect effect on the *mCry1::luc* promoter.

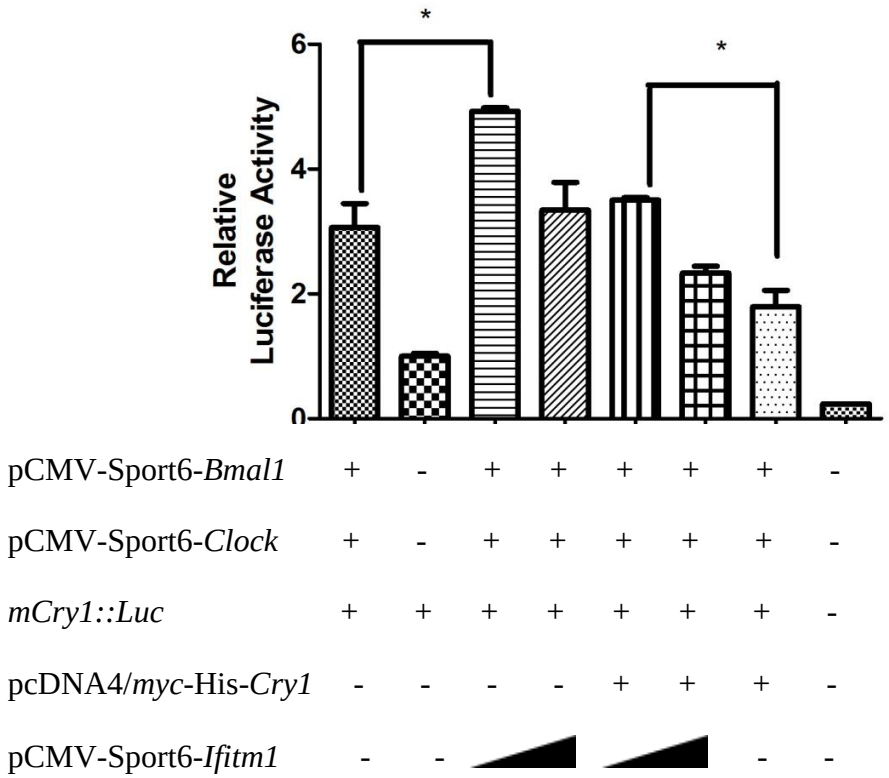


Figure 4.13. Dose dependent IFITM1 has a significant effect on *mCry1::Luc* promotor with BMAL1-CLOCK complex. HEK 293T cells were co-transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and increasing doses of pCMV-Sport6-*Ifitm1* plasmids. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *mCry1::Luc.* Student's t test shows statistically significant results (*$p<0.5$). Error bars represent mean ±SEM.

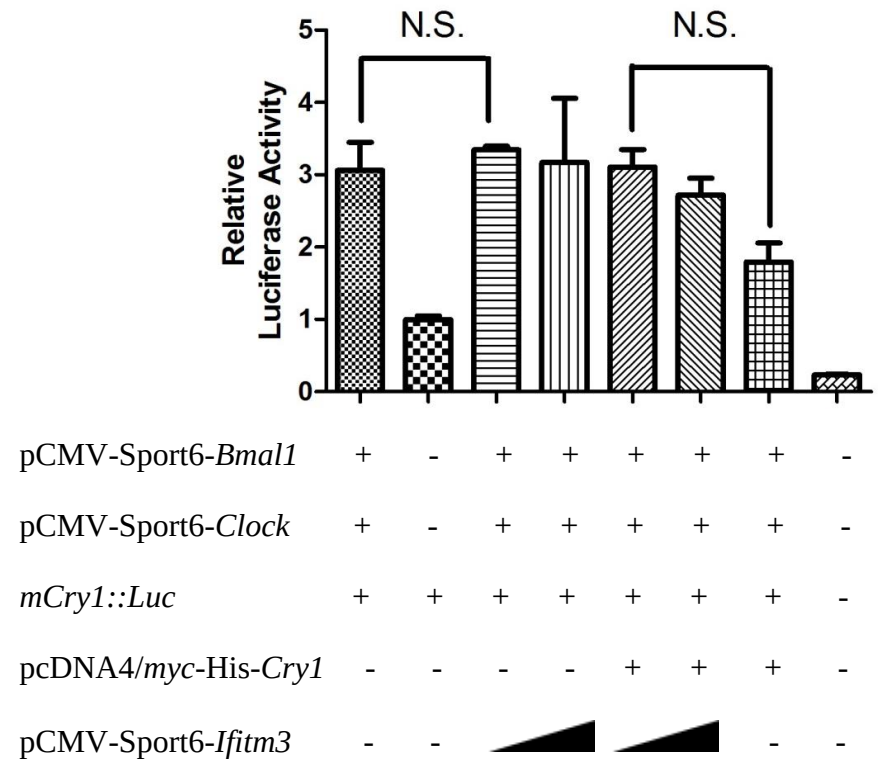
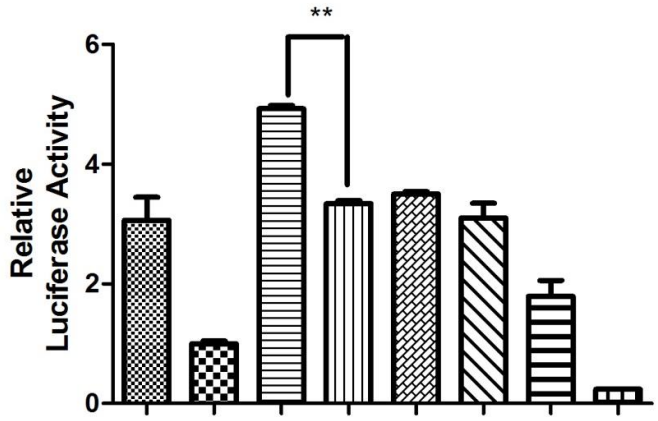


Figure 4.14. IFITM3 does not have a significant effect on *mCry1::Luc* promotor with BMAL1-CLOCK complex. HEK 293T cells were co-transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and increasing doses of pCMV-Sport6-*Ifitm3* plasmids. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *mCry1::Luc.* Student's t test shows statistically nonsignificant results. Error bars represent mean ±SEM. N.S. means nonsignificant.



pCMV-Sport6-*Bmal1*	+	-	+	+	+	+	+	-
pCMV-Sport6-*Clock*	+	-	+	+	+	+	+	-
mCry1::Luc	+	+	+	+	+	+	+	-
pcDNA4/*myc*-His-*Cry1*	-	-	-	-	+	+	+	-
pCMV-Sport6-*Ifitm1*	-	-	+	-	+	-	-	-
pCMV-Sport6-*Ifitm3*	-	-	-	+	-	+	-	-

Figure 4.15. IFITM1 has a significant effect on *mCry1::Luc* promotor with BMAL1-CLOCK heterodimer, but IFITM3 does not. HEK 293T cells were co-transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*Clock* and increasing doses of pCMV-Sport6-*Ifitm1*and pCMV-Sport6-*Ifitm3* plasmids. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *mPer1::Luc.* Student's t test shows statistically significant results (**$p<0.05$). Error bars represent mean ±SEM.

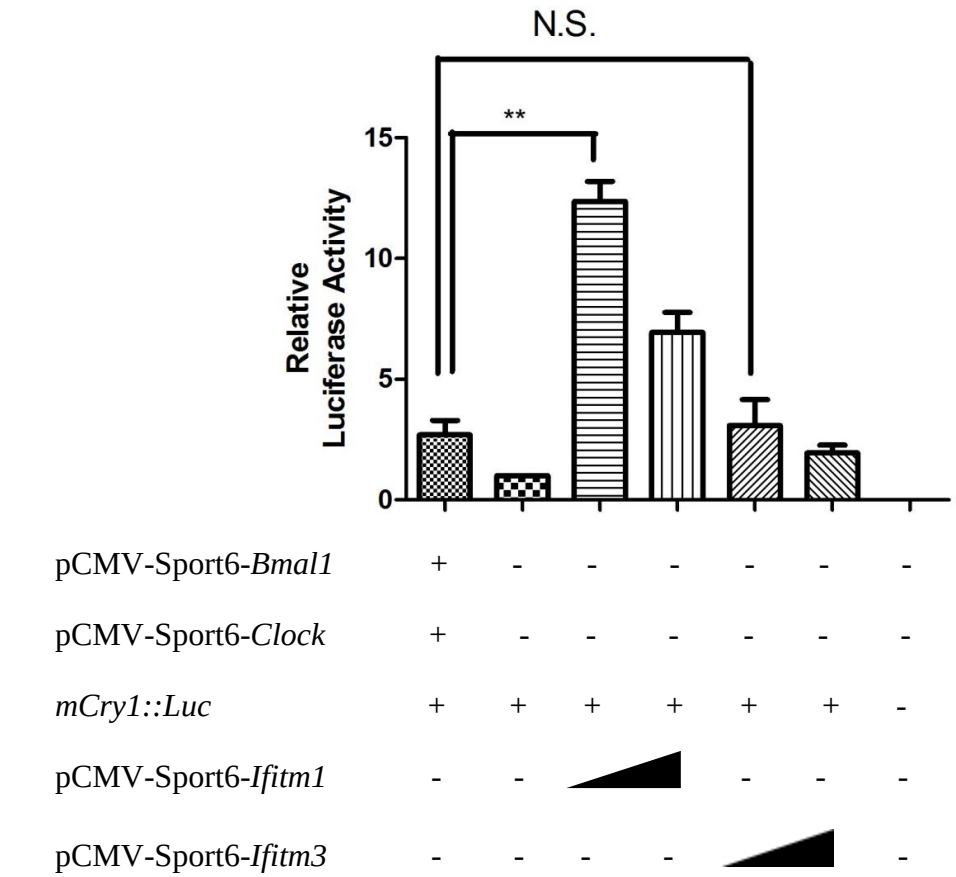


Figure 4.16. Either IFITM1 or IFITM3 does not have a direct effect on *mCry1::Luc* promotor. HEK 293T cells were transfected with increasing doses of pCMV-Sport6-*Ifitm1*and pCMV-Sport6-*Ifitm3* plasmids. For each experiment (n=9), values obtained for cells transfected with the luciferase reporter alone were normalized to *mCry1::Luc.* Student's t test shows statistically significant results (**p<0.005). Error bars represent mean ±SEM. N.S. means nonsignificant.

4.9. *Ifitm1* binding to CRY1 did not result in dissociation from BMAL1-CLOCK complex

Next, we want to see effects of interaction between IFITM1 and CRY1 on BMAL1-CLOCK heterodimer complex. For this reason, we designed an experiment whether IFITM1 binds and inhibits CRY1 to bind BMAL1-CLOCK complex or not. To clarify our hypothesis, we used GAL4-binding pGL5 promoter which is used in mammalian two-hybrid system. In this assay, we used increasing doses of pCMV-Sport6-*Ifitm1* and pCMV-Sport6-*Ifitm3* plasmids to BMAL1-CRY1 heterodimer complex in HEK 293T cells. The reason behind selecting BMAL1-CRY1 interaction is i) we know that there is an interaction between BMAL1 and CRY1. [122] ii) we know that IFITM1 interacts with CRY1. Because of these two supporting idea, we performed this assay on pACT-*Bmal1*-VP16 and pBIND-*GAL4-Cry1* interaction and the results showed that increasing doses of IFITM1 elevates the interaction between BMAL1 and CRY1, but IFITM3 does not have any effect on pGL5 promoter like in the previous experiments.

By taking this result into account, we can clearly say that IFITM1 does not inhibit CRY1 binding to BMAL1-CLOCK heterodimer. (Figure 4.17) However, this result suggested that IFITM1 might have a positive impact on BMAL1-CRY1 interaction. In a previous study, a similar result was observed with increasing amounts of *Clock* expression with *Gal4-Bmal1* and *Cry1-VP16* [122] [123]. Consequently, we can say that IFITM1 may stabilize the interaction between BMAL1-CRY1, possibly by inducing conformational changes in CRY1 that facilitate BMAL1 binding.

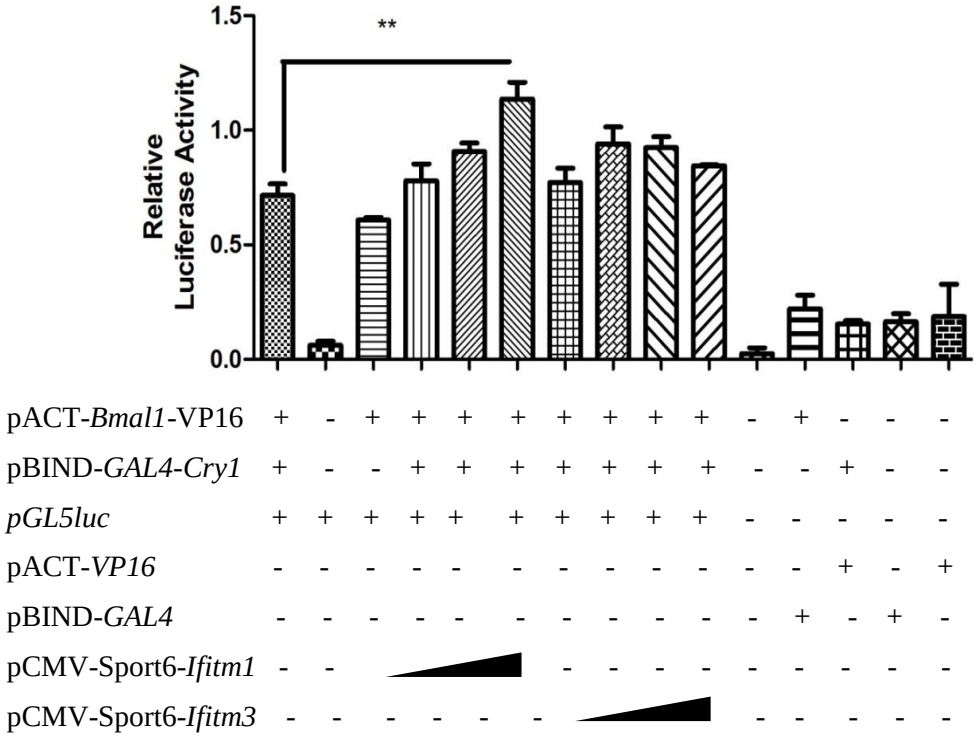


Figure 4.17. .IFITM1 and IFITM3 effect on pGL5 promotor. HEK 293T cells were co-transfected with pACT-*Bmal1*-VP16, pBIND-*GAL4-Cry1*, *pGL5luc* and increasing doses of pCMV-Sport6-*Ifitm1* and 3 plasmids. For each experiment (n=3), values obtained for cells transfected with the luciferase reporter alone were normalized to *pGL5luc*. Student's t test shows statistically significant results (**$p<0.05$). Error bars represent mean ±SEM.

4.10. The Effects of *Ifitm1* and *Ifitm3* on Circadian Rhythm

To consider the effect of *Ifitm1* and *Ifitm3* on the wild type core component oscillations in the cell, U2OS *Bmal1::Luc* cells were transfected with siRNAs designed against both *Ifitm1* and *Ifitm3.* Bioluminescence oscillations were recorded from luciferase reporter plasmid. According to previous studies, knock down of *Ifitm1* resulted in amplitude damping and period lengthening in NIH 3T3 cells [116]. Here, we repeated siRNA knock-down in U2OS *Bmal1::Luc* cells. As it is seen in Figure 4.19., knock-down of *Ifitm1* resulted in amplitude damping and period lengthening. For *Ifitm3*, the amplitude damping is not as clear as *Ifitm1*. Amplitude damping is the result of decrease in the expression of the circadian reporter gene *Bmal1*, while the period lengthening means one cycle of the oscillation takes more than 24 hours [124]. Here in this study, depletion of *Ifitm1* and *Ifitm3* causes a decrease in *Bmal1* circadian reporter gene and prolonged the oscillation in U2OS cells.

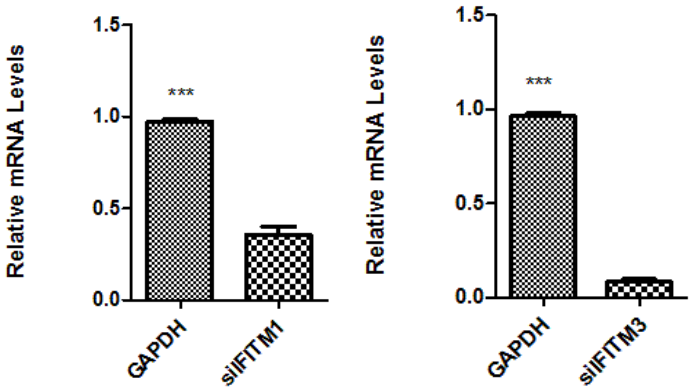


Figure 4.18. Relative mRNA levels of IFITM1 and IFITM3 knockdown HEK 293T cells (n=4)

Table 4.2. Period lengths after siRNA knock-down

	Period Length (hrs.)
WT	24,022
siIFITM1	24,503
siIFITM3	24,29

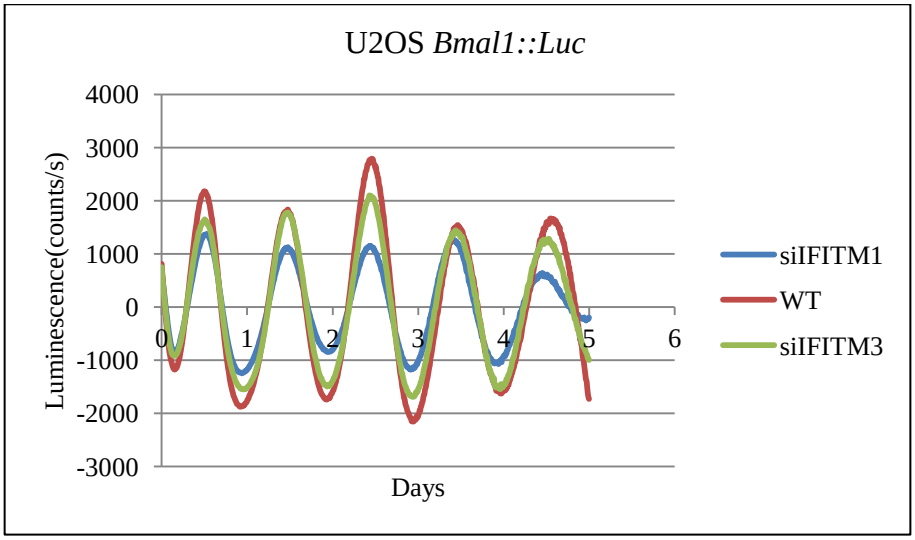


Figure 4.19. The effect of *Ifitm1* and *Ifitm3* depletion generation of rhythmic oscillations. This experiment was repeated for four times. (n=4)

CHAPTER 5

DISCUSSION

In the last two decades, several studies were carried out to identify additional clock genes and modulators. In these studies, plenty of proteins were found to be either directly or indirectly related with molecular machinery of the circadian clock. In order to discover new clock components of modifiers, the basic experimental methods such as forward mutagenesis, reverse genetics, behavioral phenotyping and molecular cloning have been used in the last decade. Although these experimental methods are still in use, computational methods superseded the experimental techniques by the development in genome-wide screens [124]. In order to further accelerate clock gene discovery, the computer assisted approaches were used to prioritize candidate clock components.

In the study by Anafi et. al., a modified version of the Naïve Bayes learning algorithm was used to quantify the evidence provided by each feature that a given gene is a member of the core circadian network. Three of these candidates (*Gm129*, *Ifitm1* and *Cbs*) demonstrated both physical binding with at least one of the clock components and statistically significant change in circadian reporter period after knock down in the NIH 3T3 cells [116]. *Ifitm1*was found to be one of these genes that choose criteria to be either a clock component or a clock modifier. Then the mRNA level oscillations of *Ifitm* gene family were checked from Circa DB and it was found that both *Ifitm1* and *Ifitm3* are oscillating in the SCN [117].

The IFITM gene family was initially identified more than 20 years ago with a particular interest on interferon-stimulated response elements (ISRE) they contained [125]. They belong to CD225 protein superfamily ranging from bacteria to primates [126]. The basic role of these protein family is to restrict the replication of a number of enveloped and non-enveloped viruses [127]. The previous studies showed that knock down of IFITM3 led to enhanced viral replication and knock down of IFITM1 and IFITM2 inhibited early viral replication [128]. Considering the close relationship of the circadian clock with immune system, it is very likely that IFITM1 has a modulatory function on the circadian clock machinery. In terms of an anti-viral protein, it inhibits viral replications which cause serious viral infections like HIV-1, H9N2, Hepatitis C, Influenza A H1N1 Virus, West Nile Virus, and Dengue Virus [105] [128]. For this reason, it might be a pharmalogical target to inhibit the spread of viral infections. Additionally, it is very promising drug target to accelerate recovery time of viral infection carrying patients by using the core clock mechanism.

By taking all these information into account, we decided to work with these to genes to see their role in circadian clock. It has been known that "the physical interaction with a core clock component" is compulsory to consider a candidate protein as a core clock component or clock modifier. For example, CHRONO (*Gm 129*) interacts with both BMAL1 and PER2 [116]. To investigate whether *Ifitm1*and *Ifitm3* interact with any core clock proteins, they were cloned and their interaction with CRY1 protein was determined by M2H assay. Despite that IFITM1 interacts with CRY1, this interaction was not seen between IFITM3 and CRY1 proteins. To confirm the initial interaction screen result of M2H assay, Co-immunoprecipitation was performed and the physical interaction between IFITM1 and CRY1 was proved. In addition, M2H assay was performed to find the exact interaction region between IFTIM1 and CRY1. The result showed that the last 20 amino acid of CRY1 is important for the interaction with IFITM1 and this result was also confirmed by Co-IP. In order to find the important amino acids in the C-termini of CRY1 which mediates the interaction with IFITM1, degenerate CRY1 constructs were prepared which carries amino acid changes. The

M2H result showed that Arg change to Pro or Leu at the position of 602 cause disruption of the interaction between IFITM1 and CRY1 proteins.

From interaction experiments between IFITM1 and CRY1, the effects of IFITM1 and IFITM3 were investigated on molecular core clock mechanism. The repression assay result on *mPer1::luc* promotor showed that, CRY1 repressor activity was reduced by approximately 15%, in the presence of the IFITM1. On the contrary, under the same conditions IFITM3 did have any effect on CRY1 activity. Besides inhibition activity on CRY1 repressor, only IFITM1 enhances the transcription of BMAL1-CLOCK complex by 25%. It was also found that, IFITM1 has no direct effect on *mPer1::luc* promoter. The same experimental procedure was followed to see the effects of IFITM1 and IFITM3 on a different E-Box carrying promoter. The results were similar with the experiments, which were done on *mPer1*. In summary, repression assay data indicated that, IFITM1 binds to CRY1 and represses its repression activity and this consequence leads to high expression on the genes which carry E-Box elements on their promoter region. As it is indicated in the current model, IFITM1and CRY1 interaction may occur in the cytoplasm and this interaction may block CRY1 to go into nucleus. This situation may be the reason of repression of core clock repressor CRY1. When CRY1 does not block BMAL1 and CLOCK heterodimerization, both core clock components and clock controlled gene expressions will increase. (Figure 2.3.)

Next, I wanted to test the dose-dependent effect of IFITM1 and IFITM3 on BMAL1-CRY1 interaction. I observed that IFITM1 did not inhibit CRY1 to bind BMAL1-CLOCK heterodimer. (Figure 4.18) However, this result proved that IFITM1 might have a positive impact on BMAL1-CRY1 interaction. IFITM1 may stabilize the interaction between BMAL1-CRY1, possibly by inducing conformational changes in CRY1 that facilitate BMAL1 binding. On the contrary, IFITM3 did not change the BMAL1-CRY1 interaction capacity like in the previous experiments we did on different promoters.

In the present study, the interaction between IFITM1 and CRY1 was identified for the first time. In addition to interaction assays, the inhibition effect on repressor activity of CRY1 was showed on different E-Box element carrying promoters *mPer1::luc* and *mCry1::luc*. Taken together, our results provide new insights into circadian clock.

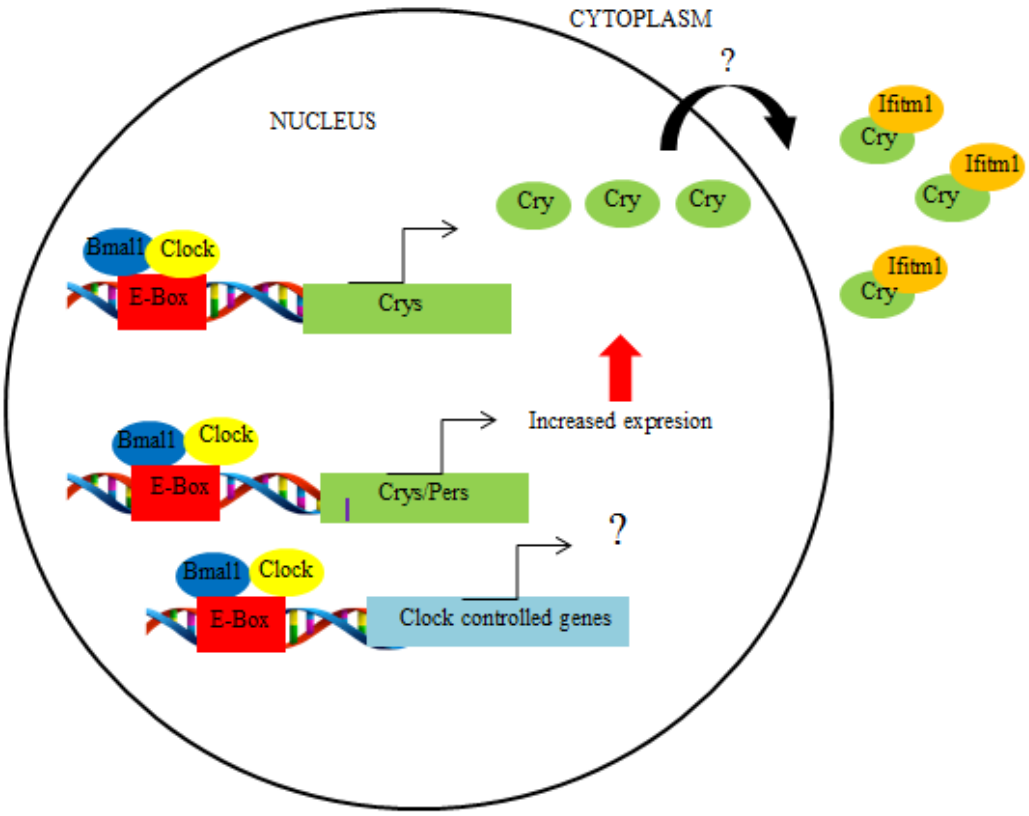
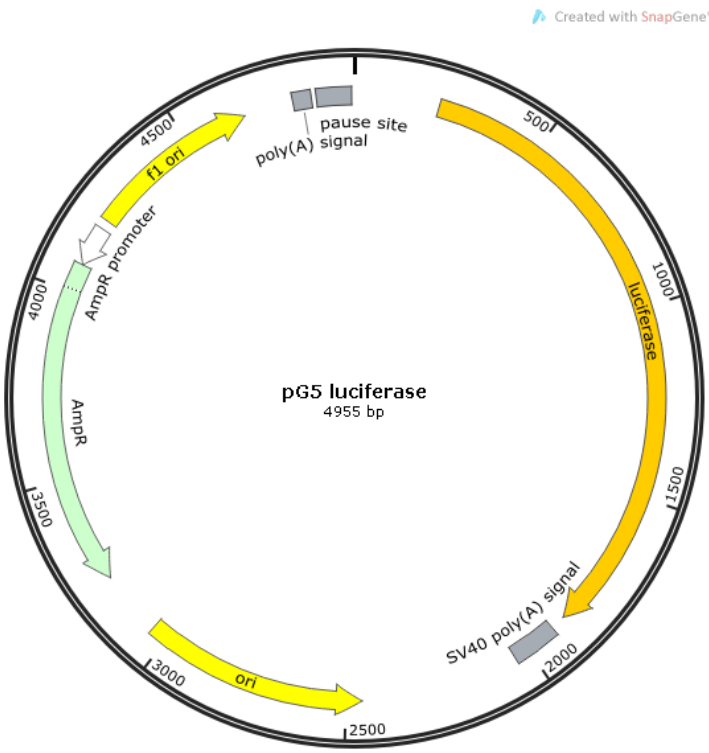


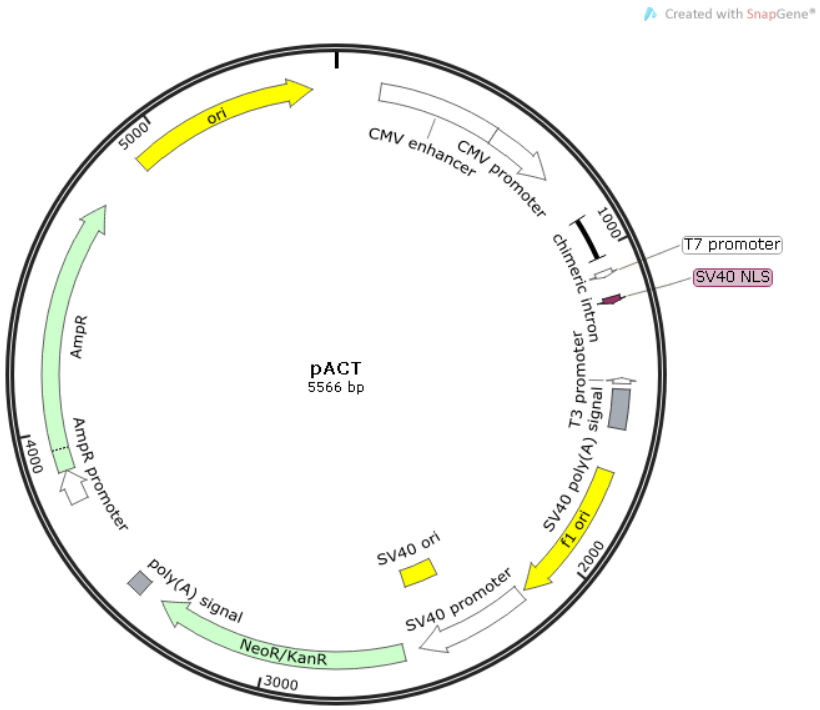
Figure 5.1. A model which shows a possible effect mechanism of IFITM1 on molecular mechanism of the circadian clock

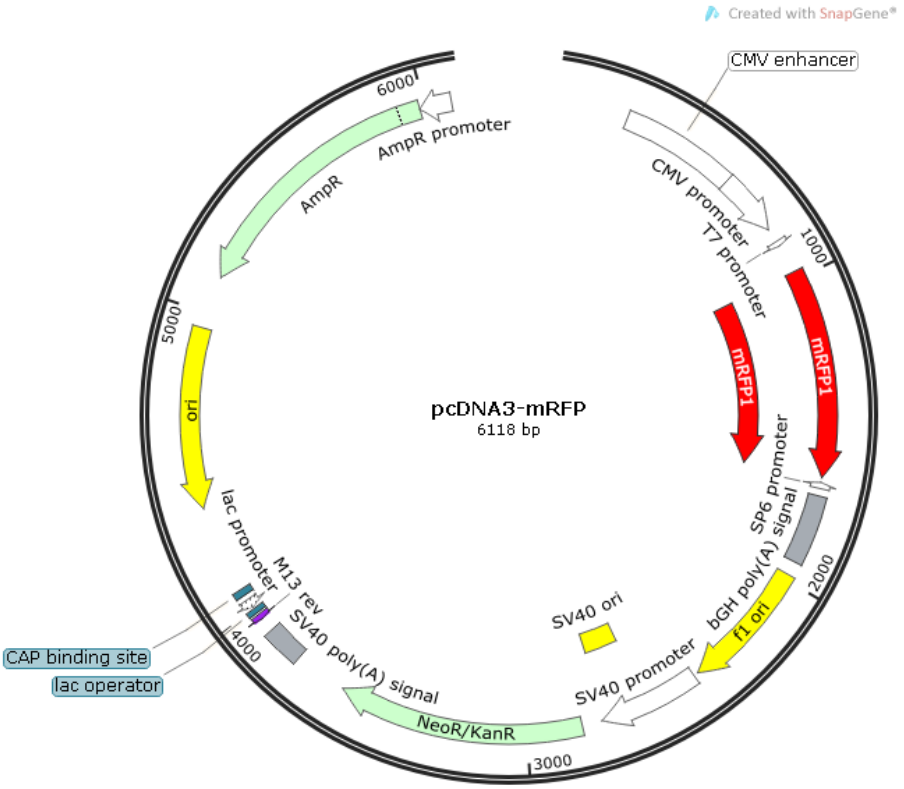
VITA

Eylem Külköylüoğlu was born in Istanbul, Turkey, on February 1, 1990. She received her B.Sc. Degree in Molecular Biology and Genetics and Chemical Engineering from Istanbul Technical University, Istanbul, in 2013. From September 2013 to August 2015 she worked as teaching and research assistant at Koç University, Istanbul, Turkey as a member of Molecular Biology and Biochemistry Laboratory. She has worked on "IFITM-1 (Interferon Induced Transmembrane Protein 1) as a Candidate Clock Regulator Gene" during her M.Sc. study. She will continue her education with Ph.D. at University of Illinois Urbana-Champaign, USA.

APPENDIX 1: Plasmid Maps



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AmpR promoter
AmpR
CMV promoter
T7 promoter
1000
5000
ori
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mRFP1
pcDNA3-mRFP
6118 bp
Sp6 promoter
SV40 poly(A) signal
lac promoter
M13 rev
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SV40 ori
bGH poly(A) signal
f1 ori
CAP binding site
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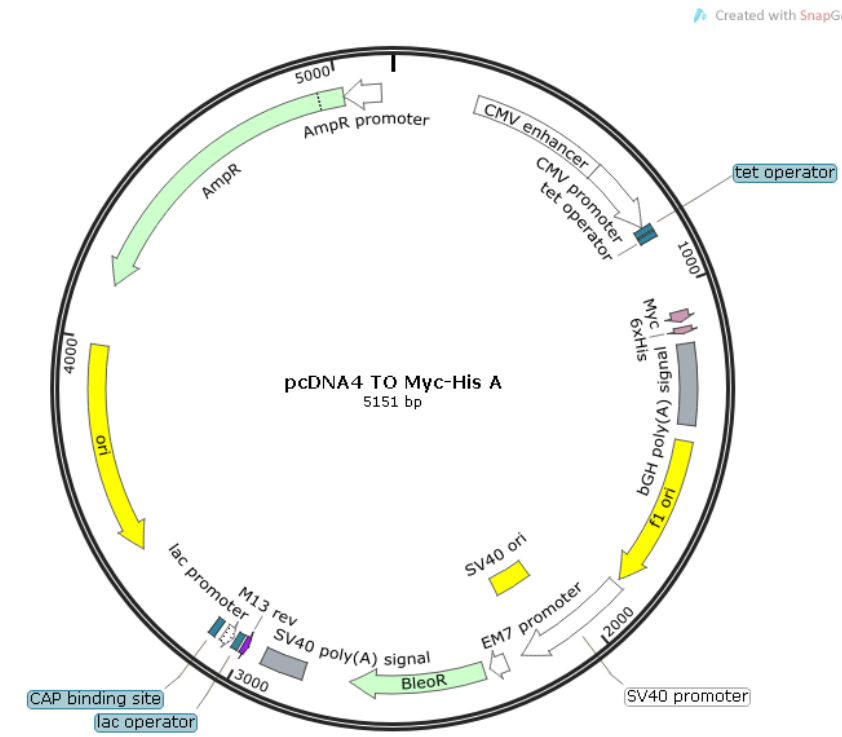
lac operator
SV40 poly(A) signal
SV40 promoter
NeoR/KanR
3000


Created with SnapGene®
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AmpR promoter
CMV enhancer
AmpR
CMV promoter
tet operator
tet operator
1000
Myc
6xHis
4000
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5151 bp
ori
bGH poly(A) signal
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lac promoter
M13 rev
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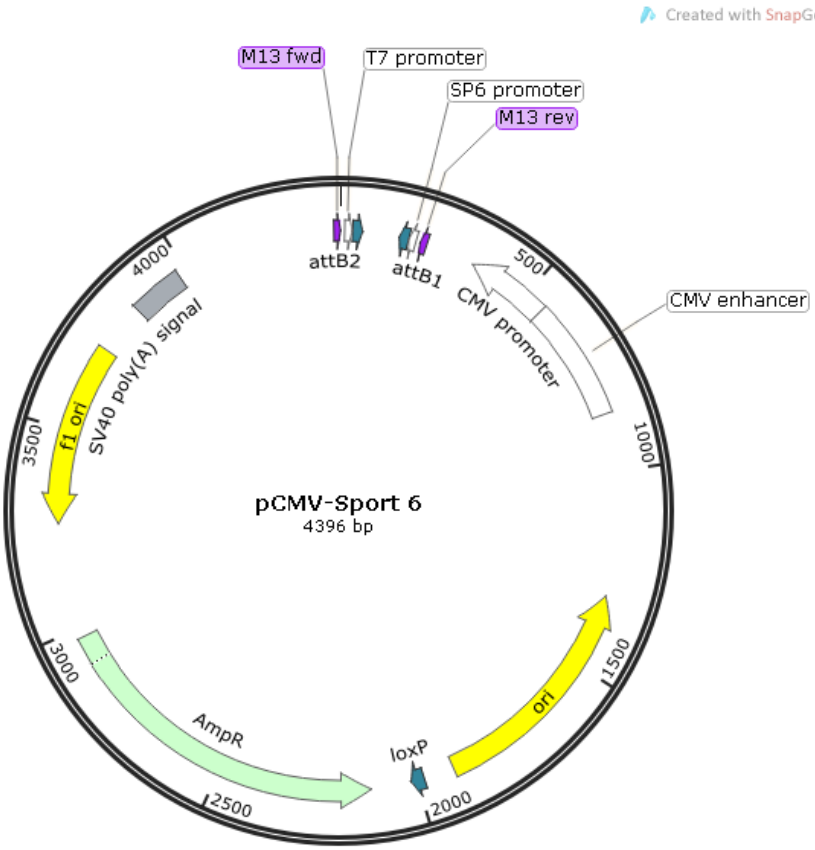
lac operator
SV40 promoter


Created with SnapGene®
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SP6 promoter
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attB1
CMV promoter
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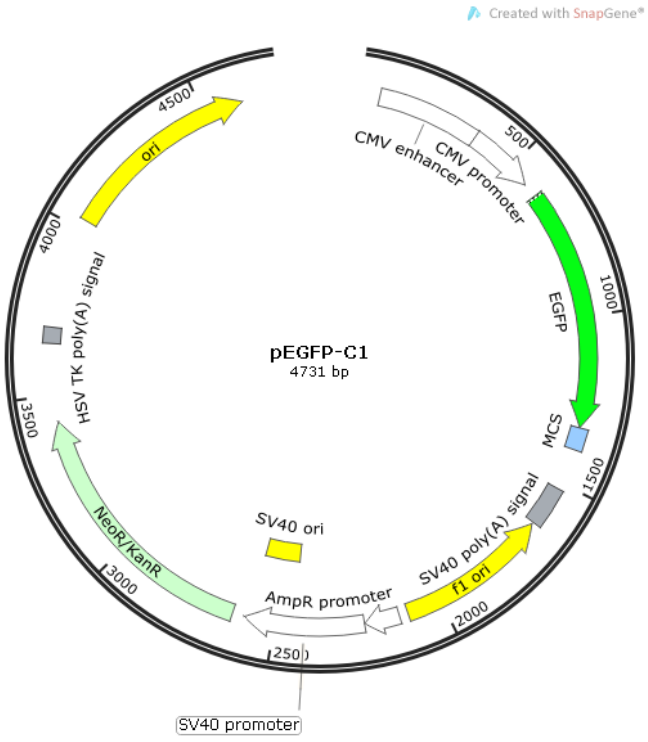
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CMV promoter
EGFP
MCS
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f1 ori
AmpR promoter
SV40 ori
NeoR/KanR
HSV TK poly(A) signal
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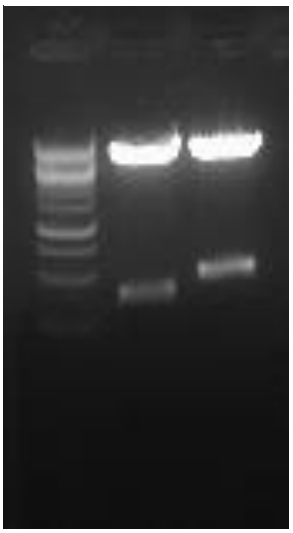
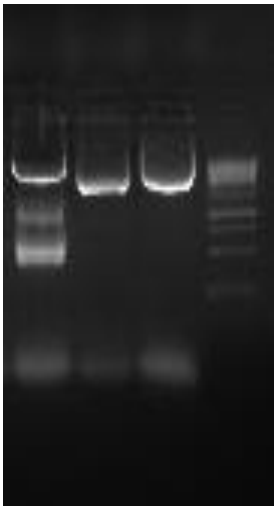
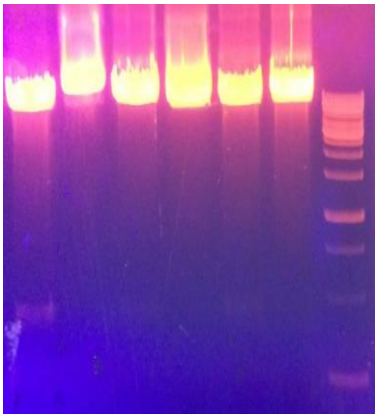
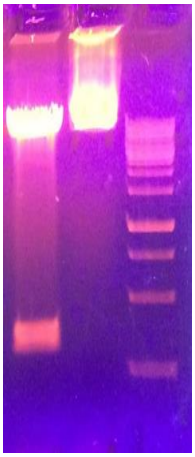
1000
1500
2000
2500
3000
3500
4000
4500

APPENDIX 2: Restriction Digest of Cloned Inserts









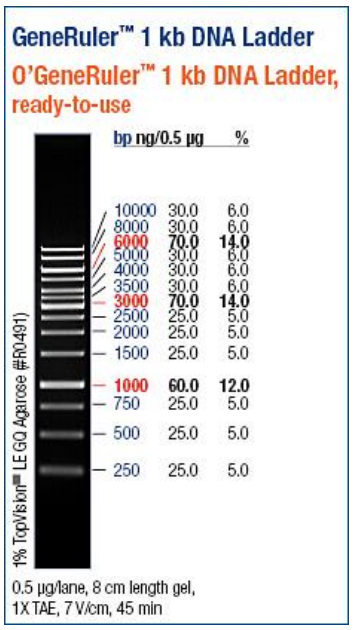
APPENDIX 3: Primer Sequences

Gene (Mouse)	Plasmid	Forward Primer	Reverse Primer
Ifitm1	pcDNA™4/*myc*-His A	5'GAATTCATGGATTACAA GGATGACGACGATAAGATG CCTAAGGAGCAGCAAGAG3'	5'CGCGTAAGCTTGTCACTTGT CGTCATCGTCTTTGTAGTCTCTAA TGGCACAGACAAC3'
Ifitm1	pACT	5'CGTTGATATCAATGCC TAAGGAGCAGCAAG3'	5'CCTGCGGCCGCTCATC TAATGGCACAGACA3'
Ifitm3	pACT	5'CCAGGGATCCGTATGAATCAC ACTGTCCAAACCTTCTTC3'	5'AGATGATATCAACTATCCATAG GCCTGGAAGATCAG3'
Ifitm1	pcDNA3-mRFP	5'AAGCTTGGTACCGAGA TGCCTAAGGAGCAGCAA3'	5'TCTGCAGAATTCTCA TCTAATGGCACAGAC3'
Ifitm3	pcDNA3-mRFP	5'AAGCTTGGTACCGAGA TGAACCACACTTCTCAA3'	5'TCTGCAGAATTCTTAA GTGTGAAGGTTTTG3'
Ifitm1	pBiFC-VN173	5'GACGACAAGCTTATGCCT AAGGAGCAGCAAGAG3'	5'CAGATCTATCGATGAATTC GCTCTAATGGCACAGACAAC3'
Ifitm3	pBiFC-VN173	5'GACGACAAGCTTATGAA CCACACTTCTCAAGCC3'	5'CAGATCTATCGATGAATTC GCAGTGTGAAGGTTTTGAGC3'
Cry1	pcDNA™4/*myc*-His A	5'TGCAGATATCCAATGGGG GTGAACGCCGTGCAC3'	5'GAGCGGGCCGCCAGTT ACTGCTCTGCCGCT3'
*Cry1*T1	pcDNA™4/*myc*-His A	5'TGCAGATATCCAATGGGG GTGAACGCCGTGCAC3'	5'GAGCGGCCGGCCAACG CTTCCCACTGCTGA3'
Cry1	pBIND	5'GCGTTGATATCAGGGGTGAA CGCCGTGCACTG3'	5'ACCTGCGGCCGCTCAGTT ACTGCTCTGCCGCTG3'
*Cry1*T1	pBIND	5'GCGTTGATATCAGGGGTGAA CGCCGTGCACTG3'	5'ACCTGCGGCCGCTCAAACG CTTCCCACTGCGAG3'
*Cry1*T2	pBIND	5'GCGTTGATATCAGGGGTGAA CGCCGTGCACTG3'	5'ACCTGCGGCCGCTCACTG CTGACTGTCCCCGTG3'

*Cry1*T3	pBIND	5’GCGTTGATATCAGGGGTGAA CGCCGTGCACTG3’	5’ACCTGCGGCCGCTCAGCTA CAGCTCGGGACGTT3’
*Cry1*T4	pBIND	5’GCGTTGATATCAGGGGTGAA CGCCGTGCACTG3’	5’ACCTGCGGCCCGCTCAGCT ACAACTCGGGACAT3’
*Cry1*T5	pBIND	5’GCGTTGATATCAGGGGTGAA CGCCGTGCACTG3’	5’ACCTGCCGGCCGCTCAGTTA GAAGGGACCGAGG3’
*Cry1*T6	pBIND	5’GCGTTGATATCAGGGGTGAA CGCCGTGCACTG3’	5’ACCTGCGGCCGCCACTGC TTCATTCTTTCAATG3’
*Cry1*T7	pBIND	5’GCGTTGATATCAGGGGTGAA CGCCGTGCACTG3’	5’ACCTGCGGCCGCTCAGTAA TTAACTCCTATCAA3’

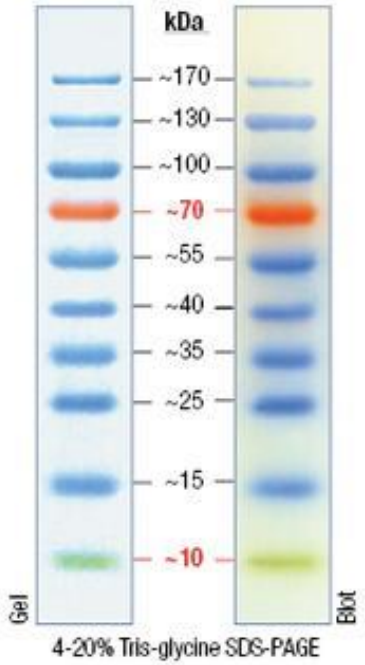
APPENDIX 4: DNA and Protein Markers

DNA Molecular Weight Marker



The ladder is a mixture of chromatography-purified individual DNA fragments.

Protein Marker



PageRuler Prestained Protein Ladders

APPENDIX 5: Laboratory Equipment

Autoclaves	**:** CL-40S/SDP (60L) ALP autoclave
Centrifuges	**:** 4K15, Sigma Laboratory
	: Microfuge 14-15, Sigma Laboratory
Deep freezes and refrigerators	**:** Heto Polar Bear 4410 ultra-freezer, JOUAN Nordic A/S, catalog# 003431.
	: 2021 D deep freezer, Arcelik.
	: 1061 M refrigerator, Arcelik.
Electrophoresis equipment	**:** E-C Mini Cell Primo EC320, E-C Apparatus.
	: Mini-PROTEAN 3 Cell and Single-Row AnyGel Stand, Catalog# 165-3321, Bio-Rad.
Gel documentation system	**:** UVIpro GAS7000, UVItec Limited.
Ice Machine	**:** AF 10, Scotsman.
Shaker	**:** Innova 4300 incubator shaker
Magnetic stirrer	**:** Heidolph MR 3001
Pipettes	**:** Pipetteman P10, P 100, P1000, Eppendorf
pH meter	**:** Inolab pH level 1, order# 1A10-1113,
Power supply	**:** PowerPac Basic (300V, 400mA, 75W) Bio-Rad
Pure water systems	**:** DV25 Pure Lab Option ELGA
Spectrophotometer	**:** W-1700 PharmaSpec, Shimadzu Corporation.
Vortexing machine	**:** Reax Top, Heidolph2
Lumicycle	: LumiCycle luminometer (Actimetrics)
Fluorescence Microscope	: Labophot 2 Fluorescence Microscope

APPENDIX 6: siRNA SEQUENCES

Hs_IFITM1_1 Catalog Number: QIAGEN SI00054117	CCTGTCTACAGTGTCATTCAA
Hs_IFITM1_2 Catalog Number: QIAGEN SI00054124	ACCATATTATGTTACAGATAA
Hs_IFITM1_5 Catalog Number: QIAGEN SI02631482	ACAGTCTACCATATTATGTTA
Hs_IFITM1_9 Catalog Number: QIAGEN SI04384359	TCCAAGGTCCACCGTGATCAA
Hs_IFITM3_5 Catalog Number: QIAGEN SI04179721	TCCCACGTACTCCAACTTCCA
Hs_IFITM3_6 Catalog Number: QIAGEN SI04336717	AGGAAACTGTTGAGAAACCGA
Hs_IFITM3_7 Catalog Number: QIAGEN SI04345446	GCACGTGTTTCTGGTGCTAAA
Hs_IFITM3_8 Catalog Number: QIAGEN SI04361329	TCCAGGCCTATGGATAGATCA

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