

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

THE EFFECTS OF SUPPRESSION OF CRY1
INTERACTORS BY RNA INTERFERENCE

EDA DEMİRÖZCAN KURUN

A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

ADVISOR: PROF. DR. NURİ ÖZTÜRK

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GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

CRY1 İNTERAKTÖRLERİNİN RNA İNTERFERANS
İLE BASKILANMASININ ETKİLERİ

EDA DEMİRÖZCAN KURUN

YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK
ANABİLİM DALI

DANIŞMAN: PROF. DR. NURİ ÖZTÜRK

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To My Family, Friends

And to Those Inspiring Minds...

ABSTRACT

The circadian clock governs daily rhythms in physiology and behavior via transcription–translation feedback loops (TTFLs). Within this system, CRY1 functions as a core repressor of the CLOCK:BMAL1 transcriptional complex. To identify novel regulators of CRY1 activity, we selected six interacting candidate proteins PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1 based on BioID proximity labeling and co-immunoprecipitation (Co-IP) results.

We used U2OS p(BMAL1)-dLuc reporter cells, which stably express luciferase under the control of the BMAL1 promoter, to monitor circadian oscillations in real time. Knockdowns were performed using FlexiTube siRNAs targeting each gene at three different doses (1, 2.5, and 5 pmol) and two cell densities (5,000 and 10,000 cells/well) with Lipofectamine RNAiMAX as the transfection reagent. Following synchronization, bioluminescence was recorded at hourly intervals over five days using a Synergy H1 multimode plate reader.

Circadian parameters period, phase, and amplitude were extracted using ChronoStar and BioDare2 platforms. Knockdown efficiencies were validated via RT-qPCR using the Luna Cell Ready One-Step RT-qPCR Kit, with GAPDH as the housekeeping gene.

Knockdown analyses of CRY1 interactors revealed that GLYR1 robustly shortens the circadian period, highlighting its strong role in accelerating core clock function. AURKAIP1 and CCT2 significantly lengthened the period and delayed phase, suggesting they slow down and shift the molecular clock. TNIP1 specifically caused a phase delay without affecting period or amplitude, implying a role in timing regulation. LPCAT1 knockdown led to moderate period extension and reduced amplitude, pointing to weakened rhythmic strength. Lastly, PURG knockdown mildly extended the period and delayed phase, indicating a subtle modulatory role. These findings demonstrate the functional diversity of CRY1 interactors in shaping circadian rhythm parameters.

Keywords: Biological clocks, Circadian rhythms, Cryptochrome (CRY), RNA interference (RNAi), BMAL1 oscillations.

ÖZET

Sirkadiyen saat, organizmaların çevresel ışık-karanlık döngüsüne uyum sağlamasını mümkün kılan endojen bir zamanlayıcıdır ve birçok fizyolojik süreci düzenleyen transkripsiyon-ters transkripsiyon döngüleriyle çalışır. Bu mekanizmanın baskılayıcı kolunun temel bileşenlerinden biri olan CRY1 proteininin etkileşim ağı, saatin hassas çalışmasını etkileyen önemli düzenleyicileri içerebilir. Bu çalışmada, BioID (Biotin Identification) ve Ko-immunopresipitasyon (Co-IP) ile daha önce belirlenen altı CRY1 etkileşimli aday genin (PURG, AURKAIP1, CCT2, TNIP1, GLYR1 ve LPCAT1) sirkadiyen ritim üzerindeki işlevleri araştırılmıştır.

Bu amaçla, insan osteosarkom hücre hattı U2OS p(BMAL1)-dLuc, siRNA aracılığıyla susturulmuş ve gen dozuna (1, 2.5 ve 5 pmol) ve hücre yoğunluğuna (5.000 ve 10.000 hücre/kuyu) göre deneysel varyasyonlar oluşturulmuştur. Transfeksiyon sonrası hücreler dexametazon ile senkronize edilerek gerçek zamanlı biyoluminesans ölçümleri 5 gün boyunca kaydedilmiş, BMAL1 promoterine bağlı luciferase aktivitesi üzerinden amplitüd, faz ve periyot parametreleri değerlendirilmiştir. Ölçümler Synergy H1 cihazı ile yapılmış; veriler ChronoStar, BioDare2 ve GraphPad Prism ile analiz edilmiştir. Susturma verimliliği ise RT-qPCR yöntemiyle (Luna Cell Ready One-Step RT-qPCR Kit) doğrulanmıştır ve Knockdown % = $(1 - [\text{siRNA fold change} / \text{kontrol fold change}]) \times 100$ formülüyle hesaplanmıştır.

Gerçek zamanlı biyoluminesans ölçümleri ve istatistiksel analizler sonucunda, CRY1 interaktörlerinin sirkadiyen ritim üzerindeki etkilerinin doz ve parametre bazında farklılık gösterdiği gözlemlenmiştir. 2.5 pmol siRNA ile yapılan knockdown deneylerinde, GLYR1, AURKAIP1, CCT2, LPCAT1 ve PURG genlerinin periyot üzerinde anlamlı etkileri olduğu bulunmuştur. GLYR1 kısa periyotlara neden olurken, diğer genler daha uzun periyotlarla ilişkilendirilmiştir. Bu etkiler, genlerin saat döngüsünün hızını doğrudan etkileyebilecek potansiyel düzenleyici roller taşıdığını göstermektedir. Bununla birlikte, 5 pmol siRNA dozunun sitotoksik etki gösterdiği, 1 pmol dozunun ise bazı genler için yetersiz olduğu belirlenmiştir. En tutarlı sonuçlar 1.25 ve 2.5 pmol dozlarında elde edilmiştir.

Faz ve amplitüd analizlerinde ise benzer şekilde çeşitlilik gözlenmiştir. AURKAIP1, CCT2, PURG ve TNIP1 knockdown'ları fazı anlamlı şekilde geciktirmiştir; bu da bu genlerin saat genlerinin pik zamanlamasını etkileyebileceğini düşündürmektedir. Amplitüd parametresinde ise bazı genler, örneğin, LPCAT1 knockdown'u amplitüdü düşürmüştür. Elde edilen sonuçlar, bu interaktörlerin sirkadiyen saat üzerinde farklı mekanizmalarla etkili olabileceğini ve her birinin özgün düzenleyici özellikler taşıdığını ortaya koymaktadır. Bu çeşitlilik, CRY1 etkileşim ağı içindeki genlerin hem çekirdek saat mekanizmasında hem de çevresel saat çıktılarında rol oynayabileceğini düşündürmektedir.

Anahtar Kelimeler: Biyolojik saatler, Sirkadiyen ritimler, Kriptokrom (CRY), RNA girişimi (RNAi), BMAL1 osilasyonları.

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LIST OF SYMBOLS AND ABBREVIATIONS

BMAL1	Brain and Muscle ARNT-Like 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CRY	Cryptochrome
PER	Period
TIM	Timeless
TTFL	Transcription-Translation Feedback Loop
SCN	Suprachiasmatic Nucleus
Co-IP	Co-Immunoprecipitation
BioID	Proximity-Dependent Biotin Identification
siRNA	Small Interfering RNA
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
DXM	Dexamethasone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer)
Pen/Strep	Penicillin/Streptomycin Antibiotic Mix
qPCR	Quantitative Polymerase Chain Reaction
CCG	Clock-Controlled Genes
AMPK	AMP-Activated Protein Kinase
CK1 δ/ϵ	Casein Kinase 1 delta/epsilon
FBXL3, FBXL21	F-box and Leucine-Rich Repeat Proteins 3 and 21
SAINTexpress	Significance Analysis of INTeractome (software)
PBS	Phosphate Buffered Saline
μ M	Micromolar (micromoles per liter)
μ g/mL	Micrograms per milliliter
μ L	Microliter
1L	One Liter
BioDare2	Biological Data Repository (biodare2.ed.ac.uk)
HA-tag	Hemagglutinin Epitope Tag
FLAG-tag	FLAG Epitope Tag

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1. INTRODUCTION

1.1. Biological Clocks: Concept, Importance, and Origins

Life on Earth evolved under the regular cycle of day and night caused by the planet's rotation. To anticipate these environmental rhythms, organisms developed internal biological clocks that regulate daily physiological processes such as sleep, feeding, hormone secretion, and metabolism (Figure 1.1.).

A biological clock is an internal timing mechanism built from molecular and cellular feedback processes. The best-known example is the circadian clock, operating on a ~24-hour cycle. Other rhythms include ultradian (shorter) and infradian (longer) cycles.

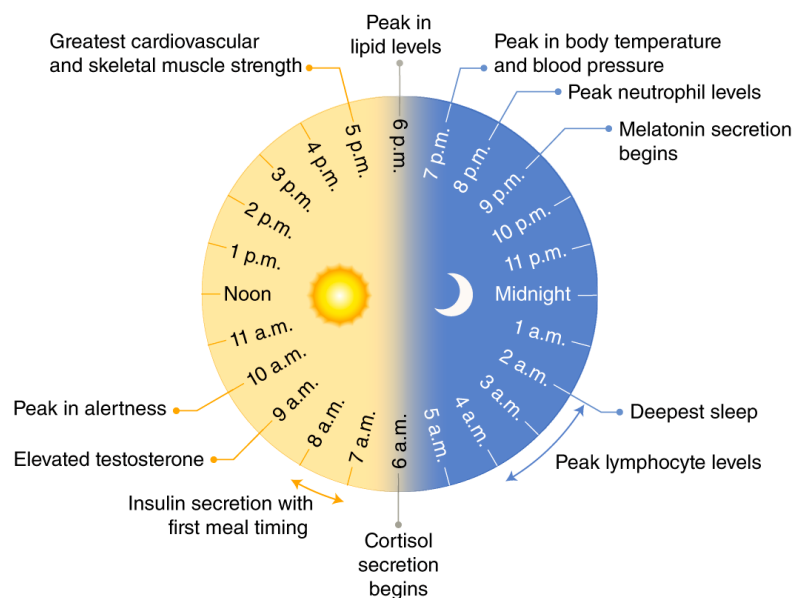


Figure 1.1: Biological Clock Timing System. This figure shows circadian patterns in human physiology, with peaks and troughs in body temperature, alertness, and hormone levels (Reproduced from Masri, S., & Sassone-Corsi, P. (2018) with permission from Springer Nature. License No: 6086450499848).

Recognition of internal biological clocks dates back to the 18th century, when Jean-Jacques d'Ortous de Mairan showed that *Mimosa* plants maintained rhythmic leaf movements even in constant darkness (Dunlap, 1999). Biological clocks are critical

for homeostasis. In humans, the circadian clock coordinates the sleep-wake cycle, hormone secretion, metabolism, and immune function (Panda et al., 2002). In animals, clocks regulate migration, reproduction, and feeding behaviors (Helm et al., 2013), while in plants, they control photosynthesis and flowering (Harmer, 2009).

Clock mechanisms are also present in microorganisms. Cyanobacteria time photosynthesis to daylight (Iwasaki & Kondo, 2004); yeast coordinate cell division; birds regulate mating behaviors; and humans experience mood and performance changes across the day (Takahashi et al., 2008).

1.2. The Mechanism of the Mammalian Biological Clock

The mammalian circadian clock is driven by a highly conserved transcription-translation feedback loop (TTFL) operating within almost every cell. At its core, two transcription factors, CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT-Like 1), form a heterodimer that binds to E-box elements (5'-CACGTG-3') in the promoter regions of target genes to activate transcription (Hardin, 2011; Takahashi, 2017).

Among their primary targets are the Period (PER1, PER2, PER3) and Cryptochrome (CRY1, CRY2) genes.

- PER and CRY mRNAs are translated in the cytoplasm, and their protein products accumulate over several hours.
- Once they reach a threshold, PER-CRY complexes form and translocate into the nucleus.
- Inside the nucleus, these complexes inhibit CLOCK-BMAL1 activity, repressing their own transcription in a classic negative feedback loop.

This core feedback loop takes approximately 24 hours to complete, defining the circadian rhythm (Figure 1.2.).

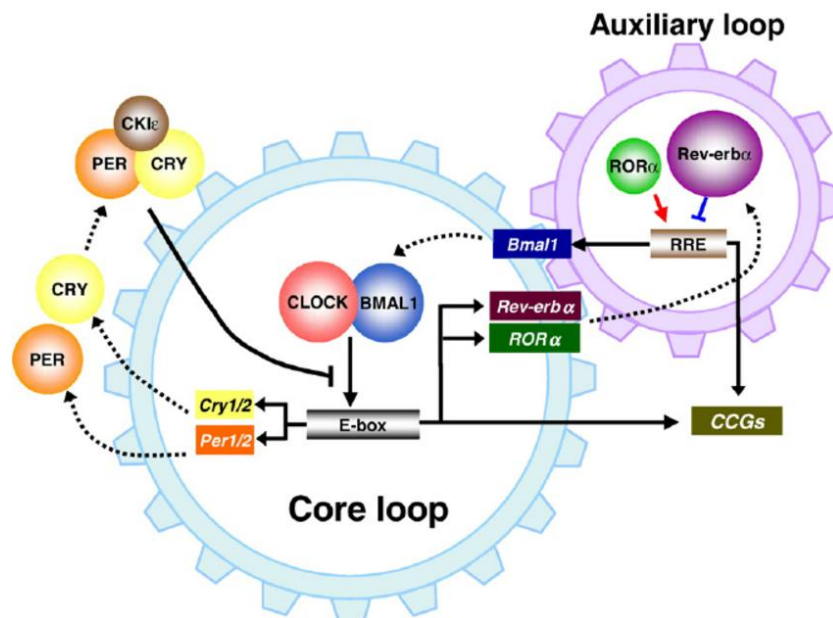


Figure 1.2: Molecular Mechanism of the Mammalian Clock. This figure depicts the primary negative feedback loop and auxiliary loops involving additional regulatory factors (Reproduced from Chung, S., Son, G. H., & Kim, K. (2011) with permission from Elsevier (License No: 1640033).).

To stabilize and fine-tune the clock, auxiliary regulatory loops operate alongside the core mechanism:

- The genes *Rev-Erba*/ β and *Rora*/ β / γ are directly activated by CLOCK-BMAL1.
- REV-ERBs repress *Bmal1* transcription, while RORs activate it, thus forming a secondary loop that regulates BMAL1 levels rhythmically.
- This system ensures robustness, stability, and temperature compensation of the circadian period.

Post-translational regulation is essential for accurate timing:

- Casein Kinase 1 δ/ϵ (CK1 δ/ϵ) phosphorylates PER proteins, marking them for ubiquitin-mediated degradation via the proteasome.
- CRY proteins are similarly regulated by ubiquitination, notably involving FBXL3 and FBXL21, two F-box proteins that modulate CRY stability.

GSK3 β kinase, AMPK, and other kinases further modify PER, CRY, and other clock proteins, affecting their localization, degradation, and function (Takahashi, 2017). Such post-translational processes delay the nuclear accumulation of inhibitory complexes, fine-tuning the phase and period of the oscillations.

Once established, the molecular clock rhythmically controls the expression of thousands of downstream clock-controlled genes (CCGs) that regulate processes such as metabolism, DNA repair, cell division, and immune function. Thus, the core clock not only maintains its own cycle but also coordinates a broad array of physiological rhythms across the organism.

1.3. Central and Peripheral Clocks, Environmental Synchronization

The SCN receives light input via melanopsin-containing retinal ganglion cells through the retinohypothalamic tract. Light exposure resets the clock by inducing *Per1* and *Per2* transcription (Panda et al., 2002; Takahashi, 2017).

The SCN coordinates peripheral clocks in tissues like the liver, lungs, and kidneys through:

- Neural signals (controlling sleep-wake cycles and hormone release),
- Hormonal outputs (e.g., melatonin, cortisol),
- Behavioral rhythms (e.g., feeding, activity patterns) (Takahashi et al., 2008; Turek & Gillette, 2004; Panda et al., 2002).

Although peripheral clocks operate autonomously, they require SCN synchronization to maintain systemic coherence (Asher & Schibler, 2011).

Environmental time cues, or zeitgebers, such as light, feeding, and temperature, entrain biological clocks.

Light remains the dominant zeitgeber (Panda et al., 2002; Takahashi, 2017), while feeding times and social activity fine-tune peripheral oscillators (Bass & Takahashi, 2010; Asher & Schibler, 2011).

1.4. Evolutionary Perspective, Disruptions, and Health Implications

Biological clocks likely evolved to synchronize internal functions with environmental cycles, enhancing survival (Hardin, 2011; Helm et al., 2013; Harmer, 2009). Even ancient organisms like cyanobacteria display circadian gene regulation (Iwasaki & Kondo, 2004; Young & Kay, 2001). Disruptions to these rhythms, caused by genetic mutations, shift work, or irregular schedules, are associated with sleep disorders, metabolic diseases, cardiovascular problems, and psychiatric conditions (Takahashi et al., 2017; Bass & Lazar, 2016). Advances in chronobiology, including genomics and chronotherapy, offer new therapeutic approaches aligned with circadian biology (Zhang et al., 2014; Hirota et al., 2010). Proper alignment of biological clocks through medical interventions, occupational planning, and lifestyle adjustments can significantly improve health outcomes (Turek et al., 2005; Arendt, 2010; Walker, 2017; Owens et al., 2014).

1.5. The Role of CRY Proteins in the Biological Clock

Cryptochromes (CRYs) are essential components of the mammalian circadian clock, acting as key transcriptional repressors that inhibit the CLOCK-BMAL1 complex and sustain 24-hour rhythmicity. By regulating gene expression and controlling the stability of other clock proteins through interactions with kinases and ubiquitin ligases, CRYs ensure the precision and robustness of circadian oscillations. They are also important for light responsiveness and clock entrainment.

Recent studies have further refined the understanding of CRY function. Research led by Professor Aziz Sancar's group has shown that CRY not only directly represses CLOCK-BMAL1 activity but also serves as a carrier for the CK1 δ /CK2 kinase complex. This interaction facilitates the phosphorylation of CLOCK while bound to DNA, promoting the displacement of CLOCK-BMAL1 from chromatin. This mechanism introduces a novel layer of transcriptional repression that operates independently of the classical transcription-translation feedback loop (TTFL), suggesting that the canonical model of circadian regulation is being revised to include these additional CRY-mediated processes (Cao, Yang, Selby, Liu, & Sancar, 2021).

In this study, the aim is to investigate how knockdown of CRY interactors by siRNA affects BMAL1 oscillations. By suppressing the expression of selected CRY-associated proteins, the study seeks to reveal their functional contributions to circadian clock regulation and to better understand the molecular mechanisms underlying BMAL1 rhythmic activity.

1.6. Circadian Parameters

Circadian rhythms can be characterized by three fundamental parameters: period, phase, and amplitude. The period is the duration of one complete oscillation cycle, typically close to 24 hours in mammalian cells. The phase refers to the timing of a specific point in the rhythm such as the peak of gene expression relative to a reference time, often the synchronization point. The amplitude reflects the strength of the rhythm and is defined as the difference between the peak and trough of the oscillation.

These parameters are essential for assessing the integrity and dynamics of the circadian clock. In this study, changes in period, phase, and amplitude were analyzed following siRNA-mediated knockdown of CRY1-interacting proteins.

2. PURPOSE OF THE STUDY

2.1. Purpose of the Experiment

In this experiment, six distinct CRY interactors, which had been previously identified through the BioID (Biotin Identification) method (Roux et al., 2012) and Co-Immunoprecipitation (Co-IP), were chosen for detailed analysis. These interactors were selected based on their potential involvement in circadian rhythm regulation and their physical interaction with the CRY protein. To investigate their roles, small interfering RNA (siRNA) molecules were designed and synthesized to specifically target and reduce the expression of each interactor gene in the experimental cell system (Elbashir et al., 2001).

To confirm that the siRNA-mediated suppression was successful, reverse transcription quantitative PCR (RT-qPCR) was performed to evaluate knockdown efficiency for each target gene.

After achieving efficient knockdown, the impact on the circadian clock was assessed by monitoring BMAL1 oscillation, a core element of the circadian transcription-translation feedback loop. This was accomplished using a luciferase reporter assay, a sensitive and dynamic method for quantifying transcriptional activity in real-time (Yoo et al., 2004).

The luciferase assay provided high-resolution measurements of amplitude, period, and phase of BMAL1-driven circadian oscillations, allowing for comparative analysis of how suppression of each interactor influenced circadian parameters. Through this integrated approach, the study aimed to uncover the distinct roles of individual CRY interactors in modulating circadian clock function.

2.2. Understanding Cryptochrome (CRY) Interactors and Their Discovery

Cryptochromes (CRYs) are a class of flavoproteins found across various organisms, including plants and animals. These proteins play critical roles in circadian rhythms, acting as transcriptional repressors in animals and photoreceptors in plants. The

discovery and study of CRY interactors have been pivotal in understanding the molecular basis of circadian clocks and light signaling.

The BioID (Proximity-Dependent Biotin Identification) method has been employed to identify CRY interactors. BioID involves the fusion of a biotin ligase to CRY, enabling the labeling of nearby proteins with biotin in living cells. These biotinylated proteins are then isolated using streptavidin beads and identified through mass spectrometry. This method has been particularly useful for uncovering transient or weak interactions that might be missed by traditional techniques, offering a more comprehensive view of the CRY interactome (Roux et al., 2012).

Mass spectrometry was also extensively used to identify CRY-bound complexes. Following isolation of these complexes, mass spectrometry revealed associated proteins, leading to the identification of several CRY interactors across different organisms (Koike et al., 2012).

Another essential method was co-immunoprecipitation (Co-IP), where CRY complexes were isolated from cellular extracts. By analyzing these complexes, we identified proteins physically associated with CRY. This method confirmed interactions with circadian regulators. (Takahashi, 2017).

2.3. BioID as a Superior Approach for Identifying Protein-Protein Interactions in Circadian Clock Proteins

The BioID (Proximity-Dependent Biotin Identification) technique was used to identify potential protein-protein interactions (PPIs) of CRY1 in mammalian cells. This method fuses a biotin ligase mutant to the bait protein, enabling biotin labeling of nearby proteins, which are then identified via mass spectrometry. A control group expressing only the ligase helps account for background labeling, and proteins enriched 4-fold are considered potential interactors (Teo et al., 2014). SAINTexpress analysis is used to assess interaction probability, though direct binding must still be validated with co-immunoprecipitation (Kavakli et al., 2022).

Compared to the yeast two-hybrid system, BioID offers more physiologically relevant interaction mapping in living mammalian cells, avoiding common false positives in yeast (Roux et al., 2012; Young & Kay, 2001). It is particularly effective for detecting transient or weak interactions and capturing dynamic regulators in native contexts

(Koike et al., 2012). BioID also enables the study of membrane-bound and nuclear proteins, which are less accessible by traditional methods (Hardin, 2011). While it requires specific reagents and can generate background noise, BioID provides a strong balance of specificity and sensitivity, making it a powerful tool for mapping circadian clock protein networks and revealing dynamic regulatory pathways.

2.4. Investigation of Functional Roles of CRY1 Interactors with CoIP

The circadian clock protein CRY1 plays a crucial role in regulating rhythmic gene expression and maintaining cellular timekeeping (Griffin et al., 1999; Ye et al., 2014). Understanding its interactions with other proteins is essential for uncovering its broader functional significance in circadian rhythm modulation. To identify and characterize its key interactors, an initial screening of 38 potential CRY1 interactors was previously conducted, after which six of the most promising candidates were selected for in-depth functional analysis.

For this purpose, all candidate interactor genes were cloned into the pcDNA3.1-3xHA expression plasmid, enabling their transient expression as HA-tagged fusion proteins in mammalian cells (Li et al., 2013). To validate these interactions, Co-immunoprecipitation (Co-IP) experiments were performed in HEK293T cells, which were transfected with CRY1-3xFLAG along with 3xHA-tagged interactor plasmids. This approach has been widely utilized to study protein-protein interactions in circadian biology (Koike et al., 2012).

Following transfection, FLAG-tagged CRY1 proteins were immunoprecipitated using anti-FLAG antibodies bound to magnetic beads. The presence of co-immunoprecipitated interactors was then detected by Western blotting using an anti-HA antibody, confirming which candidate proteins physically associate with CRY1 (Hardin & Panda, 2013). To ensure specificity, control samples using IgG-coated magnetic beads were processed in parallel, where no interaction is expected. However, since some proteins may exhibit non-specific binding to the beads due to their sticky nature, optimization steps such as pre-blocking with normal cell lysate were implemented to minimize background noise. By integrating co-immunoprecipitation with subsequent functional assays, this study aims to elucidate the role of CRY1 and

its key interactors, contributing to a deeper understanding of how circadian rhythm-related molecular networks are formed and regulated (Partch et al., 2014).

2.5. Rationale for Using siRNA-Mediated Knockdown Instead of Gene Knockout

In this study, siRNA-mediated knockdown was chosen over gene knockout to investigate the functions of CRY1-interacting candidate genes due to several practical and biological considerations. First, some of the targeted genes may be essential for cell viability. Complete knockout of such genes could result in cell death, preventing any downstream analysis of circadian phenotypes. In contrast, knockdown allows for partial suppression of gene expression, enabling the study of gene function without compromising cell survival.

Second, siRNA knockdown offers a faster and more flexible approach compared to the time-intensive process of generating stable knockout cell lines using CRISPR/Cas9. This is especially advantageous when screening multiple genes in parallel, as was done here with six different CRY1 interactors. Furthermore, knockdown provides the opportunity to assess dosage-dependent effects on circadian parameters, which may not be observable in complete loss-of-function models.

Collectively, the use of siRNA-based gene silencing in this context provides a robust, efficient, and biologically informative strategy for functional screening of circadian regulators. Genes that exhibit strong effects upon knockdown can later be prioritized for more detailed mechanistic studies using permanent gene knockout models.

2.6. Direct siRNA: A More Flexible and Immediate Approach

Direct siRNA delivery provides a fast, flexible, and technically straightforward approach for gene silencing, which makes it especially useful for studying time-sensitive processes like circadian rhythms. Unlike vector-based systems that require time-consuming cloning or stable integration, synthetic siRNAs act quickly and transiently, allowing researchers to observe immediate effects without the complications of long-term expression (Elbashir et al., 2001; Kay et al., 2001). One of its greatest advantages is dose control, scientists can fine-tune gene suppression simply

by adjusting siRNA concentrations, making experiments more precise and adaptable (Rao et al., 2009).

This method also avoids potential immune responses and genomic risks tied to viral vectors, since it doesn't require genetic integration (Judge et al., 2005). Chemical modifications further improve siRNA stability and reduce off-target effects without the need for complex vector engineering (Rao et al., 2009). While repeated applications may be needed due to natural degradation, advances in lipid-based transfection and nanoparticle delivery systems have improved siRNA uptake and made consistent gene knockdown more achievable (Scherr & Eder, 2007; Liu et al., 2008). Overall, direct siRNA administration is a reliable and efficient tool for short-term studies in cellular systems.

2.7. Why Independent siRNA Knockdown Experiments Are Necessary Despite BioGPS Data

While BioGPS, an online gene annotation portal and expression database (Wu et al., 2009), offers valuable siRNA knockdown data for circadian genes, running the experiment independently is still crucial. Public datasets often don't reflect the unique conditions of a specific cell line like U2OS p(Bmal1)-dLuc, where cell-type differences and lab-specific variables can alter results. Reliable conclusions require that observed changes are reproducible and genuinely reflect how these interactors influence the circadian clock.

3. EXPERIMENT

3.1. Experimental Design: Investigating the Impact of CRY Interactors on BMAL1 Oscillation and Knockdown Validation with qPCR.

In this experiment, six distinct CRY interactors that were previously identified by BioID and co-immunoprecipitation (CoIP) were selected for analysis. Small interfering RNA (siRNA) molecules were designed and synthesized to specifically target and knockdown the expression of these interactors in the experimental system. U2OS cells stably expressing a destabilized luciferase under the control of the BMAL1 promoter U2OS p(BMAL1)-dLuc cells (Ulusan et al., 2021) were used as the model system for these analyses. After achieving efficient knockdown, the impact on circadian rhythm regulation was assessed by monitoring BMAL1 oscillations using a luciferase reporter assay. This approach enabled the evaluation of changes in BMAL1 activity and provided insights into the role of each CRY interactor in modulating circadian clock function. To ensure the efficiency and specificity of gene silencing, knockdown levels of each target gene was validated using quantitative PCR (qPCR). Figure 3.1. shows the workflow of the experiment.

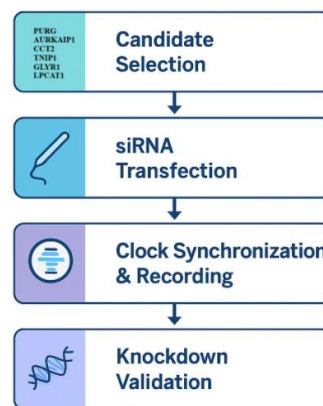


Figure 3.1: Experimental Workflow for Functional Analysis of CRY1 Interactors. This flowchart outlines the step-by-step experimental procedure used to investigate the functional roles of six CRY1-interacting proteins (PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1) in circadian regulation.

3.2. Selection of six representative interacting candidates for functional analysis.

The selection of six representative interacting candidates, PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1, for functional analysis was based on carefully defined criteria to ensure a diverse and meaningful investigation. These candidates were chosen from distinct biological pathways to allow exploration of varied mechanisms involved in circadian regulation. Their localization in different cellular compartments was also considered to provide insight into spatial aspects of clock modulation. Additionally, all selected genes were known to be under circadian regulation, further supporting their potential relevance to the circadian system. Their impact on circadian oscillation was supported by prior siRNA screening studies available in the literature. All selected genes were found to exhibit tissue-specific expression in organs known to harbor functional circadian clocks, such as the liver, brain, and muscle. This was confirmed using the BioGPS database (Wu et al., 2009), supporting the likelihood that these genes may be under circadian regulation. This expression pattern, in combination with data from public siRNA screening studies, increased the rationale for their selection. To promote novelty, preference was given to genes that had not been previously investigated in the context of the circadian clock. Furthermore, only candidates that demonstrated strong and specific co-immunoprecipitation (co-IP) with CRY1 were included, ensuring high confidence in their physical interaction. These features were determined using the UniProt (The UniProt Consortium, 2019), BioGPS (Wu et al., 2009) databases and CoIP results. Figure 3.2 explains how oscillatory behavior was confirmed using data obtained from BioGPS. Figure 3.3 illustrates how the candidates were selected based on strong positive co-immunoprecipitation (co-IP) results. Figure 3.4 presents the known biological functions of the selected interactors.

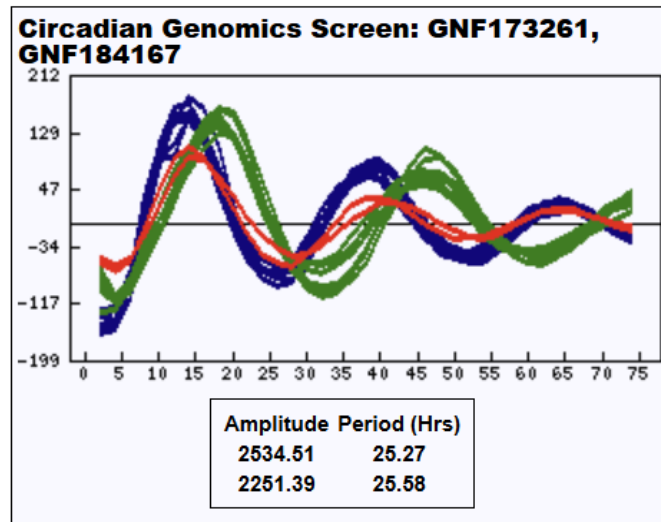


Figure 3.2: Circadian expression profile of GLYR1 from the BioGPS Circadian Genomics Screen. Bioluminescence expression data for GLYR1 derived from two independent probes (GNF173261 and GNF184167) are shown across a 72-hour time course. Rhythmic fluctuations in transcript levels are evident, with calculated circadian periods of 25.27 and 25.58 hours and corresponding amplitudes of 2534.51 and 2251.39, respectively. These findings indicate that GLYR1 exhibits robust circadian oscillation, supporting its selection for further functional characterization. (Retrieved from <http://biogps.org/#goto=genereport&id=84656> Data accessed on 11 August 2025 from BioGPS.)

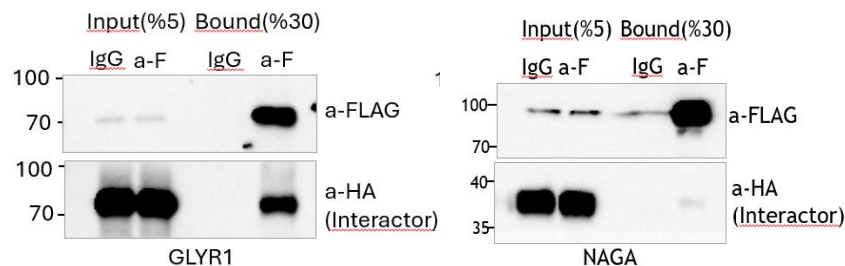


Figure 3.3: Comparison of strong and weak CRY1 protein interactions validated by co-immunoprecipitation (co-IP). To assess the strength of interaction between CRY1 and candidate proteins, co-immunoprecipitation (co-IP) assays were conducted in HEK293T cells co-transfected with FLAG-tagged CRY1 and HA-tagged interactor constructs. Immunoprecipitation was performed using anti-FLAG antibody (α -F) or control IgG, followed by western blot analysis with anti-FLAG and anti-HA antibodies. In the left panel, GLYR1 serves as a strong interactor: HA-tagged GLYR1 was efficiently co-immunoprecipitated with CRY1 in the α -F bound fraction, with minimal signal in the IgG control. In contrast, the right panel shows NAGA as a weak interactor, where only faint HA signal is observed in the α -F lane of the bound fraction, suggesting a low-affinity or transient interaction.

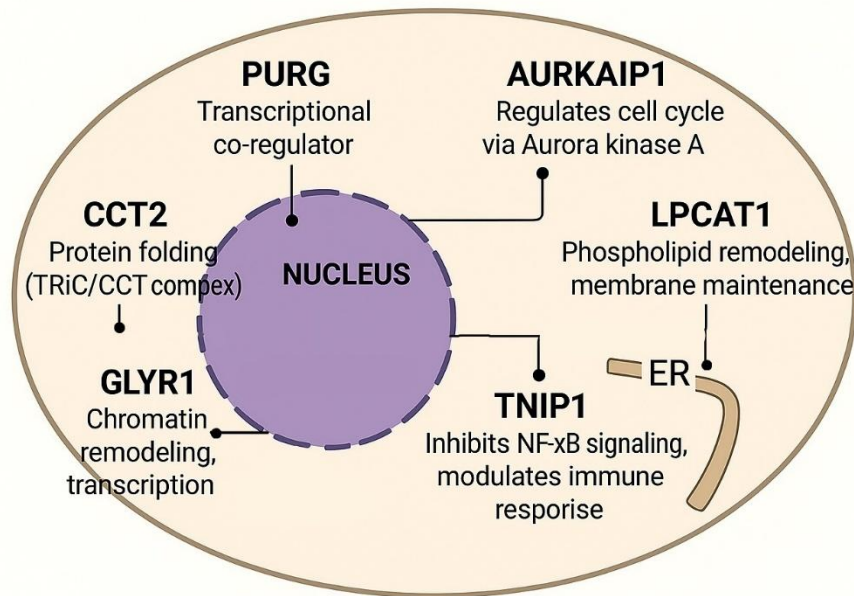


Figure 3.4: Subcellular Localization and Functions of Selected CRY1 Interactors. This schematic shows the subcellular localization and functions of six CRY1-interacting proteins. PURG acts as a transcriptional co-regulator in the nucleus and cytoplasm. AURKAIP1 localizes to the cytoplasm and mitochondria, regulating the cell cycle via Aurora kinase A. CCT2 functions in the cytoplasm as part of the TRiC/CCT complex, aiding in protein folding. TNIP1, found in the nucleus and cytoplasm, inhibits NF- κ B signaling and modulates immune responses. GLYR1, in the nucleus, remodels chromatin and regulates transcription. LPCAT1 resides in the ER and is involved in phospholipid remodeling and membrane maintenance. These proteins reflect diverse roles in circadian regulation across cellular compartments.

3.3. Selection of siRNA

A search was conducted on GeneGlobe under Qiagen FlexiTube using the mRNA accession numbers of the target genes, and the top-ranked siRNA, which is claimed to be functional, was selected for each gene. To ensure that an equal amount of siRNA is transfected into both the experimental and control cells, a negative control siRNA was also ordered. The negative control siRNA (5 nmol, Cat. No.: 1022076) was included to ensure consistency in siRNA transfection across all experimental conditions.

Table 3.1: List of Selected siRNAs Targeting CRY Interactors.

Target Gene	siRNA Name	Catalog No.	GeneGlobe ID	Target Accession Number
PURG	Hs_PURG_12	1027415	SI05035240	NM_001015508, NM_013357
AURKAIP1	Hs_AKIP_2	1027415	SI00120022	NM_001127229, NM_001127230, NM_017900
CCT2	Hs_CCT2_5	1027415	SI02655387	NM_001198842, NM_006431
TNIP1	Hs_TNIP1_6	1027415	SI04315815	NM_001252385, NM_001252386, NM_001252390
GLYR1	Hs_N-PAC_6	1027415	SI04365564	NM_032569
LPCAT1	Hs_AYTL2_3	1027415	SI04330704	NM_024830
Negative Control	Negative control siRNA	1022076	-	-

3.4. siRNA Knockdown and qPCR Analysis of PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1 in U2OS p(Bmal1)-dLuc Cells

This experiment aims to knock down six target genes (PURG, AURKAIP1, CCT2, TNIP1, GLYR1, LPCAT1) using FlexiTube siRNAs to see circadian clock function and validate the knockdown efficiency via qPCR. The impact of gene silencing on circadian oscillations will be monitored.

The effects of suppressing interactors using the siRNA method on circadian clock function were assessed using a Synergy H1 Multi-Mode Plate Reader. The experiment employed the U2OS-p[Bmal1]-dLuc stable cell line (Uluslan et al., 2021), which expresses a destabilized luciferase enzyme under the control of the BMAL1 promoter, enabling the real-time monitoring of circadian oscillations through luminescence (Yoo et al., 2004). To measure circadian clock activity, luciferin, a luciferase substrate, was added to the nutrient medium, facilitating continuous tracking of luminescence.

Luminescence readings were recorded at intervals of 15–30 minutes over the course of one week, generating detailed oscillation data. The data were analyzed to assess the impact of gene knockdown via siRNA (Elbashir et al., 2001) on key circadian parameters, including, period length, phase shifts and amplitude of the oscillations.

3.4.1. siRNA Preparation and Transfection Process

The siRNA mixtures used in this study were procured from Qiagen. Qiagen-developed siRNAs, targeting all human genes, had been previously tested and confirmed to be effective. After detaching the cells from plates using trypsin, cells were seeded.

On the following day, 0.3 μ L of Lipofectamine RNAiMAX transfection reagent (Invitrogen, Cat. No.: 13778075) per well, the appropriate siRNA mixture (1pmol, 2.5pmol and 5pmol), and Opti-MEM I Reduced Serum Medium (Gibco, Cat. No.: 31985062) were prepared. This mixture was incubated at room temperature for 5 minutes to allow complex formation before being added to the 96-well plates containing the cells.

The next day, the cells were treated with dexamethasone (DXM, 0.1 μ M) for 2 hours to reset the circadian clocks. After synchronization, the medium was replaced with a fresh bioluminescence recording medium. The recording medium per 1 liter was prepared using DMEM powder (Sigma, D-2902, 10X 1L), 0.35 g sodium bicarbonate (Sigma, S5761), 3.5 g D(+) glucose powder (Sigma, G7021), 10 mL 1 M HEPES buffer (Gibco, 15,140–122), 2.5 mL Penicillin/Streptomycin solution (100 μ g/mL), 50 mL 5% FBS, and sterile Milli-Q water to complete the volume. Fresh luciferin was added to the medium to achieve a final concentration of 0.1 mM.

The plates were sealed with an optically transparent film to prevent evaporation and maintain gas exchange, thereby supporting cellular homeostasis. Luminescence values were recorded every 15–30 minutes at 32°C, with a 15-second integration time, over the course of one week.

Circadian parameters, including period length, amplitude, and phase, were calculated using the BioDare2 platform (<https://biodare2.ed.ac.uk>) and ChronoStar. Statistical significance was assessed using an unpaired t-test with Welch's correction. GraphPad Prism (version 7, GraphPad Software, USA) was used for graphical representation and statistical analysis.

To determine the optimal siRNA concentration and cell density for effective gene knockdown without inducing cytotoxicity, we conducted five independent optimization experiments using U2OS p(BMAL1)-dLuc cells in a 96-well format. In the initial experiment, 10,000 cells/well were transfected with 1 pmol siRNA.

Subsequently, in the second experiment, we reduced the cell density to 5,000 cells/well and tested 1, 2.5, and 5 pmol concentrations. In the third experiment, we reverted to 10,000 cells/well and re-evaluated the same concentration range.

In the fourth and fifth experiments, we focused specifically on 1.25 and 2.5 pmol siRNA at a constant seeding density of 10,000 cells/well, based on prior findings. These two concentrations consistently resulted in effective and reproducible knockdown effects without compromising cell viability.

In contrast, the 5 pmol concentration was found to decrease overall bioluminescence and introduce variability, suggesting potential cytotoxic effects. On the other hand, 1 pmol siRNA did not produce a sufficient response to induce detectable phenotypic changes. Therefore, 1.25 and 2.5 pmol concentrations were selected as optimal doses for subsequent experiments.

3.4.2. Validation of siRNA Function:

Quantitative PCR (qPCR) was performed to evaluate the knockdown efficiency of selected CRY1 interactors following siRNA transfection. U2OS p(BMAL1)-dLuc cells were transfected with siRNAs targeting PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1, while a non-targeting siRNA (NC) was used as a negative control. The six strongest CRY1 interactors were selected based on prior Co-Immunoprecipitation (Co-IP) results, and primer sequences specific to these genes were obtained from PrimerBank (Table 3.2).

Table 3.2: List of Selected Primers for RT-qPCR.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
PURG	GCTCTGGCCTAAGCAAGAGTA	GCTTCACGTCTAGGTAAAACCTC
AURKAIP1	CAGACTGGATACCGGGACC	CTTCAGCACGTTTTTGCACTG
CCT2	TCTACAGCAAAGTTGCAGAAA	GACAAGAGCTAGGCGTTCCAC
TNIP1	GTTCAACCGACTGGCATCCAA	AGACGCACCCTCTTTGTTGC
GLYR1	AGCGCAAACCTTAGCCTGTCTG	TCCGGGATGGTGAGATCCTTC
LPCAT1	ACATCCCGATCTGGGGAAC	GGCCACTTTCCGTTGGACT

Quantitative PCR was performed using the Luna Cell Ready One-Step RT-qPCR Kit (NEB, E3030S), according to the manufacturer's instructions. At 48 hours post-siRNA

transfection, the culture medium was aspirated, and the cells were gently washed once with phosphate-buffered saline (PBS) to eliminate residual medium and serum. Each well was then lysed using 40 μ L of lysis buffer, followed by incubation at 37 °C for 10 minutes. To stop the lysis reaction and stabilize the released RNA, 5 μ L of stop solution was added to each well, and the plate was incubated for an additional 5 minutes at room temperature.

RT-qPCR reactions were prepared in a final volume of 20 μ L. From each lysed well, 1 μ L of cell lysate was used as the RNA input. Reactions were carried out using gene-specific probes for both target genes and the reference gene GAPDH.

The following thermal cycling conditions were applied:

- cDNA synthesis (reverse transcription): 55 °C for 10 minutes
- Initial denaturation: 95 °C for 1 minute
- Amplification (45 cycles):
 - 95 °C for 10 seconds
 - 60 °C for 30 seconds

qPCR analysis was performed using a single lysate per condition, without biological replicates. Gene expression levels were quantified using the $2^{-\Delta\Delta C_t}$ method, where each siRNA-treated sample was compared to its respective non-targeting control. Knockdown efficiency was initially expressed as fold change relative to the control group. The fluorescence threshold was set within the logarithmic (exponential) phase of amplification, where the amplification curves showed parallel slopes across all samples, ensuring accurate and consistent C_t determination.

In addition, percent knockdown efficiency (% Knockdown) was calculated based on qPCR fold change values using the following formula:

$$\text{Knockdown \%} = (1 - \text{siRNA fold change} / \text{control fold change}) \times 100$$

Data visualization and knockdown efficiency plotting were performed using GraphPad Prism (version 7, GraphPad Software, USA).

4. RESULTS

4.1. Functional Analysis of CRY1 Interactors via 1.25 and 2.5 pmol siRNA Knockdown in 10,000 U2OS p(BMAL1)-dLuc Cells

To investigate the roles of six CRY1-interacting candidate genes (PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1) in circadian rhythm regulation, U2OS p(BMAL1)-dLuc cells were seeded at a density of 10,000 cells per well and transfected with 1.25 and 2.5 pmol of gene-specific FlexiTube siRNAs (Qiagen). A non-targeting siRNA served as the negative control. Transfection was performed using Lipofectamine RNAiMAX, and following an overnight incubation, cells were synchronized using 0.1 μ M dexamethasone for 2 hours. The medium was then replaced with recording medium containing 0.1 mM luciferin, and real-time luminescence was recorded for up to five days using a Synergy H1 plate reader. To ensure reliability and statistical robustness, experiment was conducted using three independent biological replicates, allowing for reproducibility assessment and significance testing. Circadian oscillation parameters, amplitude, period, and phase were analyzed using ChronoStar and BioDare2, and statistical comparisons were conducted in GraphPad Prism using unpaired t-tests with Welch's correction. This approach enabled the identification of gene-specific effects on BMAL1-driven circadian rhythms.

4.1.1. Results of 1.25 pmol siRNA Knockdown:

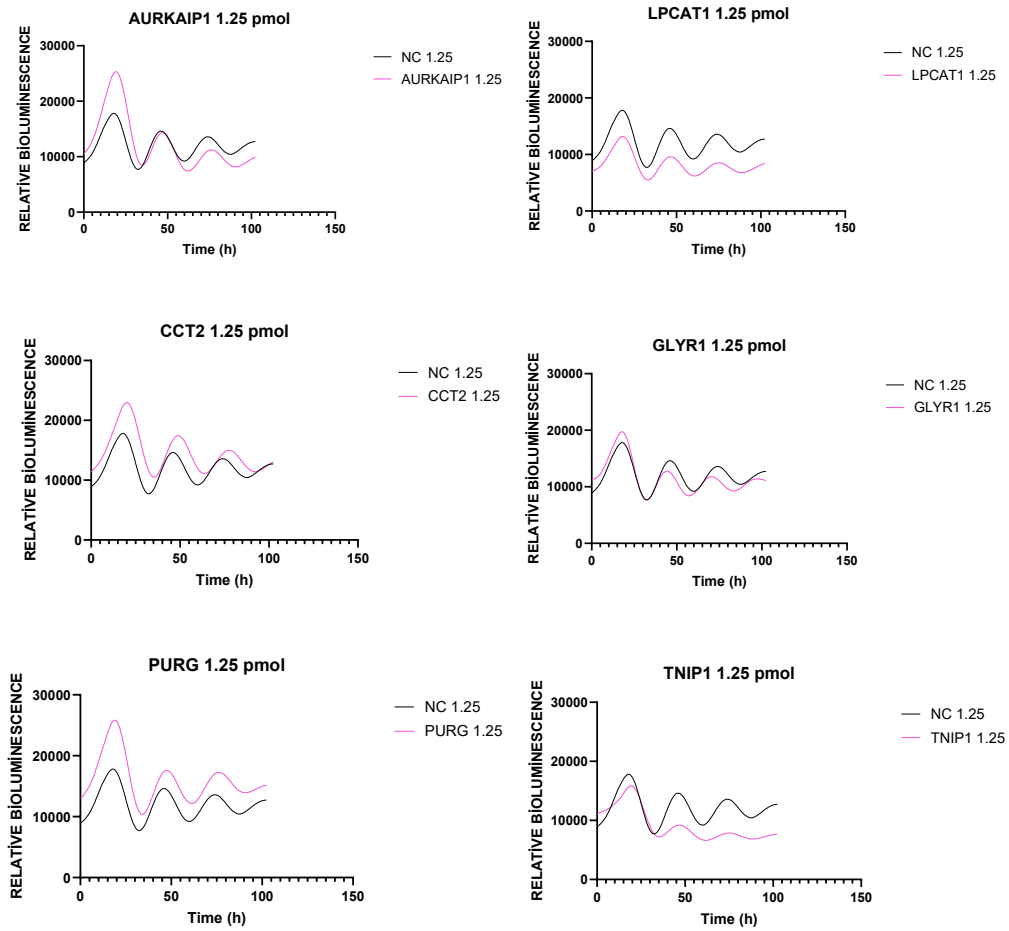


Figure 4.1: BMAL1-driven Oscillation Profiles Following 1.25 pmol siRNA Knockdown of CRY1 Interactors in 10.000 U2OS p(BMAL1)-dLuc Cells. Real-time bioluminescence recordings of U2OS p(BMAL1)-dLuc cells transfected with siRNAs targeting individual CRY1-interacting genes (PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1). Knockdowns were performed using 1.25 pmol siRNA per well, and synchronized cells were recorded over a period of 5 days using a Synergy H1 Multi-Mode Plate Reader. Black lines represent control cells treated with non-targeting siRNA (NC), and pink lines show oscillatory traces from cells in which the respective gene was silenced.

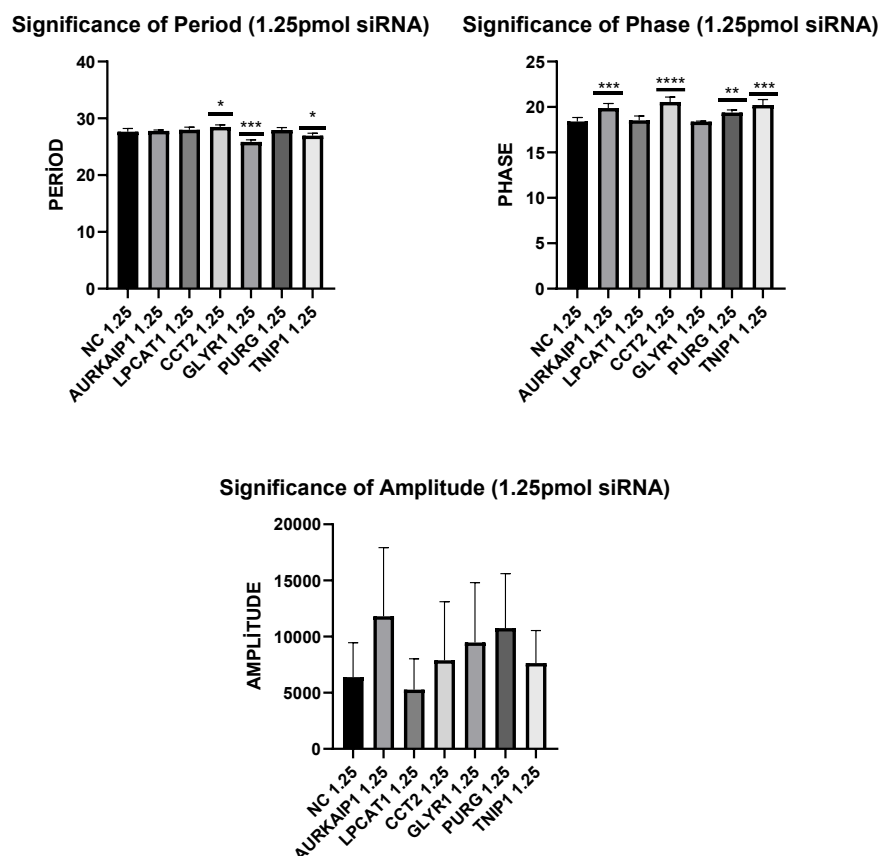


Figure 4.2: Effects of CRY1 Interactor 1.25 pmol siRNA Knockdown on Circadian Parameters in 10.000 U2OS p(BMAL1)-dLuc Cells. Bar graphs illustrate the impact of 1.25 pmol siRNA-mediated knockdown of six CRY1 interactors (PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1) on circadian oscillation parameters, amplitude, phase, and period compared to non-targeting control (NC).

Using real-time bioluminescence recordings and subsequent analysis with GraphPad, ChronoStar, and BioDare2, three core circadian parameters, amplitude, phase, and period were evaluated following knockdown of CRY1 interactors using 1.25 pmol siRNA (Figure 4.1 and Figure 4.2). At the 1.25 pmol siRNA concentration, knockdown of GLYR1 led to a significant shortening of the circadian period ($p < 0.001$), while TNIP1 knockdown also caused a milder but significant period shortening ($p < 0.05$). In contrast, knockdown of CCT2 resulted in a significant lengthening of the period ($p < 0.05$). Regarding phase changes, knockdown of CCT2 induced a highly significant phase delay ($p < 0.0001$). AURKAIP1 knockdown also caused a substantial phase

delay ($p < 0.001$), as did TNIP1 ($p < 0.001$). PURG knockdown led to a moderate but significant delay in phase ($p < 0.01$). No significant changes were observed in the amplitude of BMAL1-driven oscillations in any of the knockdown conditions.

4.1.2. Results of 2.5 pmol siRNA Knockdown:

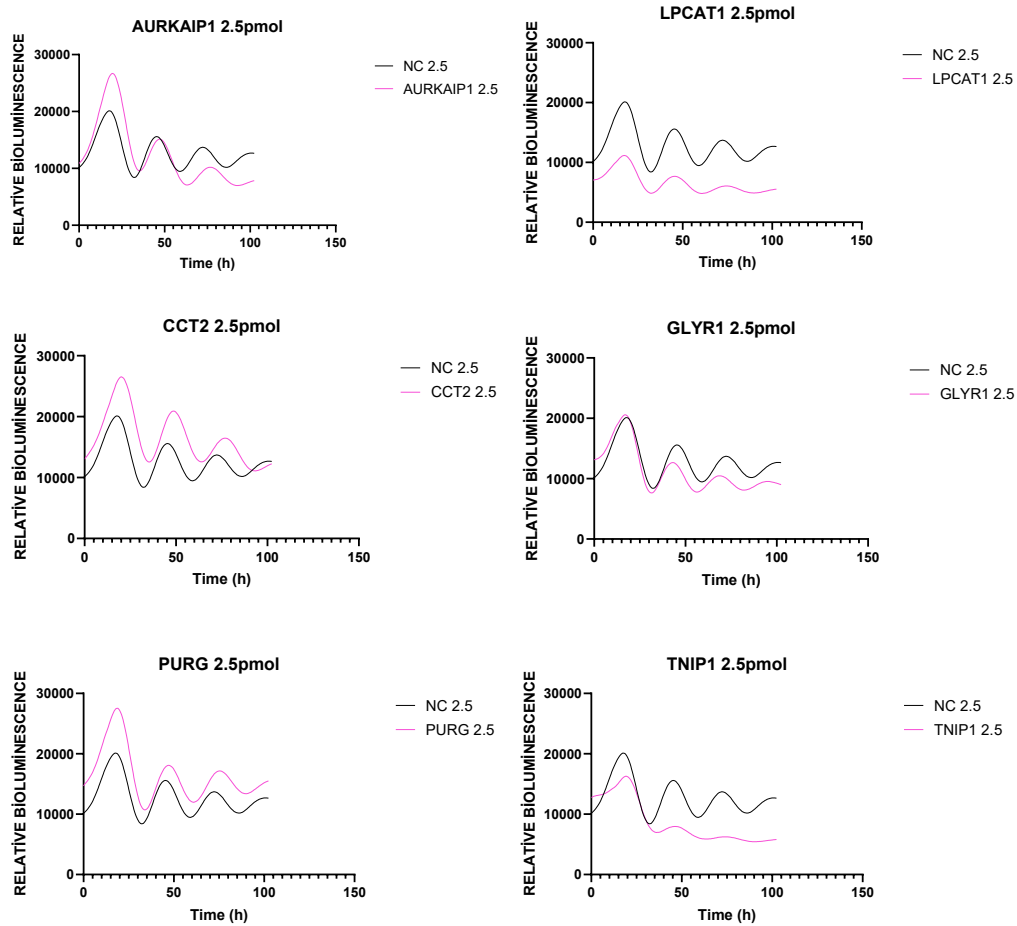


Figure 4.3: BMAL1-driven Oscillation Profiles Following 2.5 pmol siRNA Knockdown of CRY1 Interactors in 10,000 U2OS p(BMAL1)-dLuc Cells. Real-time bioluminescence recordings of U2OS p(BMAL1)-dLuc cells transfected with siRNAs targeting individual CRY1-interacting genes (PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1). Knockdowns were performed using 2.5 pmol siRNA per well, and synchronized cells were recorded over a period of 5 days using a Synergy H1 Multi-Mode Plate Reader. Black lines represent control cells treated with non-targeting siRNA (NC), and pink lines show oscillatory traces from cells in which the respective gene was silenced.

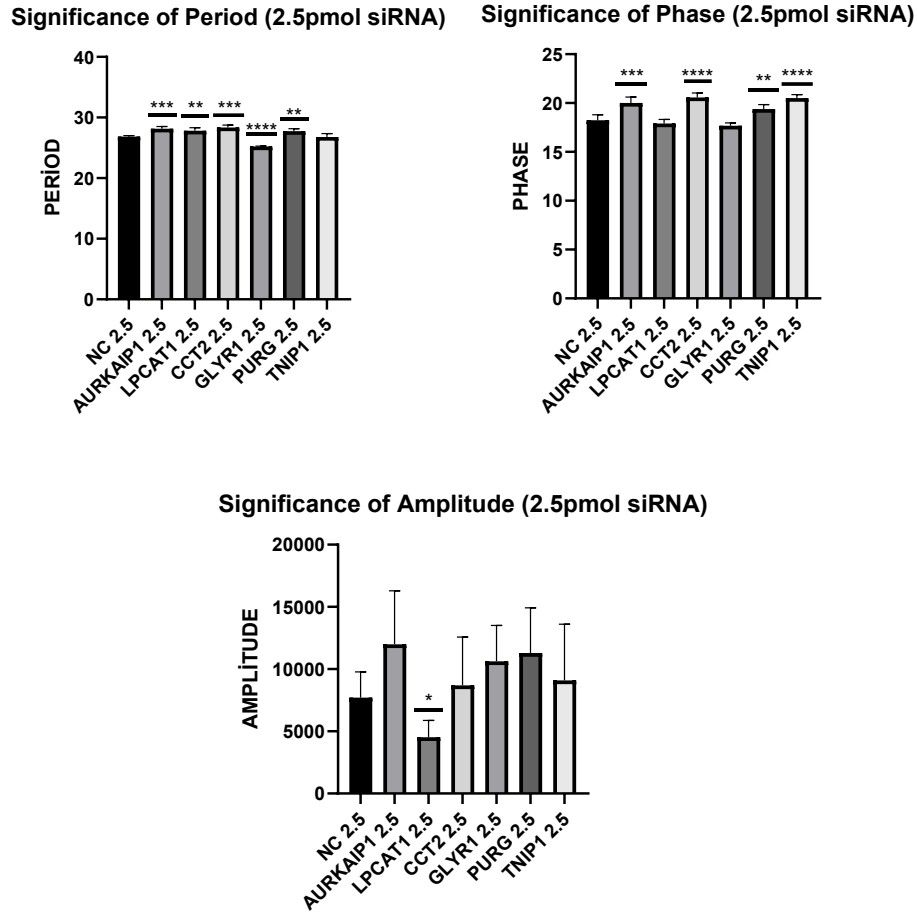


Figure 4.4: Effects of CRY1 Interactor 2.5 pmol siRNA Knockdown on Circadian Parameters in 10.000 U2OS p(BMAL1)-dLuc Cells. Bar graphs illustrate the impact of 2.5 pmol siRNA-mediated knockdown of six CRY1 interactors (PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1) on circadian oscillation parameters, amplitude, phase, and period compared to non-targeting control (NC).

Using real-time bioluminescence recordings and subsequent analysis with GraphPad, ChronoStar, and BioDare2, three core circadian parameters, amplitude, phase, and period were evaluated following knockdown of CRY1 interactors using 2.5 pmol siRNA (Figure 4.3 and Figure 4.4). At the 2.5 pmol siRNA concentration, knockdown of AURKAIP1 ($p < 0.001$), LPCAT1 ($p < 0.01$), CCT2 ($p < 0.001$), and PURG ($p < 0.01$) led to a significant increase in the circadian period, while knockdown of GLYR1 caused a marked reduction in period duration ($p < 0.0001$). Regarding phase changes, AURKAIP1 knockdown induced a significant phase delay ($p < 0.001$), as did CCT2 ($p < 0.0001$), PURG ($p < 0.01$), and TNIP1 ($p < 0.0001$), indicating consistent phase-

delaying effects among these genes. In terms of amplitude, only LPCAT1 knockdown caused a statistically significant reduction ($p < 0.05$), while no significant amplitude changes were observed in other conditions. These findings suggest that GLYR1 plays a critical role in shortening the circadian period, whereas AURKAIP1, CCT2, PURG, and LPCAT1 contribute to the lengthening of the period. Additionally, multiple genes appear to modulate phase timing.

To validate the circadian relevance of the six selected CRY1 interactors (AURKAIP1, LPCAT1, CCT2, GLYR1, PURG, and TNIP1), we compared their previously reported circadian characteristics with experimental outcomes from this study (Table 4.1). Publicly available data from the BioGPS Circadian Genomics Screen provided reference period and amplitude values under baseline conditions, while our siRNA knockdown experiments measured the same parameters at two different concentrations: 1.25 pmol and 2.5 pmol.

Table 4.1: Comparison of Circadian Parameters Between BioGPS Data and Experimental Results.

Gene	Source	Period (hrs)	Amplitude	siRNA Dose
AURKAIP1	BioGPS	27,545	1630,185	—
	This Study	27,773717	11804,99	1.25 pmol
		28,135033	11985,03	2.5 pmol
LPCAT1	BioGPS	25,575	3021,055	—
	This Study	27,96865	5282,805	1.25 pmol
		27,814933	4526,717	2.5 pmol
CCT2	BioGPS	26,03	3712,39	—
	This Study	28,4824	7886,885	1.25 pmol
		28,338317	8690,225	2.5 pmol
GLYR1	BioGPS	25,425	2392,95	—
	This Study	25,840417	9470,792	1.25 pmol
		25,201083	10619,9	2.5 pmol
PURG	BioGPS	25,875	1033,845	—
	This Study	27,952767	10764,12	1.25 pmol
		27,754183	11299,66	2.5 pmol
TNIP1	BioGPS	27,09	5577,28	—
	This Study	26,9353	7648,447	1.25 pmol
		26,788383	9094,312	2.5 pmol

As summarized in Table 4.1, the period values obtained from our study generally showed deviations from the BioGPS predictions, indicating that siRNA-mediated suppression of these genes has measurable effects on the core clock oscillation.

These results not only confirm the circadian regulation of the selected genes but also demonstrate that knockdown of these CRY1 interactors can significantly modulate the period and amplitude of BMAL1-driven circadian rhythms. This supports their potential role as regulators or modulators of the molecular clock machinery.

4.2. RT-qPCR for Knockdown Validation

To evaluate the knockdown efficiency of selected CRY1 interactors, U2OS p(BMAL1)-dLuc cells were transfected with siRNAs targeting PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1. A non-targeting siRNA (NC) was used as a negative control. For normalization of gene expression levels, GAPDH was employed as the internal reference gene. Gene expression was quantified using the Luna® Cell Ready Probe One-Step RT-qPCR Kit (New England Biolabs), following the manufacturer's protocol.

At 48 hours post-siRNA transfection, culture medium was aspirated and cells were washed once with phosphate-buffered saline (PBS) to remove residual medium and serum. Each well was then lysed using 40 µL of lysis buffer, and the plate was incubated at 37 °C for 10 minutes to ensure complete lysis. Subsequently, 5 µL of stop solution was added to each well, and the plate was incubated for an additional 5 minutes at room temperature to stabilize RNA and terminate the lysis reaction.

RT-qPCR reactions were prepared in a final volume of 20 µL per well. From each lysed well, 1 µL of lysate was added as the RNA input for the reaction. Reactions were carried out using gene-specific probes in technical replicates for both target and reference genes.

Gene expression levels were analyzed using the $2^{-\Delta\Delta C_t}$ method, where each siRNA-treated sample was compared to its corresponding non-targeting control. Knockdown efficiency was expressed as fold change relative to the control group.

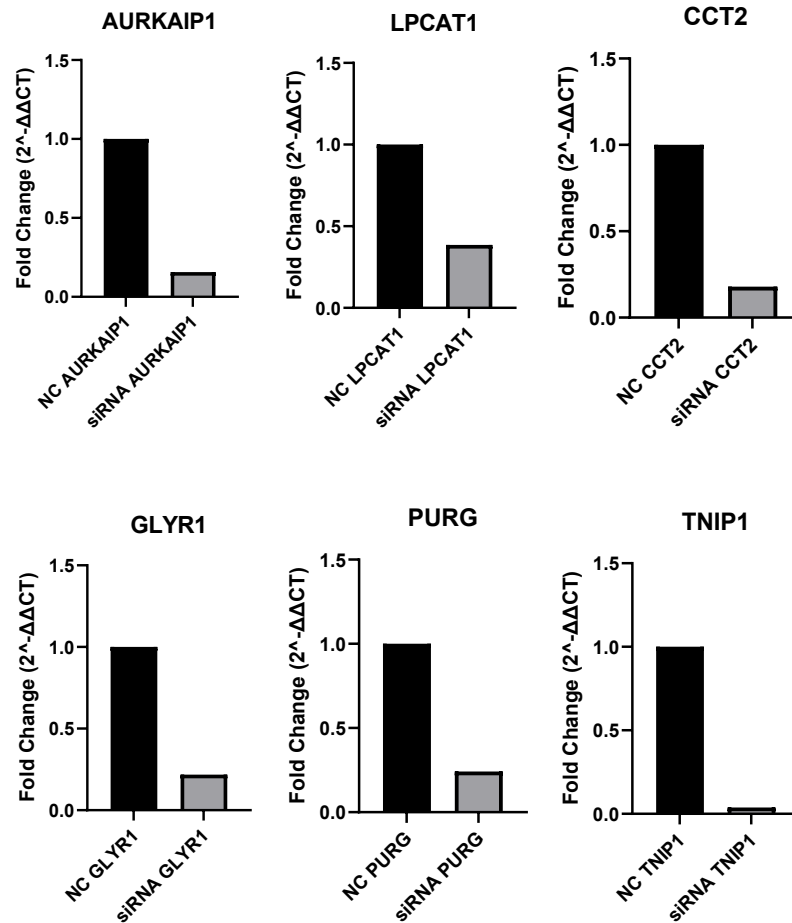


Figure 4.5: Validation of siRNA-Mediated Knockdown of Selected CRY1 Interactors by RT-qPCR. Gene expression levels of six CRY1-interacting genes (AURKAIP1, LPCAT1, CCT2, GLYR1, PURG, and TNIP1) were measured in U2OS p(BMAL1)-dLuc cells transfected with gene-specific siRNAs. Cells transfected with a non-targeting siRNA (NC) served as negative controls. Total RNA was obtained through direct cell lysis, and quantitative reverse transcription PCR was performed using the Luna Cell Ready One-Step RT-qPCR Kit (NEB, E3030S). GAPDH was used as the internal control gene. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method and are presented as fold change relative to the control. A clear reduction in expression was observed for all target genes following siRNA treatment, indicating successful knockdown.

The qPCR results demonstrated that the fold change values of siRNA-treated samples were consistently lower than those of the corresponding non-targeting controls for all six CRY1 interactors examined (Figure 4.5). This reduction in mRNA expression indicates that the gene-specific siRNAs effectively suppressed the expression of AURKAIP1, LPCAT1, CCT2, GLYR1, PURG, and TNIP1. The observed

downregulation confirms the successful knockdown of the targeted genes and validates the efficiency of the siRNA transfection protocol used in this study.

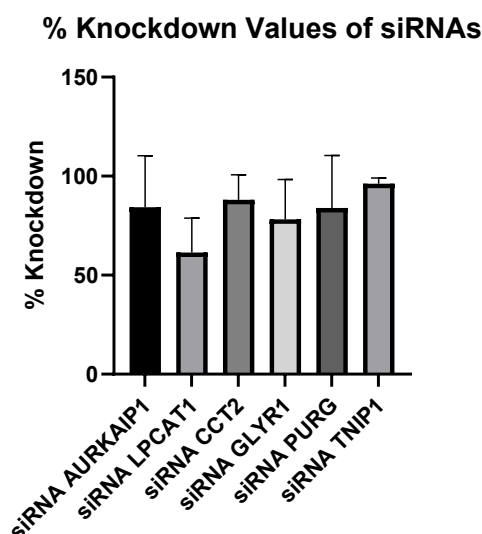


Figure 4.6. Percent Knockdown Efficiency of Gene-Specific siRNAs. Bar graph showing the knockdown efficiency of siRNAs targeting AURKAIP1, LPCAT1, CCT2, GLYR1, PURG, and TNIP1 in U2OS p(BMAL1)-dLuc cells.

% Knockdown efficiency was calculated based on qPCR fold-change values using the formula:

$$\text{Knockdown \%} = (1 - \text{siRNA fold change} / \text{control fold change}) \times 100$$

All siRNAs resulted in reduced expression of their respective target genes compared to non-targeting control. The highest knockdown was observed for TNIP1 (~90%), followed by CCT2 and GLYR1, while LPCAT1 siRNA showed the lowest knockdown efficiency (Figure 4.6). These results confirm variable but measurable gene silencing activity of the siRNAs used.

5. CONCLUSION

This study investigated the functional contributions of six CRY1-interacting proteins, PURG, AURKAIP1, CCT2, TNIP1, GLYR1, and LPCAT1 in regulating circadian rhythm dynamics using siRNA-mediated knockdown in U2OS p(BMAL1)-dLuc cells. A range of siRNA doses (1, 2.5, and 5 pmol) was applied in two different cell densities (5,000 and 10,000 cells per well), and circadian parameters (amplitude, phase, and period) were quantitatively assessed using real-time bioluminescence recording. The findings were substantiated by RT-qPCR-based knockdown validation and statistical analyses conducted in GraphPad Prism, ChronoStar, and BioDare2.

In addition to identifying functional roles of CRY1 interactors, our optimization experiments revealed important dose-dependent effects. The 5 pmol siRNA concentration consistently failed to produce meaningful rhythmic changes and often resulted in reduced bioluminescence signal, suggesting possible cytotoxic effects at higher siRNA doses. Conversely, 1 pmol siRNA was insufficient to elicit consistent or statistically significant alterations in circadian parameters, indicating that low doses may not achieve effective knockdown. These findings highlight the importance of carefully calibrating siRNA concentration and cell density to achieve reliable and physiologically relevant outcomes.

GLYR1 knockdown consistently led to a significant shortening of the circadian period, with strong significance at both 1.25 pmol ($p < 0.001$) and 2.5 pmol ($p < 0.0001$) concentrations, suggesting its essential role in period regulation. No significant changes were observed in phase or amplitude under either condition.

TNIP1 knockdown resulted in moderate period shortening at 1.25 pmol ($p < 0.05$), but no effect on period at 2.5 pmol. However, it caused highly significant phase delays at both concentrations ($p < 0.001$ and $p < 0.0001$), implicating TNIP1 primarily in phase regulation. No amplitude effects were detected.

CCT2 knockdown caused a lengthening of the period at both 1.25 ($p < 0.05$) and 2.5 pmol ($p < 0.001$). It also produced the strongest and most consistent phase delay across all genes tested, with highly significant values ($p < 0.0001$ at both doses). These results indicate a central role for CCT2 in both period and phase regulation.

AURKAIP1 knockdown led to a significant period lengthening at 2.5 pmol ($p < 0.001$), but not at the lower dose. Phase delay was observed at both concentrations, with high significance ($p < 0.001$). These results point to AURKAIP1 as a modulator of both period and phase in a dose-dependent manner.

PURG knockdown induced a moderate increase in period at 2.5 pmol ($p < 0.01$) and a significant phase delay at both concentrations ($p < 0.01$ and $p < 0.01$), suggesting its involvement in both period extension and phase delay. No amplitude changes were observed.

LPCAT1 knockdown led to a significant lengthening of the period at 2.5 pmol ($p < 0.01$), with no effect on phase. However, it was the only gene whose knockdown significantly reduced the amplitude of BMAL1-driven oscillations ($p < 0.05$), indicating a potential role in maintaining the robustness of circadian output.

In summary, these results demonstrate that CRY1-interacting proteins differentially regulate circadian dynamics.

To validate gene silencing, RT-qPCR was performed for all target genes. Knockdown efficiencies were calculated using the formula:

$$\text{Knockdown \%} = (1 - [\text{siRNA fold change} / \text{control fold change}]) \times 100.$$

All six siRNAs effectively reduced the mRNA levels of their respective targets compared to the non-targeting control. The siRNA-mediated knockdown efficiencies for the selected CRY1 interactors were consistently high, except LPCAT1, which showed a moderate reduction of approximately 61%. Among the genes analyzed, TNIP1 exhibited the highest knockdown efficiency (96.2%), followed by CCT2 (88.1%), AURKAIP1 (84.3%), PURG (83.9%), and GLYR1 (78.2%).

These efficiency levels indicate that the observed alterations in circadian parameters such as period and phase are likely a direct consequence of the specific depletion of the target genes, rather than insufficient knockdown. Although LPCAT1 had the lowest knockdown efficiency, the amplitude reduction observed upon its silencing was still statistically significant, suggesting that even partial suppression of LPCAT1 can influence BMAL1-driven circadian oscillations.

Collectively, these results support the conclusion that effective gene silencing was achieved for the majority of targets, validating the functional effects observed in the circadian rhythm phenotypes.

These findings contribute to the broader understanding of post-translational regulation of circadian components and identify new targets potentially relevant to chronotherapeutic applications. Further investigations in more complex systems and in vivo models will be needed to delineate the molecular pathways by which these interactors modulate the biological clock. Nonetheless, this study establishes a strong foundation for future functional analyses of the CRY1 interactome.

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BIOGRAPHY

Eda DEMİRÖZCAN KURUN completed her Master's degree in Molecular Biology and Genetics at Gebze Technical University (GTÜ), Türkiye, where she also received her Bachelor's degree in 2023. During her undergraduate studies, she conducted research on circadian regulation of DNA damage responses in cancer cells under the TÜBİTAK 2209-A Research Project Support Program. Her Master's research focused on the biological clock, participating in the TÜBİTAK 2247-A National Outstanding Researchers Program and conducting thesis work under a TÜBİTAK 1001 project on the functional characterization of CRY1 interactors. She was supported by the TÜBİTAK BİDEB Graduate Fellowship throughout her Master's studies. Eda presented her thesis research at the 9th Gebze Technical University Graduate Research Symposium. Her future goal is to investigate how circadian rhythms relate to psychological and physiological disorders.

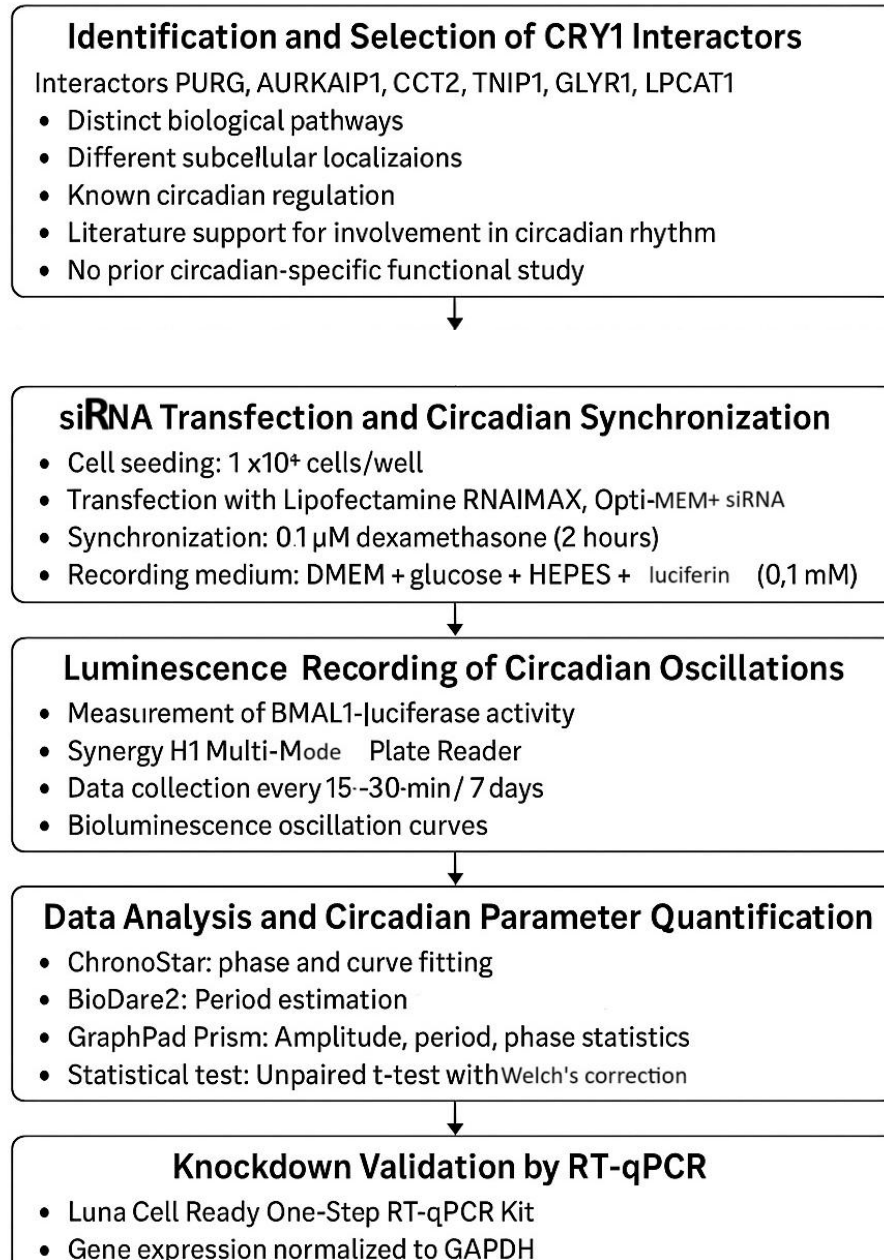
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APPENDICES

APPENDIX A: Experimental layout.

Table A.1. Overview of Experimental Workflow.



APPENDIX B: License Information.

Table B.1. License Information for Figure 1.1.

Item	Details
Figure Number	1.1
Original Source	Masri, S., & Sassone-Corsi, P. (2018). The emerging link between cancer, metabolism, and circadian rhythms. <i>Nature Medicine</i> , 24, 1795–1803. Springer Nature.
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Table B.2. License Information for Figure 1.2.

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Original Source	Chung, S., Son, G. H., & Kim, K. (2011). Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications. <i>Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease</i> , 1812(5), 581–591. Elsevier.
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