

# **Characterization of the Role of CITED2 in Circadian Clock Mechanism**

by

**Çağla Çakmak**

A Dissertation Submitted to the  
Graduate School of Sciences and Engineering  
in Partial Fulfilment of the Requirements for  
the Degree of  
Master of Science  
in  
Molecular Biological and Genetics



August 2020

**Characterization of the Role of CITED2  
in Circadian Clock Mechanism**

Koç University

Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a master's thesis by

**Çağla Çakmak**

and have found that it is complete and satisfactory in all respects,  
and that any and all revisions required by the final  
examining committee have been made.

Committee Members:

---

Prof. İ. Halil Kavaklı

---

Asst. Prof. Necla Birgül İyison

---

Asst. Prof. Serkan Kır

Date:



*to the loving memory of Semra akmak (1958 - ∞)*

## ABSTRACT

Circadian rhythm is biochemical internal clock controlling physiological, behavioral and biochemical activities in a broad array of organisms from cyanobacteria to human. Mammalian circadian clock oscillates with 24 hours of period and environmental factors contribute to the entrainment of the rhythm. Many physiological variables are under the control of circadian clock in mammals such as sleep-wake cycles, body temperature, immune responses, cognitive functions, and hormone secretion. At the cellular level, similar clock mechanisms are found to be conserved among species. In mammals, molecular clock is composed of interlocked transcriptional-translational feedback loops (TTFL) which are controlled by positive and negative regulations. Positive regulation is modulated by the activity of BMAL1 and CLOCK so that BMAL1/CLOCK complex activates the transcription of CRYs and PERs. CRYs and PERs translocate into the nucleus and inhibit the transactivation of BMAL1/CLOCK thus CRY and PER constitute the negative arm of the molecular clock. In the last 20 years, genetics studies revealed that more proteins take part in the molecular clock mechanism. Previous high-throughput screening of transcription factor library identified novel molecular clock regulators that have the potential to affect BMAL1/CLOCK activity on E-box. *CITED2* is one of the top candidates of this study that modulates BMAL1/CLOCK transactivation on *Per1* promoter.

Here in this thesis study, I aimed to characterize the role of *CITED2* in the molecular clock mechanism at the cellular level. Initially, I showed that *CITED2* has a repressor activity on BMAL1/CLOCK driven transcription on E-box element in a dose dependent manner. Knocking down of *CITED2* altered the circadian phenotype by increasing circadian period by 1.5 hours and resulted in high levels of *DBP* and *PER2* mRNA expression levels in U2-OS cell line. Affirmatively, *CITED2* over expression lengthened the period and enhanced the amplitude of circadian rhythm in U2-OS cell line. At the molecular level, *CITED2* over expression affected the transcriptional regulation and caused a reduction in *DBP* and *PER2* levels. Finally, I showed that *CITED2* reduces the physical interaction between BMAL1/CLOCK and it directly interacts with CLOCK. For the first time in this thesis, I presented evidence that *CITED2* acts as a negative transcription factor on BMAL1/CLOCK driven transcription and it is a component of the

circadian molecular clock. Prospectively, these findings will be beneficial for a better understanding of the link between the circadian clock and cellular activities.



## ÖZET

Sirkadiyen ritimler, siyanobakterilerden insanlara, canlıların metabolik, davranışsal ve fizyolojik faaliyetlerini 24 saatlik dilimlerde düzenleyen biyokimyasal döngüler bütünüdür. Bu ritim aslen doğuştan gelmekte olup sıcaklık, ışık gibi çevresel faktörler ve sosyal faktörlerden de etkilenecek 24 saatte bir tekrarlayan içsel saati oluşturur. İnsanlarda sirkadiyen ritim bağışıklık sistemi, uyku ve uyanıklık hali, vücut sıcaklığı, bilişsel düzey ve hormon salgılaması gibi birçok fizyolojik aktiviteyi düzenler. Memeli fizyolojisinde dokusal saatleri kimyasal ve sinirsel iletim yolları üzerinden süprakiazmatik çekirdekte bulunan merkezi saat senkronize eder. Hücresel düzeyde moleküler saat canlılar arasında korunmuş olup, benzer mekanizmalar ile düzenlenir. Moleküler saat, memeli hücresinde transkripsiyonel-translasyonel iç içe geçmiş geri bildirim döngülerinden oluşmaktadır. Bu döngülerde görev alan dört ana saat proteinleri, BMAL1, CLOCK, CRY ve PER, negatif ve pozitif geri bildirimleri oluşturur. BMAL1 ve CLOCK ikili yapı oluşturup aktifleştirici olarak pozitif kolda yer alır, CRY ve PER protein ekspresyonunu başlatır. CRY ve PER sitoplazmada biriktikten sonra çekirdeğe lokalize olarak, BMAL1/CLOCK kompleksi üzerinde negatif etki oluşturur ve kendi ekspresyonlarının baskılanmasını sağlar. Bu sebepten CRY ve PER negatif kolda baskılayıcı olarak görev alır. Önceden yürütülen yüksek kapasiteli (high-throughput) lusiferaz tarama yöntemi ile BMAL1/CLOCK transkripsiyonu üzerinde etkili aday saat proteinleri belirlenmiştir. CITED2, bu çalışma sonunda belirlenen aday saat proteinleri arasındadır.

Bu tez çalışmasını CITED2'nin saat mekanizmasındaki görevini bulmayı amaçlayarak yürüttüm. İlk olarak CITED2'nin BMAL1/CLOCK kompleksinin aktivasyonu üzerinde baskılayıcı etkiye sahip olduğunu gösterdim. Bu baskılayıcı etkinin CITED2'nin CLOCK ile bağlanması sonucunda oluştuğunu ve BMAL1/CLOCK arasındaki etkileşimi azalttığını gösterdim. CITED2 fazla üretiminin U2-OS hücre hattında sirkadiyen saat periyodunu uzattığını ve genliğini artırdığını gösterdim. Moleküler boyutta, CITED2'nin fazla üretildiği hücre hattında DBP ve PER seviyelerinin azalışını, bunu destekleyecek şekilde, CITED2'nin az üretildiği hücre hattında DBP ve PER2 mRNA seviyelerinin artışı gözlemlendim. Sonuç olarak, İlk kez bu tez çalışması ile CITED2'nin CLOCK ile bağlanarak BMAL1/CLOCK transaktivasyonu üzerinde baskılayıcı etkiye sahip bir saat proteini olduğuna dair kanıt sunulmuştur.

## ACKNOWLEDGMENTS

Foremost, I would like to express my sincere gratitude to my thesis advisor Prof. İ. Halil Kavaklı for accepting me to his laboratory. I am thankful for his support, guidance, and mentoring throughout my master's study. I had a chance to study in different fields of biochemistry and molecular biology under his guidance.

I would like to thank my thesis committee members Asst. Prof. Necla Birgül İyison and Asst. Prof. Serkan Kır for their valuable comments and insight on my work. I want to sincerely thank to Dr İbrahim Barış for his support and endless patience, I would not be able to make it through these two years without his advices.

I owe the deepest gratitude to my beloved friend Bilge Bahar Çamur. She was my best friend, my family, and my everything for these two years. She was the reason for me going to the lab with excitement and happiness every day. She was the reason for me staying at the lab until late hours. She was always with me during my good times and my bad times unconditionally. We had the best coffee breaks, best dinners, and best holidays together. I certainly know she will always be an irreplaceable part of my life.

I would like to sincerely thank to my lab mate and home mate Melis Çelik for her great support. She was also my private psychologist during our late-night talks at our sweet home. I also specially thank to Şeref Gül and all the other members of HK lab for being such great lab mates, I learned a lot from them.

I feel so lucky to spend seven years of my life with the funniest friend Umut Batman. He was always there for me, he always stood behind me. We had amazing times even when we stayed up all night to study for the finals. I want to specially thank to Ezgi Memiş for laughing at my jokes every single time and questioning the life with me even at the most inconvenient times. I also would like to thank Fatmanur Tiryaki and Hüseyin Karabürk for being such good friends to me.

Finally, I want to specially thank to my beloved family Nihan Çakmak Eğinli, Mehmet Çakmak and Beyhan Çakmak. They always supported and encouraged me throughout my personal and academic life. I could not do it without their endless love. I feel the luckiest person to have such a great sister, she is always my greatest support. She raised me with endless love and patience. At last but not the least, I would like to express my deepest

feelings for my beloved fiancé Mert Durmaz, my better half. I would like to thank him for his respect and understanding for my work life, I appreciate it a lot. His unconditional love, support, and patience made everything easier. I know that we will be together forever and ever.



## TABLE OF CONTENTS

|                                                                                                    |      |
|----------------------------------------------------------------------------------------------------|------|
| ABSTRACT .....                                                                                     | iv   |
| ÖZET .....                                                                                         | vi   |
| ACKNOWLEDGMENTS .....                                                                              | vii  |
| TABLE OF CONTENTS .....                                                                            | ix   |
| LIST OF TABLES .....                                                                               | xii  |
| LIST OF FIGURES .....                                                                              | xiii |
| NOMENCLATURE .....                                                                                 | xv   |
| Chapter 1 INTRODUCTION .....                                                                       | 1    |
| Chapter 2 LITERATURE REVIEW .....                                                                  | 4    |
| 2.1 Biological Timing Systems .....                                                                | 4    |
| 2.1.1 Infradian and Ultradian Rhythms.....                                                         | 4    |
| 2.1.2 Circadian Rhythms.....                                                                       | 5    |
| 2.2 Circadian Rhythms of Prokaryotes to Higher Organisms .....                                     | 6    |
| 2.2.1 Circadian Clock in Cyanobacteria .....                                                       | 6    |
| 2.2.2 Circadian Clock in Plants.....                                                               | 7    |
| 2.2.3 Circadian Clock in Invertebrates .....                                                       | 8    |
| 2.3 The Historical Overview of Mammalian Circadian Clock.....                                      | 9    |
| 2.4 Mammalian Circadian Clock.....                                                                 | 11   |
| 2.4.1 Central Pacemaker and Peripheral Clocks .....                                                | 11   |
| 2.4.2 The Importance of Circadian Rhythm in Human Physiology and Health                            | 14   |
| 2.5 Mammalian Circadian Clock at the Molecular Level .....                                         | 15   |
| 2.5.1 Core Clock Proteins and Clock mutants .....                                                  | 15   |
| 2.5.2 Molecular Architecture of Mammalian Circadian Clock .....                                    | 16   |
| 2.5.3 BMAL1/CLOCK Transactivation.....                                                             | 19   |
| 2.6 CITED2 (CBP/p300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2) ..... | 20   |

|           |                                                                   |    |
|-----------|-------------------------------------------------------------------|----|
| 2.6.1     | The Cellular Activities of <i>CITED2</i> .....                    | 21 |
| 2.6.2     | <i>CITED2</i> in Physiology and Health .....                      | 22 |
| Chapter 3 | MATERIALS AND METHODS .....                                       | 24 |
| 3.1       | Cell lines and Regular Cell Culture Procedures .....              | 24 |
| 3.1.1     | Cell Lines and Maintenance.....                                   | 24 |
| 3.1.2     | Cell Passaging and Cell Stocking .....                            | 24 |
| 3.1.3     | Cell Harvest.....                                                 | 24 |
| 3.1.4     | Cell Synchronization.....                                         | 25 |
| 3.2       | Plasmid Construct and Molecular Cloning .....                     | 25 |
| 3.2.1     | Sub-cloning.....                                                  | 25 |
| 3.2.2     | Molecular Cloning.....                                            | 26 |
| 3.3       | Repression Assay .....                                            | 27 |
| 3.4       | Mammalian Two Hybrid Assay .....                                  | 28 |
| 3.5       | Pull-Down Assay.....                                              | 29 |
| 3.6       | Establishment of <i>CITED2</i> Over Express Stable Cell line..... | 30 |
| 3.7       | Protein isolation and Western Blotting .....                      | 32 |
| 3.7.1     | Protein Isolation.....                                            | 32 |
| 3.7.2     | Western Blotting .....                                            | 32 |
| 3.8       | Real Time Quantitative PCR.....                                   | 34 |
| 3.8.1     | RNA Isolation.....                                                | 34 |
| 3.8.2     | cDNA Synthesis.....                                               | 34 |
| 3.8.3     | Quantitative Real-Time PCR (RT-qPCR).....                         | 35 |
| 3.9       | siRNA Knock-Down of <i>CITED2</i> in U2-OS cells .....            | 35 |
| 3.10      | Real-Time Tracking of Circadian Oscillations .....                | 36 |
| 3.10.1    | <i>Bmal1:dluc</i> Lentivirus Production.....                      | 36 |
| 3.10.2    | Real Time Bioluminescence Monitoring.....                         | 37 |
| Chapter 4 | RESULTS.....                                                      | 38 |

|            |                                                                                              |    |
|------------|----------------------------------------------------------------------------------------------|----|
| 4.1        | Cloning of Human <i>CITED2</i> cDNA into Mammalian Expression Vector.....                    | 39 |
| 4.2        | <i>CITED2</i> Represses BMAL1/CLOCK Heterodimer on <i>mPer1</i> Promotor.....                | 40 |
| 4.3        | Repression Activity of <i>CITED2</i> is not Dependent on CBP/p300 Complex.....               | 44 |
| 4.4        | <i>CITED2</i> Knock-Down Results in Altered Circadian Period in U2-OS .....                  | 47 |
| 4.5        | <i>CITED2</i> siRNA Knock-Down Increases the mRNA Levels of DBP and PER2 .<br>.....          | 48 |
| 4.6        | Generation of <i>CITED2</i> Over Express U2-OS Stable Cell Line .....                        | 52 |
| 4.7        | <i>CITED2</i> Over Expression Lengthens the Period of U2-OS Cells.....                       | 53 |
| 4.8        | <i>CITED2</i> Over Expression Decreases both mRNA and Protein Levels of DBP<br>and PER2..... | 55 |
| 4.9        | <i>CITED2</i> Reduces the Physical Interaction Between BMAL1 and CLOCK ....                  | 57 |
| 4.10       | <i>CITED2</i> Physically Interacts with CLOCK.....                                           | 58 |
| Chapter 5  | DISCUSSION .....                                                                             | 61 |
|            | VITA.....                                                                                    | 67 |
|            | BIBLIOGRAPHY.....                                                                            | 68 |
|            | APPENDICES .....                                                                             | 90 |
| APPENDIX A | Primer Sequences.....                                                                        | 90 |
| APPENDIX B | Maps of Expression Vectors .....                                                             | 91 |
| APPENDIX C | DNA and Protein Ladder .....                                                                 | 94 |
| APPENDIX D | Permissions and Copyrights.....                                                              | 95 |
| APPENDIX E | Laboratory Equipment.....                                                                    | 97 |

## LIST OF TABLES

|                                                                             |    |
|-----------------------------------------------------------------------------|----|
| Table 1: PCR protocol details to amplify <i>Cited2</i> for cloning.....     | 26 |
| Table 2: Firefly luciferase and Renilla luciferase buffer compositions..... | 28 |
| Table 3: Compositions of SDS-PAGE gels used in Western Blot analysis.....   | 33 |



## LIST OF FIGURES

|                                                                                                                                                                  |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1. Simplified illustration of the molecular clock mechanism.....                                                                                          | 2  |
| Figure 2. Schematic representation of ultradian, circadian and infradian rhythms and their biological examples.....                                              | 5  |
| Figure 3. Molecular mechanism of <i>Synechococcus elongatus</i> circadian clock.....                                                                             | 7  |
| Figure 4. Schematic illustration of <i>Drosophila melanogaster</i> molecular clock.....                                                                          | 9  |
| Figure 5. Schematic illustration of circadian clock parameters period, phase and amplitude.....                                                                  | 11 |
| Figure 6. Location of RHT and SCN in the brain. SCN is located in the anterior hypothalamus and RHT is a transmission tract located between the eye and SCN..... | 13 |
| Figure 7. Schematic illustration of the mammalian circadian clock mechanism.....                                                                                 | 17 |
| Figure 8. Schematic representation of pull down assay protocol. ....                                                                                             | 30 |
| Figure 9. The circadian expression pattern of CITED2 in mouse liver (A), and kidney (B) tissues.....                                                             | 39 |
| Figure 10. Confirmation of <i>CITED2</i> -pcDNA4/myc-His A cloning with PCR and restriction digestion experiments. ....                                          | 40 |
| Figure 11. Schematic representation of repression assay and working principal. ....                                                                              | 41 |
| Figure 12. Inhibitory effect of CITED2 on BMAL1/CLOCK driven activation.....                                                                                     | 42 |
| Figure 13. CITED2 does not have any effect on <i>mPer1:dluc</i> in the absence of BMAL1/CLOCK protein complex. ....                                              | 42 |
| Figure 14. Western Blot analysis of CITED2 and CRY1. ....                                                                                                        | 43 |
| Figure 15. Repression activity of CITED2 was dampened upon CBP/p300 transfection. ....                                                                           | 45 |
| Figure 16. CBP and p300 do not activate <i>mPer1:dluc</i> promoter.....                                                                                          | 46 |
| Figure 17. p300 counteracts the repression activity of CITED2 in a dose dependent manner.....                                                                    | 46 |
| Figure 18. <i>CITED2</i> knock down altered circadian phenotype in U2-OS ( <i>Bmal1:dluc</i> ). ....                                                             | 48 |
| Figure 19. <i>CITED2</i> siRNA transfection has no effect on mRNA levels of core clock genes in unsynchronized U2-OS cells. ....                                 | 50 |

|                                                                                                                                                       |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 20. <i>CITED2</i> knock down was the most successful at 12 <sup>th</sup> hour after dexamethasone treatment.....                               | 51 |
| Figure 21. <i>CITED2</i> siRNA transfection increased mRNA levels of <i>DBP</i> and <i>PER2</i> upon cell synchronization. ....                       | 52 |
| Figure 22. <i>CITED2</i> over express U2-OS cell line stably express <i>CITED2</i> . ....                                                             | 53 |
| Figure 23. <i>CITED2</i> over expression extended the period of U2-OS cells.....                                                                      | 54 |
| Figure 24. <i>CITED2</i> over expression results in decreased level of <i>DBP</i> and <i>PER2</i> . ....                                              | 56 |
| Figure 25. <i>CITED2</i> interferes with the physical interaction between <i>BMAL1</i> and <i>CLOCK</i> according to Mammalian two hybrid assay. .... | 58 |
| Figure 26. <i>CITED2</i> interacts with <i>CLOCK</i> through pull down assay.....                                                                     | 60 |
| Figure 27. Circadian expression profile of p300 in the mouse lung.....                                                                                | 63 |
| Figure 28. The proposed model for <i>CITED2</i> repression on <i>BMAL1/CLOCK</i> driven transcription on E-box containing promoters.....              | 66 |

## NOMENCLATURE

|                            |                                                                                 |
|----------------------------|---------------------------------------------------------------------------------|
| AMPK                       | AMP-activated protein kinase                                                    |
| AVP                        | Arginine vasopressin                                                            |
| bHLH                       | Basic helix-loop-helix domain                                                   |
| BMAL1/ ARNTL1              | Aryl hydrocarbon receptor nuclear translocator-like protein 1                   |
| CBP                        | CREB-binding protein                                                            |
| CCA1                       | Circadian clock associated 1 protein                                            |
| CH1/TAZ1                   | Cysteine/histidine-rich 1 domain                                                |
| CITED2                     | CBP/P300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2 |
| CKI $\epsilon$ /I $\delta$ | Casein kinase 1 epsilon/1 sigma                                                 |
| CLOCK                      | Circadian locomotor output cycles kaput                                         |
| CR                         | Circadian rhythm                                                                |
| CREB                       | cAMP response element-binding                                                   |
| CRISPR/Cas9                | Clustered regularly interspaced short palindromic repeats                       |
| CRY                        | Cryptochrome                                                                    |
| CYC                        | Cycle                                                                           |
| DBP                        | D site of albumin promoter (albumin D-box) binding protein                      |
| DSPD                       | Delayed sleep phase disorder                                                    |
| ELF                        | Early flowering                                                                 |
| FASP                       | Familial advanced sleep phase syndrome                                          |
| FBXL3/21                   | F-box/LRR-repeat protein 3/21                                                   |
| Glu                        | Glutamate                                                                       |

|                |                                            |
|----------------|--------------------------------------------|
| GSK-3 $\beta$  | Glycogen synthase kinase 3 beta            |
| HAT            | Histone acetyl transferase                 |
| HEK 293T       | Human embryonic kidney cells               |
| HIF1- $\alpha$ | Hypoxia-inducible factor 1- $\alpha$       |
| HPX            | Hypoxia                                    |
| LHY            | Late elongated hypocotyl                   |
| MEF            | Mouse embryonic fibroblast cells           |
| MLL1           | Mixed lineage leukaemia protein-1          |
| NAMPT          | Nicotinamide phosphoribosyl transferase    |
| NFIL3          | Nuclear factor, interleukin 3 regulated    |
| NF- $\kappa$ b | Nuclear factor $\kappa$ B                  |
| NMDA           | N-methyl-D-aspartate                       |
| NPAS2          | Neuronal PAS domain protein 2              |
| NRX            | Normoxia                                   |
| OE             | Over express                               |
| p300           | Histone acetyltransferase p300             |
| PACAP          | Pituitary adenylate cyclase-activating     |
| PAS            | Per-arnt-sim domain                        |
| PER            | Period                                     |
| PPAR           | Peroxisome proliferator-activated receptor |
| pRGC           | Photosensitive retinal ganglion cells      |
| PRR5           | Pseudo response regulator                  |
| PTM            | Post translational modification            |
| REV-ERB        | Nuclear receptor subfamily 1, group D      |

|              |                                                  |
|--------------|--------------------------------------------------|
| RHT          | Retinohypothalamic tract                         |
| ROR          | RAR-related orphan receptor                      |
| RORE         | ROR/REV-ERB-response element                     |
| RT-qPCR      | Real time quantitative PCR                       |
| SCN          | Suprachiasmatic nucleus                          |
| siRNA        | Small interfering RNA                            |
| SIRT1        | Sirtuin 1                                        |
| SLIMB        | Supernumerary limbs                              |
| SP1          | Specificity protein 1                            |
| SRJ          | Serine-rich junction                             |
| STAT         | Signal transducer and activator of transcription |
| TFAP2        | Transcriptional regulation by the AP-2           |
| TGF- $\beta$ | Transforming growth factor $\beta$               |
| TIM          | Timeless                                         |
| TOC1         | Timing of CAB Expression 1                       |
| TTFL         | Transcriptional-translational feedback loops     |
| U2-OS        | Human bone osteosarcoma epithelial cells         |
| WB           | Western Blotting                                 |
| ZT           | Zeitgeber time                                   |

## Chapter 1

### INTRODUCTION

Circadian rhythm (CR) is an endogenous biological clock oscillating throughout the day and regulating behavioural, biochemical, and physical activities of an organism (Lowrey et al., 2004). Rhythmicity is observed in a broad array of organisms from cyanobacteria to human (Dunlap et al., 1999; Panda et al., 2002). Mammalian circadian clock oscillates with 24 hours of the period and environmental factors contribute to entrainment of the rhythm. A wide range of events occurring in mammalian physiology including sleep-wake cycles, body temperature, immune responses, cognitive functions, and hormone secretion are under the control of the circadian clock. Central pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, is responsible for regulation and synchronization of the peripheral clocks through neuronal signals and hormones (Moore & Eichler, 1972).

At the cellular level, similar clock mechanisms are found to be conserved among species. In mammals, the molecular clock is composed of interlocked transcriptional-translational feedback loops (TTFL) which are controlled by positive and negative regulators. The positive arm of the main TTFL is comprised of Brain and Muscle ARNT-Like-1 (*BMAL1*) and Circadian Locomotor Output Cycles Kaput (*CLOCK*) transcription factors (**Figure 1**). Prior to transactivation, *BMAL1* and *CLOCK* heterodimerize then bind to a specific target on DNA which is a canonical E-box element in some clock-related genes. *BMAL1/CLOCK* binding activates the transcription of *PERIOD* (*PER*) and *CRYPTOCHROME* (*CRY*) which are the members of the negative arm of the TTFL. *CRY*s and *PER*s heterodimerize in the cytosol and translocate back into the nucleus upon complex formation with CKI $\epsilon$  and Casein kinase  $\epsilon$  and  $\delta$  (CKI $\delta$ ). In the nucleus *CRY/PER* complex exerts a negative effect on *BMAL1/CLOCK* transactivation and eventually repress their own expression (Ueda et al., 2005; Ko et al., 2008). Other two TTFLs also

have a similar molecular mechanism. *BMAL1* and *NFIL3* activation are under the control of the second TTFL in which RORE-box transcription factors RORs (Retinoic acid receptor-related orphan receptors) and REV-ERBs activate and repress their own transcription, respectively. The last TTFL is structured by the activity of *NFIL3* and *DBP* transcription factors which bind to the D-box element to up regulate the expression of RORs and REV-ERBs (Ueda et al., 2005; Takahashi, 2017). These three interlocked TTFLs generates the circadian rhythmicity on the molecular level that takes approximately 24 hours to complete in mammalian physiology.

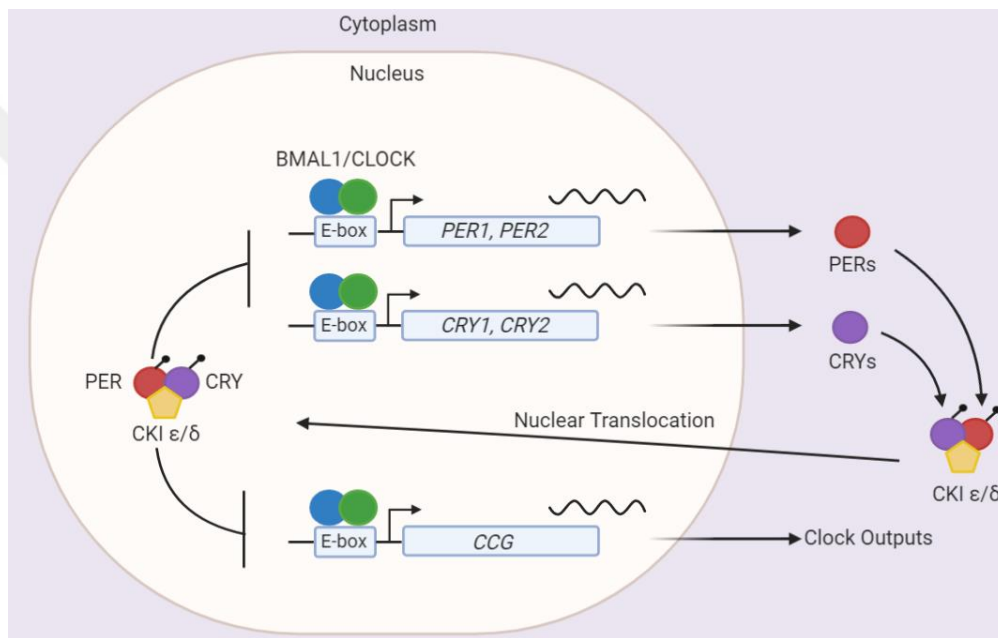


**Figure 1. Simplified illustration of the molecular clock mechanism.**

Since a great variety of physiological and metabolic events are under the control of circadian rhythmicity, robust clock is vital for both physical and mental health. As it is expected, disturbance in circadian clock may result in a variety of diseases in human. Therefore, it is essential to characterize the circadian clock and circadian clock-related mechanisms in great detail.

Apart from the known core clock proteins and other clock regulator proteins, many proteins are found to be connected to the circadian clock as new studies are conducted. A previous study conducted by Prof. Halil Kavaklı identified candidate clock regulators which have a potential to modulate *BMAL1/CLOCK* transactivation by using high-

throughput luciferase screening of transcription factor library. Subsequently, some of the candidate proteins are already shown to be a clock regulator by further studies. CITED2 (CBP/p300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2) was designated as one of the top candidates that have the potential to modulate BMAL1/CLOCK activity. Here in this thesis, I investigated the role of candidate clock protein CITED2 in the circadian clock mechanism.

CITED2 is a transcriptional coactivator carrying i) three conserved domains which are common in CITED family proteins and ii) distinctive serine-glycine rich junction (SRJ) (Shioda et al., 1997; Dunwoodie et al., 1998). CITED2 is involved in the modulation of a variety of cellular and developmental processes at the molecular level. CITED2 stimulates TFAP2-mediated transcriptional activation by acting as a bridge between TFAP2 transcription factors and the p300/CBP transcriptional coactivator complex (Bragança et al., 2003). CITED2 also negatively regulates HIF-1 transactivation through disrupting the complex of HIF-1 $\alpha$  and CBP/p300 upon binding to the CBP/p300 (Freedman et al., 2003; Bakker et al., 2007). CITED2 mutations are implicated in a variety of human diseases from ventricular and atrial septal defects (Sperling et al., 2005) to breast cancer (Van Agthoven et al., 2009; Mhatre et al., 2013). This wide spectrum of activities leads CITED2 into molecular biology research.

Chapter 2 provides a detailed literature review about the structure and molecular mechanism of the mammalian circadian clock and its importance in mammalian physiology. Additionally, CITED2 function and CITED2 involved cellular events are also reviewed.

Chapter 3 provides information about the materials and methods used in this study.

Chapter 4 presents all results and a detailed explanation of the results obtained during the study.

Chapter 5 discusses all the results and addresses future directions to further characterize the function of CITED2 in the circadian clock mechanism.

## Chapter 2

### LITERATURE REVIEW

#### 2.1 Biological Timing Systems

Temperature, light, seasons, humidity, day, and night durations all are dependent on the earth's shape and rotation on daily basis and throughout the year. Almost all organisms developed a great variety of mechanisms to adapt these fluctuations which are studied by chronobiology field (Giebultowicz, 2004). These mechanisms constitute biological rhythms which studied under three categories; i) infradian rhythms, ii) ultradian rhythms and iii) circadian rhythms (**Figure 2**).

##### 2.1.1 Infradian and Ultradian Rhythms

Infradian rhythm is defined as the whole of events which take longer than 24 hours to complete. Infradian rhythms can be yearly such as hibernation and migration of animals, monthly such as menstrual cycles or weekly (Laje et al., 2018). Additionally, we can observe events that repeat three or four times a year or some events that cycling takes more than a year. These all events are classified under infradian rhythms. Ultradian rhythms are the whole of events that take shorter than 24 hours to be completed. Ultradian rhythms are not dependent on environmental changes but instead, they are endogenously generated. Breathing, urination, blinking, heart rate or sleep are examples of ultradian rhythms. Even though sleep seems to be repeating once in each 24-hour, it is comprised of repetitive two stages (Weber, 2017).

The other timing systems, the circadian rhythm is defined as the whole of events and oscillations which takes 24-hours to cycle. It is explained in greater detail in the next section.

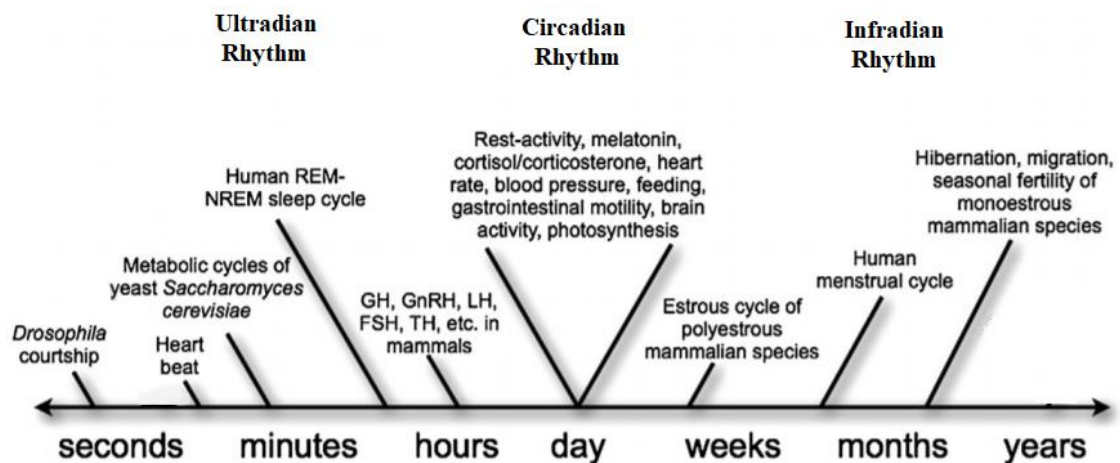


**Figure 2. Schematic representation of ultradian, circadian and infradian rhythms and their biological examples.**

This figure is adopted from Lamont & Amir, 2016. Copyright 1997, with permission from ELSEVIER.

### 2.1.2 Circadian Rhythms

The circadian word comes from *circa* which means ‘about’ and *diem* which means ‘a day’ in Latin. The whole of behavioural, physiological, and biochemical events which repeat once in 24 hour constitutes the circadian rhythm. In other words, circadian rhythms are endogenous time-keeping systems with distinct characteristics. Circadian rhythms are encoded by the genome. Circadian rhythms are entrainable systems by external factors such as light or feeding even though they are also self-sustainable without external cues (Pittendrigh, 1960). Another hallmark of circadian rhythms is that oscillations can sustain periodicity over a temperature range (Eckardt, 2010). Circadian rhythmicity itself is highly conserved among organisms with a variety of mechanisms from unicellular organisms to insects, plants, and mammals (Dunlap, 1999).

Circadian rhythm is entrained by external factors such as light and temperature and forms biological clock also known as the circadian clock. The circadian clock is synchronized to the solar system through light and other external cues thus it adjusts organisms to environmental conditions (Aschoff, 1984). Additionally, external factors which affect the circadian rhythmicity is also named as *zeitgeber* (time-giver in German). Since each

organism has a variety of physiological regulations, the circadian clock may vary between 23 to 25 hours depending on the organism. Circadian clock can also be conceived as a protection mechanism that prevents organisms' fitness upon the fluctuant environment (Aton et al., 2005).

## 2.2 Circadian Rhythms of Prokaryotes to Higher Organisms

Circadian rhythm has shown to be a common feature across a great array of organisms with cumulative studies. Even though rhythmic control is commonly observed, each species developed different molecular mechanisms to adapt environmental fluctuations and solar system. Circadian rhythm also provides an evolutionary advantage since internal clocks convey behavioural or physiological adaptation to external changes (Hut & Beersma, 2011). This adaptation may be beneficial in terms of energy storage, mating cycles or survival. Additionally, some clock proteins share similar functions among organisms. Also, in some cases clock proteins may have different functions in different organisms even though they share homology. For instance, photolyase serves as a photoreceptor in *Drosophila melanogaster* where it is a member of the core clock mechanism in human instead of photoreception (Miyamoto & Sancar, 1999).

### 2.2.1 Circadian Clock in Cyanobacteria

In 1990s, there was a misconception that only eukaryotic organisms have a circadian rhythm. However, the study, done by Kondo et al. in 1993, disproved this dogma and proved that cyanobacterium *Synechococcus elongatus* has a circadian rhythm. Cyanobacteria is a well-studied prokaryote and the simplest organism that have a robust circadian clock. Gene expression patterns, cell division, photosynthesis, respiration and chromosome compaction are under the control of circadian clock genes *kaiA*, *kaiB*, and *kaiC* (Liggett, 2008; Ishiura et al., 1998). During the day, KaiA and KaiC mostly reside in the cytosol. Upon KaiA and KaiC interaction, KaiC autophosphorylates and gives rise to RpaA phosphorylation (**Figure 3**). Accumulated RpaA-P, activates the expression of dusk peaking genes where it represses the expression of dawn peaking genes. During the night, KaiB interacts with KaiA/KaiC complex so that this interaction results in dephosphorylation of RpaA. Subsequently, RpaB replaces RpaA and completes the cycle of dawn peaking genes (Cohen & Golden, 2015).

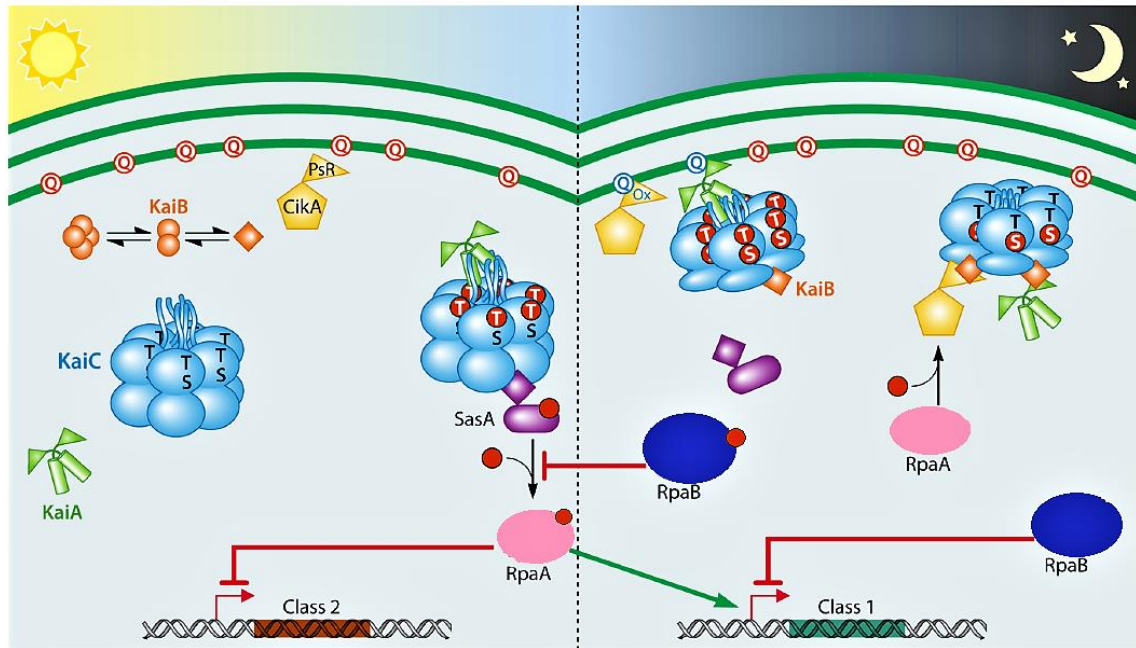


**Figure 3. Molecular mechanism of *Synechococcus elongatus* circadian clock.**

This figure is adopted from Cohen & Golden, 2015.

## 2.2.2 Circadian Clock in Plants

For many centuries, plants served as a major nutrition supply for human, therefore human got a chance to observe and examine plant physiology more than other organisms. So, the first cues about the presence of circadian rhythm emerged from plant observations in the 18<sup>th</sup> century. Not surprisingly, plants possess highly complicated circadian regulations depending on the environmental factors. Since *Arabidopsis thaliana* is the most studied plant, clock studies also focus on this species. In plants photosynthesis, respiration, flowering, water uptake, defoliation, pollination and many other essential metabolic processes are under the control of circadian rhythm (Sanchez & Kay, 2016). The circadian clock of plants consist of three main transcriptional-translational feedback loops as following; i) central loop, ii) morning loop and iii) evening loop (Harmon et al., 2005). Central feedback loop comprised of Circadian Clock Associated 1 (CCA1) and Late Elongated Hypocotyl (LHY), which are expressed during the day and responsible for the repression of other clock proteins. These central loop members are repressed during the night by Timing of CAB Expression 1 (TOC1) (Millar et al., 1995). During the day,

Pseudo Response Regulators (PRR5, PRR7 and PRR9) are expressed and regulate circadian clock through repression of CCA1 and LHY. In the evening loop LUX, Early Flowering 3 and 4 (ELF3 and ELF4) transcription factors are activated. These proteins repress PRRs where they induce expression of central loop members CCA1 and LHY (Sanchez & Kay, 2016). Although these feedback loops and proteins are the main drivers in plant circadian clock, this is just an oversimplification of this highly complicated system.

### 2.2.3 Circadian Clock in Invertebrates

Discovery of *per* was a high impact and milestone study in chronobiology and it was carried on *Drosophila melanogaster* model organism by Konopka (Konopka & Benzer, 1971). This finding paved the way for further clock studies and discoveries. The molecular clock of *D. melanogaster* is driven by four main clock proteins Cycle (CYC), CLOCK, PER, and Timeless (TIM) (**Figure 4**). CYC and CLOCK carry PAS domains and DNA-binding domains so that they activate the expression of TIM and PER upon binding to the E-box of target genes at midday (Harmer et al., 2001; Hardin, 2004). TIM and PER are expressed at night and they form a heterodimer which also protects PER from ubiquitination and degradation by double time kinase (DBP) and supernumerary limbs (SLIMB) (Panda et al., 2002; Hardin, 2011). CRY/PER heterodimer translocates to the nucleus subsequently they interact with CYC/CLOCK complex and inhibit their own transcription. This feedback loop has also common and similar elements with the mammalian circadian clock. *D. melanogaster* can sense light through different tissues which contain opsin-based photopigments in common. Additionally, CRY is also found in photoreceptive elements and it is also involved in photoreception and clock synchronization (Yoshii et al., 2008).

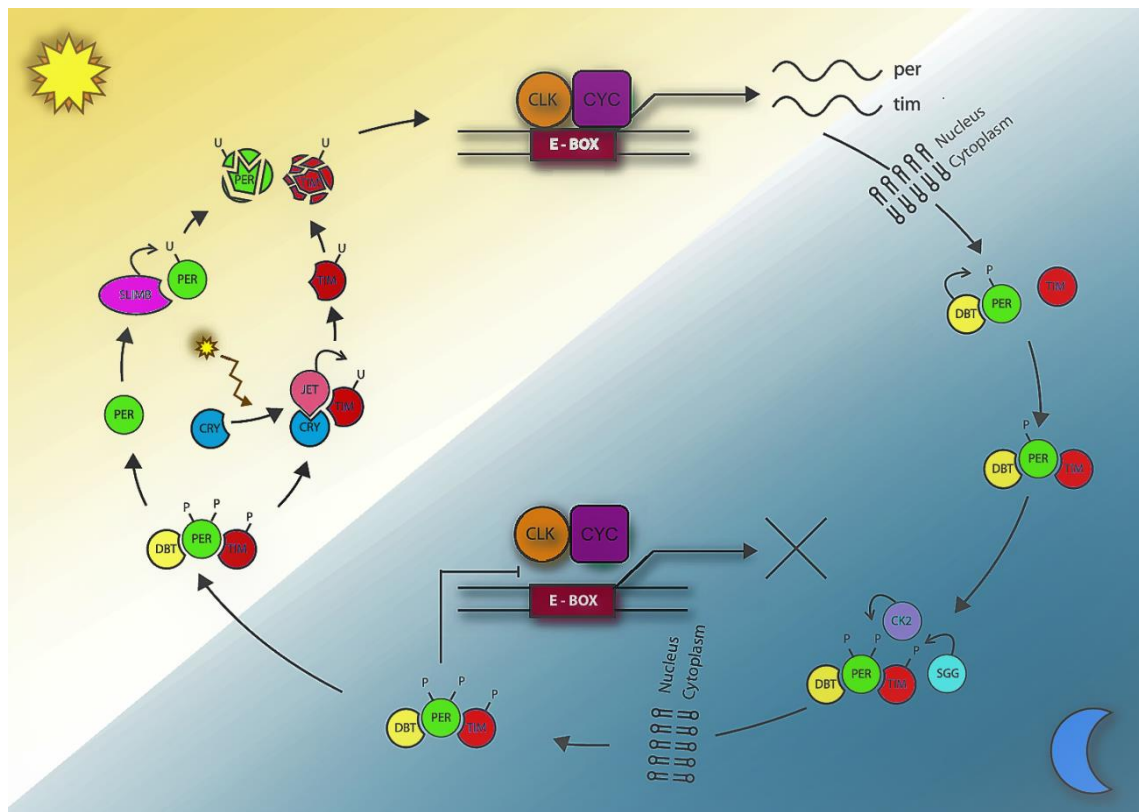


**Figure 4. Schematic illustration of *Drosophila melanogaster* molecular clock.**

This figure is adopted from Muraro & Ceriani, 2014.

### 2.3 The Historical Overview of Mammalian Circadian Clock

Chronobiology studies first started with the French scientist Jean-Jacques d'Ortous de Mairan who observed the *Mimosa pudica* leaves. He realised that the leaves of the plant was opened during day-time and closed during night-time. He conducted a small experiment and kept the plant at constant dark conditions. What he saw is that the movement of the leaves sustained even under constant dark conditions (D'Ortous de Mairan, 1729). de Marian was the very first scientist that presented proof of endogenous rhythm in plants. The discovery of de Marian was also proved by Swiss scientist Augustin Pyramus de Candolle. Candle studied on the leaf folding period of *Mimosa pudica* and he realised the period of *M. pudica* was not exactly 24 hours ben instead shorter than 24 hours (Candolle, 1798). Subsequently, he reasoned that the biological clock is under the control of both endogenous clock and environmental factors. After many years, German scientist Jürgen Aschoff described the term 'zeitgeber' and described the endogenous

rhythm of organisms (Hobson & Aschoff, 1965). In the 19<sup>th</sup> century, the first attempt to determine the period of a mammalian organism was carried by Maynard S. Johnson in 1939. He measured the endogenous free-running locomotor activity of mice (Johnson, 1939).

The discovery of genetic elements in circadian regulation started with a pioneer study done by Konopka and Benzer in 1971. Konopka, as a graduate student, worked with forward genetics methods in fruit flies and identified three mutants which have i) longer period with 28 hours, ii) shorter period with 19 hours and iii) abolished period. These three mutations were all mapped to the same location on X-chromosome and identified as alleles of the same gene *period*. *Period* was isolated and characterized 15 years later as the DNA sequencing technologies advanced. The other core clock gene *Clock* was discovered by Takahashi and Vitaterna in 1994 via forward genetics analysis carried on *Mus Musculus*. Takahashi's group also revealed that CLOCK regulates period length sustains the rhythmicity of the molecular clock (Antoch et al., 1997). The discovery of genetic elements of circadian rhythm was continued with the discovery of another core clock gene *Bmal1* (also named as *Arntl1*) by Hogenesch in 1997. His group also conducted wide studies where they characterized BMAL1 function.

The history of CRY, another core clock protein, was more debated than the other discoveries. In 1996, two photolyase orthologs were identified in human by Sancar and Ikenaga (Hsu et al., 1996; Todo et al., 1996). It was known that photolyases act as a photoreceptor and induced DNA repair under the blue light in a variety of species from bacteria to plants (Sancar, 1996). The discovery brought a heated debate about the function of these two ortholog proteins in human. The debate was concluded when the function of CRYs were identified as clock regulators (Miyamoto & Sancar, 1999). In 2002, a study done by Berson in 2002 revealed that melanopsin photopigment in retinal ganglion cells sense the light and transmit the signal to the central pacemaker, SCN, to adjust circadian rhythmicity in the human body.

## 2.4 Mammalian Circadian Clock

The circadian mechanism is modelled with three main components i) a centre that produces periodic oscillations, ii) input pathways that receive external cues and iii) output pathways which generate rhythmic changes. Circadian clocks are depicted by plotting a particular oscillator such as body temperature versus time. This sinusoidal-shaped plot brings three main parameters of a circadian oscillation i) period, ii) amplitude and iii) phase. Period is defined as the time interval for one cycle to be completed. Amplitude is the distance from the peak point to the bottom point. Phase can be described as a specific location of a point in a repetitive waveform. In **Figure 5**, all parameters are schematically explained.

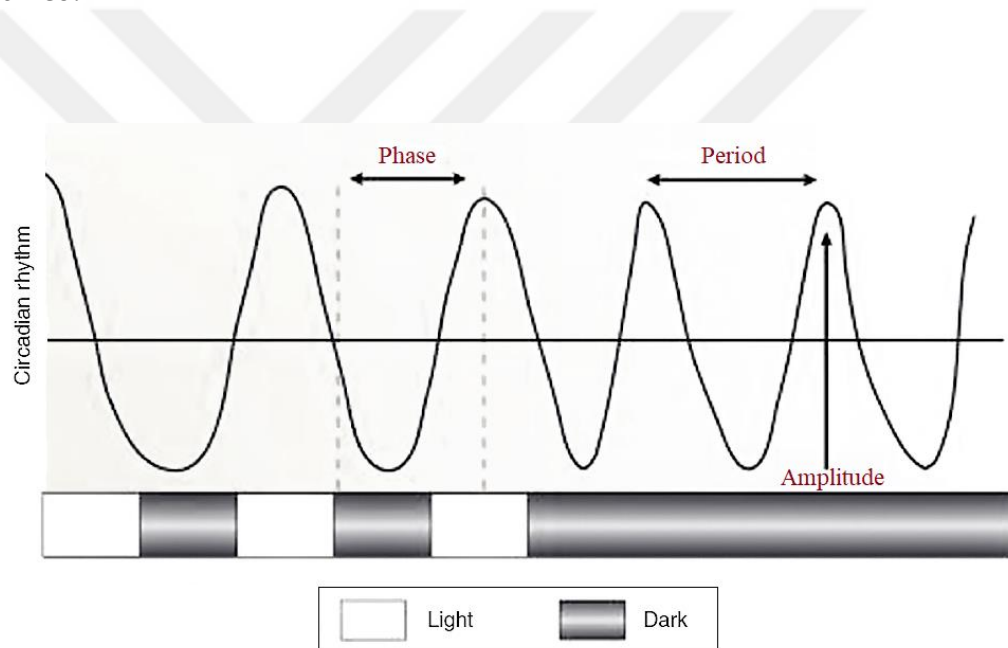


**Figure 5. Schematic illustration of circadian clock parameters period, phase, and amplitude.**

This figure is adopted from Nydegger et al., 2014.

### 2.4.1 Central Pacemaker and Peripheral Clocks

SCN is located in the anterior hypothalamus and it is comprised of approximately 20,000 neurons. Through neuronal signalling pathways and hormonal release, SCN orchestrates all peripheral clocks. Light is the most dominant external factor and it is received by photosensitive retinal ganglion cells (pRGC) which are a type of neuronal cell in the

mammalian retina. pRGCs were discovered in 1923 when rodless and coneless mice reacted to light stimuli by pupil movement (Keeler, 1928). Photopigment melanopsin is highly expressed in pRGCs and transmits light signals to SCN by a chemical tract (Do & Yau, 2010; Dijk & Archer, 2009). Other photoreceptor cells, rods and cones are also responsible for light perception and they also relay light information through electrical impulses. However, melanopsin has a higher capacity to receive (wavelength up to 500 nm) and transmit light to SCN compared to other photoreceptors (Do & Yau, 2010). Light perception is projected to SCN through a tract called retinohypothalamic tract (RHT) (**Figure 6**). RHT is comprised of ipRGCs axons and it lies along with ganglion cells and inner nuclear layer (Gooley, 2001; Berson et al., 2002).

Signal transduction pathway through RHT includes the release of glutamate (Glu),  $\alpha$ -aminobutyric acid GABA, pituitary adenylate cyclase-activating protein (PACAP), arginine vasopressin (AVP) and some other neurotransmitters (Morin & Allen, 2006). Several studies showed that expression of *Period* is also controlled as a result of this signal transduction pathways. Glutamate release turns on N-methyl-D-aspartate (NMDA) receptor and results in high levels of  $\text{Ca}^{+2}$  concentration. CREB (cAMP response element-binding protein) responds to increased  $\text{Ca}^{+2}$  levels and binds to the PER promoter thereafter starts PER expression (Travnickova et al., 2002; Shigeyoshi et al., 1997). As a result of this sensitive signalling mechanism of SCN, SCN neurons create a web where they relay signals to form a unified clock throughout the body. Apart from this essential role of SCN, SCN itself displays rhythmic oscillations ranging from 20 hours to 30 hours (Herzog, 2007; Liu et al., 1997).

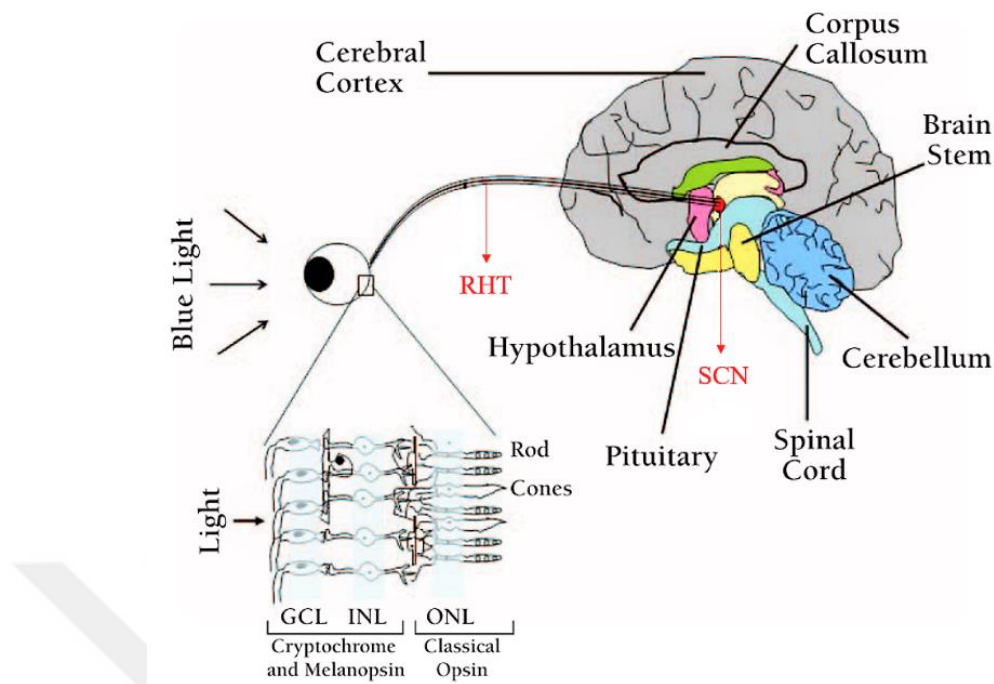


**Figure 6. Location of RHT and SCN in the brain. SCN is located in the anterior hypothalamus and RHT is a transmission tract located between the eye and SCN.**

This figure is adopted from Kavakli & Sancar, 2002.

Peripheral organs receive signals from SCN, and each organ has another level of regulation through a specialized clock also known as the peripheral clock or slavery clocks. Peripheral clocks produce organ-specific oscillations through regulation of mRNA expressions (Yamazaki et al., 2000; Zhang & Hogenesch, 2014). Since each organ has different function, activity, physiology and biochemistry, each peripheral tissue developed a delicate clock although the molecular mechanism of the clock is shared among all tissues. Cumulative studies proved that each organ has a different expression pattern with different number of genes and phases as a homeostatic response. A previous study examined 12 organs of mice through DNA microarrays and RNA sequencing analysis and demonstrated that 43% of all protein coding genes are controlled by the circadian clock in mammals (Zhang & Hogenesch, 2014). Peripheral clock-controlled physiological functions include, lipid, amino acid and carbohydrate metabolism, heart rate and blood pressure, mitochondrial biogenesis and energy production, digestive function and detoxification by liver and kidney (Gachon & Schibler, 2004; D. Feng & Lazar, 2012)

Hormonal oscillations are essential to maintain homeostasis, and hormone secretion is also controlled by peripheral clocks (Cajochen et al., 2003). Melatonin secretion, by the pineal gland, occurs at night in both diurnal and nocturnal animals. Melatonin participates in the regulation of energy metabolism (Pévet, 2003) and it provides a neat sleep. Another clock-controlled hormone, leptin, is mostly produced by enterocytes and adipose tissue and responsible for adjustment of energy balance by inhibiting food intake (Froy, 2010). When the fat stores are elevated, leptin levels increase to control appetite. Several studies indicated disrupted leptin regulation is associated with obesity (Pan et al., 2014).

#### **2.4.2 The Importance of Circadian Rhythm in Human Physiology and Health**

As it is mentioned above, many physiological processes are regulated by the circadian clock. Therefore, it is not surprising that dysregulation of the circadian clock may be pathogenic to human health. To our best knowledge, circadian dysregulation is associated with sleep deprivations, poor immune system, decreased cognitive functions, accelerated aging, diabetes and obesity, altered metabolic functions, high risk of cardiovascular diseases and wide range of cancer types (Krishnaiah et al., 2017; Ercolani et al., 2015).

Familial advanced sleep phase disorder (FASPD) results from a heterozygous mutation on PER3 and causes early-onset and offset of sleep in the afflicted individuals (Toh et al., 2001). A wide study was conducted in 1999 by Jones et al., they studied 3 families with 29 FASP patients and 46 unaffected individuals by polysomnography, in other words, sleep study. The study revealed that all FASP patients exhibit 3 to 4 hours phase advance compared to unaffected individuals. Additionally, a recent study revealed that CRY1 and CRY2 are also associated with FASPD and delayed sleep phase disorder (DSPD) (Hirano, Shi, et al., 2016).

Circadian clock dysregulation is also highly associated with a broad range of human cancer such as metastatic breast cancer and prostate cancer. Among the cancer types, breast cancer draws the most attention since it is clearly shown that PER1 and PER2 expression in primary breast tumors is significantly reduced (Chen et al., 2005; Winter et al., 2007). Moreover, large-scale genomic screening of breast tumors showed that PER1 or PER2 mutations are shared among the majority of the cases (Sjöblom et al., 2006). Another study presented that in prostate cancer, rhythmic oscillations of core clock proteins are significantly impaired (Qi et al., 2009). Overexpression of PERs in breast

tumor and colorectal tumor cultures reduced proliferation rate of the cells where siRNA knock down of PERs increased the growth rate in culture which in turn reveals the tumor suppressor role of PERs (Yang et al., 2009; Gery et al., 2007). Other cancer types including lung cancer, endometrial cancer, glioma, hepatocellular carcinoma, ovarian and pancreatic cancer have shown to be associated with dysregulated circadian clock genes to the best of our knowledge (Gery et al., 2006; Lin et al., 2008; Hsu et al., 2012).

## 2.5 Mammalian Circadian Clock at the Molecular Level

### 2.5.1 Core Clock Proteins and Clock mutants

At the cellular level, core clock proteins BMAL1, CLOCK, CRYs and PERs drive cell-autonomous circadian system. These proteins are also called core clock proteins. BMAL1 and CLOCK constitute the positive arm of the molecular clock by the transactivation of E-box containing target genes. BMAL1 and CLOCK are the members of bHLH-PAS (Helix-Loop-Helix PER-ARNT-SIM) family. CRY and PER belong to the negative arm of the molecular clock since they have the ability to repress BMAL1/CLOCK driven transactivation.

Due to the fact that core clock proteins drive circadian clock at the molecular level, mutant clock genes cause changes on the circadian phenotype. As it is shown with previous studies, *Cry1*<sup>-/-</sup> mutant mice exhibit shortened period length (Van Der Horst et al., 1999) where *Cry2*<sup>-/-</sup> mice display lengthened period (Van Der Horst et al., 1999). Another study showed that the circadian clock in *Cry1*<sup>-/-</sup> / *Cry2*<sup>-/-</sup> double mutant mice was completely abolished (Van Der Horst et al., 1999). Another negative arm protein PER was also studied in mouse. *per* knock out mouse models showed that shortened period is common for each *Per* mutant (*Per1*<sup>-/-</sup>, *Per2*<sup>-/-</sup> and *Per3*<sup>-/-</sup>) but each mutant was further affected from a different aspect. In addition to a shorter period, *Per1*<sup>-/-</sup> and *Per2*<sup>-/-</sup> mice also exhibit dampened amplitude (Zheng et al., 2001) where *Per3*<sup>-/-</sup> mice also exhibit loss of sensitivity to light (Pereira et al., 2014). Nonetheless, constitutive expression of PER1 or PER2 completely abolished rhythm in MEF cells (R. Chen et al., 2009). Under free-running conditions, *Bmal1*<sup>-/-</sup> mice showed arrhythmic behaviour and abolished clock (Bunger et al., 2000). *Clock*<sup>-/-</sup> mouse model was firstly studied by the same group that

discovered CLOCK. They showed that *Clock*<sup>-/-</sup> mice had a longer period and arrhythmic behaviour under constant conditions (Vitaterna et al., 1994).

### 2.5.2 Molecular Architecture of Mammalian Circadian Clock

At the molecular level, the clock mechanism is driven by three interlocked transcriptional-translational feedback loops (TTFL) (Dunlap, 1999). First and the main feedback loop is generated by BMAL1 (also named as ARNTL1), CLOCK, CRYs and PERs (**Figure 7**). During the daytime, positive arm members BMAL1 and CLOCK heterodimerize via bHLH-PAS domains then bind to the E-box elements on *CRY1*, *CRY2*, *PER1*, *PER2* and *PER3* promoters. In the afternoon, BMAL1/CLOCK binding initiates the transcription of *CRYs* and *PERs*. Upon transactivation, negative arm members CRYs and PERs accumulate in the cytosol in the late afternoon and interact with each other along with the serine/threonine kinases casein kinase 1 $\delta$  (CK1 $\delta$ ) and casein kinase 1 $\epsilon$  (CK1 $\epsilon$ ). This interaction leads to the translocation of CRY/PER into the nucleus together with CK1 $\epsilon/\delta$  at night. Upon translocation, CRY/PER heterodimer interacts with BMAL1/CLOCK complex to suppress their own transcription and other BMAL1/CLOCK driven transactivations during the night (Takahashi, 2017; Ko & Takahashi, 2008; Zhang et al., 2014). However, repression activity of CRY/PER declines in time since half-lives of CRYs and PERs are relatively short. During the repression process, FBXL3 and FBXL21 facilitate CRY polyubiquitination and degradation where CK1 $\epsilon/\delta$  mediates PER degradation (Gallego & Virshup, 2007; Lee, 2001). As negative arm elements are degraded, the molecular clock starts to a new cycle in the next morning with BMAL1/CLOCK activity.

Secondary BMAL1/CLOCK driven feedback loop includes the retinoic acid-related orphan nuclear receptors REV-ERB $\alpha$  (encoded by NR1D1), REV-ERB $\beta$  (encoded by NR1D2), ROR $\alpha$ , ROR $\beta$  and ROR $\gamma$ . BMAL1/CLOCK heterodimer initiates the transcription of REV-ERBs and RORs. As REV-ERBs and RORs translocate into the nucleus, they compete with each other to bind RORE elements on *BMAL1* and *NFIL3* promoter. REV-ERBs exert an inhibitory effect on RORE promoter where RORs act as an activator on the same element. Consequently, BMAL1 expression is regulated by an additional feedback loop (Jolley et al., 2014; Ueda et al., 2005).

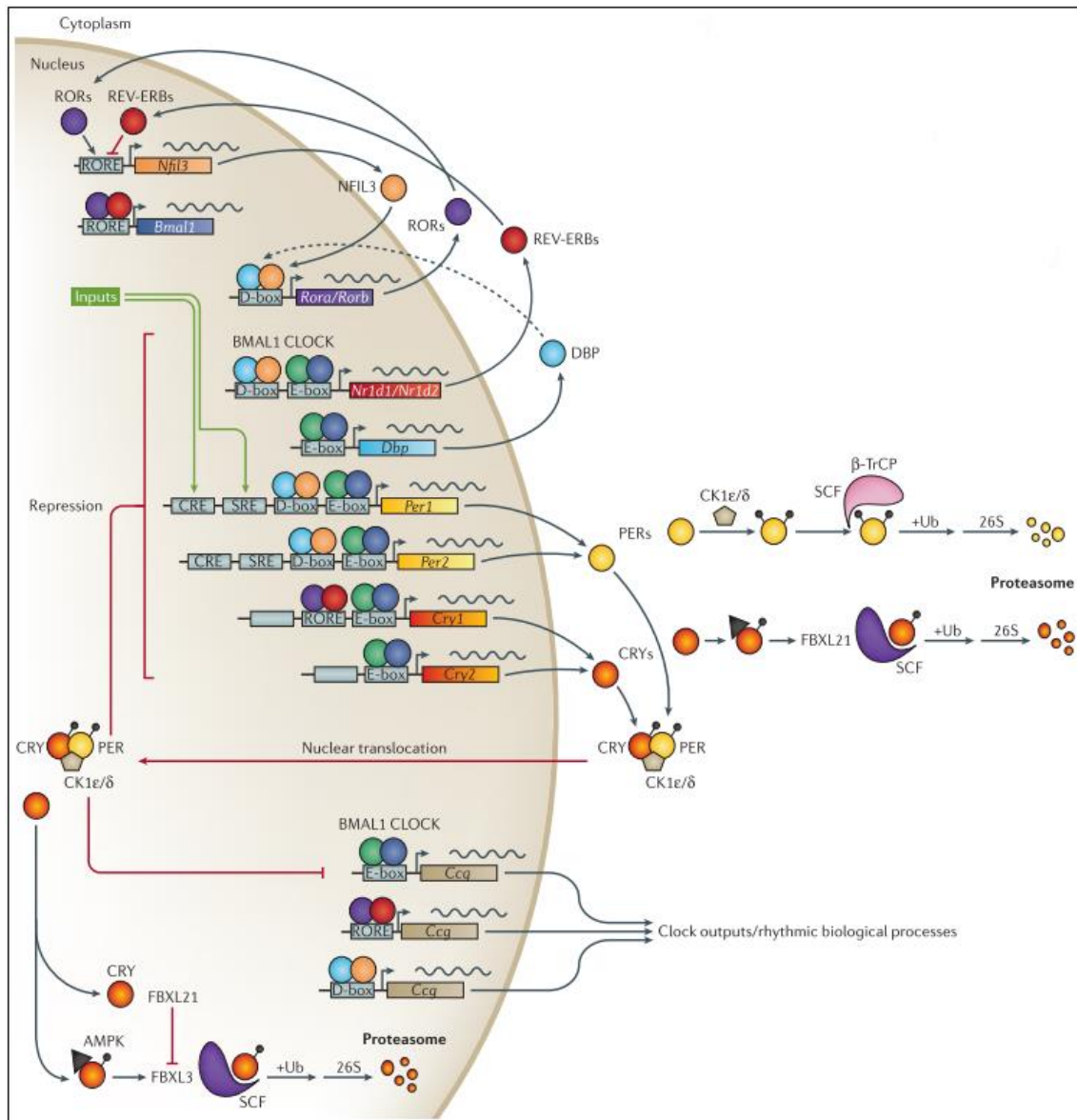


**Figure 7. Schematic illustration of the mammalian circadian clock mechanism.**

Copyright 2016, with permission from Springer Nature.

Another level of clock regulation is controlled by the third feedback loop which includes transcription factors of D-box containing genes. D-box binding protein (DBP), nuclear factor interleukin 3 (NFIL3), proline and acidic amino acid rich basic leucine zipper (PARbZip), thyrotroph embryonic factor (TEF) and hepatic leukaemia factor (HLF) are involved in the third feedback loop (Cowell, 2002; Gachon et al., 2006). NFIL3 and DBP

transcription factors bind to the D-box element to upregulate the expression of RORs and REV-ERBs (Ueda et al., 2005). Among these clock proteins and regulators, DBP is directly controlled by BMAL1/CLOCK activity, therefore it is conceived as an output of BMAL1/CLOCK activity on E-box element. It is concluded that TTFLs drives the cyclic expression of core clock proteins and they have a key role in rhythmic oscillations.

Post-translational modifications (PTM) also have an essential role in maintaining rhythmic oscillations. Phosphorylation by CKI $\alpha$ , CKI $\delta$ , and CKI $\epsilon$  kinases, dephosphorylation by PP1 and PP5 phosphatases and ubiquitination by FBXL3 and FBLX21 regulates the stability and degradation of core clock proteins. PERs are phosphorylated by CKI $\delta$  and CKI $\epsilon$  which leads to the ubiquitination by  $\beta$ -transducin-repeat containing protein ( $\beta$ -TrCP). Ubiquitinated PERs are degraded by 26S proteasomes. The stability of PER is regulated also by acetylation and deacetylation. Deacetylation of PER2 by SIRT1 destabilizes PER2 and leads to the proteasomal degradation (Eide, 2005; Shirogane et al., 2005). It has been shown that a rare SNP in *PER2*, p.Ser662Gly, leads to increased PER2 degradation and results in a shortened circadian period in human. This SNP is also associated with Familial advanced sleep phase syndrome (FASP) (Toh et al., 2001).

Repression activity of CRYs on BMAL1/CLOCK complex is stronger than PERs (Gallego & Virshup, 2007). Therefore, PTMs on CRYs are essential for the sustainability of circadian oscillations. Stability of CRYs is mainly driven via phosphorylation by AMP-activated protein kinase (AMPK) and ubiquitination by FBXL3 and FBXL21 (Lamia et al., 2009). CRYs are phosphorylated by AMPK that enhances the poly-ubiquitination by SKP1-CUL1-F-box together with FBXL3 (SCF<sup>FBXL3</sup>) in the nucleus. Poly-ubiquitinated CRYs are degraded through 26S proteasomes. Another ubiquitin ligase 3, FBXL21, has shown to stabilize CRYs through antagonizing FBXL3. FBXL3 and FBXL21 ubiquitinate CRYs at different residues so that ubiquitination by FBXL21 results in CRY modification and prevents FBXL3 binding to CRYs (Hirano et al., 2013; Ko et al., 2007).

PTMs of BMAL1 includes acetylation, phosphorylation and SUMOylation. Transactivity of BMAL1 is promoted through phosphorylation by CKI  $\epsilon$  and SUMOylation. However, BMAL1 transactivation is downregulated upon phosphorylation by CK2 $\alpha$  and MAPK (Tamaru et al., 2009; Hirano et al., 2016). Surprisingly, a previous study showed that

mutation in a SUMOylation site of BMAL1 (Lys<sup>259</sup>), suppressed both ubiquitination and proteolysis of BMAL1 which is reversed with SUMO3 attachment to the C-terminus of the protein (Lee et al., 2008). Apart from the other core clock proteins, CLOCK does not exhibit significant rhythmic oscillations in protein level instead PTMs of CLOCK exhibit oscillations. Nuclear translocation, stability and transcriptional activity of CLOCK is dependent on phosphorylation (Hirano, Fu, et al., 2016).

### 2.5.3 BMAL1/CLOCK Transactivation

BMAL1 and CLOCK interact and heterodimerize through bHLH-PAS domains then bind to the E-box elements on target genes. BMAL1/CLOCK complex interacts with p300 and CREB-binding protein (CBP), which are known histone acetyltransferases (HATs). It is shown by Hosoda et al. in 2009 that CBP/p300 activity on BMAL1/CLOCK complex is cell type specific. According to the same study, CBP/p300 complex either enhances or represses BMAL1/CLOCK mediated transcription through CBP activity. In COS-I cells, where pCAF coactivator is highly expressed, CBP/p300 complex enhanced BMAL1/CLOCK transactivation. However, in NIH3T3 cells, where co-repressor HDAC3 is highly expressed, CBP/p300 complex represses BMAL1/CLOCK complex. They also proposed that repression activity of CBP/p300 is achieved by CBP by targeting CLOCK.

A previous study showed that intrinsic HAT activity of CLOCK also provides access for transcription by acetylating histone H3 on Lys9 and Lys14 residues (Doi et al., 2006). Another level of BMAL1/CLOCK activity regulation is facilitated by BMAL1/CLOCK driven *NAMPT* transcription. NAMPT activity generates oscillations in NAD-dependent deacetylase Sirtuin 1 (SIRT1) suppression on BMAL1/CLOCK complex. Eventually, SIRT1 is released from the complex and BMAL1/CLOCK activity is enhanced (Asher et al., 2008; Ramsey et al., 2009; Nakahata et al., 2009). Therefore, acetylation and deacetylations also oscillate as a result of BMAL1/CLOCK activity. It is shown that BMAL1/CLOCK complex also interacts with mixed lineage leukaemia 1 methyltransferase (MLL1) which is associated with H3K4 trimethylation, a hallmark for active genes. Indeed, MLL1 is also thought to be involved in CLOCK recruitment to DNA (Katada & Sassone-Corsi, 2010).

## 2.6 CITED2 (CBP/p300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2)

Finding new regulators of the TTFLs has been the center of interest for the better elucidation of the mammalian circadian clock. A previous study conducted by Prof. Halil Kavaklı identified candidate clock regulators. In this study, transcription factors were screened through high-throughput luciferase assay to identify candidate clock proteins which have a potential to modulate BMAL1/CLOCK transactivation. CBP/p300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2 (CITED2) was designated as one of the top candidates that has a potential to regulate BMAL1/CLOCK activity. This finding paved the way for us to characterize the role of CITED2 in mammalian circadian clock mechanism. In this section, CITED2 is explained by any measure.

CITED2 was identified in human as a result of database search of murine *msg1* (also known as *Cited1*) cDNA by Shioda et al., 1996. In this study for the first time, Shioda isolated full length CITED2 cDNA from melanocytes via 5' RACE-PCR. They also showed that subcellular localization of CITED2 is predominantly nuclear. (Shioda et al., 1996).

CITED2 (also known as MRG1, MRG-1 and p35SRJ) is a transcription factor, it acts as both corepressor and coactivator in various transcriptional activities although it does not directly bind to DNA. CITED2 is encoded by *CITED2* which is located in the 6<sup>th</sup> chromosome and encompasses 9.39 kb of DNA with three exons and two introns. *CITED2* promoter does not include a TATA box but it comprises hypoxia response elements, specificity protein 1 (SP1) binding domains, signal transducer and activator of transcription (STAT) binding motifs and a NF- $\kappa$ B (nuclear factor  $\kappa$ B) binding site (Leung et al., 1999). CITED2 has two isoforms which are generated through different splicing processes. First transcript, *p35SRJ*, one of the introns are not spliced out and codes for a serine-rich junction (SRJ) and the other transcript, *MRG1*, does not contain the SRJ coding intron. Therefore, *p35SRJ* transcript is translated into 270 aminoacid polypeptide with 27 kDa molecular weight where *MRG1* transcript is translated into 213 aminoacid polypeptide with 23.7 kDa molecular weight (Leung et al., 1999). CITED2 has 32-amino acid sequence at the C-terminus which is a shared characteristic of CITED family members. A particular domain that is composed of 12 aspartic acid and glutamic acid

residues has shown to be highly conserved among human and mouse CITED family members (Shioda et al., 1997). mRNA expression patterns of CITED2 show that CITED2 is widely expressed amongst major tissues, overexpressed in fetal heart, and it is a developmentally regulated protein (GTEx Analysis Release V8; dbGaP Accession phs000424.v8.p2; ENSG00000164442.9).

### **2.6.1 The Cellular Activities of CITED2**

In 1999, Bhattacharya showed that CITED2 interacts with general transcriptional coactivators CBP and p300, in U2-OS cells via immunoprecipitation. Also, mutation analysis proved that conserved 32 aminoacid residues on the C-terminus of CITED2 interact with both CBP, p300 and CBP/p300 complex through CH1 domain (cysteine/histidine-rich 1 domain, also known as TAZ1) of both CBP and p300. (Bhattacharya et al., 1999). This wide study also revealed the function of CITED2 in HIF1- $\alpha$  (hypoxia inducible factor 1- $\alpha$ ) pathway. HIF1- $\alpha$  is known to be the master regulator of hypoxic response (Iyer et al., 1998). Under hypoxic conditions, HIF1- $\alpha$  is upregulated and controls the expression of more than 60 genes which are involved in cellular hypoxic response. HIF1- $\alpha$  also stimulates oxygen transmission to hypoxic locations (Lee et al., 2004). CITED2 is one of the upregulated proteins by HIF1- $\alpha$  as a response to hypoxia. However, CITED2 acts as a negative regulator for HIF1- $\alpha$  driven expression. At the molecular level, transactivation of HIF1- $\alpha$  is dependent on CBP/p300 interaction on the promoter of target genes. HIF1- $\alpha$  interacts with the CH1 domain of CBP/p300 complex and CITED2 competes with HIF1- $\alpha$  for binding to the CH1 domain of CBP/p300. Consequently, CITED2 and CBP/p300 interaction results in inhibition of HIF1- $\alpha$  transactivation (Bhattacharya et al., 1999; Berlow et al., 2017). This negative feedback regulation enables the fine tuning of hypoxic response at the cellular level.

As it is mentioned before, CITED2 is able to act as a coactivator or corepressor in various transcriptional activities. In contrast to the HIF1- $\alpha$  pathway, CITED2 positively regulates TFAP2 (Transcriptional regulation by the AP-2) mediated transcription activity. TFAP2 is a transactivator with a consensus DNA binding motif acting on metallothionein and SV40 promoters. TFAP2 is highly expressed in neural crest cells and ectoderm which suggests that TFAP2 plays a part in differentiation and development. A previous study conducted by Bragança in 2003 showed that TFAP2 is activated by CBP/p300 only in the presence of CITED2. They proved that physical interaction between TFAP2 and p300 is

dependent on CITED2 co-transfection via mammalian two hybrid assay. Moreover, it is shown that CITED2 and TFAP2 has a strong physical interaction through conserved residues of C-terminal of CITED2 and N-terminal of TFAP2. In this wide research they also studied p300<sup>+/-</sup> and CBP<sup>+/-</sup> mouse embryonic fibroblasts (MEF), as a result, they have observed TFAP2 driven transcription is impaired in p300<sup>+/-</sup> and CBP<sup>+/-</sup> MEFs. Apart from these two key roles of CITED2, it has a key role in the regulation of left-right patterning during embryogenesis by stimulating NODAL-mediated transcription (Schneider et al., 2004). Besides, it is shown that CITED2 acts as a coactivator in TGF- $\beta$  signalling (Chou et al., 2012), peroxisome proliferator-activated receptor (PPAR) activity (Tien et al., 2004) and estrogen-dependent transactivation (Ho et al., 2013). It is also involved in early gonad development as well as sex determination (Buaas, Val, & Swain, 2009).

### 2.6.2 CITED2 in Physiology and Health

CITED2 mouse model was established and studied by several groups from different physiological aspects. In 2001 Bamforth et al. reported that *Cited2*<sup>-/-</sup> mice developed exencephaly, defective cranial ganglia and neural crest, and high levels of apoptosis in the midbrain as well as cardiac malformations with a defective cardiac tube, neural tube, adrenal tube, and cranial ganglia (Bamforth et al., 2001). Several years later in 2011, Dunwoodie et al. showed that *Cited2*<sup>-/-</sup> mice die during gestation as a result of septal defects and laterality defects. Since CITED2 is essential in NODAL-mediated transactivation, it is reasoned that laterality defects resulted from loss of NODAL-mediated transactivation. Other cardiac defects and neuronal defects are thought to be a consequence of reduced TFAP2 signalling as it was shown that TFAP2 signalling is essential in neural crest cells and ectoderm. It is also suggested that aberrant HIF1- $\alpha$  and NODAL expression may contribute to these malformations (Bamforth., 2004; Macdonald et al., 2008). Another study conducted on *Cited2*<sup>+/-</sup> and *Cited2*<sup>-/-</sup> mice showed pulmonary immaturity with defective terminal sacs and alveolar epithelial cells (Xu et al., 2009).

In human physiology, CITED2 has shown to be implicated in hypoxic response, adrenal development, cardiac and neuronal function, eye development (Chen et al., 2009), fracture healing (Chen et al., 2009), liver and lung development (Xu et al., 2009), and haematopoiesis (Kranc & Bamforth, 2003).

*CITED2* mutations were first indicated in 2005 by Sperling et al., in patients with septal defects. In this report, a 27-bp deletion in exon 2 was reported in a patient with ventricular septal defect and two mutations; 27-bp insertion and 6-bp deletion were identified in patients suffering from atrial septal defects. Using HIF1- $\alpha$  responsive reporter and TFAP2 reporter systems, they also revealed that loss of repression activity on HIF1- $\alpha$  and reduced TFAP2 activation were the underlying reasons. *CITED2* mutations were also associated with various cancer types including breast cancer, colorectal cancer, prostate cancer, oesophageal cancer, osteosarcoma, ovarian carcinoma, and adrenocortical carcinoma. In invasive breast cancer cells, *CITED2* has shown to be highly expressed (Chou et al., 2006), in contrast, in ovarian carcinoma (Young et al., 2007) and osteosarcoma (Daino et al., 2009), the expression of *CITED2* is significantly decreased. This variation signifies that *CITED2* defects have a wide range of consequences in varying disease mechanisms. Moreover, a recent study hypothesizes that deterioration of *CITED2*/HIF1- $\alpha$  regulation may cause cancer initiation, progression, or prevention depending on the cancer type and cellular state (Oncol & Bragança, 2020).

For many cancers, hypoxia is a common property and *CITED2* plays a key role in the hypoxic response. HIF1- $\alpha$  is accepted as the master regulator of hypoxic response and *CITED2* is upregulated by HIF1- $\alpha$  and FOXO3 under hypoxic conditions. *CITED2* negatively regulates adaptive responses by inhibiting HIF1- $\alpha$  target genes (Bakker et al., 2007). This negative feedback regulation enables the fine tuning of hypoxic response at the cellular level. Previous studies addressed the interplay between circadian clock and hypoxia signalling pathway. A recent study demonstrated that HIF1- $\alpha$  is important for synchronization of the circadian rhythm. According to this study, HIF1- $\alpha$  deficient mice exhibit downregulated *Per1* and *Cry1* transcripts and failed to adapt a jet-lag protocol under hypoxic conditions compared to WT mice (Adamovich, Ladeuix, Golik, Koeners, & Asher, 2017). Negative arm element PER2 enhances the recruitment of HIF1- $\alpha$  to hypoxia response element (HRE) by direct interaction (Kobayashi et al., 2017). Another negative arm elements CRY1 exerts a negative regulation on HIF1- $\alpha$  signalling. CRY1 reduces the half-life of HIF1- $\alpha$  by binding to the bHLH domain. As a result of this interaction, HIF1- $\alpha$  cannot bind to target gene promoters (Dimova et al., 2019). Another recent study revealed that hypoxic response is time and tissue dependent and it results in intertissue misalignment of the circadian clock (Manella et al., 2019).

## Chapter 3

### MATERIALS AND METHODS

#### 3.1 Cell lines and Regular Cell Culture Procedures

##### 3.1.1 Cell Lines and Maintenance

Human embryonic kidney cell line (HEK293T) and human bone osteosarcoma epithelial cell line (U2-OS) were maintained in DMEM with 10% fetal bovine serum (FBS; Gibco, Cat. No. 11573397) and 100 µg/mL penicillin and streptomycin (P/S; Gibco, Cat. No. 15140122). The cells were incubated at a sterile humidified cell culture incubator at 37 °C with 5% CO<sub>2</sub>.

##### 3.1.2 Cell Passaging and Cell Stocking

As a regular maintenance protocol, 80%-100% confluent cells were passaged. To do so, cells were first washed with 5 ml of 1X phosphate buffered saline buffer (PBS) and trypsinized with 1ml of Trypsin-EDTA (Wisent Multicell, Cat. No. 325-043-CL). Cells were kept in the cell culture incubator for 2-3 minutes until detachment and resuspended with fresh medium. 1-2 ml of cell suspension were transferred into fresh medium containing plates. For the nitrogen stock, cells were centrifuged at 400xg for 5 minutes, resuspended in freezing medium (90% FBS and 10% DMSO) and kept in the liquid nitrogen.

##### 3.1.3 Cell Harvest

HEK293T cells and U2-OS cells were harvested with different methods. HEK293T cells were harvested from 35 mm culture dishes by pipetting and cell suspensions were transferred into Eppendorf tubes. U2-OS cells, in 35 mm culture dishes, were trypsinized and detached from the plate with 200 µl of trypsin-EDTA for 4-5 minutes in a cell culture incubator. 1 ml of DMEM was added onto detached U2-OS cells and the cell suspension

was transferred into Eppendorf tubes. The rest of the procedure is the same for both cell lines. The cell suspension was centrifuged at 5000xg and 4 °C for 7 minutes. The supernatant was removed, and the cells were washed with ice cold 1X PBS and centrifuged at 5000xg and 4 °C for 7 minutes, the supernatant was removed after centrifugation.

### 3.1.4 Cell Synchronization

Cell synchronization was performed 2 hours before siRNA transfection and 0.1  $\mu$ M Dexamethasone (Sigma Aldrich, Cat. No. D4902) containing DMEM with 10% FBS was replaced with media of the cells.

## 3.2 Plasmid Construct and Molecular Cloning

*CITED2* was cloned into pcDNA4/myc-His A and pLenti-III-HA plasmids.

### 3.2.1 Sub-cloning

To achieve successful cloning, sub-cloning was performed with pGEM-T-ccdB plasmid vector. First, *CITED2* was amplified with specific primer pairs in separate reactions for 2 different cloning setups i) *CITED2*\_BamHI\_F and *CITED2*-EcoRI\_R primer pair for pcDNA4/myc-His A, ii) *CITED2*\_EcoRI\_F and *CITED2*\_SalI\_R primer pair for pLenti-III-HA cloning via Polymerase Chain Reaction (PCR). Primer sequences are listed in Appendix A. PCR reaction was carried out with homemade Phusion polymerase enzyme and amplification confirmed with agarose-gel electrophoresis. Thermocycler protocol for the PCR reaction is detailed in the Table 1. *CITED2* amplicons were purified with Nucleospin Gel and PCR Clean-up kit (Macherey Nagel, Cat. No. 740609.50) pGEM-T-ccdB plasmid was restricted with SmaI enzyme following manufacturer's protocol (Thermo Fisher Scientific, Cat. No. FD0663) and purified with the Nucleospin Gel and PCR Clean-up kit. *CITED2* amplicons and restricted pGEM-T-ccdB plasmid were ligated with Rapid DNA Ligation Kit (Thermo Fisher Scientific, Cat. No. K1422) at room temperature for 2 hours. Ligation product was transformed into competent cells and grown in 100  $\mu$ g/mL ampicillin (AMP; Sigma Aldrich, Cat. No. 10835242001) and 20  $\mu$ g/mL chloramphenicol (Sigma Aldrich, Cat. No. C1919). For confirmation, plasmids were sequenced.

Table 1: PCR protocol details to amplify *CITED2* for cloning.

| Stage              | Initial Denaturation | Denaturation | Annealing  | Extension | Final Extension |
|--------------------|----------------------|--------------|------------|-----------|-----------------|
| <b>Temperature</b> | 98 °C                | 98 °C        | 62 °C      | 72 °C     | 72 °C           |
| <b>Duration</b>    | 1 min                | 30 seconds   | 30 seconds | 2 minutes | 5 minutes       |
| <b>Repeat</b>      | 1 time               | 30 cycles    |            |           | 1 time          |

### 3.2.2 Molecular Cloning

3000 ng of *CITED2* in pGEM-T-ccdB plasmid and 3000 ng of the host plasmid pcDNA4/myc-His A were digested with FastDigest BamHI (Thermo Fisher Scientific, Cat. No. FD0054) and FastDigest EcoRI (Thermo Fisher Scientific, Cat. No. FD0274) enzymes at 37 °C for 1 hour according to manufacturer's protocol with the addition of FastAP enzyme and FastAP reaction buffer. Next, both digested *CITED2* and digested host plasmid ran on the agarose gel at 100 V for 45 minutes to separate and purify the correct fragments. Purification of the fragments from agarose gel was performed with the Nucleospin Gel and PCR Clean-up kit. In order to ligate *CITED2* into pcDNA4/myc-His A plasmid, Rapid DNA Ligation Kit was used at room temperature for 2 hours. Ligation product was transformed into Top10 competent cells via heat shock procedure. Competent cells stored at -80 °C and thawed on ice for 15 minutes. 5 µl of ligation product was transferred into 50 µl of competent cells and kept on ice for another 15 minutes. Then cells were exposed to 42 °C for 45 seconds as a usual heat shock transformation procedure and transferred back on ice for 2 minutes. Next, 1 ml of Lysogeny Broth (LB) was added into cells and incubated at 37 °C, 200 rpm for 1 hour. After the incubation, cells were centrifugated at 4000xg, 4 °C for 6 minutes. Supernatant was discarded and the cells were resuspended with 100 µl of LB followed by spreading onto LB-agar plates containing 1 µg Ampicillin. After 16-18 hours of incubation, 5-10 colonies appeared on the agar plate. 5 colonies were selected and grown in separate test tubes containing 5 ml of LB broth with 1 µg AMP for 16-18 hours at 37 °C and 200 rpm. For each screening, 5 colonies were grown. Plasmid isolation was performed with Nucleospin Plasmid kit (Macherey Nagel, Cat. No. 740588.50). Plasmid concentrations were measured with NanoDrop 2000

(Thermo Fisher Scientific) and candidate plasmid vectors were digested with BamHI and EcoRI restriction enzymes for screening. Digestion products were run on the gel and screened for correctly cloned plasmid. As an extra confirmation step, some of the cloning products were sequenced. For sequencing, total 4000 ng of plasmid vector in 20 µl of ddH<sub>2</sub>O was sent to Macrogen, Inc. Sequencing results were analysed with Chromas Lite 2.1 and NCBI BLAST tool.

Other *CITED2* cloning construct with pLenti-III-HA host plasmid vector, was also cloned with the same protocol explained above. For pLenti-III-HA-*CITED2* cloning, EcoRI and SalI restriction sites were used.

### 3.3 Repression Assay

On day zero, HEK293T cells were passaged so that they would reach 80-90% confluency on the day of transfection. On day two, cells were washed with PBS and detached from the plate. Cells were counted and  $4 \times 10^4$  cells (per 1 well) were seeded in 50 µl medium into 96-well plate. For each different transfection condition, total DNA amounts were equalized to 300 ng. The following plasmids were used for the transfection solutions; 50 ng of pCMV-Sport6-*Bmal1*, 125 ng of pCMV-Sport6-*CLOCK*, 50 ng of *mPer1:dluc*, pcDNA4/myc-His-A plasmid with *CRY1* (2 and 20 ng), pcDNA4/myc-His-A plasmid with *CITED2* (5, 12.5, 25, 50 and 100 ng), 50 ng of pcDNA3β

-FLAG-HA-*CBP*, 50 ng of pCMVβ-Myc-*p300*, 5 ng of pGL4-Renilla and empty pCMV-Sport6 vector (to equalize DNA concentration to 300 ng). Total volume of transfection solutions was equalized to 50 µl with DMEM and 0.6 µl of PEI was added into transfection solutions. The solutions were incubated for 20 minutes at room temperature, later solutions were added onto cells and mixed gently. After the reverse transfection, cells were incubated in a cell culture incubator. 24-hour post transfection, cells were lysed with LUC buffer (Table 2) and incubated for 20 minutes. Firefly luciferase activity was measured with Fluoroskan Ascent. Later, cells were mixed with Renilla buffer (Table 2) and incubated for 20 minutes. Renilla measurements were also taken with Fluoroskan Ascent and luciferase measurements were normalized with Renilla measurements.

Table 2: Firefly luciferase and Renilla luciferase buffer compositions.

| Firefly Luciferase Buffer      |           |                            |          |
|--------------------------------|-----------|----------------------------|----------|
| A. Buffer Components (3X)      |           | B. Triton Lysis Buffer     |          |
| DTT:                           | 15 mM     | Tris-HCl:                  | 0.1082 M |
| Coenzyme A:                    | 0.6 mM    | Tris-base:                 | 0.0419 M |
| ATP:                           | 0.45 mM   | NaCl:                      | 75 mM    |
| Luciferin:                     | 4.2 mg/ml | MgCl <sub>2</sub> :        | 3 mM     |
| <i>in Triton lysis buffer</i>  |           | Triton X-100:              | 0.25%    |
|                                |           | <i>in ddH<sub>2</sub>O</i> |          |
| Renilla Luciferase Buffer      |           |                            |          |
| A. Buffer Components (3X)      |           | B. Renilla Salts Buffer    |          |
| PTC124 (in DMSO):              | 0.06 mM   | Na <sub>2</sub> EDTA:      | 45 mM    |
| h-CTZ (in ethanol):            | 0.01 mM   | Na pyrophosphate:          | 30 mM    |
| <i>in Renilla Salts buffer</i> |           | NaCl:                      | 1.425 M  |
|                                |           | <i>in ddH<sub>2</sub>O</i> |          |

### 3.4 Mammalian Two Hybrid Assay

HEK293T cells were used for mammalian two hybrid assay. Cells were passaged two days before reverse transfection. On the reverse transfection day,  $4 \times 10^4$  cells in 50  $\mu$ l medium are seeded into 1 well of 96-well plate. For each transfection solution, 3 technique replicates were prepared. All transfection solutions are prepared such that total DNA concentration was 300 ng/  $\mu$ l and total transfection solution volume was 50  $\mu$ l per 1 well. 50 ng of pACT-VP16-Bmal1- vector, 125 ng of pBIND-GAL4-CLOCK vector, 50 ng of pG5Luc vector, 20 ng of pcDNA4/myc-His-A plasmid with *CRY1*, 50 ng of pcDNA4/myc-His-A plasmid with *CITED2*, 50 ng of pcDNA3 $\beta$ -FLAG-HA plasmid with *CBP*, 50 ng of pCMV $\beta$ -Myc plasmid with *p300*, 50 ng of pACT vector, 125 ng of pBIND, empty pCMV-Sport6 vector (different amounts for each condition to equalize DNA concentration to 300 ng) and 5 ng of pGL4-Renilla were used for different transfection conditions. Transfection solutions were equalized to 50  $\mu$ l with DMEM. For each well, 0.6  $\mu$ l of polyethylenimine (PEI; Polysciences, Cat. No. 23966-1) was added and incubated for 20 minutes. Transfection solution were transferred into wells after incubation and mixed gently with the cell suspension. Transfected cells were incubated in a cell culture incubator for 24 hours. After 24-hour incubation, cells were lysed with 50  $\mu$ l of homemade firefly luciferase buffer (Table 2) and incubated for 15 minutes. Next, luciferase signal was measured with a luminometer (Thermo Fluoroskan Ascent FL).

After the first measurements, 75  $\mu$ l of homemade Renilla luciferase buffer (Table 2) was added into the wells and incubated for 15 minutes, measurements were taken with the luminometer. Renilla readings were used to normalize luciferase signals.

### 3.5 Pull-Down Assay

HEK293T cells were passaged on day zero, so that the confluency would reach 80% on the next day. On day one,  $30 \times 10^4$  cells were seeded into 35 mm culture dishes with 2 replicates for each transfection. For the forward transfection (on day two), 3 transfection solutions were prepared with pcDNA4/myc-His-A plasmid with *CITED2* (1000 ng), FLAG-tagged pCMV-Sport6-*Bmal1* (2000 ng) and pCMV-Sport6-*CLOCK* 350 ng). Total DNA amount was equalized to 2000 ng with an empty pCMV-Sport6 vector and total volume of the transfection solution was equalized to 200  $\mu$ l with DMEM. As a transfection agent, 6  $\mu$ l of PEI was added to each transfection solution and incubated for 20 minutes at room temperature. Transfection solution was added onto the cells dropwise gently. Transfected cells were kept in a cell culture incubator. 36 hours post transfection, the cells were harvested.

One day before the cell harvest, Ni-NTA resin (Thermo Fischer Scientific, Cat. No. 88223) was equilibrated (**Figure 8**). For each binding, 15  $\mu$ l of Ni-NTA resin was used. To equilibrate the resin, TBS300 with 25 mM imidazole (300 mM NaCl, 5 mM Tris base) solution was prepared. The resin was washed with 75  $\mu$ l of TBS300/25 mM imidazole and centrifuged for 3 minutes at 1000xg and 4 °C. Next, the resin was washed with 75  $\mu$ l of PLB for two times. To eliminate the nonspecific binding to the resin, equilibrated resin was incubated with HEK293T cell lysate at 4 °C with rotation overnight. The next day, the resin was centrifuged for 3 minutes at 1000xg and 4 °C and the supernatant was removed.

After the cell harvest, cell pellet was resuspended with 300  $\mu$ l of home-made passive lysis buffer (PLB; 15 mM HEPES, 5 mM NaF, 300 nM NaCl, 1% NP40, 1% Protease inhibitor cocktail and 2% phenylmethylsulfonyl fluoride, ddH<sub>2</sub>O). Resuspended cells were gently handled kept on ice for 10 minutes and vortexed for one second. The cells were kept on ice for 10 minutes more and centrifuged for 25 minutes at 14000xg and 4 °C. The whole cell protein lysate was in the supernatant after the centrifugation and the pellet was

discarded. 10% (30  $\mu$ l) volume of the protein lysate was saved for WB step and named as input. On the day of protein isolation. For pull-down assay 4 different set ups were planned as follow i) CITED2, ii) BMAL1 and CLOCK, iii) CITED2 and BMAL1, iv) CITED2 and CLOCK. 100  $\mu$ l of corresponding protein lysate was added onto the resin with 25 mM imidazole. Protein mix and the resin were incubated with rotation at 4  $^{\circ}$ C for 2 hours. Following the incubation, resin and the protein mix were centrifuges for 3 minutes at 1000xg and 4  $^{\circ}$ C. 30  $\mu$ l of supernatant (also named as flow-through) was saved for WB analysis. The resin was washed 4 times with 350  $\mu$ l of TBS300/60 mM imidazole solution. For the protein elution, 50  $\mu$ l of home-made elution buffer (50mM  $\text{Na}_2\text{HPO}_4$ , 300mM NaCl, 250mM imidazole, 6M guanidine·HCl, pH=8) was used. Following the elution, resin was centrifuged, and supernatant was used for WB analysis. CITED2 was probed with Anti-Myc, BMAL1 and CLOCK were probed with Anti-FLAG antibodies by following the WB procedures.

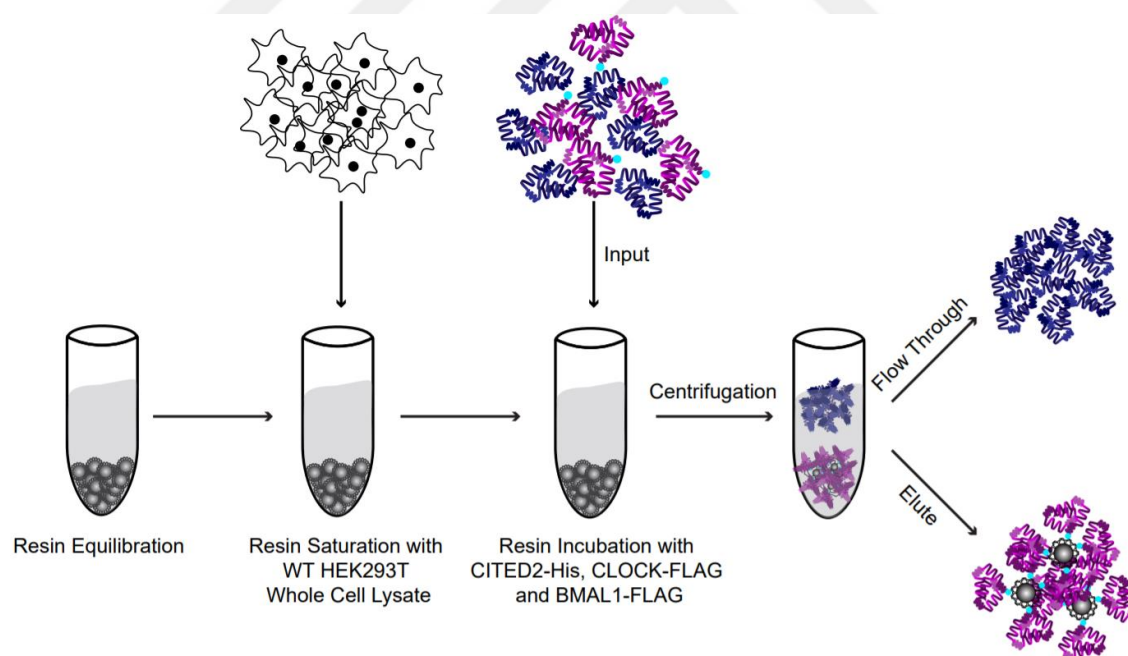


**Figure 8. Schematic representation of pull down assay protocol.**

### 3.6 Establishment of CITED2 Over Express Stable Cell line

*CITED2* was cloned to pLenti-III-HA to achieve a stable overexpression cell line as explained above. U2-OS cells were electrotransfected with Neon® Transfection System

(Invitrogen, Cat. No. MPK5000). U2-OS cells were passaged 2 days before the transfection such that the cells are 70–90% confluent on the day of the electroporation. Medium was discarded and the cells were washed with 1X PBS. Cells were trypsinized and resuspended with 5 ml of DMEM. Next cells were counted with hemocytometer,  $1 \times 10^5$  cells were centrifuged for 5 minutes at 300xg and room temperature. Meanwhile, electroporation tube was set up with 3 ml of Electrolytic Buffer E (Invitrogen Neon® Kit, Cat. No. MPK1025). After centrifugation, cell pellet was resuspended with 9  $\mu$ l of Resuspension buffer R (Invitrogen Neon® Kit, Cat. No. MPK1025) and 500 ng of pLenti-III-HA-*CITED2* plasmid vector in 1  $\mu$ l of Tris was transferred onto resuspended cells. 10  $\mu$ l of cell and plasmid mixture was loaded into electroporation tip (Invitrogen Neon® Kit, Cat. No. MPK1025). The cells were electroporated with (1400 pulse voltage, 20 pulse width). After the procedure, cells were transferred into 24-well plate containing 0.5 ml of DMEM per well. One well was designated as control well and control cells were not electroporated, directly transferred into the 24-well plate. Cells were kept in the cell culture incubator for 2 days and medium was changed with DMEM with 10% FBS and 100  $\mu$ g/mL P/S. Cells were maintained for almost 1 week in the cell culture incubator to recover because the electroporation itself was damaging to the cells. After the physiology of the cells recovered and the number of the cells increased, antibiotic selection procedure was started. pLenti-III-HA plasmid vector carries puromycin resistance gene for successful selection of positive cells. 2  $\mu$ g /ml puromycin antibiotic in 0.5 ml of DMEM with 10% FBS and 100  $\mu$ g/mL P/S was used to select positive cells. Cells were maintained with 2  $\mu$ g /ml puromycin for almost 1 month such that the cells were 70-90% confluent and the medium was regularly changed with fresh medium during this process. Next the cells were transferred into 12-well plate with a regular passaging procedure and as they reach 70-90% confluency they were transferred into 6-well plate. As the cells reached 100% confluency in 6-well plate, cells were harvested. To confirm successful overexpression of *CITED2*, protein lysate was probed via Western Blot analysis with HA antibody (Santa Cruz Biotechnology Inc., Cat. No. sc-7392).

### **3.7 Protein isolation and Western Blotting**

#### **3.7.1 Protein Isolation**

To lyse the cells, cell pellet was resuspended with 90  $\mu$ l of RIPA buffer (0.1% SDS, 150mM NaCl, 50mM Tris, 1% Triton-X, 1% Protease inhibitor cocktail and 2% phenylmethylsulfonyl fluoride) and incubated on ice for 15 minutes. The cells were vortexed for two seconds and incubated on ice for 15 more minutes. Next, cells were centrifuged for 20 minutes at 15000xg and 4 °C. Following the centrifugation, whole cell protein lysate was in the supernatant, so the pellet was discarded. Protein concentration was measured with Pierce assay (Thermo Fisher Scientific, Cat. No. 22660). 1  $\mu$ l of protein lysate was mixed with 9  $\mu$ l of ddH<sub>2</sub>O and 150  $\mu$ l of Pierce reagent in a 96 well plate. The measurements were taken with a plate reader at 660 nm. To be able to store the protein lysate, 30  $\mu$ l of 4X Laemmli Buffer (Tris HCl at pH:6.8 and 250 mM, Glycerol 40 %, SDS 4 %, Bromophenol Blue 0.02 %, Beta-mercaptoethanol 10 %) was added into 90  $\mu$ l of protein lysate. The proteins were boiled at 95 °C for 5 minutes on a heat block and stored at -20 °C.

#### **3.7.2 Western Blotting**

The protein lysates are thawed on ice and boiled at 95 °C for 1 minute, and spin down for 1 minute. Protein composition of the lysate was separated with SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) depending on their molecular sizes. SDS-PAGE was performed with 5% stacking gel and 6% or 10% separating gel (10% gel for CITED2, BMAL1, CLOCK, CRY1 and DBP, 6% gel for PER2) Table 3. The samples were run at 100V for 20 minutes then the voltage was increased to 120V for approximately 1 hour. After the separation, the gel was transferred to a Polyvinylidene fluoride membrane (PVDF; Millipore, Cat. No. IEVH00005). Membrane transfer were run at 100V for 1.5 hours. Freshly transferred membrane was handled carefully and washed with 1X TBS-T (Tris-buffered saline with 0.1% Tween 20; Thermo Fischer Scientific, Cat. No. 003005) for 1 minute and blocked with blocking solution (5% non-fat dry milk (NFDM) in TBS-T) for 1 hour at room temperature. The blocked membrane was incubated at 4 °C overnight with a primary antibody depending on the protein of interest; Anti-FLAG (Sigma Aldrich, Cat. No. SAB4200071), Anti-Myc (abcam, Cat. No. ab18185), Anti-PER2 (Bethyl, Cat. No. A303-109A), Anti-DBP (Santa Cruz

Biotechnology Inc., Cat. No. sc-365441), Anti-CITED2 (abcam, Cat. No. EPR3416(2)). All antibodies were prepared with 5% NFDm in 1X TBS-T. Following the overnight incubation with a primary antibody, the membrane was washed with 1X TBST-T for 3 times and 5 minutes for each wash at room temperature. The membrane was incubated with a secondary HRP conjugated antibody (secondary mouse (Santa Cruz, Cat. No. SC-358920) or rabbit (Cell Signaling, Cat. No. 7074) at room temperature for 1 hour before imaging. For the imaging, secondary antibody was removed, and the membrane was washed for 5 minutes and 3 times. To visualize the protein of interest, ECL HRP substrate buffer (Advansta, Cat. No. K-12045-D50) was spread over the membrane and HRP chemiluminescence was detected with BioRad ChemiDoc Touch.

Table 3: Compositions of SDS-PAGE gels used in Western Blot analysis.

| Percentage                    | Solution Compositions                                                                                                                                                                                         |
|-------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 6% Separating gel (for 5 ml)  | dH <sub>2</sub> O: 2.6 ml<br>30 % acrylamide mix: 1.0 ml<br>1.5 M Tris (pH=8.8): 1.3 ml<br>10% SDS: 0.05 ml<br>10% ammonium persulfate: 0.05 ml<br>TEMED (Thermo Fischer Scientific Cat. No. 17919): 0.004 ml |
| 10% Separating gel (for 5 ml) | dH <sub>2</sub> O: 1.9 ml<br>30 % acrylamide mix: 1.7 ml<br>1.5 M Tris (pH=8.8): 1.3 ml<br>10% SDS: 0.05 ml<br>10% ammonium persulfate: 0.05 ml<br>TEMED: 0.002 ml                                            |
| 5% Stacking gel (for 1 ml)    | dH <sub>2</sub> O: 0.68 ml<br>30 % acrylamide mix: 0.17 ml<br>1.5 M Tris (pH=6.8): 0.13 ml<br>10% SDS: 0.01 ml<br>10% ammonium persulfate: 0.01 ml<br>TEMED: 0.001 ml                                         |

### **3.8 Real Time Quantitative PCR**

#### **3.8.1 RNA Isolation**

Before starting the RNA isolation procedure, every surface and material was wiped with RNaseZAP (Thermo Fischer Scientific, Cat. No. AM9780). Cells were harvested according to cell harvest protocols and the cell pellets were resuspended by pipetting and vortexing with 1 ml of TRIzol reagent (Invitrogen, Cat. No. 15-596-018) under a chemical hood. The tubes were incubated for 5 minutes at room temperature. 200 µl of chloroform was added onto the cell suspension and gently mixed by inverting the tubes. To separate the RNA layer, the tubes were centrifuged for 15 minutes at 14000xg and 4 °C. Following the centrifugation, top layer (approximately 400 µl, includes RNA) was carefully pipetted into a new tube. 400 µl of 100% ice cold isopropanol and 0.6 µl of 20 mg/ml glycogen were mixed with RNA by gently inverting the tube 10 times. This mixture was stored at -80 °C for 24 hours. The next day, the mixture was thawed on ice and centrifuged for 20 minutes at 14000xg and 4 °C. RNA must be slightly visible pellet at this point. Supernatant was removed and the RNA pellet was washed with 1 ml of ice cold 75% ethanol. Following the supernatant removal, the tubes were left for air-dry for 15-30 minutes. When the pellet dried, it was resuspended with 30 µl of nuclease-free ddH<sub>2</sub>O. To check the quality of the RNA and detect any DNA remains, 500 ng of RNA was run on a 0.8% agarose gel at 100V for 30 minutes.

#### **3.8.2 cDNA Synthesis**

Freshly isolated RNA was used for 2-step cDNA synthesis. For the first step, 500 ng of RNA was mixed with 2 µl of 100 100 µM oligo(dT)<sub>18</sub> primer (Thermo Fisher Scientific, Cat. No. SO131), 1 µl of 10mM dNTP and the mixture was equalized to 10 µl with nuclease-free ddH<sub>2</sub>O. The mixture was kept at 65 °C to denature secondary structures of primers and RNA and it is followed by 5 minutes of 4 °C in a thermocycler. For the second step, 0.2 µl of murine RNase Inhibitor (Biolabs, Cat. No. M0314S), 1 µl of RevertAid Reverse Transcriptase enzyme (Thermo Fischer Scientific, Cat. No. EP0441), 4 µl of 5X reaction buffer (Thermo Fischer Scientific, Cat. No. EP0441) and 4.8 µl of ddH<sub>2</sub>O were added into the reaction tubes. The final volume after the second step was 20 µl for each reaction. The reaction was kept at 42 °C for 1 hour followed by 65 °C for 20 minutes in a

thermocycler. Following the cDNA synthesis, cDNAs were diluted 1:5 with 80 µl of ddH<sub>2</sub>O and stored at -20 °C.

### 3.8.3 Quantitative Real-Time PCR (RT-qPCR)

Quantitative Real-Time PCR (RT-qPCR) was performed with SYBR green master mix. For each RT-qPCR mix, 1:5 diluted cDNA samples were further diluted to 1:40 with nuclease-free ddH<sub>2</sub>O. For each reaction, 8 µl of SYBR green mix, 8 µl of ddH<sub>2</sub>O, 3 µl of cDNA and 1 µl of forward and reverse primer mix (10 µM) were mixed. All primers used in RT-qPCR are listed in Appendix A. All reactions had a 3 technique replicates and as an internal control *RPLP0* was used. The cycling protocol for RT-qPCR reaction was as following; denaturation at 95°C for 10 seconds, annealing at 60°C for 20 seconds and elongation at 72°C for 30 seconds, 40 cycles. The results were analysed relative to *RPLP0* transcript level with the  $2^{-\Delta\Delta CT}$  method.

### 3.9 siRNA Knock-Down of *CITED2* in U2-OS cells

siRNA knock-down of *CITED2* was performed with two different approaches. First, cells were not synchronized before the siRNA treatment, and the effect of siRNA knock-down was analysed via RT-qPCR for *CITED2* mRNA level. According to the first results, second experimental approach was designed, and the cells were synchronized 2 hours before the siRNA treatment followed by the same transfection procedure explained below.

3x10<sup>5</sup> U2-OS cells were seeded in 35-mm dishes in DMEM with 10% FBS, without any antibiotics. 24 hours later, cells reached %80-90 confluency and they were transfected with *CITED2* siRNA mixes (Qiagen; FlexiTube GeneSolution GS10370 for *CITED2*) using Lipofectamine RNAiMAX Reagent (Invitrogen, Cat. No. 13778-150) and following manufacturer's protocol. siRNA mixes and lipofectamine mixes were prepared separately. For lipofectamine mixes, 9 µl of lipofectamine was added into 150 µl of Opti-MEM (Thermo Fisher Scientific, Cat. No. 11058021) in an Eppendorf tube and incubated at room temperature for 5 minutes. Final concentration of 150 µM *CITED2* siRNA mixes were prepared in 150 µl of Opti-MEM and incubated at room temperature for 5 minutes. Next, 150 µl lipofectamine mix was transferred into 150 µl of siRNA mix and incubated at room temperature for 10 minutes. In the meantime, cells were prepared for transfection.

The medium was removed, and the cells were washed with 1 ml of 1X PBS. Lipofectamine and siRNA mix was added into 2 ml of DMEM with 10% FBS and transferred onto cells. As a control group, AllStars negative control siRNA (also known as scrambled siRNA) (Qiagen, Cat. No. 1027280) was used for one group of cells in each experiment. Cells were incubated in the cell culture incubator and harvested 24 hours after the transfection.

siRNA transfection was performed with the same protocol as unsynchronized cells. For synchronized group, cells were harvested at 3 time points which are 6<sup>th</sup> hour, 12<sup>th</sup> hour and 18<sup>th</sup> hour. Results were analysed via RT-qPCR to measure *CITED2*, *BMAL1*, *CLOCK*, *CRY1*, *PER2*, *REV-ERB* and *ROR $\alpha$*  mRNA levels.

### **3.10 Real-Time Tracking of Circadian Oscillations**

#### **3.10.1 *Bmal1:dluc* Lentivirus Production**

On day zero, HEK293T cells were passaged such that confluency would reach 80% on the next day. On day one, the medium on the cells were removed, the cells were washed with 1X PBS and 5 ml of DMEM was added onto cells before transfection. Transfection solution was prepared as following vectors; 10  $\mu$ g of pLV6-Bmal-luc vector, 9  $\mu$ g of pCMV- $\Delta$ R8.2dvpr packaging vector and 1  $\mu$ g of pCMV-VSVG envelope vector (Appendix B). 20  $\mu$ l of PEI was added into the vector mixture and the mixture was equalized to 400  $\mu$ l total volume with DMEM. Transfection mix was incubated for 20 minutes and added onto the cells. 16 hours post transfection, 3ml of DMEM was added into the plate. On day three, the medium of the cells was changed. The lentiviral particles were ready to collect after 72 hours of transfection, particles in the medium were collected on day four and five. The lentiviral particles were sterilized with 0.45  $\mu$ m filter and stored at -80 °C.

### 3.10.2 Real Time Bioluminescence Monitoring

CITED2-OE/U2-OS and U2-OS cells passaged two days before seeding.  $30 \times 10^4$  cells were seeded into 35 mm culture dishes. On the next day, 750  $\mu$ l of *Bmal1:dluc* lentiviral particles were transduced into cells with 250  $\mu$ l of DMEM and 1  $\mu$ l of 8 mg/ml protamine sulphate. 16 hours post transduction, medium of the cells were changed with fresh medium. 2 days later, the cells were synchronized two hours before the measurements with dexamethasone. For the measurements, the medium of the cells was replaced with lumicycle medium (10 mM HEPES, 2 mM L-Glutamine, 0.35 mg/ml  $\text{NaHCO}_3$ , 100  $\mu$ g/ $\mu$ l P/S, 1 mM luciferin in DMEM at pH=7.2). The edges of the 35 mm culture were sealed with grease. Then the culture dishes replaced into the LumiCycle luminometer. LumiCycle luminometer provides required 5%  $\text{CO}_2$ , humidity at 37 °C. Bioluminescence recordings were taken with photomultiplier for 70 seconds every 10 minutes.

## Chapter 4

### RESULTS

Genetic, biochemical, and computational tools indicate that there is likely to be a rich network of clock modulating factors and additional clock components. Additional genetic and epigenetic mechanisms regulate tissue-specific clock functions (Robles, 2010; Anafi et al., 2014). To identify these factors a high throughput screening is performed in our lab to identify transcription factors which have the potential to modulate BMAL1/CLOCK transcription activity through high-through luciferase screening (Bulut, 2017). To determine the positive or negative effect on the BMAL1/CLOCK transactivation, cells were co-transfected with pCMV-Sport6-*Bmal1* and pCMV-Sport6-*Clock* along with TFs separately. As a reporter vector, *mPer1:dluc* was used. To eliminate any false positive activity, luciferase activity of BMAL1/CLOCK and pBIND co-transfected cells were also measured, and it was shown that pBIND vector (without any TFs) does not affect BMAL1/CLOCK transactivation on *mPer1:dluc* promoter. For the positive control of repression activity, CRY1 was co-transfected with BMAL1/CLOCK. Luciferase activity was measured with a luminometer. TFs which have a repression or activation activity on BMAL1/CLOCK driven transcription were identified as candidate clock proteins. CITED2 was one of the top candidates of this study, therefore, my study is based upon these findings.

Prior to investigate the function of CITED2 in clock mechanism, I investigated the mRNA expression patterns of CITED2. CITED2 has a low tissue specificity and it is found in almost all tissues from endocrine tissue to the brain (GTEx Analysis Release V8; dbGaP Accession phs000424.v8.p2). According to CircaDB database (Pizarro et al., 2013) (<http://circadb.hogeneschlab.org/>), *CITED2* is oscillating in mouse liver and kidney

**(Figure 9).** Therefore, we decided to characterize the role of *CITED2* in circadian clock regulation at the molecular level.

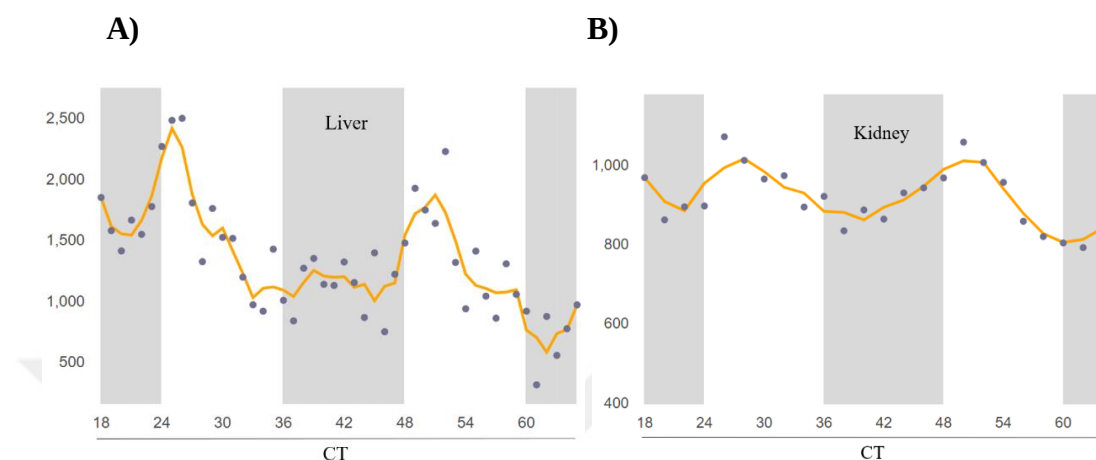


**Figure 9. The circadian expression pattern of *CITED2* in mouse liver (A), and kidney (B) tissues.**

The expression profile revealed that *CITED2* has circadian rhythmicity. This figure is adopted from CircaDB database.

#### 4.1 Cloning of Human *CITED2* cDNA into Mammalian Expression Vector

To investigate the role of *CITED2* in mammalian cells for reporter assays and pull-down assay, *CITED2* was cloned into mammalian expression vector pcDNA4/myc-His A. *CITED2* was first amplified using PCR with *CITED2*-BamHI\_F and *CITED2*-EcoRI\_R primers (Appendix A) and subcloned into pGEM-T-ccdB plasmid. Then, pGEM-T-ccdB-*CITED2* and pcDNA4/myc-His A plasmids were digested with EcoRI and BamHI. Digested pGEM-T-ccdB vector and insert *CITED2* fragment were ligated and transformed into competent cells. Several colonies were picked and screened for the presence of *CITED2* with both PCR and restriction digestion. I selected a plasmid with *CITED2* based on PCR and restriction digestion results. PCR result of pcDNA4/myc-His-A plasmid with *CITED2* (left column) and a positive control *CITED2* (right column) is shown in the **Figure 10 A**. As another confirmation I performed restriction digestion with EcoRI and BamHI, expected fragment sizes were around 5 kb corresponding to the backbone and 0.8 kb corresponding to the *CITED2* as it is shown in the **Figure 10 B**.

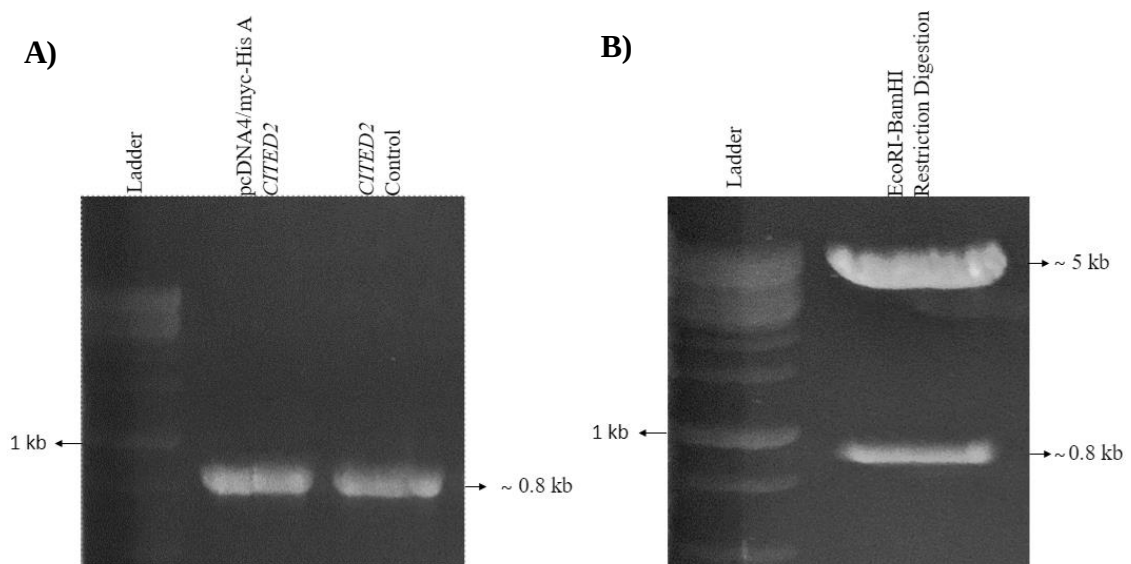


**Figure 10. Confirmation of *CITED2*-pcDNA4/myc-His A cloning with PCR and restriction digestion experiments.**

A) PCR result of pcDNA4/myc-His-A plasmid with *CITED2* (left column) and a positive control *CITED2* (right column) on agarose gel. B) Restriction digestion result of pcDNA4/myc-His-A plasmid with *CITED2* cut with EcoRI and BamHI on agarose gel.

#### 4.2 CITED2 Represses BMAL1/CLOCK Heterodimer on *mPer1* Promotor

The mammalian circadian clock has four core clock proteins BMAL1, CLOCK, CRYs and PERs which are involved in three interlocked transcriptional feedback loops as described in Chapter 2. BMAL1 and CLOCK forms heterodimer and act as activators by binding the E-box elements on *CRYs*, *PERs* and clock-controlled gene promoters. CRYs and PERs act as a negative arm as they accumulate on the cytosol and translocates into the nucleus to repress their own transcription which is driven by BMAL1/CLOCK complex (Takahashi, 2017). Repression assay is a commonly used reporter assay in the field. *mPer1* promoter (contains 3 E-box motifs) is fused with a destabilized form of luciferase gene named as *mPer1:dluc* plasmid (**Figure 11**). BMAL1/CLOCK complex binds to the E-box element on *mPer1* promoter and any activation or repression activity of CITED2 on BMAL1/CLOCK complex can be quantified through luciferase activity level. I hypothesized that if CITED2 acts as a repressor on BMAL1/CLOCK transactivation, I can show this inhibitory effect with repression assay since repressors

decrease the output luciferase signal relative to the positive control in this construct (**Figure 10**).

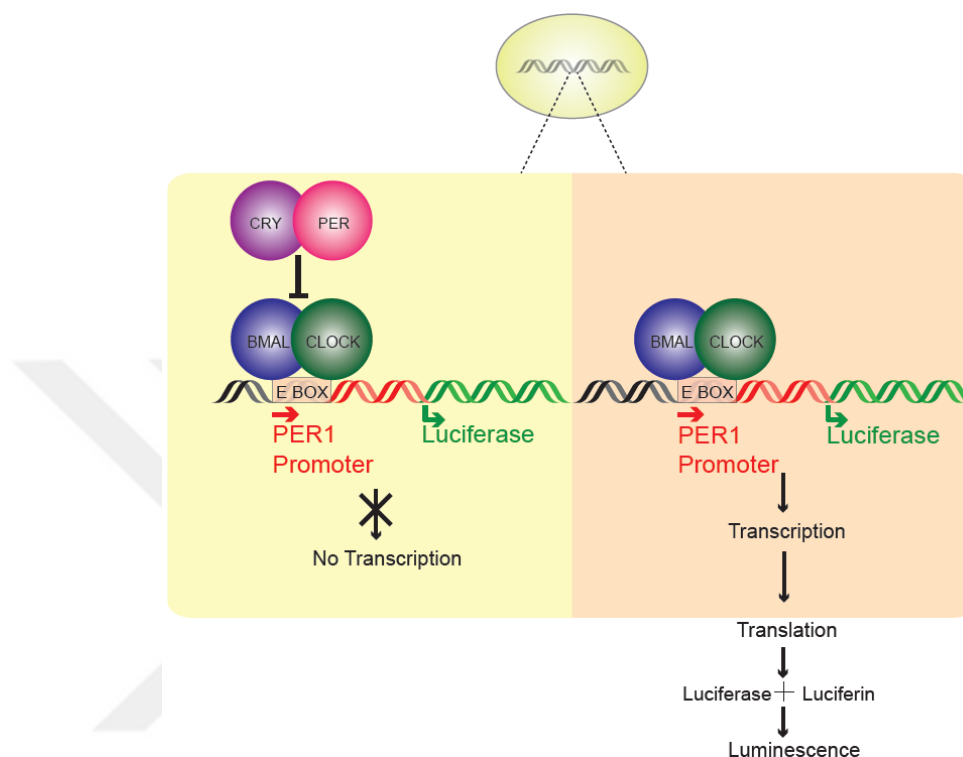


**Figure 11. Schematic representation of repression assay and working principal.**

HEK293T cells were transiently transfected with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*CLOCK* and *mPer1:dluc* with increasing doses of pcDNA4/myc-His-A plasmid with *CITED2* (5 ng, 12.5 ng, 25 ng, 50 ng and 100 ng). As a positive control for the repression activity, I used two doses of pcDNA4/myc-His A plasmid with *CRY1* along with pCMV-Sport6-*Bmal1*, pCMV-Sport6-*CLOCK* and *mPer1:dluc* vectors (**Figure 12**). For the negative control conditions, again I used increasing doses of pcDNA4/myc-His-A plasmid with *CITED2* and two doses of pcDNA4/myc-His-A plasmid with *CRY1* (2 ng and 20 ng) with and *mPer1:dluc* vector separately (**Figure 13**). Luciferase activity was normalized to *Renilla* to eliminate any variance such as cell number or transfection efficiency between biological replicates.

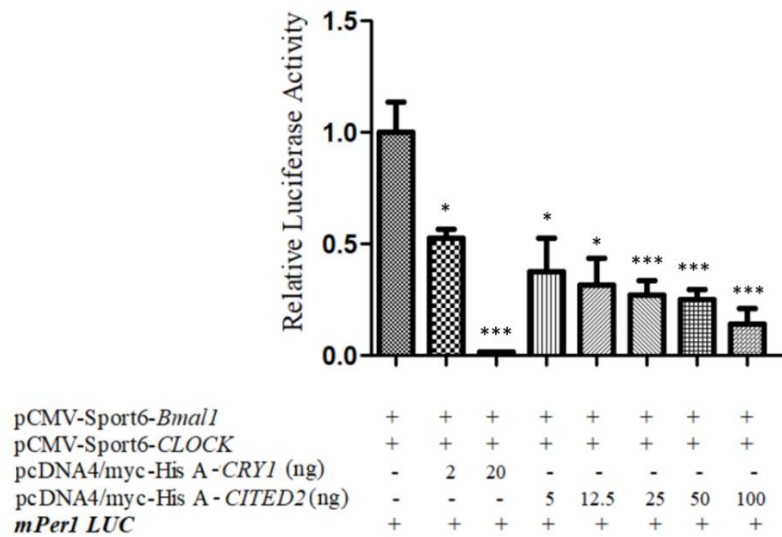


**Figure 12. Inhibitory effect of CITED2 on BMAL1/CLOCK driven activation.**

CITED2 represses BMAL1/CLOCK activity on E-box in a dose dependent manner. Firefly luciferase activity signals were normalized with Renilla luciferase activity. CRY1 used as a positive control for the repression assay. Statistical significance was calculated compared to the control group (first bar) for each condition. (Data represent the mean  $\pm$  SEM,  $n \geq 3$  biological replicates. \*:  $p < 0.05$ , \*\*\*:  $p < 0.0001$  by student t-test)

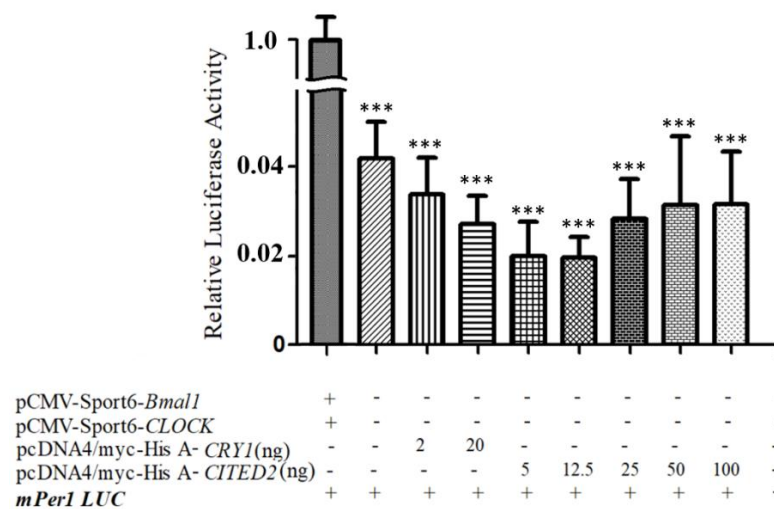


**Figure 13. CITED2 does not have any effect on *mPer1:dluc* in the absence of BMAL1/CLOCK protein complex.**

Neither CITED2 nor CRY1 does not start transcription upon binding to E-box promoter. Statistical significance was calculated compared to the control group (first bar) for each condition. (Data represent the mean  $\pm$  SEM,  $n \geq 3$  biological replicates. \*\*\*:  $p < 0.0001$  by student t-test)

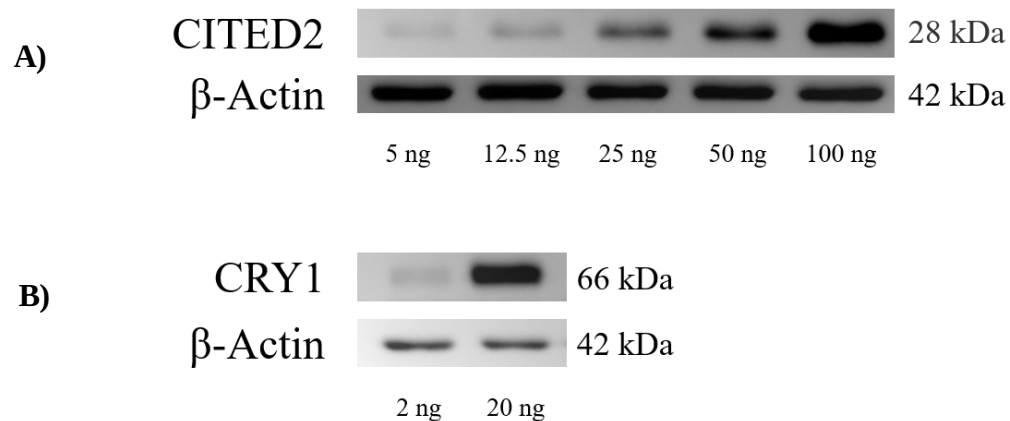


**Figure 14. Western Blot analysis of CITED2 and CRY1.**

CITED2 and CRY1 showed the dose dependent increase on the protein level. (n=2 biological replicates.)

CITED2 decreased relative luciferase activity compared to positive control (pCMV-Sport6-*Bmal1*, pCMV-Sport6-*CLOCK* and *mPer1:dluc*) in a dose dependent manner as well as control CRY1 repression activity. The lowest dose (5 ng) of CITED2 decreased relative luciferase signal to 50% and the highest dose (100 ng) of CITED2 decreased the signal to 20%. Student's t-test showed statistically significant results for the all doses of CITED2 suggesting that CITED2 acts as a repressor on BMAL1/CLOCK activity on E-box element of *mPer1* promoter (**Figure 12**). Negative control conditions of repression assay suggested that neither CRY1 nor CITED2 is able to activate or repress transcription on *mPer1:dluc* without BMAL1/CLOCK. This result eliminates the possible false positive result and validates that CITED2 acts on BMAL1/CLOCK (**Figure 13**). Additionally, I showed the increasing doses of CITED2 and CRY1 with Western Blot analysis with a parallel scaled-up experiment set up as shown in **Figure 14**.

All these results suggested that CITED2 repress CLOCK/BMAL1 transactivation in a dose dependent manner and acts on the negative arm of TTFL.

### 4.3 Repression Activity of CITED2 is not Dependent on CBP/p300 Complex

CITED2 interacts with general transcriptional coactivators CBP and p300 through conserved 32 amino acid residues on the C-terminus. A mutagenesis study showed that CITED2 interacts with CBP and p300 CH1 domain (cysteine/histidine-rich 1 domain, also known as TAZ1) of both CBP and p300. (Bhattacharya et al., 1999). In HIF1- $\alpha$  and TFAP-2 pathways, CITED2 requires CBP/p300 complex to display its positive or negative regulation (Berlow et al., 2017; Bragança et al., 2003). It was also shown that CBP/p300 complex modulates BMAL1/CLOCK transactivation positively or negatively in a cell type dependent manner (Hosoda et al., 2009). Therefore, I reasoned that CITED2 may require CBP/p300 complex to exert its inhibitory activity on BMAL1/CLOCK transactivation.

To this end, we used repression assay to investigate the effect of CBP and p300 on the repression activity of CITED2 in the molecular clock. HEK293T cells were transiently transfected with pcDNA3 $\beta$ -FLAG-HA-CBP and pCMV $\beta$ -Myc-p300 separately and together with pcDNA4/myc-His-A plasmid with CITED2. For the negative control, I used the same transfection conditions without BMAL1 and CLOCK, in this way I tested whether CBP or p300 activates *mPer1:dluc* promoter alone. Repression assay revealed that CBP/p300 complex enhanced BMAL1/CLOCK activation on E-box in HEK293T cells (**Figure 15**). Moreover, CBP and p300 were still able to enhance BMAL1/CLOCK activity when they are transfected separately. Strikingly, CITED2 mediated inhibition on BMAL1/CLOCK was dampened when co-transfected with CBP/p300 complex. For the negative control, CBP and p300 had higher luciferase activity on *mPer1:dluc* promoter compared to CRY1 and CITED2 in the absence of BMAL1/CLOCK, however, this activity was dramatically low compared to BMAL1/CLOCK driven activation (**Figure 16**). Endogenous BMAL1 and CLOCK are most likely the reason for this activity. Therefore, I concluded that CBP and p300 did not have significant activity on *mPer1:dluc* promoter. These results suggest that CITED2 does not require CBP/p300 complex to exhibit its inhibitory effect but instead, CITED2 and CBP/p300 complex exert an opposite effect on BMAL1/CLOCK driven transcription on E-box.

Next, I tested the effect of p300 on BMAL1/CLOCK transactivation in a dose dependent manner. HEK293T cells were transfected with 3 doses of pCMV $\beta$ -Myc-p300 (25 ng, 50 ng, 100 ng) with and without 50 ng of pcDNA4/myc-His-A plasmid with CITED2. p300

activated the transcription dose dependently without CITED2 as expected, however, repression activity of CITED2 was also dampened with the same trend upon increasing doses of p300 (**Figure 17**). These results suggest that p300 counteracts the inhibitory effect of CITED2 on BMAL1/CLOCK driven transcription in a dose dependent manner.

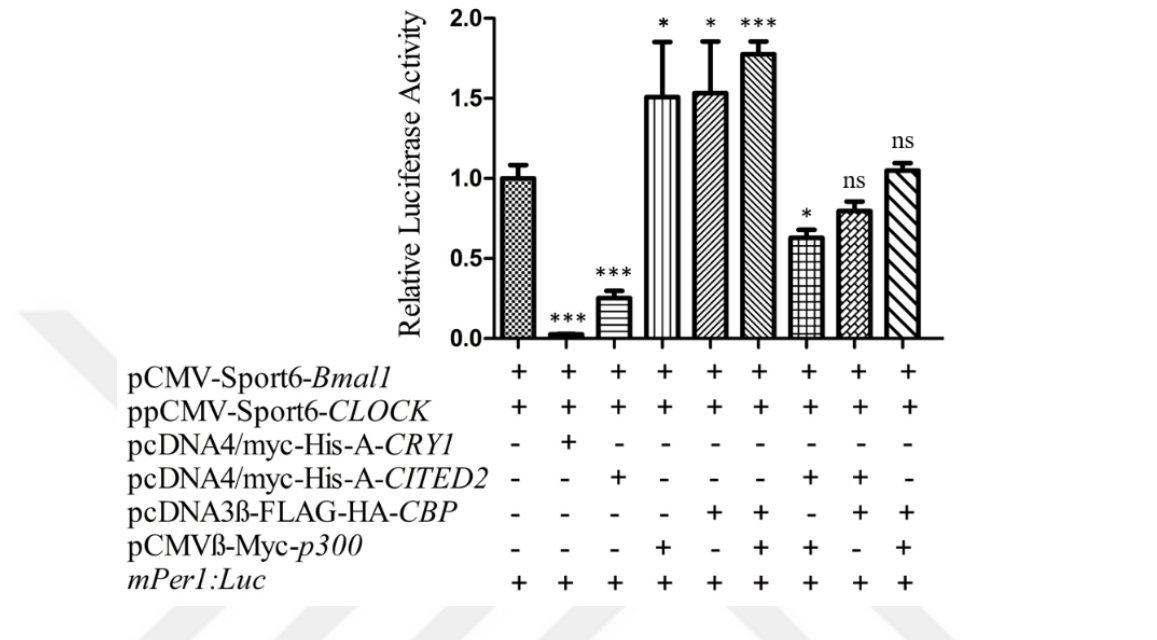


**Figure 15. Repression activity of CITED2 was dampened upon CBP/p300 transfection.**

CBP and p300 activate BMAL1/CLOCK driven transcription on E-box and repression activity of CITED2 was dampened when HEK293T cells were co-transfected with CBP or p300. Firefly luciferase activity was normalized with Renilla activity. CRY1 used as a positive control for the repression assay. Statistical significance was calculated compared to the control group (first bar) for each condition. (Data represent the mean  $\pm$  SEM, n=3 biological replicates. \*:  $p < 0.05$ , \*\*\*:  $p < 0.0001$  by student t-test)

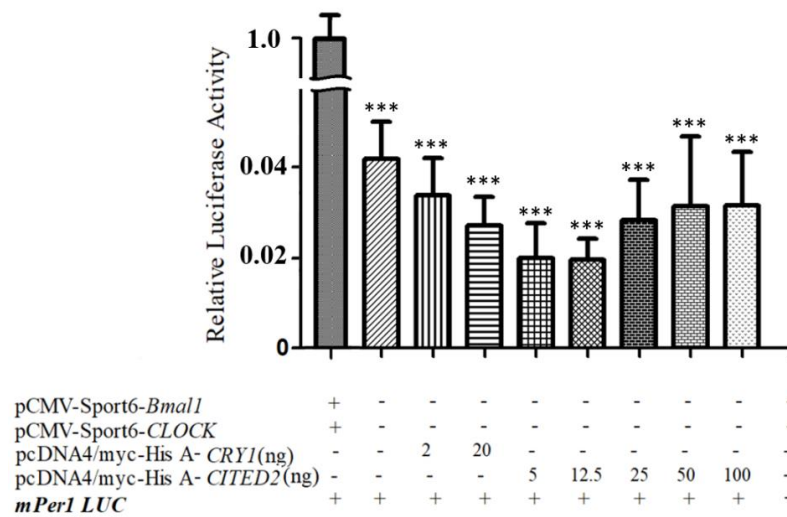


**Figure 16. CBP and p300 do not activate *mPer1:dluc* promoter.**

Neither CBP nor p300 does not activate transcription upon binding to E-box on *mPer1:dluc* promoter in the absence of BMAL1/CLOCK. Statistical significance was calculated compared to the control group (first bar) for each condition. (Data represent the mean  $\pm$  SEM, n=3 biological replicates. \*\*\*:  $p < 0.0001$  by student t-test)

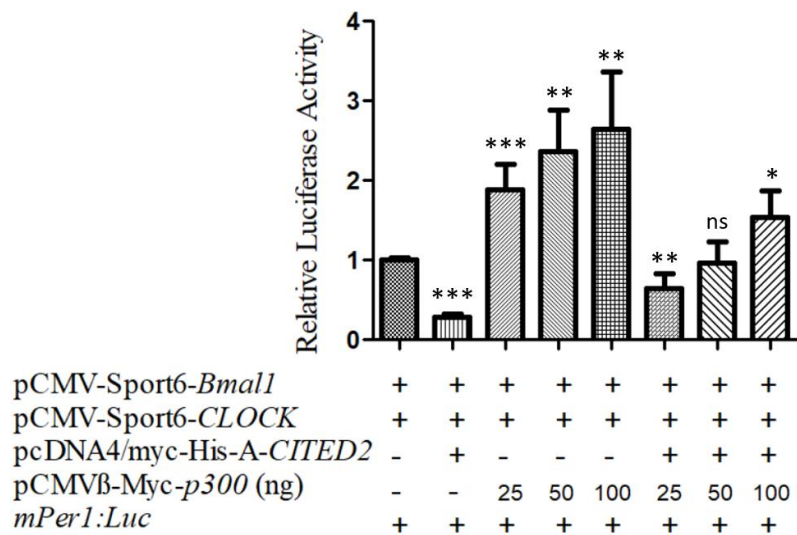


**Figure 17. p300 counteracts the repression activity of CITED2 in a dose dependent manner.**

Statistical significance was calculated compared to control group (first bar) for each condition. Data represent the mean  $\pm$  SEM, n=3 biological replicates. \*:  $p < 0.05$ , \*\*:  $p < 0.005$ , \*\*\*:  $p < 0.0001$  by student t-test.

#### 4.4 *CITED2* Knock-Down Results in Altered Circadian Period in U2-OS

As mammalian circadian clock exhibits 24-hour cycles, it was shown that mutant forms of negative arm members CRYs and PERs result in a change in the period length. *Cry1*<sup>-/-</sup> mice exhibits almost 1-hour shorter rhythm and *Cry2*<sup>-/-</sup> mice have a 1-hour longer rhythm compared to wild type mice (Van Der Horst et al., 1999). The mutations on both *PER1* and *PER2* caused more drastic effects on rhythm. Either constitutive expression or knock out of PERs resulted in severe defects on circadian rhythm (Chen et al., 2009; Cermakian et al., 2001). CBS enzyme also acts as a circadian regulator and *Cbs*<sup>-/-</sup> mice exhibits reduced amplitude despite the fact *Cbs*<sup>-/-</sup> mice do not show any period length change (Cal-Kayitmazbatir et al., 2020). Taking these facts into account, it is expected to see a rhythm change when a clock regulator protein is over expressed or knocked out/down. Considering the repressor activity of *CITED2* on BMAL1/CLOCK transcriptional activation, I hypothesized that *CITED2* knock down results in a change in the molecular rhythm of the cells. Therefore, RNA interference, *CITED2* siRNA (small interfering RNA), was used to knock down *CITED2* in U2-OS (*Bmal1:dluc*) cells.

Control group was transfected with a scrambled siRNA mix which does not target and knock down any genes. Cells were synchronized with 0.1μM dexamethasone 2 hours before the measurements. The real time measurements were taken with LumiCycle for 7 days. The measurements of the first 24 hours and the last 24 hours were subtracted. The first 24 hours are not taken into measurements since the cells barely adapt to the new medium and the new environment, the last 24 hours are subtracted because the signal weakens and may cause miscalculation of rhythm.

Initially, the level of the *CITED2* mRNA level was measured by qPCR to make sure siRNA method effectively working in U2-OS cells (**Figure 18 A**). Then, I analysed lumicycle of the results, which indicated that, *CITED2* knock down in U2-OS cell line caused 40 minutes longer period (23.40 hours) compared to control U2-OS cells (23 hours) (**Figure 18 B-C**). Additionally, the amplitude of circadian rhythm was significantly decreased 28% upon siRNA transfection compared to control group (**Figure 18 D**). These results suggest that *CITED2* is required for robust circadian rhythm and reduction of *CITED2* alters the circadian phenotype of U2-OS cells.

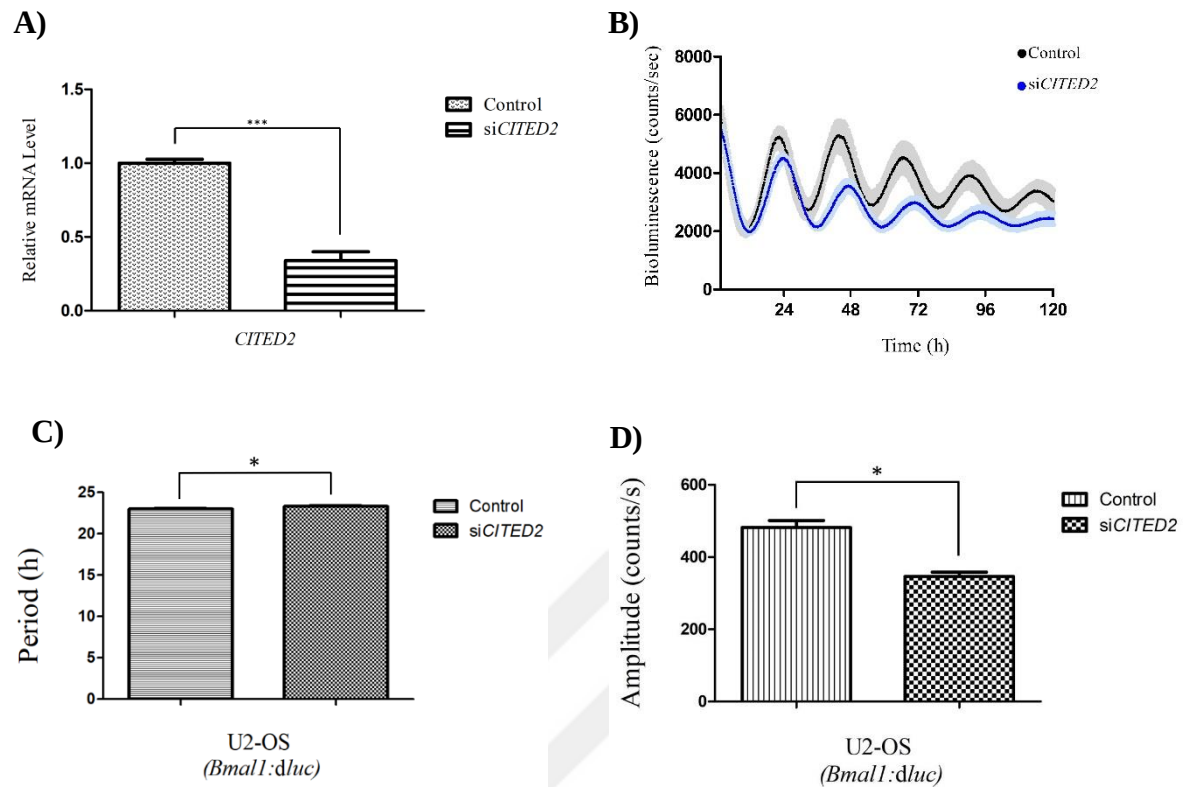


**Figure 18. *CITED2* knock down altered circadian phenotype in U2-OS (*Bmal1:dluc*).**

A) *CITED2* expression was knocked to 30 % according to RT-qPCR. (Error bars represent  $\pm$  SEM,  $n \geq 3$  biological replicates. \*\*\*:  $p < 0.0001$ ) B) U2-OS (*Bmal1:dluc*) cells were transfected with siRNAs and synchronized with 0.1  $\mu$ M dexamethasone containing medium for 2 hours. The medium was replaced with lumicycle medium and cells were placed into LumiCycle device. The luminometric measurements were taken for 7 days, with photomultiplier for 70 seconds every 10 minutes. C-D) Change in the period length and the amplitude upon *CITED2* knock down. (Data represent the mean  $\pm$  SEM,  $n=2$  biological replicates with duplicates. \*:  $p < 0.05$ , \*\*\*:  $p < 0.0001$ ). (İlkem Sevgili, unpublished data)

#### 4.5 *CITED2* siRNA Knock-Down Increases the mRNA Levels of DBP and PER2

As I showed that *CITED2* knock down alters the period of circadian rhythm in U2-OS cells. I wish then to explore the effect of *CITED2* knock down at the molecular level. Since *CITED2* has a repression activity on BMAL1/CLOCK driven transactivation, I hypothesized that *CITED2* knock down leads to reduce in BMAL1/CLOCK repression and results in higher levels of clock output genes. To test that, I first transfected unsynchronized U2-OS (*Bmal1:dluc*) cells with *CITED2* siRNAs and harvested cells 48

hours post-transfection. I used scrambled siRNA mix which contains siRNA molecules without interfering with any gene expression for the control group. Even though *CITED2* was knocked down to 30 % according to qPCR results shown in **Figure 18 A**, I did not observe any statistically significant change in the mRNA levels of BMAL1/CLOCK driven genes *DBP*, *PER2* or other clock genes (**Figure 19 A-G**). I reasoned that unsynchronized cells might be masking the effect of siRNAs on *DBP* and *PER2* mRNA levels.



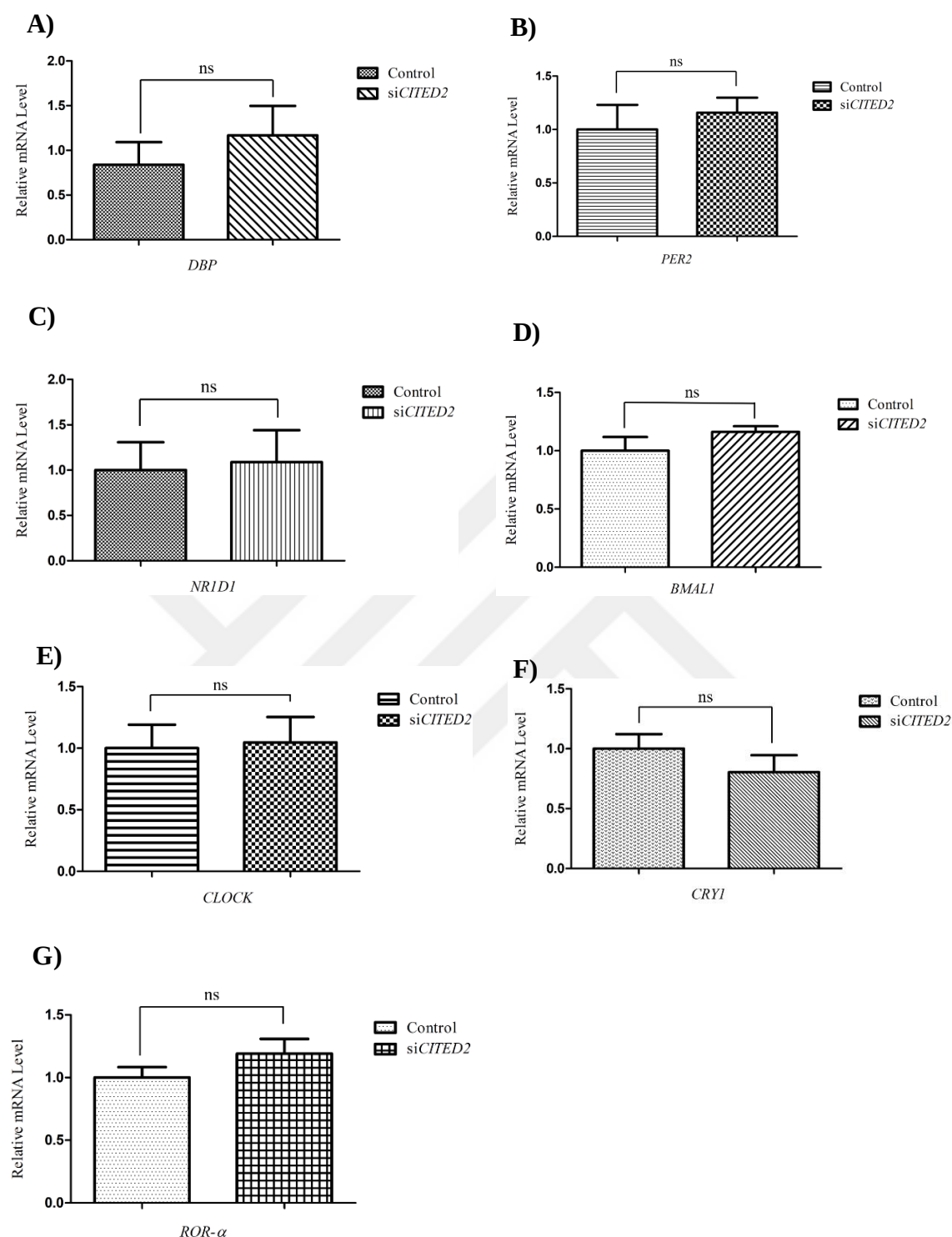


**Figure 19. CITED2 siRNA transfection has no effect on mRNA levels of core clock genes in unsynchronized U2-OS cells.**

A-G) RT-qPCR analysis did not result in any significant change in *DBP*, *PER2*, *NR1D1*, *BMAL1*, *CLOCK*, *CRY1* or *ROR- $\alpha$*  relative mRNA levels. (Data indicates  $\pm$  SEM with n=3 biological replicates, by student t-test)

I synchronized U2-OS cells after siRNA transfection and harvested cells at 3 time points: 6<sup>th</sup> hour, 12<sup>th</sup> hour and 18<sup>th</sup> hour. I performed qPCR analysis for *CITED2*, and I observed that *CITED2* was also knocked down at different levels on different time points. At the 6<sup>th</sup> hour, *CITED2* mRNA expression level was reduced to 16.3%, and the reduction was 20.6% at the 18<sup>th</sup> hour. Knock down was the most effective at the 12<sup>th</sup> hour at which *CITED2* mRNA expression level was reduced to 8.6% (**Figure 20**). So, I continued with cell synchronization and chose the 12<sup>th</sup> hour time point specifically for further analysis. Next, I performed qPCR analysis to determine *Dbp* and *Per2* relative mRNA levels of 12<sup>th</sup> hour samples. I observed that *DBP* relative mRNA levels were increased almost 40%, and the increase was 20 % for *PER2* levels upon siRNA transfection also shown in **Figure 21 A-B**. For the other core clock genes, relative mRNA level change was not statistically significant.

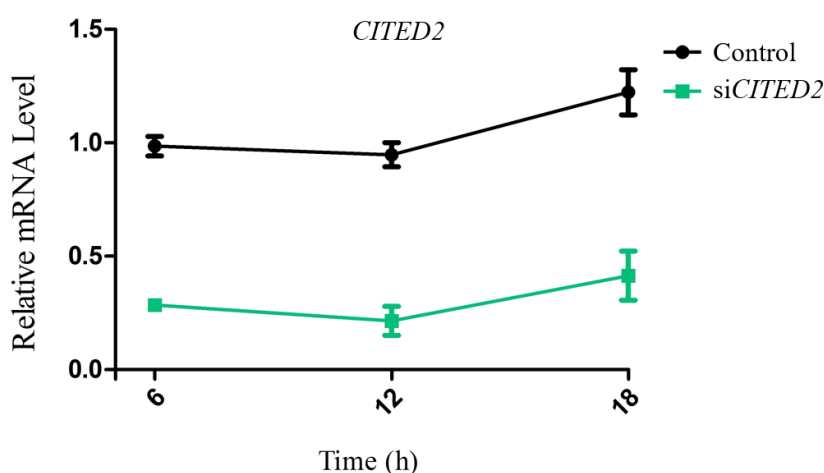


**Figure 20. *CITED2* knock down was the most successful at 12<sup>th</sup> hour after dexamethasone treatment.**

U2-OS cells were synchronized after siRNA transfection and sampled at three time points; 6<sup>th</sup> hour, 12<sup>th</sup> hour and 18<sup>th</sup> hour. RT-qPCR revealed that *CITED2* mRNA expression was reduced to 16.3% at the 6<sup>th</sup> hour, 8.6% at the 12<sup>th</sup> hour and 20.6% at the 18<sup>th</sup> hour. (Error bars indicate  $\pm$  SEM with n=2 replicates)

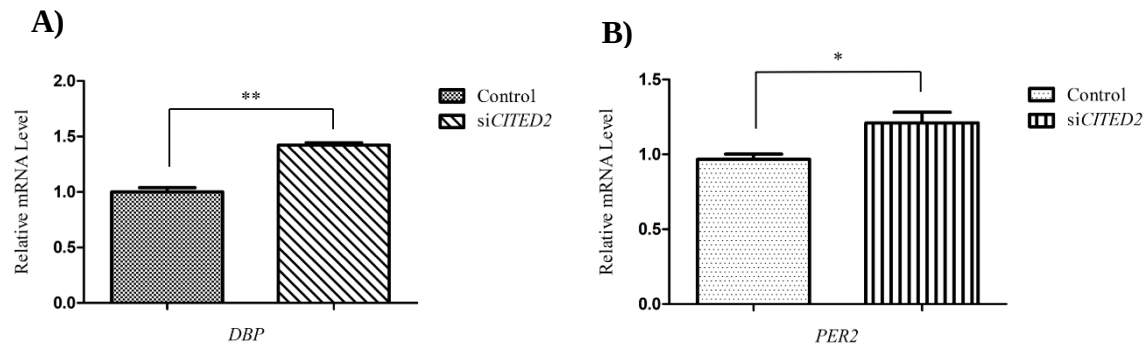


**Figure 21. CITED2 siRNA transfection increased mRNA levels of *DBP* and *PER2* upon cell synchronization.**

A) siCITED2 transfected U2-OS cells exhibit 40% increase in *DBP* mRNA expression at 12<sup>th</sup> hour time point upon dexamethasone treatment. B) *PER2* mRNA levels exhibit 20% increase at 12<sup>th</sup> hour time point upon dexamethasone treatment compared to control. (Error bars indicate  $\pm$  SEM with n=3 replicates. \*: p < 0.05, \*\*: p < 0.005 by student t-test)

#### 4.6 Generation of *CITED2* Over Express U2-OS Stable Cell Line

U2-OS is commonly used as a model cell line due to its robust circadian rhythm (Baggs et al., 2009). The robust oscillation is mediated by positive and negative arm elements of the circadian clock which are critical for the molecular clock mechanism and the regulation of other clock related genes. As it is shown with previous studies, constitutive expression of *PER1* or *PER2* completely abolished rhythm in MEF cells (R. Chen et al., 2009). Since I have already shown that *CITED2* knock down altered circadian phenotype in U2-OS (*Bmal1:dluc*) cells, I hypothesized that over expression of *CITED2* may also change the circadian rhythm in U2-OS cells.

To this end, *CITED2* was cloned into pLenti-III-HA lentiviral vector which has an ability to integrate into genomic DNA of the host cells and carries puromycin resistance for mammalian selection (Appendix B). *CITED2* was amplified with appropriate primers which possess EcoRI and SalI restriction sites (Appendix A). After obtaining PCR products, both insert, and pLenti-III-HA were digested with EcoRI and SalI and then ligated. I transformed ligation product into *E.coli* DH5 $\alpha$  competent cells. 10 colonies were screened to find a plasmid with *CITED2*. Once the presence of the *CITED2* was confirmed with restriction digestion and PCR, I named the construct as pLenti-III-HA-

*CITED2*. I used electroporation transfection to transfect U2-OS cells with pLenti-III-HA-*CITED2* since U2-OS cells yield very low transfection efficiency with regular chemical transfection methods. After the transfection, cells were selected with puromycin and analysed with WB and qPCR for *CITED2* over expression. I named *CITED2* over expressing cells as *CITED2*-OE/U2-OS. Compared to the U2-OS cells, *CITED2*-OE/U2-OS cell line yielded 8-fold more relative mRNA level (**Figure 22 A**) and 10-fold more *CITED2* as it is clearly seen in **Figure 22 B**.

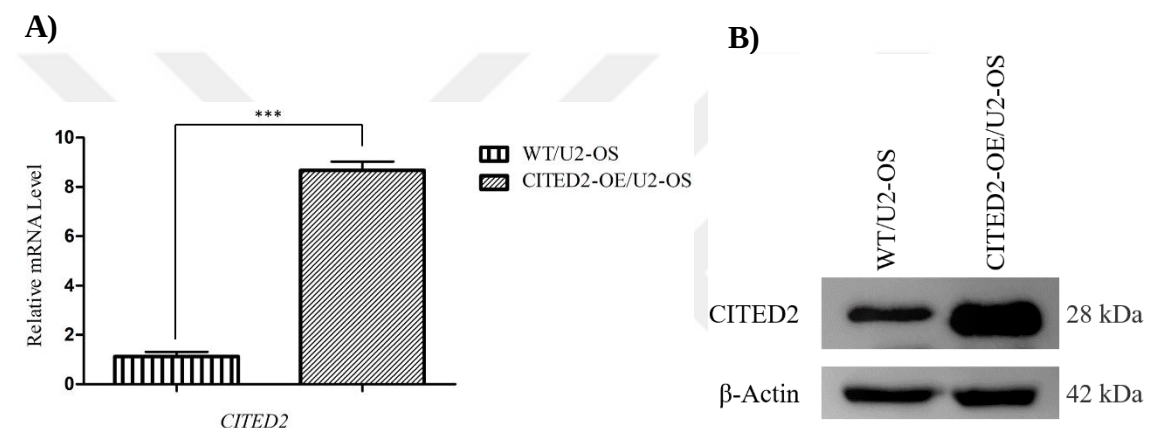


**Figure 22. *CITED2* over express U2-OS cell line stably express *CITED2*.**

A) *CITED2* relative mRNA levels indicate *CITED2* transcribed 8-fold higher in over express stably cell line (*CITED2*-OE/U2-OS) (Error bars represent  $\pm$  SEM,  $n \geq 3$  biological replicates. \*\*\*:  $p < 0.0001$ ). B) WB analysis reveals that *CITED2* levels are almost 10-fold higher in over express cells compared to WT cells.

#### 4.7 *CITED2* Over Expression Lengthens the Period of U2-OS Cells

To see the effect of *CITED2* over expression on the circadian rhythm, I monitored real time bioluminescence measurements on *CITED2*-OE/U2-OS cells and U2-OS cells as a control. I used *Bmal1:dluc* lentiviral plasmid that contains luciferase reporter gene and the cells were maintained with special luciferase buffer containing luciferin which in turn will be degraded and emits bioluminescence. I transduced *CITED2*-OE/U2-OS and U2-OS cells with *Bmal1:dluc* lentiviral particles and synchronized with 0.1  $\mu$ M dexamethasone before taking *in vivo* bioluminescence measurements. As a positive control for signal

emission, I used U2OS *Bmal1:dluc* cell line without transduction since this cell line can express *Bmal1:dluc* stably. For the negative controls, I used CITED2-OE/U2-OS and U2-OS cells but without lentiviral transduction, which did not show any luciferase activity. I performed this experiment with I calculated period length according to 5-days (120 hours) of measurements. CITED2-OE/U2-OS cells exhibit 1.5 hours longer period (24.2 hours) compared to the WT U2-OS cells with 22.7 hours of period (**Figure 23**). Also, CITED2-OE/U2-OS cells display 2.5 fold enhanced amplitude compared to WT U2-OS cells. This result suggests that *CITED2* over expression causes statistically significant period length alterations in the circadian rhythm of the U2-OS cells.

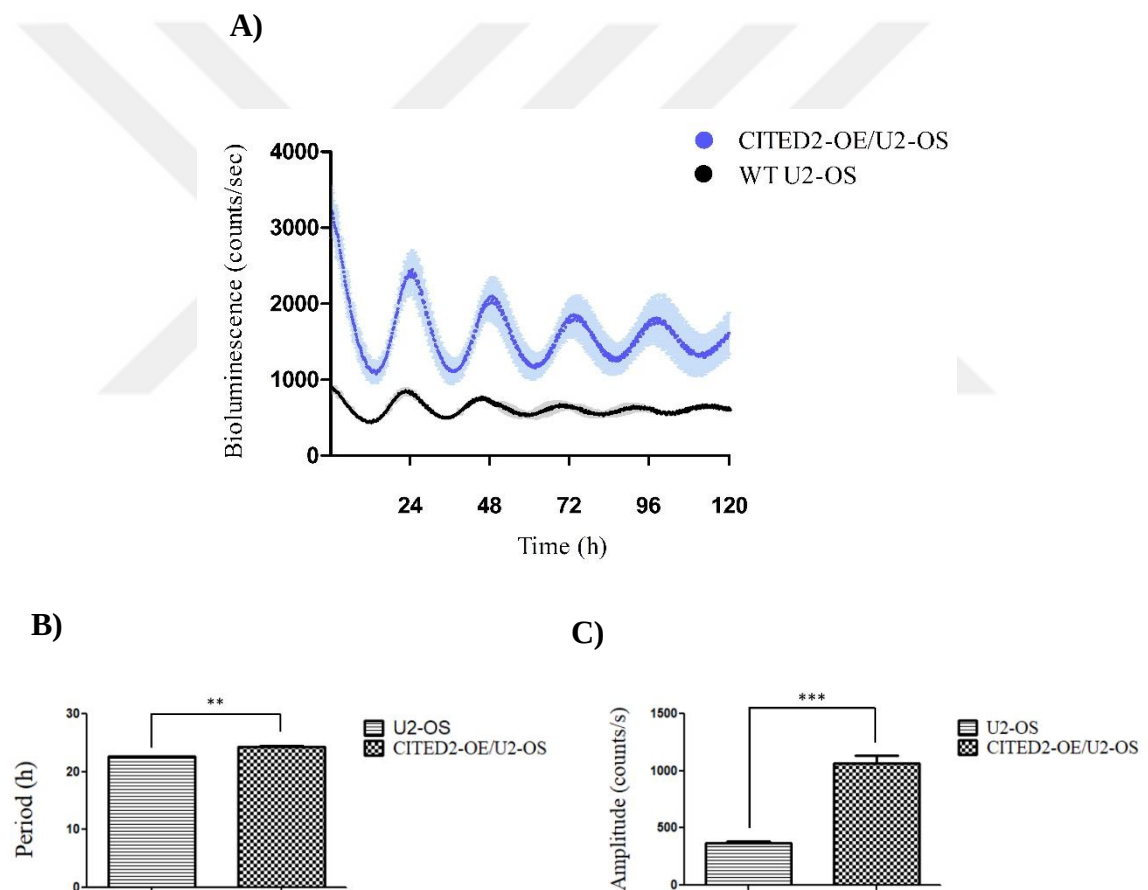


**Figure 23. *CITED2* over expression extended the period of U2-OS cells.**

WT U2-OS and CITED2-OE/U2-OS cells were transduced with *Bmal1:dluc* lentiviral particles and synchronized with 0.1  $\mu$ M dexamethasone containing medium for 2 hours. The medium was replaced with lumicycle medium and cells were placed into LumiCycle device. The luminometric measurements were taken for 7 days, with photomultiplier for 70 seconds every 10 minutes. (Data indicates  $\pm$  SEM n=2 with duplicates, \*\*: p < 0.005, \*\*\*: p < 0.0001, by student t-test).

#### 4.8 *CITED2* Over Expression Decreases both mRNA and Protein Levels of DBP and PER2

All my data so far suggested that *CITED2* has a repressor activity on BMAL1/CLOCK driven transcriptional activation. If this is true, I expected to see a reduction in the transcription of BMAL1/CLOCK driven genes. To investigate the changes in the transcriptional activation at the molecular level, I used *CITED2*-OE/U2-OS and WT U2-OS cells. The mRNA levels of *CRY1*, *PER2*, *DBP*, *REV-ERB $\alpha$*  and *ROR $\alpha$*  were measured with qPCR. I used *RPLP0* to normalize mRNA levels of clock genes in all experiments. Analysis of the qPCR results revealed that *DBP*, *PER2* and *NR1D1* relative mRNA levels are significantly decreased in *CITED2*-OE/U2-OS cell line compared to wild type cells (**Figure 24 A-C**). The reduction was %48 for *PER2*, 25% for *DBP* and 15% for *NR1D1*. On the contrary, *ROR- $\alpha$*  showed 27% increased mRNA expression (**Figure 24 D**). Relative mRNA levels of *CRY1* did not show statistically significant results (**Figure 24 E**). To further confirm this observation, I performed WB analysis for DBP and PER2 with Anti-DBP and Anti-PER2 antibodies. WB analysis showed that DBP and PER2 levels were reduced to 47% and 54% in *CITED2*-OE/U2-OS cells respectively (**Figure 24 F**) as in correlation with qPCR results. As it supports the hypothesis, over expression increased the inhibitory effect of *CITED2* on BMAL1/CLOCK heterodimer and resulted in decreased levels of DBP and PER2 expression.

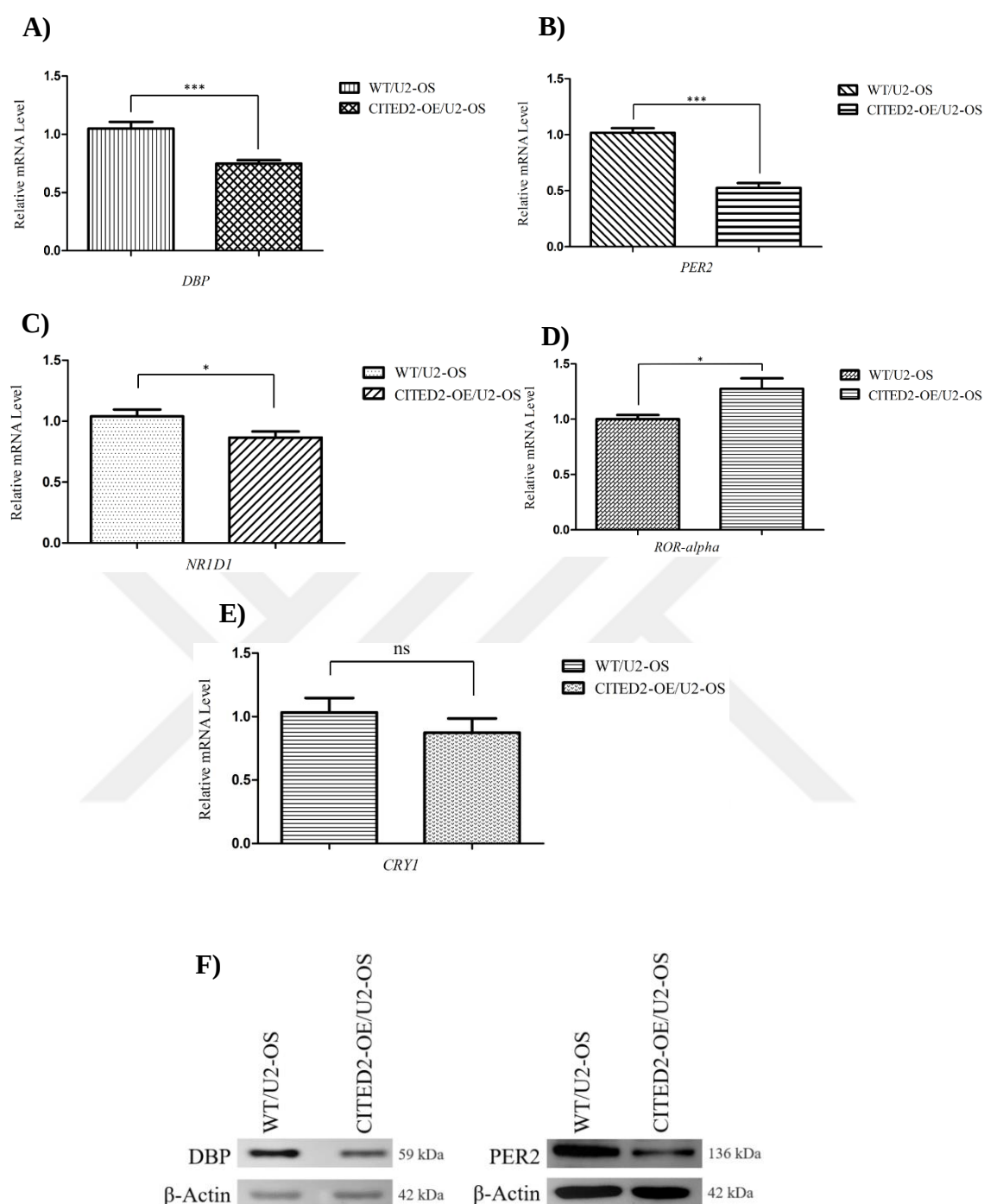


**Figure 24. *CITED2* over expression results in decreased level of DBP and PER2.**

A-C) Relative mRNA level of *DBP*, *PER2* and *NR1D1* decreases upon *CITED2* over expression in *CITED2*-OE/U2-OS cell line. D) *ROR- $\alpha$*  showed increased mRNA expression. E) *CRY1* did not show any significant change. F) WB analysis revealed that DBP and PER2 levels are decreased in *CITED2*-OE/U2-OS cell line. (Data indicates  $\pm$  SEM with n=3 biological replicates. \*:  $p < 0.05$  ;\*\*\*:  $p < 0.0001$ , by student t-test)

By taking these results into account, *CITED2* over expression and knock down experiments' findings validate each other and support the hypothesis. We can conclude that *CITED2* has an inhibitory effect on BMAL1/CLOCK heterodimer and acts as a repressor taking part in the negative arm of the molecular clock.

#### 4.9 CITED2 Reduces the Physical Interaction Between BMAL1 and CLOCK

Knocking down and overexpressing *CITED2* in U2-OS cell line caused changes in the transcriptional regulation of the molecular clock. I wish then to explore whether *CITED2* itself interacts CLOCK/BMAL1 cause such circadian phenotype changes or it requires other factors to modify circadian rhythm. To test that, we used mammalian two-hybrid assay (M2H). M2H is a commonly used method to detect protein-protein interactions in transiently transfected mammalian cells. M2H assay is based on the reconstruction of a functional transcription system and a reporter gene is transcribed because of the direct interaction of two proteins (Dang et al., 1991; Feng et al., 2001). I hypothesized that if *CITED2* is interacting with BMAL1 or CLOCK, it may reduce the physical interaction between BMAL1 and CLOCK, and M2H assay may explain the molecular mechanism of *CITED2* repression activity.

HEK293T cells were transfected with pACT-*Bmal1*-VP16, pBIND-GAL4-CLOCK, pG5Luc and pcDNA4/myc-His-A plasmid with *CITED2*. I used pcDNA4/myc-His-A plasmid with *CRY1* as a negative control for BMAL1/CLOCK complex disruption because it was already shown that *CRY1* repress BMAL1/CLOCK transactivation by binding to the BMAL1/CLOCK complex and it does not interfere with the physical interaction between these proteins during repression (Ye et al., 2011). I also tested the activation of pG5Luc plasmid without any additional vector as a negative control. In all experiments, pGL4 plasmid, which contains *Renilla* gene, was used for the normalization purpose. As shown in **Figure 25**, *CITED2* reduces BMAL1 and CLOCK interaction as student's t-test showed statistically significant change where *CRY1* did not disrupt BMAL1 and CLOCK interaction. *mPer1:dluc* repression assay and M2H results suggested that *CITED2* has a repressor activity on BMAL1/CLOCK transactivation on E-box by interfering with the direct interaction between BMAL1 and CLOCK.

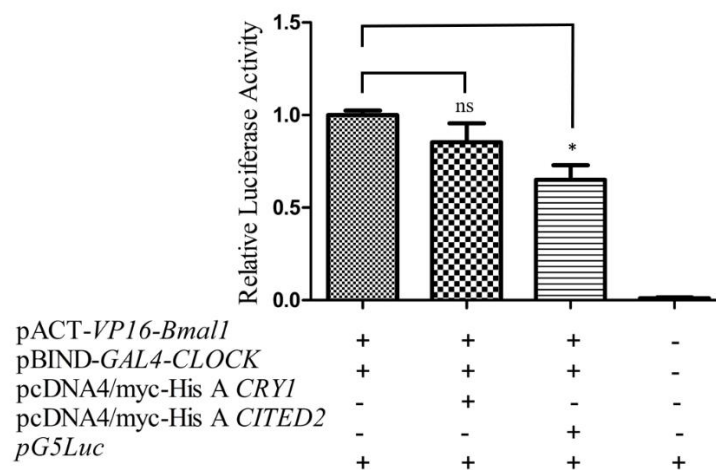


**Figure 25. CITED2 interferes with the physical interaction between BMAL1 and CLOCK according to Mammalian two hybrid assay.**

Increasing doses of CITED2 did not result in a decrease in luciferase activity where CRY1 interrupted the physical interaction between BMAL1/CLOCK. Firefly luciferase activity was normalized to Renilla luciferase activity. (Data is shown with  $\pm$  SEM,  $n=3$  biological replicates. \*:  $p < 0.05$  according to students t-test)

#### 4.10 CITED2 Physically Interacts with CLOCK

CITED2 has the repression activity on BMAL1/CLOCK complex and it interferes with the physical interaction between BMAL1 and CLOCK. In order to address any direct interaction of CITED2 with BMAL1 or CLOCK, pull-down assay was used. Pull-down assay is based on the purification of a protein of interest (Bornhorst et al., 2010). The protein carries a tag which has an affinity for specific bead or column. The cells are transiently transfected with genes of interest and purified with a specific resin. The purified protein lysate can be analysed for the presence of other proteins. This method can be used to detect protein-protein interaction since other interaction partners are also pulled down with the purified protein.

To see whether CITED2 is physically interacting with BMAL1 or CLOCK, I performed pull-down assay with Ni-NTA. I used pcDNA4/myc-His-A plasmid with CITED2 where CITED2 is fused with 6xHis tag. HEK293T cells were transiently transfected with pcDNA4/myc-His-A plasmid with CITED2 and other plasmids consist of BMAL1 and CLOCK cDNA. For the experiment set up, CITED2 protein lysate was mixed with

BMAL1 protein lysate and CLOCK protein lysate separately, then it was purified with Ni-NTA. As a negative control, BMAL1 and CLOCK lysates pulled down which in turn should not bind to the Ni-NTA resin. For the WB analysis, I probed CITED2 with Anti-myc antibody, BMAL1 and CLOCK with FLAG antibody. At first, I analysed the whole cell lysates for the presence of CITED2, BMAL1 and CLOCK. Next, I analysed flow-through lysates after the Ni-NTA resin binding for the presence or absence of these proteins and the purified cell lysates for CITED2, BMAL1 and CLOCK presence.

CITED2 is present in the whole cell lysates (input) but absent in the flow-through lysates, which indicates a successful pull-down of CITED2 (**Figure 26 A**, lower panel). CITED2 is also present in purified lysates in a large amount (**Figure 26 B**, lower panel). When I probed the purified lysates to look for the presence of BMAL1 or CLOCK, I observed CLOCK is also pulled-down with CITED2 (**Figure 26 B**, upper panel), where it does not bind with Ni-NTA by itself according to the negative control. Since CLOCK is pulled down with CITED2, I inferred that CLOCK is a binding partner of CITED2.

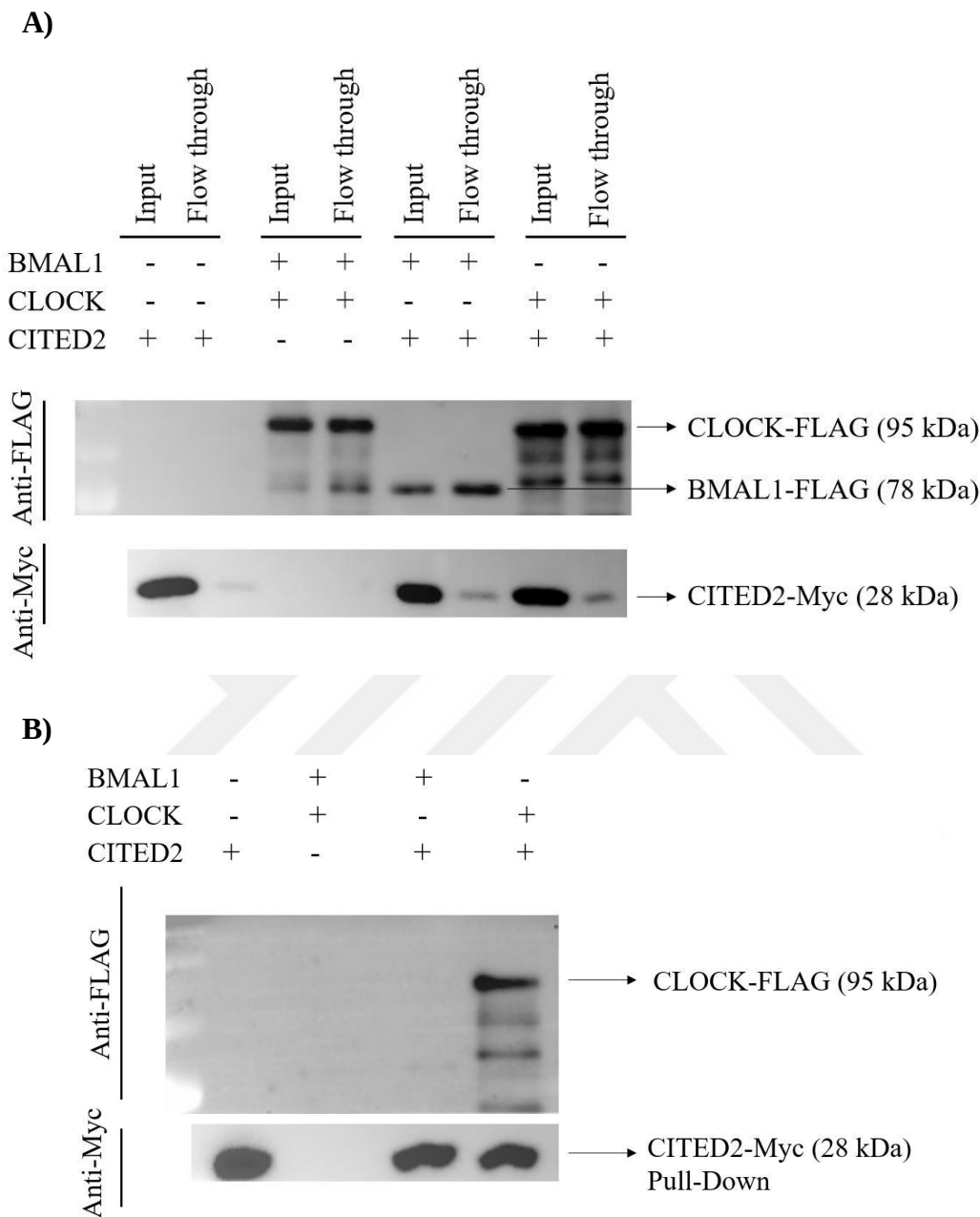


**Figure 26. CITED2 interacts with CLOCK through pull down assay.**

A) The upper panel indicates FLAG-tagged CLOCK pulled down with His/myc-tagged CITED2. The lower panel displays the purified CITED2. B) Upper panel shows the presence of FLAG-tagged BMAL1 and CLOCK in whole cell lysate (input) and flow through lysates. Lower panel indicates the presence of myc-tagged CITED2 in the whole cell lysate and absence in flow through lysate. (n≥3)

## Chapter 5

### DISCUSSION

Daily regulation of physiological, metabolic and behavioral processes are orchestrated by the circadian clock in mammals and almost all living organisms. The mammalian circadian clock is highly conserved, endogenous timers that generate 24-h oscillation throughout the body. Mammalian circadian rhythm has a central pacemaker, SCN, that receives information about external factors and orchestrates peripheral clocks through hormonal and neuronal signalling resulting in a unified clock throughout the body. The significance of a robust circadian clock for the mental and physical health is increasingly admitted, and therefore, 2017 Nobel Prize in the field of Physiology and Medicine was awarded to Michael W. Young, Michael Rosbash and Jeffrey C. Hall for their contributions to the elucidation of molecular clock mechanisms. At the cellular level, the molecular clock is composed of TTFLs driven mainly by the core clock proteins BMAL1, CLOCK, CRYs and PERs. BMAL1 and CLOCK exert positive regulation where CRYs and PERs exert negative regulation on the modulation of the molecular clock (Takahashi, 2017; Ko & Takahashi, 2008). Apart from the core clock proteins, a wide range of proteins have an effect on the modulation of molecular clock directly or indirectly. There has been an increasing interest for the identification of novel clock components modulating molecular clock for the last 20 years. For example, a machine learning study identified CHRONO as molecular clock component and it is shown that CHRONO interacts with BMAL1 and it inhibits the recruitment of CBP to the BMAL1/CLOCK complex to (Anafi et al., 2014). Another study revealed that RACK1 (Receptor for activated C kinase-1) acts on the negative limb of the molecular clock by inhibiting BMAL1/CLOCK transactivation (Robles, 2010). To determine novel molecular clock components, a previous study conducted by Prof. Halil Kavaklı, focused on the identification of novel clock proteins through high-throughput luciferase screening of transcription factor library. The study revealed candidate clock proteins which have a

potential to modulate BMAL1/CLOCK transactivation. CITED2 was one of the top candidates of this study, moreover, CITED2 has shown to be oscillating in mouse liver and kidney according to CircaDB database. These findings strengthen our hypothesis of CITED2 being a clock regulator protein and paved the way for us to further investigate its function in the molecular clock mechanism. In this thesis study for the first time, I aimed to investigate and characterize the function of CITED2 in the molecular clock through functional and biochemical assays.

*mPer1:dluc* repression assay is extensively used in the field as a first step to determine the repression or activating role of a clock regulator protein on BMAL1/CLOCK transactivation. Repression assay suggested CITED2 has a repression activity on BMAL1/CLOCK transcriptional machinery in a dose dependent manner in HEK293T cells (**Figure 12**). Previous studies revealed that CITED2 may act as a coactivator or corepressor in a variety of pathways through an interplay with CBP/p300 complex, in TFAP-2 and HIF1- $\alpha$  pathways respectively (Bhattacharya et al., 1999; Bragança et al., 2003). It was also shown that CBP/p300 modulates BMAL1/CLOCK transactivation in a cell-type specific manner. In NIH3T3 cells, CBP/p300 inhibits BMAL1/CLOCK transcriptional machinery, in contrast, they activate the transcription in COS-1 cells (Hosoda et al., 2009). I reasoned that CITED2 may require CBP/p300 to exert its activity on the molecular clock mechanisms, or CBP/p300 interference may exert an additional level of transcriptional regulation in HEK293T cells. Therefore, I tested the role of CBP/p300 on CITED2 repression activity using *mPer1:dluc* repression assay. I showed that CITED2 acts independently from CBP/p300 complex since CITED2 can repress BMAL1/CLOCK activity without CBP/p300 complex (**Figure 15**). CBP and p300 enhanced BMAL1/CLOCK transactivation, but p300 has a stronger effect compared to CBP. Importantly, I observed that inhibitory activity of CITED2 was dampened when the cells are co-transfected with CBP/p300 and CITED2 and p300 was also able to counteract repression activity of CITED2 in a dose dependent manner. This opposite effect of CITED2 and CBP/p300 on BMAL1/CLOCK transactivation can be explained by anti-phase expression patterns of CITED2 and p300. According to CircaDB database (Pizarro et al., 2013), p300 expression peaks at 36 CT in mouse lung (**Figure 27**), where CITED2 expression is at the lowest (**Figure 9 A**). These findings suggest that CITED2 inhibition and CBP/p300 activation occurs at different time periods but on the same transcriptional

regulation mechanism. I can speculate that when *CITED2* expression peaks around CT 24, it exerts the repression activity on the transcriptional machinery and that is reversed by p300 around CT 36 when p300 expression peaks.

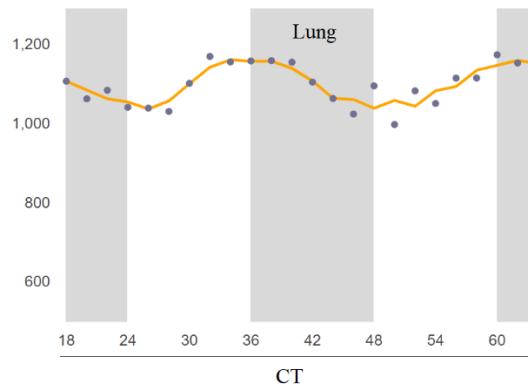


**Figure 27. Circadian expression profile of p300 in the mouse lung.**

Previous studies revealed that mutant forms of negative arm members CRYs and PERs caused altered circadian phenotype. *Cry1<sup>-/-</sup>* mice exhibits almost 1-hour shorter rhythm and *Cry2<sup>-/-</sup>* mice have a 1-hour longer rhythm compared to wild type mice (Van Der Horst et al., 1999). The mutations on *PER* caused severe defects on circadian rhythm (Chen et al., 2009; Cermakian et al., 2001). We hypothesized that, if *CITED2* is essential for a robust circadian rhythm, *CITED2* knock down may cause alterations in the circadian phenotype of U2-OS cells. According to the results, siRNA knock down of *CITED2* caused 40 minutes longer rhythm in U2-OS cells and decreased circadian amplitude almost 28% (**Figure 18**). This finding suggests that *CITED2* is important for the maintenance of a robust circadian rhythm in mammalian cells. However, I did not observe any statistically significant change in the mRNA levels of clock output genes upon *CITED2* knock down in U2-OS cells (**Figure 19**). I reasoned that these nonsignificant results may arise from multiple reasons. First, I reasoned that fluctuations among unsynchronized cells may be masking the effect of si*CITED2* on clock genes and second, *CITED2* mRNA expression was knocked down to 30% and this level of expression may be sufficient to exert its function in the clock mechanism. Therefore, I synchronized si*CITED2* transfected U2-OS cells and in this case, I achieved to knock down *CITED2* approximately 90%. I have observed a significant increase in the mRNA levels of *DBP* and *PER2* (**Figure 21**). Similar findings are presented by another study, where they

identified *FTO* (Fat mass and obesity-associated) as a negative regulator of *BMAL1/CLOCK* driven transcription. *FTO* knock down resulted in 3-fold increase in the mRNA levels of both *PER1* and *PER2* in mice liver (Wang et al., 2015).

Previous studies addressed that over expression of clock proteins also affected circadian phenotype. Constitutive expression of *PER1* or *PER2* completely abolished rhythm in MEF cells (R. Chen et al., 2009). Therefore, I hypothesized that over expression of *CITED2* may also change the circadian rhythm in U2-OS cells. My findings demonstrated that *CITED2*-OE/U2-OS cells display lengthen period with 24.8 hours where WT U2-OS cells display 23.3 hours period (**Figure 23**). Moreover, I also observed that *CITED2* over expression is increased the amplitude 2.5-fold compared to control cells. Consistently, knock down and over expression of *CITED2* had an opposite effect on the circadian amplitude. Surprisingly, both *CITED2* knock down and over expression resulted in a lengthened period in circadian rhythm where I expected to see the opposite effect on period length. A similar phenotype was also reported before where both over expression and knock down of a clock regulator protein *CIPC* caused shortened circadian period (Zhao et al., 2007). This contradiction may arise from multiple reasons. We still do not fully understand the cellular circadian mechanism and there are multiple levels of regulation to our best knowledge. *CITED2* is an essential protein and it is involved in many other cellular pathways including *HIF1- $\alpha$*  and *TFAP-2*. Apart from the circadian mechanism, down regulation of *CITED2* certainly affects the modulation of other pathways which may cause the lengthened period in circadian phenotype.

Molecular mechanisms behind the repression activity of *CITED2* remained unclear so far. Some negative arm elements directly act on *BMAL1/CLOCK* complex to inhibit transcriptional machinery such as *CRYs* (Ye et al., 2011), however previous studies revealed that repression activity can also be indirect by interfering with core clock components. For example, *CHRONO* represses *BMAL1/CLOCK* transcriptional machinery by reducing the interaction between *BMAL1* and coactivator *CBP* in HEK293T cells (Anafi et al., 2014). Another study demonstrated that *RACK1* (Receptor for activated C kinase 1) suppresses the transcriptional machinery by enhancing the phosphorylation of *BMAL1* by *PKC $\alpha$*  (Robles, 2010). I hypothesized that *CITED2* may be interfering with *BMAL1/CLOCK* physical interaction or it may be directly acting on *BMAL1/CLOCK* complex. M2H assay demonstrated that *CITED2* reduces

BMAL1/CLOCK physical interaction (**Figure 25**) where CRY1 did not interfere with BMAL1/CLOCK interaction.

Physical interaction between a core clock protein and a candidate protein is another important criterion to identify a new clock protein. As I mentioned before, CHRONO exerts repression activity by interacting with BMAL1, another clock protein CBS enhances repression activity of CRY1 by direct binding (Anafi et al., 2014; Cal-Kayitmazbatir et al., 2020). Ultimately, I demonstrated that CITED2 physically interacts with CLOCK (**Figure 26**). Similarly, a previous study reported that non-essential repressor protein CIPC (CLOCK interacting protein circadian) represses BMAL1/CLOCK transcriptional machinery in mice. Their findings suggest that CIPC interacts with CLOCK and presumably abrogates the interplay between CLOCK and its coactivators (Zhao et al., 2007). They also present evidence that additional negative feedback mechanisms are involved in the regulation of the molecular clock apart from CRYs and PERs. Even though I was not able to elucidate the complete molecular mechanism of CITED2 repression activity on BMAL1/CLOCK transcriptional machinery, I can speculate that reduction in the BMAL1 and CLOCK interaction may arise from CITED2/CLOCK binding. This binding may eventually result in the abrogation of BMAL1/CLOCK complex. However, this is an assumption and it must be proven with additional experiments.

These findings suggest that CITED2 is located on the negative arm of the molecular clock by repressing BMAL1/CLOCK driven transcription on E-box. CITED2 interacts with CLOCK and reduces the physical interaction between BMAL1 and CLOCK. I have modelled the suggested mechanism according to my findings to provide a better comprehension in **Figure 28**.

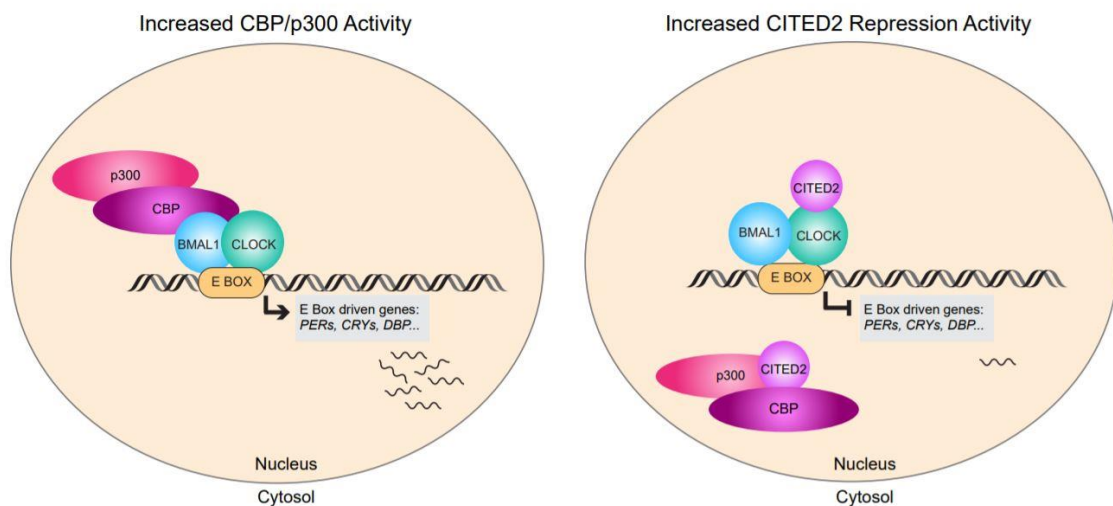


**Figure 28. The proposed model for CITED2 repression on BMAL1/CLOCK driven transcription on E-box containing promoters.**

I have suggested a model for the CITED2 repression mechanism; however, further experiments are needed in order to validate and elucidate the underlying mechanism. First, my findings suggested that CITED2 reduces the interaction between BMAL1 and CLOCK by a reporter assay, this finding must be verified with biochemical analysis. Another future aspect may be to determine whether CITED2 repression activity is cell-type specific. Similar experiments must be performed on other cell lines. Also, I was not able to discover the molecular mechanism behind CITED2/CLOCK interaction. CITED2 and CLOCK mutants would enable us to determine the binding domains of both CITED2 and CLOCK.

Establishment of a CITED2 mouse model would enable us to investigate CITED2 activity in the circadian clock *in vivo*. We can validate the alterations in the circadian rhythm and the transcriptional regulations. Another future direction can be to elucidate the connection between hypoxic signalling pathways and CITED2 repression activity on circadian clock mechanism.

## VITA

Çağla Çakmak was born in Balıkesir, Turkey on August 4, 1993. She received her B.Sc. Degree in Molecular Biology and Genetics from Boğaziçi University, Istanbul, in 2018. She continued her education at M.Sc. Program in Molecular Biology and Genetics at Koc University, as a research and teaching assistant at Prof. İ. Halil Kavaklı's Biotechnology and Circadian Clock Laboratory from September 2018 to August 2020. During her M.Sc. studies, she has worked on "Characterization of the Role of CITED2 in Circadian Clock Mechanism". She will continue on her PhD degree at the University of Cologne Human Genetics Institute, Germany.

**BIBLIOGRAPHY**

- Adamovich, Y., Ladeuix, B., Golik, M., Koeners, M. P., & Asher, G. (2017). Rhythmic Oxygen Levels Reset Circadian Clocks through HIF1 $\alpha$ . *Cell Metabolism*, 25(1), 93–101. <https://doi.org/10.1016/j.cmet.2016.09.014>
- Anafi, R. C., Lee, Y., Sato, T. K., Venkataraman, A., Ramanathan, C., Kavakli, I. H., ... Hogenesch, J. B. (2014). Machine Learning Helps Identify CHRONO as a Circadian Clock Component. *PLoS Biology*, 12(4). <https://doi.org/10.1371/journal.pbio.1001840>
- Antoch, M. P., Song, E. J., Chang, A. M., Vitaterna, M. H., Zhao, Y., Wilsbacher, L. D., ... Takahashi, J. S. (1997). Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell*, 89(4), 655–667. [https://doi.org/10.1016/S0092-8674\(00\)80246-9](https://doi.org/10.1016/S0092-8674(00)80246-9)
- ASCHOFF, J. (1984). Circadian Timing. *Annals of the New York Academy of Sciences*, 423(1), 442–468. <https://doi.org/10.1111/j.1749-6632.1984.tb23452.x>
- Asher, G., Gatfield, D., Stratmann, M., Reinke, H., Dibner, C., Kreppel, F., ... Schibler, U. (2008). SIRT1 Regulates Circadian Clock Gene Expression through PER2 Deacetylation. *Cell*, 134(2), 317–328. <https://doi.org/10.1016/j.cell.2008.06.050>
- Aton, S. J., & Herzog, E. D. (2005). Come together, right...now: Synchronization of rhythms in a mammalian circadian clock. *Neuron*, 48(4), 531–534. <https://doi.org/10.1016/j.neuron.2005.11.001>
- Baggs, J. E., Price, T. S., Ditacchio, L., Panda, S., Fitzgerald, G. A., & Hogenesch, J. B. (2009). Network features of the mammalian circadian

- clock. *PLoS Biology*, 7(3), 0563–0575.  
<https://doi.org/10.1371/journal.pbio.1000052>
- Bakker, W. J., Harris, I. S., & Mak, T. W. (2007). FOXO3a Is Activated in Response to Hypoxic Stress and Inhibits HIF1-Induced Apoptosis via Regulation of CITED2. *Molecular Cell*, 28(6), 941–953.  
<https://doi.org/10.1016/j.molcel.2007.10.035>
- Bamforth, S. D., Bragança, J., Eloranta, J. J., Murdoch, J. N., Marques, F. I. R., Kranc, R., ... Bhattacharya, S. (2001). *Cardiac malformations , adrenal agenesis , neural crest defects and exencephaly in mice lacking Cited2 , a new* © 2001 Nature Publishing Group  
<http://genetics.nature.com>. 29(december), 469–474.  
<https://doi.org/10.1038/ng768>
- Berlow, R. B., Dyson, H. J., & Wright, P. E. (2017). Hypersensitive termination of the hypoxic response by a disordered protein switch. *Nature*, 543(7645), 447–451. <https://doi.org/10.1038/nature21705>
- Berson, D. M., Dunn, F. A., & Takao, M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. *Science*, 295(5557), 1070–1073. <https://doi.org/10.1126/science.1067262>
- Bhattacharya, S., Michels, C. L., Leung, M. K., Arany, Z. P., Kung, A. L., & Livingston, D. M. (1999). Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. *Genes and Development*, 13(1), 64–75. <https://doi.org/10.1101/gad.13.1.64>
- Bornhorst, J.A., Falke, J. . (2010). Purification of Proteins Using Polyhistidine Affinity Tags. *Methods Enzymol*, 2000(326), 245–254.  
<https://doi.org/10.1016/j.immuni.2010.12.017>.Two-stage

- Bragança, J., Eloranta, J. J., Bamforth, S. D., Ibbitt, J. C., Hurst, H. C., & Bhattacharya, S. (2003). Physical and functional interactions among AP-2 transcription factors, p300/CREB-binding protein, and CITED2. *Journal of Biological Chemistry*, 278(18), 16021–16029. <https://doi.org/10.1074/jbc.M208144200>
- Buaas, F. W., Val, P., & Swain, A. (2009). *The transcription co-factor CITED2 functions during sex determination and early gonad development*. 18(16), 2989–3001. <https://doi.org/10.1093/hmg/ddp237>
- Bulut, S. (2017). Identification of Novel Transcription Factors That Modulate BMAL1 : CLOCK Transactivation by. *PhD Dissertation, Koc University*.
- Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Laurel, A., Hogenesch, J. B., ... Bradfield, C. A. (2000). Mop3 Is an Essential Component of the Master Circadian Pacemaker in Mammals. *Cell*, 103(7), 1009–1017. Retrieved from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3779439/pdf/nihms517125.pdf>
- Cajochen, C., Kräuchi, K., & Wirz-Justice, A. (2003). Role of melatonin in the regulation of human circadian rhythms and sleep. *Journal of Neuroendocrinology*, 15(4), 432–437. <https://doi.org/10.1046/j.1365-2826.2003.00989.x>
- Cal-Kayitmazbatir, S., Kulkoyluoglu-Cotul, E., Growe, J., Selby, C. P., Rhoades, S. D., Malik, D., ... Kavakli, I. H. (2020). CRY1-CBS binding regulates circadian clock function and metabolism. *FEBS Journal*. <https://doi.org/10.1111/febs.15360>

- Candolle, A. P. de. (1798). *Reticularia rosea. Bibliotheca Reoia Monacensis*.  
<https://doi.org/10.1017/CBO9781107415324.004>
- Cermakian, N., Pando, M. P., & Sassone-corsi, P. (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the *Period1* gene. *EMBO J*, 20(15), 3967–3974.  
<https://doi.org/10.1002/anie.199512351>
- Chen, R., Schirmer, A., Lee, Y., Lee, H., Kumar, V., Yoo, S. H., ... Lee, C. (2009). Rhythmic PER Abundance Defines a Critical Nodal Point for Negative Feedback within the Circadian Clock Mechanism. *Molecular Cell*, 36(3), 417–430. <https://doi.org/10.1016/j.molcel.2009.10.012>
- Chen, S. T., Choo, K. B., Hou, M. F., Yeh, K. T., Kuo, S. J., & Chang, J. G. (2005). Deregulated expression of the *PER1*, *PER2* and *PER3* genes in breast cancers. *Carcinogenesis*, 26(7), 1241–1246.  
<https://doi.org/10.1093/carcin/bgi075>
- Chen, Y., Doughman, Y., Gu, S., Jarrell, A., Aota, S., Cvekl, A., ... Yang, Y. (2009). *Cited2* is required for the proper formation of the hyaloid vasculature and for lens morphogenesis. *Development*, 135(17), 2939–2948. <https://doi.org/10.1242/dev.021097.Cited2>
- Chou, Y., Hsieh, C., Chiou, S., Hsu, C., Kao, Y., Lee, C., ... Shieh, Y. (2012). *CITED2* functions as a molecular switch of cytokine-induced proliferation and quiescence. *Cell Death and Differentiation*, (2012), 2015–2028. <https://doi.org/10.1038/cdd.2012.91>
- Chou, Y., Wang, H., Chen, Y., Danielpour, D., & Yang, Y. (2006). *Cited2* modulates TGF- $\beta$ -mediated upregulation of MMP9. *Oncogene*, 25, 5547–5560. <https://doi.org/10.1038/sj.onc.1209552>

- Cohen, S. E., & Golden, S. S. (2015). Circadian Rhythms in Cyanobacteria. *Microbiology and Molecular Biology Reviews*, 79(4), 373–385. <https://doi.org/10.1128/mnbr.00036-15>
- Cowell, I. G. (2002). E4BP4/NFIL3, a PAR-related bZIP factor with many roles. *BioEssays*, 24(11), 1023–1029. <https://doi.org/10.1002/bies.10176>
- D’Ortous de Mairan, J. (1729). Observation botanique. *Histoire de l’Academie Royale de Sciences*, 35–36.
- Daino, K., Ugolin, N., & Altmeyer-morel, S. (2009). Gene expression profiling of alpha-radiation-induced rat osteosarcomas : Identification of dysregulated genes involved in radiation-induced tumorigenesis of bone. *Int. J. Cancer*, 620, 612–620. <https://doi.org/10.1002/ijc.24392>
- Dang, C. V, Barrett, J., Villa-Garcia, M., Resar, L. M., Kato, G. J., & Fearon, E. R. (1991). Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. *Molecular and Cellular Biology*, 11(2), 954–962. <https://doi.org/10.1128/mcb.11.2.954>
- Dijk, D. J., & Archer, S. N. (2009). Light, sleep, and circadian rhythms: Together again. *PLoS Biology*, 7(6), 7–10. <https://doi.org/10.1371/journal.pbio.1000145>
- Dimova, E. Y., Jakupovic, M., Kubaichuk, K., Mennerich, D., Chi, T. F., Tamanini, F., ... Kietzmann, T. (2019). The Circadian Clock Protein CRY1 Is a Negative Regulator of HIF-1 $\alpha$ . *IScience*, 13, 284–304. <https://doi.org/10.1016/j.isci.2019.02.027>
- Do, M., & Yau, K.-W. (2010). Intrinsically photosensitive retinal ganglion cells. *Reviews of Physiology, Biochemistry and Pharmacology*, 90(4),

- 1547–1581. [https://doi.org/10.1007/112\\_2011\\_4](https://doi.org/10.1007/112_2011_4)
- Doi, M., Hirayama, J., & Sassone-Corsi, P. (2006). Circadian Regulator CLOCK Is a Histone Acetyltransferase. *Cell*, 125(3), 497–508. <https://doi.org/10.1016/j.cell.2006.03.033>
- Dunlap, J. C. (1999). Molecular bases for circadian clocks. *Cell*, 96(2), 271–290. [https://doi.org/10.1016/S0092-8674\(00\)80566-8](https://doi.org/10.1016/S0092-8674(00)80566-8)
- Dunwoodie, S. L., Rodriguez, T. A., & Beddington, R. S. P. (1998). Msg1 and mrg1, founding members of a gene family, show distinct patterns of gene expression during mouse embryogenesis. *Mechanisms of Development*, 72(1–2), 27–40. [https://doi.org/10.1016/S0925-4773\(98\)00011-2](https://doi.org/10.1016/S0925-4773(98)00011-2)
- Eckardt, N. A. (2010). Temperature compensation of the circadian clock: A role for the morning loop. *Plant Cell*, 22(11), 3506. <https://doi.org/10.1105/tpc.110.221111>
- Ercolani, L., Ferrari, A., Mei, C. De, Parodi, C., Wade, M., & Grimaldi, B. (2015). Circadian clock: Time for novel anticancer strategies? *Pharmacological Research*, 100, 288–295. <https://doi.org/10.1016/j.phrs.2015.08.008>
- Erik J. Eide. (2005). Control of Mammalian Circadian Rhythm by CKIε-Regulated Proteasome-Mediated PER2 Degradation. *Molecular and Cellular Biology*, 25(7), 2795–2807. <https://doi.org/10.1128/MCB.25.7.2795>
- Feng, D., & Lazar, M. A. (2012). Clocks, Metabolism, and the Epigenome. *Mol Cell*, 47(2), 158–167. <https://doi.org/10.1038/jid.2014.371>

- Feng, X., & Derynck, R. (2001). Mammalian Two-Hybrid Assays. *Methods in Molecular Biology*, 177, 221–239.
- Floro, K. L., Artap, S. T., Preis, J. I., Fatkin, D., Chapman, G., Furtado, M. B., ... Dunwoodie, S. L. (2011). *Loss of Cited2 causes congenital heart disease by perturbing left – right patterning of the body axis*. 20(6), 1097–1110. <https://doi.org/10.1093/hmg/ddq554>
- Freedman, S. J., Sun, Z. Y. J., Kung, A. L., France, D. S., Wagner, G., & Eck, M. J. (2003). Structural basis for negative regulation of hypoxia-inducible factor-1 $\alpha$  by CITED2. *Nature Structural Biology*, 10(7), 504–512. <https://doi.org/10.1038/nsb936>
- Froy, O. (2010). Metabolism and circadian rhythms - Implications for obesity. *Endocrine Reviews*, 31(1), 1–24. <https://doi.org/10.1210/er.2009-0014>
- Gachon, F., Nagoshi, E., Brown, S. A., Ripperger, J., & Schibler, U. (2004). The mammalian circadian timing system: From gene expression to physiology. *Chromosoma*, 113(3), 103–112. <https://doi.org/10.1007/s00412-004-0296-2>
- Gachon, F., Olela, F. F., Schaad, O., Descombes, P., & Schibler, U. (2006). The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metabolism*, 4(1), 25–36. <https://doi.org/10.1016/j.cmet.2006.04.015>
- Gallego, M., & Virshup, D. M. (2007). Post-translational modifications regulate the ticking of the circadian clock. *Nature Reviews Molecular Cell Biology*, 8(2), 139–148. <https://doi.org/10.1038/nrm2106>

- Gery, S., Virk, R. K., Chumakov, K., Yu, A., & Koeffler, H. P. (2007). The clock gene Per2 links the circadian system to the estrogen receptor. *Oncogene*, 26(57), 7916–7920. <https://doi.org/10.1038/sj.onc.1210585>
- Gery, Sigal, Komatsu, N., Baldjyan, L., Yu, A., Koo, D., & Koeffler, H. P. (2006). The Circadian Gene Per1 Plays an Important Role in Cell Growth and DNA Damage Control in Human Cancer Cells. *Molecular Cell*, 22(3), 375–382. <https://doi.org/10.1016/j.molcel.2006.03.038>
- Giebultowicz, J. M. (2004). Chronobiology: Biological Timekeeping. *Integrative and Comparative Biology*, 44(3), 266. <https://doi.org/10.1016/j.sab.2004.08.004>
- Gooley, J. J., Lu, J., Chou, T. C., Scammell, T. E., & Saper, C. B. (2001). Melanopsin in cells of origin of the retinohypothalamic tract. *Nature Neuroscience*, 4(12), 1165. <https://doi.org/10.1038/nn768>
- Hardin, P. (2011). Molecular genetic analysis of circadian timekeeping in *Drosophila*. In *Adv Genet*. <https://doi.org/10.1016/B978-0-12-387690-4.00005-2>.Molecular
- Hardin, P. E. (2004). Transcription regulation within the circadian clock: The E-box and beyond. *Journal of Biological Rhythms*, 19(5), 348–360. <https://doi.org/10.1177/0748730404268052>
- Harmer, S. L., Panda, S., & Kay, S. A. (2001). *Molecular Bases of Circadian Rhythm*. 17, 215–253.
- Harmon, F. G., Imaizumi, T., & Kay, S. A. (2005). The Plant Circadian Clock: Review of a Clockwork Arabidopsis. *Annual Plant Reviews Online*, 21, 1–23. <https://doi.org/10.1002/9781119312994.apr0206>

- Herzog, E. D. (2007). Neurons and networks in daily rhythms. *Nature Reviews Neuroscience*, 8(10), 790–802. <https://doi.org/10.1038/nrn2215>
- Hirano, A., Fu, Y. H., & Ptáek, L. J. (2016). The intricate dance of post-translational modifications in the rhythm of life. *Nature Structural and Molecular Biology*, 23(12), 1053–1060. <https://doi.org/10.1038/nsmb.3326>
- Hirano, A., Shi, G., Jones, C. R., Lipzen, A., Pennacchio, L. A., Xu, Y., ... Fu, Y. H. (2016). A cryptochrome 2 mutation yields advanced sleep phase in humans. *ELife*, 5(AUGUST), 1–21. <https://doi.org/10.7554/eLife.16695>
- Hirano, A., Yumimoto, K., Tsunematsu, R., Matsumoto, M., Oyama, M., Kozuka-Hata, H., ... Fukada, Y. (2013). FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. *Cell*, 152(5), 1106–1118. <https://doi.org/10.1016/j.cell.2013.01.054>
- Hobson, J. A., & Aschoff, J. (1965). Circadian Rhythms in Man. *States of Brain and Mind*, (6), 21–22. [https://doi.org/10.1007/978-1-4899-6771-8\\_8](https://doi.org/10.1007/978-1-4899-6771-8_8)
- Hogenesch, J. B., Chan, W. K., Jackiw, V. H., Brown, R. C., Gu, Y. Z., Pray-Grant, M., ... Bradfield, C. A. (1999). Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *Journal of Biological Chemistry*, 272(13), 8581–8593. <https://doi.org/10.1074/jbc.272.13.8581>

- Hosoda, H., Kato, K., Asano, H., Ito, M., Kato, H., Iwamoto, T., ... Kida, S. (2009). CBP/p300 is a cell type-specific modulator of CLOCK/BMAL1-mediated transcription. *Molecular Brain*, 2(1), 1–18. <https://doi.org/10.1186/1756-6606-2-34>
- Hsu, C. M., Lin, S. F., Lu, C. T., Lin, P. M., & Yang, M. Y. (2012). Altered expression of circadian clock genes in head and neck squamous cell carcinoma. *Tumor Biology*, 33(1), 149–155. <https://doi.org/10.1007/s13277-011-0258-2>
- Hsu, D. S., Zhao, X., Zhao, S., Kazantsev, A., Wang, R. P., Todo, T., ... Sancar, A. (1996). Putative human blue-light photoreceptors hCRY1 and hCRY2 are flavoproteins. *Biochemistry*, 35(44), 13871–13877. <https://doi.org/10.1021/bi962209o>
- Hut, R. A., & Beersma, D. G. M. (2011). Evolution of time-keeping mechanisms: Early emergence and adaptation to photoperiod. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366(1574), 2141–2154. <https://doi.org/10.1098/rstb.2010.0409>
- Ishiura, M., Kutsuna, S., Aoki, S., Iwasaki, H., Andersson, C. R., Tanabe, A., ... Kondo, T. (1998). Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. *Science*, 281(5382), 1519–1523. <https://doi.org/10.1126/science.281.5382.1519>
- Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., ... Semenza, G. L. (1998). Cellular and developmental control of O<sub>2</sub> homeostasis by hypoxia-inducible factor 1 $\alpha$ . *Genes and Development*, 12(2), 149–162. <https://doi.org/10.1101/gad.12.2.149>
- Johnson, M. (1939). Effect of continuous light on periodic spontaneous

- activity of white-footed mice (*Peromyscus*). *Journal of Experimental Zoology*, 315–328.
- Jolley, C. C., Ukai-Tadenuma, M., Perrin, D., & Ueda, H. R. (2014). A mammalian circadian clock model incorporating daytime expression elements. *Biophysical Journal*, 107(6), 1462–1473. <https://doi.org/10.1016/j.bpj.2014.07.022>
- Jones, C. R., Campbell, S. S., Zone, S. E., Cooper, F., Desano, A., Murphy, P. J., ... Ptáček, L. J. (1999). Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans. *Nature Medicine*, 5(9), 1062–1065. <https://doi.org/10.1038/12502>
- Kavakli, I. H., & Sancar, A. (2002). Circadian photoreception in humans and mice. *Molecular Interventions*, 2(8), 484–492. <https://doi.org/10.1124/mi.2.8.484>
- Keeler, C. E. (1928). Blind mice. *Journal of Experimental Zoology*, 51(4), 495–508. <https://doi.org/10.1002/jez.1400510404>
- Ko, C. H., & Takahashi, J. S. (2008). Molecular components of the mammalian circadian clock Molecular components of the mammalian circadian clock. *Review Literature And Arts Of The Americas*, 15(September), 271–277. <https://doi.org/10.1093/hmg/ddl207>
- Ko, H. W., Jiang, J., Edery, I., Koh, K., Zheng, X., Sehgal, A., ... Schibler, U. (2007). SCFFbxl3 Controls the Oscillation of the Circadian Clock by Directing the Degradation of Cryptochrome Proteins. *Science*, 316(May), 900–905.
- Kobayashi, M., Morinibu, A., Koyasu, S., Goto, Y., Hiraoka, M., & Harada, H. (2017). A circadian clock gene, PER2, activates HIF-1 as an effector

- molecule for recruitment of HIF-1 $\alpha$  to promoter regions of its downstream genes. *FEBS Journal*, 284(22), 3804–3816. <https://doi.org/10.1111/febs.14280>
- Kondo, T., Strayer, C. A., Kulkarni, R. D., Taylor, W., Ishiura, M., Golden, S. S., & Johnson, C. H. (1993). Circadian rhythms in prokaryotes: Luciferase as a reporter of circadian gene expression in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 90(12), 5672–5676. <https://doi.org/10.1073/pnas.90.12.5672>
- Konopka, R. J., & Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 68(9), 2112–2116. <https://doi.org/10.1073/pnas.68.9.2112>
- Kranc, K. R., & Bamforth, S. D. (2003). Transcriptional Coactivator Cited2 Induces Bmi1 and Mel18 and Controls Fibroblast Proliferation via Ink4a / ARF. *Molecular and Cellular Biology*, 23(21), 7658–7666. <https://doi.org/10.1128/MCB.23.21.7658>
- Krishnaiah, S. Y., Wu, G., Altman, B. J., Growe, J., Rhoades, S. D., Coldren, F., ... Weljie, A. M. (2017). Clock Regulation of Metabolites Reveals Coupling between Transcription and Metabolism. *Cell Metabolism*, 25(4), 961–974.e4. <https://doi.org/10.1016/j.cmet.2017.03.019>
- Laje, R., Agostino, P. V., & Golombek, D. A. (2018). The Times of Our Lives: Interaction Among Different Biological Periodicities. *Frontiers in Integrative Neuroscience*, 12(March), 1–6. <https://doi.org/10.3389/fnint.2018.00010>

- Lamia, K. A., Sachdeva, U. M., Ditacchio, L., Williams, E. C., Alvarez, J. G., Egan, D. F., ... Ronald, M. (2009). AMPK Regulates the Circadian Clock by Cryptochrome Phosphorylation and Degradation. *Science*, 326(5951), 437–440. <https://doi.org/10.1126/science.1172156>. AMPK
- Lamont, E. W., & Amir, S. (2016). Circadian and ultradian clocks/rhythms. *The Curated Reference Collection in Neuroscience and Biobehavioral Psychology*, (January 2016), 257–261. <https://doi.org/10.1016/B978-0-12-809324-5.00283-2>
- Lee, C. (2001). Posttranslational Mechanisms Regulate the Mammalian Circadian Clock. *Cell*, 107, 855–867. <https://doi.org/10.1136/bjo.2008.139840>
- Lee, J., Lee, Y., Lee, M. J., Park, E., Kang, S. H., Chung, C. H., ... Kim, K. (2008). Dual Modification of BMAL1 by SUMO2/3 and Ubiquitin Promotes Circadian Activation of the CLOCK/BMAL1 Complex. *Molecular and Cellular Biology*, 28(19), 6056–6065. <https://doi.org/10.1128/mcb.00583-08>
- Lee, J. W., Bae, S. H., Jeong, J. W., Kim, S. H., & Kim, K. W. (2004). Hypoxia-inducible factor (HIF-1) $\alpha$ : Its protein stability and biological functions. *Experimental and Molecular Medicine*, 36(1), 1–12. <https://doi.org/10.1038/emm.2004.1>
- Leung, M. K., Jones, T., Michels, C. L., Livingston, D. M., & Bhattacharya, S. (1999). Molecular cloning and chromosomal localization of the human CITED2 gene encoding p35srj/Mrg1. *Genomics*, 61(3), 307–313. <https://doi.org/10.1006/geno.1999.5970>
- Liggett. (2008). A Cyanobacterial Circadian Clockwork. *Curr Biol.*, 18(17).

<https://doi.org/10.1038/jid.2014.371>

Lin, Y. M., Chang, J. H., Yeh, K. T., Yang, M. Y., Liu, T. C., Lin, S. F., ... Chang, J. G. (2008). Disturbance of circadian gene expression in hepatocellular carcinoma. *Molecular Carcinogenesis*, 47(12), 925–933. <https://doi.org/10.1002/mc.20446>

Liu, C., Weaver, D. R., Strogatz, S. H., & Reppert, S. M. (1997). Cellular construction of a circadian clock: Period determination in the suprachiasmatic nuclei. *Cell*, 91(6), 855–860. [https://doi.org/10.1016/S0092-8674\(00\)80473-0](https://doi.org/10.1016/S0092-8674(00)80473-0)

Lowrey, P. L., & Takahashi, J. S. (2004). MAMMALIAN CIRCADIAN BIOLOGY: Elucidating Genome-Wide Levels of Temporal Organization. *Annual Review of Genomics and Human Genetics*, 5(1), 407–441. <https://doi.org/10.1146/annurev.genom.5.061903.175925>

Macdonald, S. T., Bamforth, S. D., Chen, C., Farthing, C. R., Schneider, E., Saga, Y., ... Bhattacharya, S. (2008). Epiblastic Cited2 deficiency results in cardiac phenotypic heterogeneity and provides a mechanism for haploinsufficiency. *Cardiovascular Research*, 448–457. <https://doi.org/10.1093/cvr/cvn101>

Manella, G., Aviram, R., Bolshette, N., Muvkadi, S., Golik, M., & Smith, D. F. (2019). Hypoxia induces a time- and tissue-specific response that elicits intertissue circadian clock misalignment. 1–8. <https://doi.org/10.1073/pnas.1914112117>

Mhatre V. Ho, Ji-Ann Lee, and K. C. M. (2013). CITED2 Modulates Estrogen Receptor Transcriptional Activity in Breast Cancer Cells. *Biochem Biophys Res Commun.*, 437(2), 261–266.

- <https://doi.org/10.1038/jid.2014.371>
- Millar, A. J., Carré, I. A., Strayer, C. A., Chua, N. H., & Kay, S. A. (1995). Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science*, 267(5201), 1161–1163. <https://doi.org/10.1126/science.7855595>
- Miyamoto, Y., & Sancar, A. (1999). Circadian regulation of cryptochrome genes in the mouse. *Molecular Brain Research*, 71(2), 238–243. [https://doi.org/10.1016/S0169-328X\(99\)00192-8](https://doi.org/10.1016/S0169-328X(99)00192-8)
- Moore, R. Y., & Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Research Bulletin*, 72(900), 54–56.
- Morin, L. P., & Allen, C. N. (2006). The circadian visual system, 2005. *Brain Research Reviews*, 51(1), 1–60. <https://doi.org/10.1016/j.brainresrev.2005.08.003>
- Muraro, N. I., & Ceriani, M. F. (2014). Circadian rhythms. In *Cambridge University Press* (Vol. 585). <https://doi.org/10.1016/j.febslet.2011.04.055>
- Nydegger, U. E., Escobar, P. M., Risch, L., Risch, M., & Stanga, Z. (2014). Chronobiology and circadian rhythms establish a connection to diagnosis. *Diagnosis*, 1(4), 295–303. <https://doi.org/10.1515/dx-2014-0036>
- Oncol, W. J. C. (2020). CITED2 and the modulation of the hypoxic response in cancer. *Clinical Oncology*, 4333(11), 260–274.
- Pan, H., Guo, J., & Su, Z. (2014). Advances in understanding the

- interrelations between leptin resistance and obesity. *Physiology and Behavior*, 130, 157–169. <https://doi.org/10.1016/j.physbeh.2014.04.003>
- Panda, S., Hogenesch, J. B., & Kay, S. A. (2002). Circadian rhythms from flies to human. *Nature*, 417(6886), 329–335. <https://doi.org/10.1038/417329a>
- Pereira, D. S., Van Der Veen, D. R., Gonçalves, B. S. B., Tufik, S., Von Schantz, M., Archer, S. N., & Pedrazzoli, M. (2014). The effect of different photoperiods in circadian rhythms of Per3 knockout mice. *BioMed Research International*, 2014, 10–14. <https://doi.org/10.1155/2014/170795>
- Pévet, P. (2003). Melatonin: From seasonal to circadian signal. *Journal of Neuroendocrinology*, 15(4), 422–426. <https://doi.org/10.1046/j.1365-2826.2003.01017.x>
- PITTENDRIGH, C. S. (1960). Circadian rhythms and the circadian organization of living systems. *Cold Spring Harbor Symposia on Quantitative Biology*, 25, 159–184. <https://doi.org/10.1101/SQB.1960.025.01.015>
- Pizarro, A., Hayer, K., Lahens, N. F., & Hogenesch, J. B. (2013). CircaDB: A database of mammalian circadian gene expression profiles. *Nucleic Acids Research*, 41(D1), 1009–1013. <https://doi.org/10.1093/nar/gks1161>
- Qi, C., Gery, S., Dashti, A., Dong, Y., Yan, Z., Jiang, G., & Koeffler, H. P. (2009). A role for the clock gene Per1 in prostate cancer. *Cancer Research*, 69(19), 7619–7625. <https://doi.org/10.1158/0008-5472.CAN-08-4199>

- Ramsey, K. M., Yoshino, J., Brace, C. S., Abrassart, D., Kobayashi, Y., Marcheva, B., ... Bass, J. (2009). Circadian clock feedback cycle through NAMPT-Mediated NAD<sup>+</sup> biosynthesis. *Science*, 324(5927), 651–654. <https://doi.org/10.1126/science.1171641>
- Robles, M. S. (2010). Identification of RACK1 and Protein Kinase C $\alpha$  as Integral Components of the Mammalian Circadian Clock. *Science*, 463(327). <https://doi.org/10.1126/science.1180067>
- Sancar, A. (1996). No “End of history” for photolyases. *Science*, 272(5258), 48–49. <https://doi.org/10.1126/science.272.5258.48>
- Sanchez, S. E., & Kay, S. A. (2016). The plant circadian clock: From a simple timekeeper to a complex developmental manager. *Cold Spring Harbor Perspectives in Biology*, 8(12). <https://doi.org/10.1101/cshperspect.a027748>
- Sayako Katada, & Sassone-Corsi, P. (2010). The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. *Nat Struct Mol Biol*, 17(12), 1414–1421. <https://doi.org/10.1016/j.gde.2016.03.011>
- Schneider, E., Broadbent, C., Bamforth, S. D., Farthing, C. R., Michell, A. C., Clarke, K., ... Bhattacharya, S. (2004). *Cited2 controls left-right patterning and heart development through a Nodal-Pitx2c pathway*. 36(11), 1189–1196. <https://doi.org/10.1038/ng1446>
- Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., ... Okamura, H. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell*, 91(7), 1043–1053. [https://doi.org/10.1016/S0092-8674\(00\)80494-8](https://doi.org/10.1016/S0092-8674(00)80494-8)

- Shioda, Toshi, Fenner, M. H., & Isselbacher, K. J. (1997). MSG1 and its related protein MRG1 share a transcription activating domain. *Gene*, 204(1–2), 235–241. [https://doi.org/10.1016/S0378-1119\(97\)00551-9](https://doi.org/10.1016/S0378-1119(97)00551-9)
- Shioda, Toshihiro, Fenner, M. H., & Isselbacher, K. J. (1996). msg1, A novel melanocyte-specific gene, encodes a nuclear protein and is associated with pigmentation. *Proceedings of the National Academy of Sciences of the United States of America*, 93(22), 12298–12303. <https://doi.org/10.1073/pnas.93.22.12298>
- Shirogane, T., Jin, J., Ang, X. L., & Harper, J. W. (2005). SCF $\beta$ -TRCP controls Clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. *Journal of Biological Chemistry*, 280(29), 26863–26872. <https://doi.org/10.1074/jbc.M502862200>
- Sjöblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., ... Velculescu, V. E. (2006). The consensus coding sequences of human breast and colorectal cancers. *Science*, 314(5797), 268–274. <https://doi.org/10.1126/science.1133427>
- Sperling, S., Grimm, C. H., Dunkel, I., Mebus, S., Sperling, H. P., Ebner, A., ... Hammer, S. (2005). Identification and functional analysis of CITED2 mutations in patients with congenital heart defects. *Human Mutation*, 26(6), 575–582. <https://doi.org/10.1002/humu.20262>
- T Todo , H Ryo, K Yamamoto, H Toh, T Inui, H Ayaki, T Nomura, M. I. (1996). Similarity among the Drosophila (6-4)photolyase, a human photolyase homolog, and the DNA photolyase-blue-light photoreceptor family. *Science*, 272(April), 6–9.

- Takahashi, J. S. (2017). Transcriptional architecture of the mammalian circadian clock. *Nature Reviews Genetics*, 18(3), 164–179. <https://doi.org/10.1038/nrg.2016.150>
- Tamaru, T., Hirayama, J., Isojima, Y., Nagai, K., Norioka, S., Takamatsu, K., ... Medical, T. (2009). CK2 $\alpha$  phosphorylates BMAL1 to regulate the mammalian clock Teruya. *Nat Struct Mol Biol*, 16(4), 446–448. <https://doi.org/10.1038/nsmb.1578.CK2>
- Tien, E. S., Davis, J. W., & Heuvel, J. P. Vanden. (2004). Identification of the CREB-binding Protein / p300-interacting Protein CITED2 as a Peroxisome Proliferator-activated. *Journal of Biological Chemistry*, 279(23), 24053–24063. <https://doi.org/10.1074/jbc.M401489200>
- Toh, K. L., Jones, C. R., He, Y., Eide, E. J., Hinz, W. A., Virshup, D. M., ... Fu, Y. H. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science*, 291(5506), 1040–1043. <https://doi.org/10.1126/science.1057499>
- Travnickova-bendova, Z., Cermakian, N., Reppert, S. M., & Sassone-corsi, P. (2002). Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK-BMAL1 activity. *PNAS*, 99(11), 7728–7733. <https://doi.org/10.1073/pnas.102075599>
- Ueda, H. R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., ... Hashimoto, S. (2005). System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nature Genetics*, 37(2), 187–192. <https://doi.org/10.1038/ng1504>
- Van Agthoven, T., Sieuwerts, A. M., Veldscholte, J., Meijer-Van Gelder, M. E., Smid, M., Brinkman, A., ... Dorssers, L. C. J. (2009). CITED2 and

- NCOR2 in anti-oestrogen resistance and progression of breast cancer. *British Journal of Cancer*, 101(11), 1824–1832. <https://doi.org/10.1038/sj.bjc.6605423>
- Van Der Horst, G. T. J., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S. I., Takao, M., ... Yasui, A. (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*, 398(6728), 627–630. <https://doi.org/10.1038/19323>
- Vitaterna, M. H., King, D. P., Chang, A. M., Kernhauser, J. M., Lowrey, P. L., McDonald, J. D., ... Takahashi, J. S. (1994). Mutagenesis and mapping of a mouse gene, clock, essential for circadian behavior. *Science*, 264(5159), 719–725. <https://doi.org/10.1126/science.8171325>
- Wang, C., Shie, S., Hsieh, I., & Tsai, M. (2015). Biochemical and Biophysical Research Communications FTO modulates circadian rhythms and inhibits the CLOCK-BMAL1-induced transcription. *Biochemical and Biophysical Research Communications*, 1, 1–7. <https://doi.org/10.1016/j.bbrc.2015.07.046>
- Weber, F. (2017). Modeling the mammalian sleep cycle. *Current Opinion in Neurobiology*, 46, 68–75. <https://doi.org/10.1016/j.conb.2017.07.009>
- Winter, S. L., Bosnoyan-Collins, L., Pinnaduwege, D., & Andrulis, I. L. (2007). Expression of the circadian clock genes Per1 and Per2 in sporadic and familial breast tumors. *Neoplasia*, 9(10), 797–800. <https://doi.org/10.1593/neo.07595>
- Xu, B., Qu, X., & Yang, Y.-C. (2009). *Cited2* is required for fetal lung maturation. 317(1), 95–105.
- Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R. I., Ueda, M.,

- ... Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, 288(5466), 682–685. <https://doi.org/10.1126/science.288.5466.682>
- Yang, X., Wood, P. A., Oh, E. Y., Du-Quiton, J., Ansell, C. M., & Hrushesky, W. J. M. (2009). Down regulation of circadian clock gene Period 2 accelerates breast cancer growth by altering its daily growth rhythm. *Breast Cancer Research and Treatment*, 117(2), 423–431. <https://doi.org/10.1007/s10549-008-0133-z>
- Yasukazu Nakahata, Saurabh Sahar, Giuseppe Astarita, Milota Kaluzova, and P. S.-C. (2009). CIRCADIAN CONTROL OF THE NAD<sup>+</sup> SALVAGE PATHWAY BY CLOCK-SIRT1. *Science*, 324, 654–657. <https://doi.org/10.1016/j.gde.2016.03.011>
- Ye, R., Selby, C. P., Ozturk, N., Annayev, Y., & Sancar, A. (2011). Biochemical analysis of the canonical model for the mammalian circadian clock. *Journal of Biological Chemistry*, 286(29), 25891–25902. <https://doi.org/10.1074/jbc.M111.254680>
- Yoshii, T., Todo, T., Wülbeck, C., Stanewsky, R., & Helfrich-Förster, C. (2008). Cryptochrome is present in the compound eyes and a subset of *Drosophila*'s clock neurons. *Journal of Comparative Neurology*, 508(6), 952–966. <https://doi.org/10.1002/cne.21702>
- Young, T. W., Mei, F. C., Rosen, D. G., Yang, G., Li, N., Liu, J., & Cheng, X. (2007). Up-regulation of Tumor Susceptibility Gene 101 Protein in Ovarian Carcinomas Revealed by Proteomics Analyses \*. *Molecular and Cellular Proteomics*, 294–304. <https://doi.org/10.1074/mcp.M600305-MCP200>

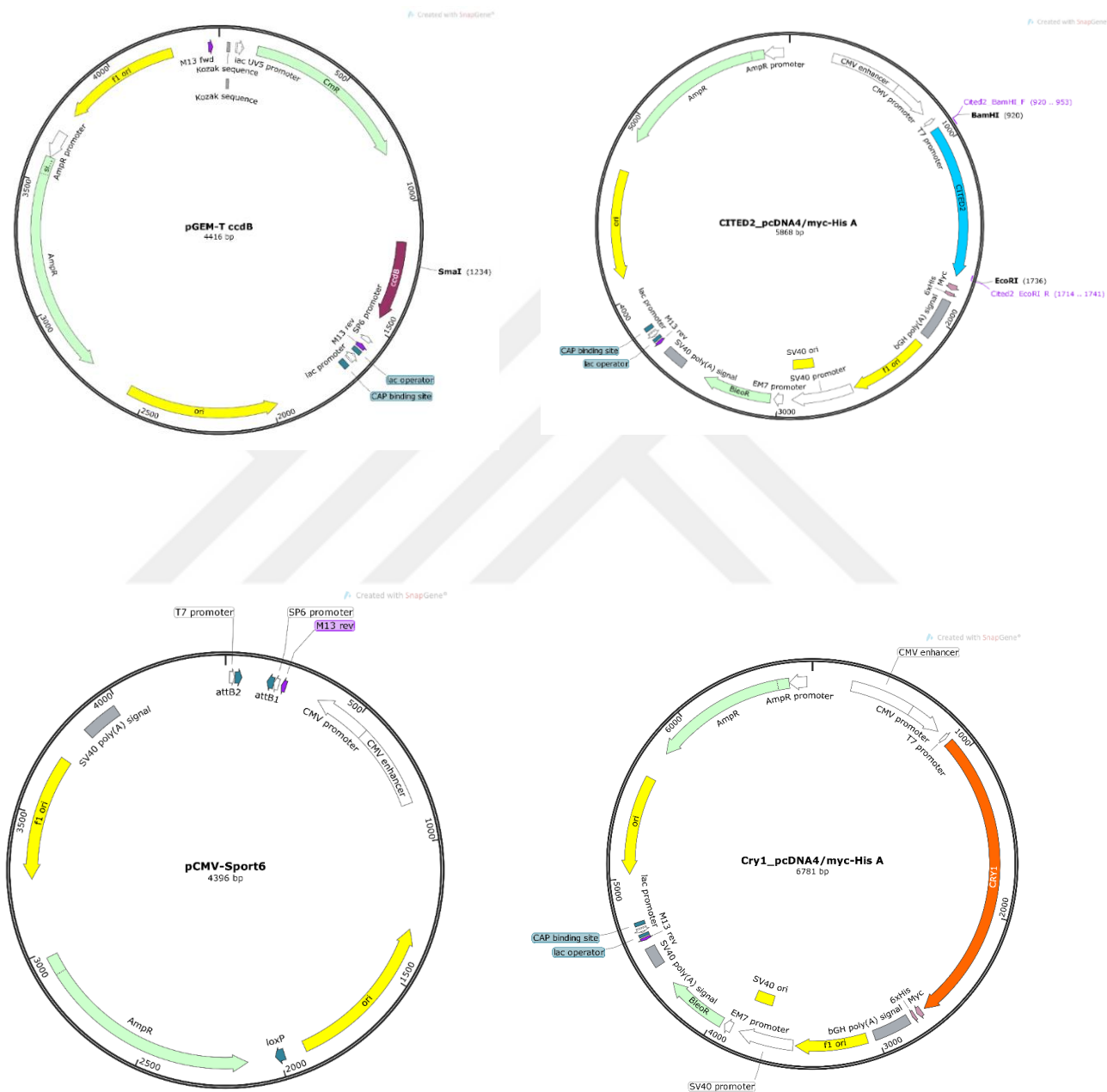
- Zhang, R., Lahens, N. F., Ballance, H. I., Hughes, M. E., & Hogenesch, J. B. (2014). A circadian gene expression atlas in mammals: Implications for biology and medicine. *Proceedings of the National Academy of Sciences of the United States of America*, 111(45), 16219–16224. <https://doi.org/10.1073/pnas.1408886111>
- Zhao, W. N., Malinin, N., Yang, F. C., Staknis, D., Gekakis, N., Maier, B., ... Weitz, C. J. (2007). CIPC is a mammalian circadian clock protein without invertebrate homologues. *Nature Cell Biology*, 9(3), 268–275. <https://doi.org/10.1038/ncb1539>
- Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., ... Lee, C. C. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell*, 105(5), 683–694. [https://doi.org/10.1016/S0092-8674\(01\)00380-4](https://doi.org/10.1016/S0092-8674(01)00380-4)

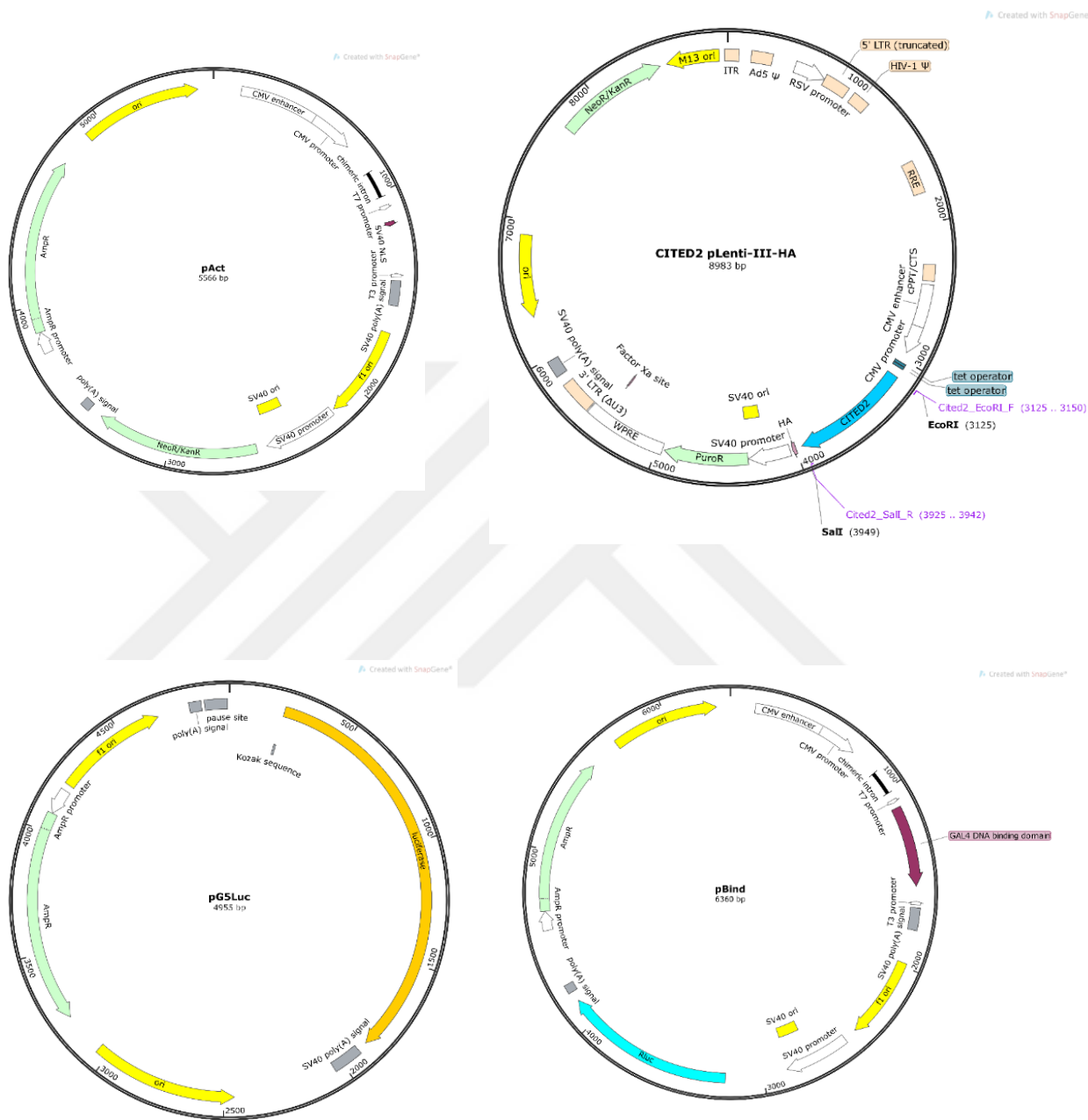
## APPENDICES

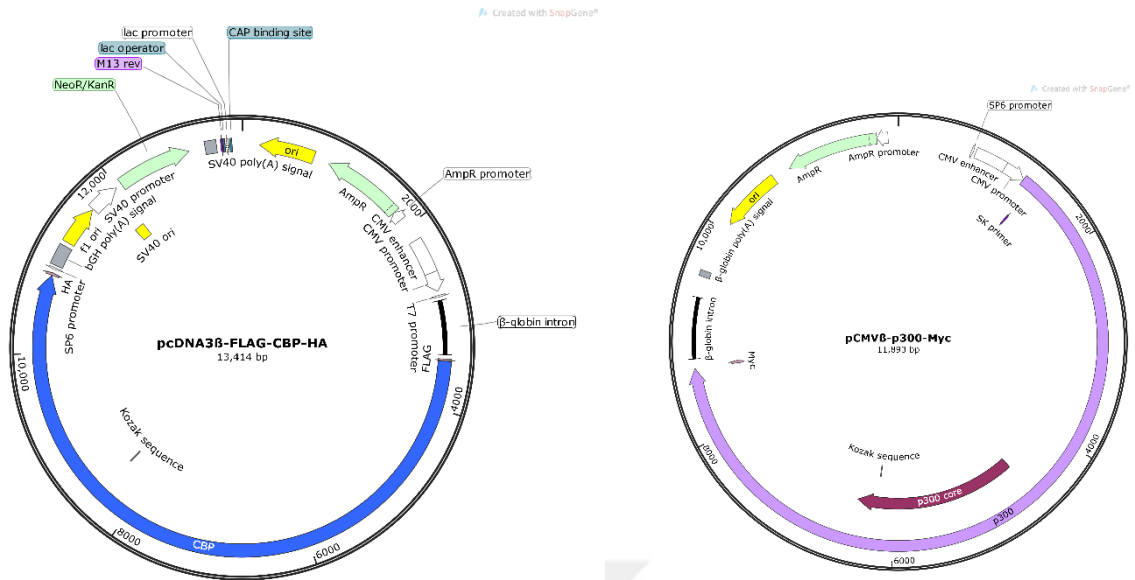
### APPENDIX A      Primer Sequences

| The Name of the Primer | The Sequence of the Primer         |
|------------------------|------------------------------------|
| <i>CITED2</i> _BamHI_F | GGATCCATGGCAGACCATATGATGGCCATGAACC |
| <i>CITED2</i> -EcoRI_R | GAATTCACAGCTCACTCTGCTGGGCTGC       |
| <i>CITED2</i> _EcoRI_F | GCGCCCGAATTTCGGATGGCAGACCATATGATG  |
| <i>CITED2</i> _SalI_R  | CGCAGAGTCGACTGGACAGCTCACTCTGCTGGG  |
| RT_ <i>hBMAL1</i> _F   | GCCCATTGAACATCACGAGTAC             |
| RT_ <i>hBMAL1</i> _R   | CCTGAGCCTGGCCTGATAGTAG             |
| RT_ <i>CITED2</i> _F   | TGCTGCCGCCCAATGTCATA               |
| RT_ <i>CITED2</i> _R   | CAAACCTCGTTTTGCCCCAGC              |
| RT_ <i>CLOCK</i> _F    | AGAGCGCGAAGGAAATCTGG               |
| RT_ <i>CLOCK</i> _R    | ACTGACCATCAGAACTCTTGCT             |
| RT_ <i>hCRY1</i> _F    | ACAGGTGGCGATTTTTGCTTC              |
| RT_ <i>hCRY1</i> _R    | TCCAAAGGGCTCAGAATCATACT            |
| RT_ <i>hDBP</i> _F     | GAGGAACTTAAGCCCCAGCC               |
| RT_ <i>hDBP</i> _R     | CTCGTTGTTCTTGTACCGCC               |
| RT_ <i>hPER2</i> _F    | GCGTGTTCCACAGTTTCACC               |
| RT_ <i>hPER2</i> _R    | GGCTTTTCCGGACACTGACA               |
| RT_ <i>REVERB</i> _F   | TGGA CTCCAACAACAACACAG             |
| RT_ <i>REVERB</i> _R   | GTGGGAAGTAGGTGGGACAG               |
| RT_ <i>RORA</i> _F     | ACTACACACCAGCATCAGGC               |
| RT_ <i>RORA</i> _R     | GCAGGTTTCCAGATGCGATT               |
| RT_ <i>RPLP0</i> _F    | TGTGGGAGCAGACAATGTGG               |
| RT_ <i>RPLP0</i> _R    | CATTCCCCCGGATATGAGGC               |

APPENDIX B Maps of Expression Vectors

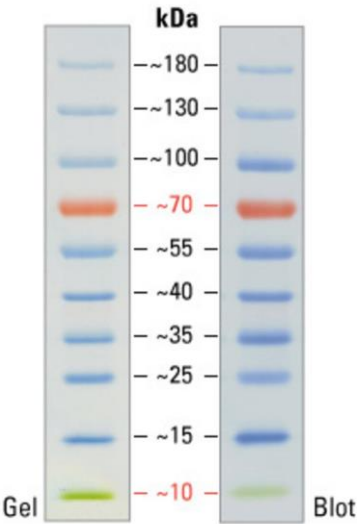
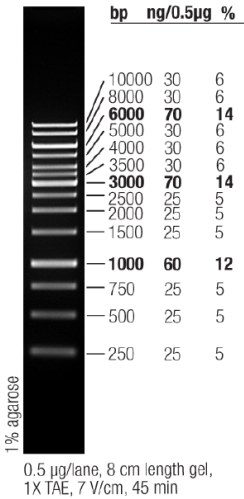






APPENDIX C      DNA and Protein Ladder

GeneRuler 1 kb DNA Ladder



## APPENDIX D Permissions and Copyrights

7/25/2020

RightsLink Printable License

### SPRINGER NATURE LICENSE TERMS AND CONDITIONS

Jul 24, 2020

This Agreement between koç university -- Cagla Cakmak ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

|                                        |                                                               |
|----------------------------------------|---------------------------------------------------------------|
| License Number                         | 4874450599153                                                 |
| License date                           | Jul 22, 2020                                                  |
| Licensed Content Publisher             | Springer Nature                                               |
| Licensed Content Publication           | Nature Reviews Genetics                                       |
| Licensed Content Title                 | Transcriptional architecture of the mammalian circadian clock |
| Licensed Content Author                | Joseph S. Takahashi                                           |
| Licensed Content Date                  | Dec 19, 2016                                                  |
| Type of Use                            | Thesis/Dissertation                                           |
| Requestor type                         | non-commercial (non-profit)                                   |
| Format                                 | print and electronic                                          |
| Portion                                | figures/tables/illustrations                                  |
| Number of figures/tables/illustrations | 1                                                             |
| High-res required                      | no                                                            |
| Will you be translating?               | no                                                            |

<https://e100.copyright.com/CustomerAdmin/PLF.jsp?ref=1364b759-45d6-4d96-8392-c3835732de1>

7/25/2020

RightsLink Printable License

|                                        |                                                                                                                  |
|----------------------------------------|------------------------------------------------------------------------------------------------------------------|
| Circulation/distribution               | 1 - 29                                                                                                           |
| Author of this Springer Nature content | no                                                                                                               |
| Title                                  | Characterization of the Role of CITED2 in Circadian Clock Mechanism                                              |
| Institution name                       | Koc University                                                                                                   |
| Expected presentation date             | Aug 2020                                                                                                         |
| Portions                               | Figure 2                                                                                                         |
| Requestor Location                     | koç university<br>Rumeli feneri street Koc University<br>Istanbul, other 34450<br>Turkey<br>Attn: koç university |
| Total                                  | 0.00 USD                                                                                                         |
| Terms and Conditions                   |                                                                                                                  |

7/26/2020

RightsLink Printable License

ELSEVIER LICENSE  
TERMS AND CONDITIONS

Jul 26, 2020

---

This Agreement between koç university -- Cagla Cakmak ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

|                                        |                                  |
|----------------------------------------|----------------------------------|
| License Number                         | 4876480045228                    |
| License date                           | Jul 26, 2020                     |
| Licensed Content Publisher             | Elsevier                         |
| Licensed Content Publication           | Elsevier Books                   |
| Licensed Content Title                 | Advances in Microbial Physiology |
| Licensed Content Author                | David Lloyd                      |
| Licensed Content Date                  | Jan 1, 1997                      |
| Licensed Content Pages                 | 48                               |
| Start Page                             | 291                              |
| End Page                               | 338                              |
| Type of Use                            | reuse in a thesis/dissertation   |
| Portion                                | figures/tables/illustrations     |
| Number of figures/tables/illustrations | 1                                |
| Format                                 | both print and electronic        |

<https://is100.copyright.com/AppDispatchServlet>

7/26/2020

RightsLink Printable License

Are you the author of this Elsevier No  
chapter?

Will you be translating? No

Title Characterization of the Role of CITED2 in Circadian  
Clock Mechanism

Institution name Koc University

Expected presentation date Aug 2020

Portions Figure 1

koç university  
Rumeli feneri street Koc UniversityRequestor Location  
Istanbul, other 34450  
Turkey  
Attn: koç university

Publisher Tax ID GB 494 6272 12

Total 0.00 USD

Terms and Conditions

**APPENDIX E      Laboratory Equipment**

For the basic laboratory practices, I used Corning Lampda pipettes, AF 10 Scotsman ice machine, Innova 4300 incubator shaker, Sigma Microfuge 1-14K and Beckman Coulter Allegra X-15R centrifuges, CL-40S/SDP (60L) ALP autoclave, Thermo Scientific GenPure ultrapure water system, Mettler Toledo SevenCompact pH/Ion meter S220 pH meter, Heidolph Reax Top vortex, Arcelik 1061 M refrigerator.

Bacterial stocks and RNA samples were kept in New Brunswick Scientific Premium U570 Ultra-Low Temperature Freezer.

Cell culture experiments were carried under ESCO Airstream® Class II Biological Safety Cabinets, Gen 3 (D-Series). Cells were kept in Incucell 111 incubator and observed with Motic AE31E microscope. Real-time bioluminescence measurements were taken with LumiCycle 32 luminometer (Actimetrics).

For gel electrophoresis, Bio-Rad Mini-Sub Cell GT, Bio-Rad Mini-PROTEAN 3 Cell and Single-Row AnyGel and BioRad PowerPac power supply were used. For gel documentation; Thomas Scientific SmartBlue™ Blue Light Table and Bio-Rad ChemiDoc Touch Imaging System were used.

Microplate measurements were taken with BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader and Thermo Scientific Fluoroskan™ Microplate Fluorometer.