Institut für Medizinische Immunologie der Medizinischen Fakultät
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DISSERTATION

Mutational scanning of CLOCK exon 19 transactivation domain

Mutationsscanning der Transaktivierungsdomäne von CLOCK Exon 19

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Ashraf Nabiel Mohamed Elsaid Abdo

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List of abbreviations

Arntl, ARNTL, ARNTL: Aryl hydrocarbon receptor nuclear translocator like

Bmal1, BMAL1, BMAL1: Brain and muscle Arntl-like 1

CIP-C: CLOCK interacting protein-Circadian

Clock, CLOCK, CLOCK: Circadian locomotor output cycles protein kaput

CLOCKΔ19: CLOCK protein missing 51 amino acids coded by exon 19 in mice.

CMV promoter: Cytomegalovirus promoter

CREB: cAMP response element binding protein

CRISPR: Clustered regularly interspaced short palindromic repeats

ENU: N-ethyl-N-nitrosourea

HEK293 cells: Human embryonic kidney cells

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

MLL1: Mixed Linear leukemia 1

PASD1: PAS Domain Containing Repressor 1

Per, PER, PER: Period circadian regulator

RIPA: radioimmunoprecipitation assay

SCN: Suprachiasmatic nucleus

TTFL: Transcriptional-translational feedback loop

U-2 OS: Human osteosarcoma cell line

UTR: Untranslated region

1. Abstract

In mammalian circadian clocks, the CLOCK:BMAL1 heterodimer transactivates E-box enhancer elements to regulate the transcription of circadian clock targets. The CLOCK exon 19 deletion (CLOCK△19) lacks 51 amino acids and exhibits an arrhythmic phenotype. For more than 27 years, we had no clear insight into the essential amino acids of CLOCK exon 19-domain that are required for normal transactivation. To investigate this, we developed a CLOCK rescue system based on a human-derived reporter cell line with CLOCK-knockout using CRISPR/Cas9 technology. We performed alanine mutation scanning for the entire exon 19-domain. The CLOCK-knockout cell line is arrhythmic, and the rhythmic phenotype was rescued by transduction with wild-type CLOCK but not with CLOCK∆19. We identified 10 CLOCK variants that recapitulate the deletion of exon 19, suggesting that these residues are essential for CLOCK protein functionality. We also identified certain residues where mutations shortened the period. Some of those mutations showed a dominant phenotype in wild-type cell line. Interestingly, many of the identified mutations play a role in the hydrophobic interaction of the predicted dimer of CLOCK exon 19-domains. These results reveal critical residues responsible for CLOCK functionality. Our data also indicate the importance of exon 19-domain dimerization to serve as a platform for activator and repressor binding, which is critical for normal circadian rhythms.

1. Zusammenfassung

In zirkadianen Uhren von Säugetieren transaktiviert das CLOCK:BMAL1-Heterodimer E-Box-Enhancer-Elemente, um die Transkription zirkadian exprimierter Zielgene zu regulieren. Der CLOCKΔ19-Mutante fehlen 51 Aminosäuren und sie weist einen arrhythmischen Phänotyp auf. Mehr als 27 Jahre lang fehlte ein klarer Einblick, welche Aminosäuren des CLOCK-Exons 19 für eine normale Transaktivierung erforderlich sind. Um dies zu untersuchen, haben wir ein CLOCK-Rescue-System entwickelt, das auf einer humanen, mittels CRISPR/Cas9-Technologie generierten *CLOCK*-Knockout-Reporterzelllinie basiert. Die CLOCK-Knockout-Zelllinie ist arrhythmisch, und der rhythmische Phänotyp wurde durch Transduktion mit Wildtyp-CLOCK, nicht aber mit CLOCKΔ19 Rescue. Mittels Alanin-Mutationsscanning konnten wir 10 CLOCK-Varianten identifizieren, die die Deletion von Exon 19 rekapitulieren, was darauf schließen lässt, dass diese Reste für die Funktionalität des CLOCK-Proteins wesentlich sind. Des Weiteren konnten wir Punktmutationen identifizieren, die die Periode verkürzten. Einige dieser Mutationen zeigten einen dominanten Phänotyp in Wildtyp-Zelllinien. Viele dieser Reste spielen eine Rolle bei der hydrophoben Interaktion eines postulierten Dimers der CLOCK-Exon-19-Domänen. Diese Studie identifiziert somit entscheidende Aminosäurereste, die für die Funktionalität von CLOCK verantwortlich sind. Unsere Daten deuten zudem darauf hin, wie wichtig die Dimerisierung des Exons 19 ist, um als Plattform für die Bindung von Aktivatoren und Repressoren zu dienen, was für normale zirkadiane Rhythmen entscheidend ist.

2. Introduction

2.1 Historical and basic concepts in chronobiology

Chronobiology is the field that studies biological rhythms in living organisms. Early 18th century, French astronomer Jean-Jacques d'Ortous de Mairan, was the first to direct our attention to the existence of circadian rhythms (J. J. De Mairan, 1729). He noted that the heliotrope plant leaves open at sunrise and closes at sunset, even if the plant is kept in darkness. This observation indicated a 24-hour cycle controlled by an internal clock. Over the past three decades, the molecular mechanism by which circadian rhythms are generated has been studied in many living organisms. Humans are a rhythmic species, as we are adapted to the environmental cycles such as daily and annual rhythms. Metabolism, physiology and eventually behaviour are temporally coordinated according to the time of day by an endogenous timing system, the so-called circadian clock (Finger et al., 2021).

The circadian clock is an endogenous, autonomous oscillator that regulates physiological and behavioural processes crucial for human health. Disrupted clocks result in attention problems, increased daytime sleepiness and higher risks of depression, metabolic disorders and cancer (Finger et al., 2020). The suprachiasmatic nucleus (SCN) is a hypothalamic nucleus that is considered the master clock. The SCN is synchronised to the light-dark cycles as it receives the light input via the retinohypothalamic tract from the eyes. Molecular clocks in all peripheral tissues are hierarchically influenced by the SCN. All other peripheral cells contain a molecular oscillator as well (Balsalobre et al., 1998; Yoo et al., 2004).

2.2 Mammalian circadian clock

The mammalian molecular circadian clock is primarily driven by a "transcriptional-translational feedback loop" (TTFL). The TTFL is generated by regulated cycles of clock gene expression with time delayed repression or activation of these genes by protein products of their own targets. Core clock genes are *CLOCK*, *BMAL1*(*ARNTL*); *CRY1/2*, *PERIOD 1/2/3*. Upon the translation of CLOCK and BMAL1, they form a heterodimer and together they form the positive arm of the loop (Partch et al., 2014). The CLOCK:BMAL1 heterodimer, which is formed in the nucleus, binds to E-box enhancer elements in the promoters of its target genes (Gekakis et al., 1998). CLOCK:BMAL1 recruits chromatin modulating and transcription activators like histone

methyltransferase MLL1 (Mixed-Lineage Leukaemia 1) which is playing a regulatory activating role for CLOCK:BMAL1 activity. Upon PER and CRY protein translation, together with kinases Casein Kinase 1δ and/or Casein Kinase 1ε, they become phosphorylated and form a macromolecular assembly (Aryal et al., 2017). This macromolecular assembly translocate into the nucleus where it blocks CLOCK:BMAL1 heterodimer transactivation activity either by direct or indirect interaction. Direct repression through blocking by CRY binding to the CLOCK:BMAL1–E-box complex, or displacement repression in which PER eliminates CLOCK:BMAL1 from the E-box in a CRY-dependent manner (Cao et al., 2021). Post-translational modifications, especially phosphorylation and ubiquitination, have been shown to govern the activity and degradation of PER and CRY proteins. Additionally, an auxiliary transcriptional negative arm was identified as a component of the molecular circadian clock. This negative arm consists of CLOCK Interacting Protein-Circadian (CIPC) and CHRONO blocking coactivators (like MLL1 or P300/CBP) mediated transactivation (Takahata et al., 2000; Etchegaray et al., 2003; Katada and Sassone-Corsi, 2010).

2.3 Role of mutational studies in understanding molecular circadian clocks

Mutational studies contribute in the determination of the role of individual amino acids in proteins. Changes in a single amino acid can result in misfolding of an entire protein, thermodynamic instability or posttranslational modifications. Mutations can offer insights into protein structures, functionality, and binding domains of such proteins. The key problem that mutational scanning techniques solve is to pick and study the most informative protein mutations. We can finally correlate the phenotype to the mutation, which can shape our understanding of the function of a certain group of amino acids in the protein.

Most of our understanding of molecular oscillators is derived from genetic loss-of-function experiments. The first clock gene (*Period*) was discovered by a mutational study. Konopka and Benzer performed a random mutagenesis study in *Drosophila*, where they isolated three mutations (Konopka and Benzer, 1971). One that increased the period, one that shortened the period and the third which abolished the rhythm. The three mutations were identified to be in the same locus, which they called *Period*.

Random mutagenesis screening was done in order to discover the first mammalian clock gene (*Clock*) in mammals was achieved using N-ethyl-N-nitrosourea (ENU) in mice (Vitaterna et al., 1994). Phenotypically, they identified a mouse with a

heterozygous *Clock* mutation that resulted in lengthening of the circadian period in constant dark conditions. Homozygous *Clock* mutant mice were arrhythmic. Genetically, they identified a mutation in an RNA splicing site, which results in a deletion of exon 19-domain in the CLOCK protein (King et al., 1997).

2.4 Background on *CLOCK* exon 19

The exon 19 codes for 51 amino acids corresponding to CLOCK domain was co-crystallized in a 2:1 stoichiometry with the repressor CIPC (Hou et al., 2017). Structural analysis of the crystalized exon 19-domain provided initial clues to the potential role of individual residues within the domain. Nevertheless, the role of each amino acid has not been systematically characterized so far regarding circadian rhythmicity. As genomic sequencing has become more feasible over the past decade, variants of unclear significance are increasingly being identified (Weile and Roth, 2018). The exon 19 homolog of CLOCK is exon 17 in humans (Steeves et al., 1999). Note that for historic reasons, we will also call human CLOCK, which lacks amino acids corresponding to exon 17, CLOCKΔ19. Exon 19 deletion is an antimorphic-dominant-negative mutation (Vitaterna et al., 1994; Gekakis et al., 1998). Dominant coding variations change coded protein properties and functionality. These mutations' effects are usually revealed by a phenotype that emerges and molecular genetics techniques are deployed to identify these mutations, which are linked later to the phenotype.

2.5 Aims of our study

The aim of the study is to investigate the phenotype of *Clock* mutant mice on an amino acid level and to transfer the accumulated knowledge in the mouse models to human circadian clock. In order to answer this question, we created a *CLOCK*-knockout reporter cell line to establish a CLOCK-rescue system. we characterised the phenotype of CLOCK exon 19 deletion in a human-derived cell line. In order to narrow down which are the crucial amino acids responsible for the changes in the circadian dynamics in CLOCKΔ19 models, we applied a mutational scanning technique to mutate individual amino acids of CLOCK exon 19-domain systematically. It is thought that amino acids encoded by CLOCK exon 19 bind to activators as well as repressors of CLOCK:BMAL1 heterodimer. We have identified several residues that are essential for CLOCK functionality. Most of these amino acids are thought to take part in the

coiled-coil interaction of CLOCK exon 19-domain dimerization. These results will lead to a better understanding of the mechanism of molecular circadian clock rhythms generation.

Materials and methods

3.1 Cell culturing

U-2 OS *Bmal1*-luciferase reporter cell line, *CLOCK*-knockout U-2 OS cell line, HEK 293 and HEK 293 T cell lines were cultured in culture medium at 37°C and 5% CO_2. Culture medium included 500 ml Dulbecco's Modified Eagle Medium (DMEM), high glucose, 5 ml streptomycin and penicillin mixture, 50 ml of fetal bovine serum and 12.5 ml of 1M HEPES buffer. Every other day, cells were passaged at a 70% confluency. Cells were washed with pre-warmed PBS and trypsin/EDTA was added and incubated at the incubator for 15 min. Detached cells were then suspended in culture medium with appropriate ratios.

3.2 CRISPR/Cas9 mediated Knockout

In order to knockout *CLOCK* in the U-2 OS reporter cell line, the genomic locus of *CLOCK* was analysed for exon and intron regions in order to induce codon frame shift. Single guide RNAs were designed using CRISPOR tool (Haeussler et al., 2016) to bind in the intronic area upstream and downstream of the targeted exonic region. A combination of two sgRNA were used to induce double strand breaks. Single guide RNAs were chosen based on their specificity, off-target score and to the vicinity of the target exon. Single guide RNAs were then phosphorylated using T4 Polynucleotide Kinase (NEB M0201S). Phosphorylated sgRNAs were then integrated into lentiCRISPR v2 plasmid (Addgene 52961) (Sanjana et al., 2014). Oligonucleotides for sgRNA are in table S1. Viruses were produced as explained in section 3.5.

3.3 Genomic DNA isolation and exon abundance assessment

Cells were grown to be confluent in a 24-well plate, washed in pre-warmed PBS, trypsinized as described above. Cells were resuspended in culture medium, where 10 µl of the suspension was added to 40 µl of DirectPCR lysis Reagent (Cell) in PCR tube. The mixture was then put into the thermocycler for 15 min at 55°C and 45 min at 85°C. The mixture was then diluted in 1:20 and stored for genomic qPCR abundance testing.

Primers targeting the targeted exons were designed. 4 µl of the genomic DNA was added to 1µl primer mix and 5µl SYBR Green Master Mix for qPCR. The acquired data was then normalised to CLOCK 3'-UTR.

3.4 Site-directed mutagenesis

Mutations were created in the region of CLOCK exon 17(corresponding to exon 19 in mice) as described in (Laible M et al., 2009). 50 ng of the CLOCK harbouring construct was used. Human *CLOCK* harbouring plasmid (addgene 82247) was cloned in lentiviral plasmid. Lentiviral plasmid is a pLenti6 backbone where CLOCK is driven by a CMV promoter. Oligonucleotides were designed in order to carry the mutated codon in a form of mismatches to the template plasmid. The oligonucleotides are complementary to each other and range between 25 and 45 nucleotides where the mismatches are centred. GC content was kept around 45% and the Tm was calculated to by around 60°C. Tables of the designed oligonucleotides for mutational scanning are listed in table S2. PCR amplification using the mutated primers was done and high-fidelity DNA polymerase was used. After initial denaturation at 95°C for 30 seconds were done, thermocycling conditions were adjusted to run 18 cycles. Annealing temperature was set at 55°C for one minute, elongation temperature was set at 72°C for 10 minutes (1 min per 1 kb). After PCR amplification, the parent plasmid was enzymatically digested using DpnI (NEB R0176S). 1µl of DpnI and 5µl Cutsmart buffer was used for 50 µl total reaction volume. The reaction was used to transform competent cells as described above. Transformation was done using heat shock method, where the bacteria were incubated with the plasmid mixture for 30 minutes on ice. The mixture was then transferred to a 42°C heating block for 1 minute. LB medium was then added for competent cell recovery from the heat shock and left at 37°C for 45 minutes. The mixture was then platted in ampicillin-rich agar plates and left overnight at 37°C incubator.

Several colonies were picked and let to grow overnight at 37°C shaker in ampicillin-rich LB medium. DNA was extracted from the competent cells using NucleoSpin plasmid Easy Pure kit for miniprep. DNA concentration was NucleoSpin plasmid easy pure kit measured using NanoDrop 2000c. Screening for mutation creation was done using Sanger sequencing.

3.5 Lentivirus production

Human embryonic kidney 293 T cells were used as described in (Maier et al., 2009) to produce lentiviruses. HEK293T cells were plated at 70% confluence in 25 cm^2 dishes. Cells were transfected using CalPhos kit (Takara Bio, 631312) with packaging and lentiviral expression plasmids (psPAX and pMD2G plasmids Addgene, 12260 and Addgene, 12259 respectively). The transfection solution consisted of 2.5 ng lentiviral expression plasmid, 2µg psPAX plasmid, 1.2µg pMD2G plasmid, 24.6µl 2M calcium solution and 200 µl 2x HBS solution. Next day, culture medium was replaced with fresh medium. Next day, supernatants were harvested and kept on ice overnight and fresh medium was added to the cells and harvested again the next day. Supernatants containing the viral particles from both days were filtered using a 0.45 µm filter (SARSTEDT). Filtered supernatants were then used for transductions and aliquots were kept at -80°C.

3.6 Viral Transduction

U-2 OS *Bmal1*-luciferase reporter cells were plated at 50-70 % confluence and transduced with the lentivirus supernatant in a 24 well plate and protamine sulphate (8 µg/ml). Cells were incubated for two days before replacing the culture medium with the suitable selection antibiotic (10 µg/ml). Selection pressure was kept for 4 days before any experiments with the proper controls. For the *CLOCK*-knockout experiment, cells were selected for CRISPR/Cas9 plasmids-receiving cells using Puromycin for 2 days.

3.7 Sub-cloning of transduced U-2 OS-BLH cells

Cells were trypsinized and counted using the Neubauer cell chamber. Cell suspensions were diluted in culture medium until the final concentration of the cells was 3 cells per 150µl. Cells were seeded in 96-well plates. Plates were incubated for 3 weeks in a cell incubator.

3.8 Bioluminescence recordings

U-2 OS *Bmal1*-luciferase reporter bioluminescence recordings were done using luminometer (TopCount) at 37°C. *Bmal1* promoter driving the expression of luciferase. Cells were seeded in a 96-well plate with a density of 20,000 cells per well, synchronised using dexamethasone (1µM) for 30 minutes using pre-warmed PBS, cells were washed and cultured in a reporter medium. 200µl of the reporter medium

was added which is a phenol-red-free medium supplemented with Penicillin-Streptomycin, 10% FBS and D-luciferin (250 µM). Live bioluminescence recordings were collected for 5 consecutive days. Circadian parameters such as period and amplitude were extracted using Topcount viewer and Chronostar software (Maier et al., 2009). Amplitude reference was determined at 24 hours. Cells were considered rhythmic based on the goodness of sine-wave curve fitting error parameter of Chronostar. Raw data was detrended by dividing raw counts by a running average of the 24 hour average. Sine-wave curve fitting error was determined using the detrended data, consequently circadian parameters were extracted. Clones with fitting errors less than 0.3 were included in circadian parameters calculations.

3.9 Transactivation assay

HEK293 cells were plated at around 60% density in a 96-well plate a day before transfection. Cells were grown in culture medium as described above. Lipofectamine 2000 was used for transfection according to the manufacturer's protocol. DNA amounts were adjusted to be equal in all the reaction conditions. Cells were transformed with a construct harbouring firefly luciferase reporter driven by 6 E-box enhancer elements (12.5 ng), 75 ng of *CLOCK*, 75 ng of *CLOCKΔ19*, 75 ng of *Bmal1* and 75ng of *CLOCK* exon 19 mutants. 0.5 ng of Renilla luciferase construct was co-transfected for normalisation. Transfected cDNA total volume was 150 ng in each transfection reaction. pDEST vector carrying lacZ was used to adjust the amounts of transfected DNA. The next day the medium was aspirated and fresh culture medium was added. The next day, cells were harvested and lysed in 50 µl passive lysis buffer (PLB) provided in the Dual-Luciferase Reporter Assay System kit (Promega). 5µl of each lysate was used to measure the luciferase activity by adding 25µl of LARII and 25 µl of Stop&Glow reagents. Using Orion II, Berthold Detection System the signal was measured. Data was collected and analysed using firefly luciferase activity after normalisation to Renilla luciferase activity.

3.10 Western Blotting

Cells were plated in a 6-well plate at 60-70% confluence and harvested next day in RIPA lysis buffer (1× PBS, 1% Igepal CA-630, 0.5% Na deoxycholate, 0.1% SDS). Protease inhibitor cocktail (1:100) was added to the RIPA buffer Protein concentrations were measured using Qubit Protein Assay Kits (Life technologies Q33211) according to the manufacturer's protocol. Equal amounts of protein were

separated by SDS-page using 4-125 Bis-Tris gels (Invitrogen). Nitrocellulose membrane was used in order to transfer the proteins from the gels. Blocking solution was prepared using 5% non-fat dry milk in PBST. Blocking was done for 1 hour at room temperature. Primary antibodies for ß-actin 1: 100,000 (Sigma) and CLOCK were added to PBST 1:1000 (Cell signalling 5157) and incubated overnight at 4°C. Next day, membranes were incubated for 1 hour at room temperature with secondary antibodies goat-anti-mouse IgG-HRP (1:1000 in TBS-T, Santa Cruz D0116) and 10ml of donkey-anti-rabbit IgG-HRP (1:1000 dilution in TBS-T, Santa Cruz D0615). Band detection was done using chemiluminescence assay with Super Signal West Pico substrate.

3.11 Graph presentation and Statistical analysis

All statistical analysis and graphs were done using GraphPad PRISM 9.3.1. Figure 2 was done using Biorender.

4.Results

4.1 *CLOCK*-knockout reporter cell line generation using CRISPR/Cas9 technology

Refer to figure 1 in the publication and supporting information

In order to characterise different phenotypes of individual CLOCK variants, we generated a *CLOCK* knockout-*CLOCK* rescue system. Firstly, we developed a human-derived cell line lacking functional CLOCK using CRISPR/Cas9 technology. Previously, we have published a workflow in order to create and analyse knockout cell lines (Börding et al., 2019). We designed single guide RNAs (sgRNAs) aiming intronic regions upstream of *CLOCK* exon 6 and downstream of *CLOCK* exon 7 using CRISPOR tool (Haeussler, M. et al., 2016) to induce a frameshift in the open reading frame of *CLOCK* gene. Oligonucleotides were integrated in the lentiCRISPRv2 plasmid (Sanjana et al., 2014). Lentiviruses were produced accordingly, and U-2 OS *Bmal1*-luciferase reporter cells were transduced with lentivirus mixtures and cultures were selected with puromycin. To confirm whether the genomic deletions did occur, we performed qPCR analysis using primers targeting the deleted region. We could not detect any signal from primers targeting exon 6 and 7 in two single clones (#49 and #112). To further confirm the deletion of the targeted exons, we extracted the genomic DNA from the selected clones and sequenced the targeted region. We could detect a

deletion of 3789 bases, which corresponds to the targeted exon-deleted region. In order to test whether the full-length CLOCK protein is still produced, we analysed the abundance of CLOCK protein by western blot using antibodies specific to CLOCK. CLOCK protein was not detected in both tested clones, while it was detected in positive controls of wild-type U-2 OS cells. I characterised the phenotype of *CLOCK*-knockout clones in *Bmal1*-luciferase reporter cell line measuring bioluminescence recording using the TopCount luminometer. Both identified clones (#112 not shown) showed arrhythmic phenotype, as expected from knockdown and *in vivo* experiments and in *CLOCK* knockdown experiments (Debruyne, J.P. et al., 2007), unlike the wild-type U-2 OS cells (Figure 1E). Moreover, we analysed the magnitude of the bioluminescence recordings (Figure S1A). The magnitudes of the *CLOCK*-knockout cell line were higher than the wild-type cell lines. This increase is probably a result of increased activity of *Bmal*1 promoter activity as a result of decreased expression of BMAL1 repressor *REV-ERBα* (*CLOCK* controlled gene). Together, we have generated a *CLOCK*-knockout reporter cell line that serves as a basis for our next experiments.

4.2 Characterizing the phenotype of CLOCKΔ19 in human-derived cell line

In order to characterise CLOCKΔ19 phenotype, we created CLOCKΔ19 human-derived reporter cell line. This was achieved by transducing CLOCK-knockout U-2 OS *Bmal1*-luciferase reporter cell line with a lentivirus harbouring CLOCKΔ19 coding sequence driven by CMV promoter. We found that expression of CLOCKΔ19 could not rescue the rhythmic phenotype while the wild-type CLOCK could (Figure 1F). Bioluminescence magnitudes of wild-type *CLOCK* rescue were reduced to wild-type U-2 OS reporter cell line levels, while the magnitude of the CLOCKΔ19 was similar to *CLOCK*-knockout cell lines (Figure S1B). This demonstrates the functional ability of the *CLOCK* knockout-*CLOCK* rescue system to phenotypically characterise different CLOCK variants. In order to test whether CLOCK is being expressed in the rescued cell lines, we performed western blotting using anti-CLOCK antibodies. CLOCK protein was detected in wild-type CLOCK rescue as well as in CLOCKΔ19 rescue cells (Figure S2A). These results suggests that CLOCKΔ19 in human-derived cell lines recapitulates its phenotype described historically.

4.3 Workflow to create and analyse alanine substitutions in CLOCK exon 19 domain

Refer to figure 2 in the publication and supporting information

To identify residues within exon 19-domain that play an important role in CLOCK functionality, I designed a workflow to create and analyse alanine substitutions for each residue within the domain (Figure 2). Alanine substitutions were performed in human *CLOCK* using site-directed mutagenesis in a lentivector backbone under CMV promoter. Primers carrying the mutated codon were designed and used to amplify the whole plasmid using PCR. Sanger sequencing was performed to verify the mutation. Lentiviral particles were produced from each plasmid. Viruses were used to transduce the *CLOCK*-knockout *Bmal1*-luciferase reporter cell line. Live cell bioluminescence recording was performed by Topcount luminometer after dexamethasone synchronisation. Rhythm analysis was done using Chronostar software. Each cell line was tested in three independent experiments. Different CLOCK variants that showed altered functionality compared to wild-type CLOCK, were tested for dominant features in the wild-type U-2 OS reporter cell line. Furthermore, selected CLOCK variants were tested for their E-box (enhancer elements) transactivation ability in HEK 293 cell line.

4.4 Alanine scanning in CLOCK exon 19 domain

Refer to figure 3 in the publication and supporting information

In order to identify residues which are functionally responsible for CLOCKΔ19 effect, we created a library of mutations in human *CLOCK* gene exon 17, which corresponds to exon 19 in mice. We individually mutated 48 residues to alanines. Since exon 19 has three alanine residues, we mutated these to arginine residues. All mutations were confirmed using Sanger sequencing. Lentiviral particles were produced to transduce the *CLOCK*-knockout U-2 OS *Bmal1*-luciferase reporter cell line with each of the CLOCK mutants individually. Three cell lines for each mutation were created and bioluminescence recording of all CLOCK variants was performed for 5 days after dexamethasone synchronisation. Using Chronostar software, bioluminescence data were analysed and extracting periods, relative amplitudes, magnitudes and sine-wave-curve fitting errors were extracted (Fig 3A, figure S3 and table S3). Percentage of amplitude rescue was measured with wild-type CLOCK

rescue set to 100. Clones with a sine-wave-curve fitting error more than 0.3 were considered arrhythmic. We identified 10 CLOCK variants that could not rescue the rhythmic phenotype when introduced into *CLOCK*-knockout cell compared to wild-type CLOCK (M523A, I537A, E538A, I541A, E547A, L548A, I551A, H559A, G562A and Q564A). In order to test whether CLOCK is expressed in arrhythmic clones, we did western blotting for CLOCK protein. All tested mutant cells showed CLOCK protein expression (Figure S2B). We suggest that these positions are essential for the functionality of CLOCK protein. We identified 4 CLOCK variants with an approximately two-fold higher amplitude than the wild-type CLOCK (A518R, A522R, A539R and R549A). We identified four residues that rescued the circadian rhythmicity with a significant lower amplitude rescue percentage (L520A, H525A, Q552A and Q564A). Remarkably, we identified four mutations that resulted in a one-hour longer period compared to wild-type CLOCK (L530A, L555A, M557A and V558A). We also identified two CLOCK variants that resulted in a one-hour shorter period than wild-type CLOCK (G521A and Q544A). Bioluminescence magnitudes of the screened CLOCK variant-expressing cells were calculated as relative magnitudes in comparison to wild-type CLOCK (Figure S3). 6 out of the 10 CLOCK variants that could not rescue the rhythmic phenotype had a comparable or higher magnitude than the *CLOCK*-knockout cell line. Interestingly, all four CLOCK variants that rescued the *CLOCK*-knockout cell line with longer period, had a comparable or higher magnitude than the wild-type *CLOCK*. We found that most of the residues that upon alanine substitution, resulted in disrupted circadian rhythms were hydrophobic. Notably, these residues were conserved across species from *Drosophila* till *Homo sapiens* (Hou et al., 2017). Period difference, percentage of amplitude rescue and magnitude difference relative to wild-type CLOCK as well as sine-wave curve fitting error values are shown in table S3. These results not only identify the most essential residues in CLOCK exon 19-domain, but also indicates that exon 19-domain has multiple functions and interacting sites whose mutation results in different circadian phenotypes.

4.5 Testing dominant feature of selected alanine mutations

Refer to figure 4 in the publication and supporting information

Clock mutant mice which lack exon 19-domain has a dominant-negative effects, which leads to long-period rhythms even in the presence of the wild-type CLOCK (Vitaterna et al., 1998). We wanted to test whether any of the created mutations can

recapitulate the dominant feature of CLOCK exon 19 deletion in mice. Hence, we transduced wild-type U-2 OS reporter cells with lentiviruses harbouring CLOCKΔ19 or selected CLOCK variants in a background of wild-type U-2 OS cell line. Mutations were selected to cover the whole range of different phenotypes. Mutations at positions 522 and 539 were tested for increasing the amplitude (Figure 4B) and at position 552 for decreasing the amplitude. Mutations at positions 530, 555 and 558 were tested for lengthening the period (Figure 4C). Mutations at positions 523, 537, 538, 541, 547, 548, 551, 559, 562 and 563 were tested for inducing arrhythmicity in wild-type U-2 OS (Figure 4D). We used wild-type CLOCK and CLOCKΔ19 as controls (Figure 4A). We transduced the U-2 OS *Bmal1*-luciferase reporter cell line with lentiviral particles harbouring each of these mutated CLOCKs, wild-type CLOCK or CLOCKΔ19 individually. Wild-type CLOCK transduction did not show any significant difference to non-transduced U-2 OS cell line, while CLOCKΔ19 resulted in arrhythmic phenotype (Figure 4A, E, F). A522R and A539R showed a dominant effect with shortening the period by around 1 hour, but not with increasing the amplitude. On the other hand, L530, L555 and V558 that previously lengthened the period, did not show any dominant effects on either the period or the amplitude. CLOCK variants that could not rescue the rhythmic phenotype in *CLOCK*-knockout cell line had a wide range of effects. E547A and L548A showed a dominant effect on wild-type cells as they have lengthened the period by 1 hour and reduced the amplitude by around 60% (Figure 4D, F). This data only suggests that CLOCK exon 17-domain in humans (exon 19 in mice) has several functions where different residues play different roles in CLOCK functionality.

4.6 Characterization of the transactivation ability of selected CLOCK variants

Refer to figure 5 in the publication and supporting information

In order to test the ability of different CLOCK residues to transactivate E-box enhancer elements along with BMAL1, we did a cotransactivation assay. We co-transfected HEK293 cells with plasmids harbouring a E-box-luciferase reporter construct, a *Bmal1* construct with different CLOCK variants (wild-type or CLOCKΔ19 or selected mutated CLOCK variants). We analysed the resulting luciferase activity. As shown previously, we found that CLOCKΔ19 transactivation ability was reduced in relation to wild-type CLOCK (Figure 5). A522R and A539R both showed similar behaviour as wild-type. L530A and L555A (long-period variants), showed a significant

reduction in CLOCK transactivation ability similar to CLOCK△19. L548A, H559A and L563A displayed a reduction in the transactivation ability of CLOCK. In summary, this data shows that CLOCK residues at 530, 548, 555, 559 and 563 are essential for the wild-type functionality of the CLOCK and suggests that 537 and 551 may play a role in interacting with negative regulators such as CIPC.

5. Discussion

Mutational studies played a very important role in discovering and understanding the molecular circadian clock. From *Drosophila melanogaster* to human-derived cell lines, mutational studies shaped our understanding of functions, binding domains, and mechanisms of certain proteins. The very first clock gene to be identified was revealed by a mutational study in *Drosophila* (Konopka et al., 1971). Furthermore, the first clock gene to be identified in mammals was discovered by a random mutagenesis technique, which identified a deletion of exon 19 in *Clock* gene (Vitaterna et al., 1994). CLOCK exon 19-domain was found to be a transactivation domain that binds to repressors like CIPC as well as activators like MLL1 (Zhao et al., 2007; Katada and Sassone-Corsi,2010). Its 51 amino acids were crystalized with the repressor CIPC (Hou et al., 2017). Despite the significance of exon 19 deletion and its drastic effect, it has never been studied in humans. CLOCK exon 19 homolog is exon 17 in humans (Steeves et al., 1999). For historical reasons, we refer to human CLOCK lacking exon 17 as CLOCK△19. CLOCK exon 19-domain lies in the glutamine-rich region of the C-terminus of the protein. CLOCK△19 previously showed a dramatic decrease in the transactivation ability of E-box enhancer elements and hence a reduction of the transcription of clock target genes (Gekakis et al., 1998). Since glutamine-rich regions are associated with the transactivation ability and the stability of its proteins, it was hypothesised that glutamine residues are crucial for exon 19-domain activity.

5.1 Conclusions and perspectives

To better understand the exon 19 function and to transfer the knowledge accumulated in the mouse models, we developed a model of a human-derived *CLOCK* knockout-CLOCK rescue system in a reporter cell line to better study different CLOCK variants. This *CLOCK*-knockout cell line showed an arrhythmic phenotype as

described previously in knockdown and in *vivo* experiments (Maier et al., 2009; Debruyne, J.P. et al., 2007). The rhythmic phenotype was rescued in CLOCK-knockout cell line upon transduction of wild-type CLOCK but not with CLOCKΔ19. We pursued a systematic mutational scanning approach to identify the essential residues in exon-19 domain using a human-derived *Bmal1*-luciferase reporter cell line as a model. Several residues have been identified that are required for the functionality of CLOCK. 10 CLOCK variants could not rescue the rhythmicity of the *CLOCK*-knockout cell line, recapitulating the phenotype of CLOCKΔ19, indicating the importance of these positions in the functionality of the CLOCK protein. Furthermore, we identified CLOCK variants that affected the mode of action of CLOCK, i.e L530A, I537A, E538A, I541A, L548A, I551A, L555A, and V558A. All these variants have hydrophobic residues mutated to alanines. Remarkably, we found that all the identified variants (L530A, I537A, E538A, I541A, E547A, L548, AI551A, and L555A) are conserved across species from *Drosophila* until *Homo sapiens* (Hou et al., 2017) which advocates that those residues have a preserved role in CLOCK protein function.

In all 51 amino acids encoded by exon 19, there are 6 leucine residues. Since it was shown that exon 19-domain was co-crystallized in 2:1 stoichiometry with CIPC (Hou et al., 2017), we suggest that exon 19 helices are a part of leucine-zipper formation, which is typical in coiled-coil helices of transcription activators that bind to enhancer elements like E-box elements. 5 leucine residues out of the 6 coded by exon 19, upon their mutation, produced arrhythmic phenotype or lengthened the period or decreased the rescue amplitude significantly (L520A, L530A, L548A, L555A, L563A). This suggests that mutating these positions disrupts coiled-coil helix formation between the two exon 19 helices, which is necessary to create binding interface for interacting proteins either repressors (CIPC), or coactivators (MLL1). (Zhao et al., 2007; katada and Sassone-Corsi, 2010). Notably, PASD1 – an evolutionarily related protein to CLOCK has a coiled-coil 1 (CC1) domain. This CC1 domian amazingly similar to the CLOCK exon 19-domain and its paralog NPAS2. It was shown that PASD1 acts as a repressor on CLOCK:BMAL1 heterodimer. Upon the deletion of the CC1-domain in the PASD1, its repression activity is relieved (Michael et al., 2015). Interestingly, L520A, L530A, L548A, L555A variants that resulted in either low amplitude rescue, longer period or arrhythmicity, are conserved in the PASD1 CC1-domain. Here we propose that these CLOCK variants that disrupt the CLOCK:BMAL1 oligomer, contribute to reduction in recruitment of coactivators or repressors (Figure

S4). This hypothesis justifies the antimorphic dominant feature of CLOCKΔ19 (Vitaterna et al., 1994; Zhao et al., 2007), where mutated leucine variants L530A, L555A, L548A (Figure 4F) destabilise the binding between the two CLOCK exon-19 domains.

CLOCK:BMAL1 binding to E-box sites appears to be cooperative (Rey et al., 2011). CLOCK:BMA1 complexes bind to E-box elements as a tandem complex, while CLOCKΔ19:BMAL1 binds as a single complex (Shimomura et al., 2013). Indeed, many CLOCK-controlled genes contain tandem E-box sites where the spacing between tandem E-box sites are 6-7 nucleotides. This would strengthen the hypothesis that CLOCK:BMAL1 oligomerization is mediated by exon 19-domain which is important for circadian dynamics. Interestingly, we identified wild-type alanine residues that mutated to arginine which results in significantly higher amplitudes. Those variants also showed a dominant feature. This might suggest that arginine mutants might disrupt the structure of exon 19-domain. More work is needed to unravel and further understand the co-binding proteins of CLOCK:BMAL1 heterodimer and understand the mechanism of which, CLOCK interacts with activators and repressors through its transactivation domain.

Taken together, our results confirm the crucial function of the exon 19-domain and shed light on the participation of individual residues in maintaining CLOCK's finely regulated function. Particularly, residues potentially disrupting the coiled-coil interaction between exon 19 dimers had a severe effect on the ability of CLOCK to mediate normal circadian behaviour. Given the known studies of hereditary forms of circadian disruption (Patke et al., 2017), it is enticing to presume that some of the interesting circadian phenotypes are a result of CLOCK variants' altered functionality. Since genome sequencing has become a lot more feasible over the past decade, variants of uncertain significance are increasingly found (Weile et al., 2018). Missense mutations in *CLOCK* exon 17 were identified in most of CLOCK residues (Table S4), and the role of each residue can only be revealed by systematic analyses of such variants.

5.2 Limitations of the study

It is known that the CLOCKΔ19 mice suffer from several metabolic pathologies such as hyperlipidemia, hepatic steatosis, hyperglycemia, and hyperinsulinemia. These

metabolic pathologies are probably due to the altered circadian rhythm which disrupts the diurnal feeding rhythm (Turek et al., 2005) or through the regulation of insulin secretion (Tian et al., 2022). Additionally, it has been shown that the CLOCKΔ19 mouse model shows an altered dopaminergic receptors behaviour. Therefore, CLOCKΔ19 mouse is widely used as a model for bipolar disorder representing the manic phase (Spencer et al., 2012; Kristensen et al., 2018). Putting this all into consideration, one of the limitations of this study is that none of the identified CLOCK variants were tested in *vivo*. It will be interesting to test whether any of these pathologies can be detected in a single mutant mouse. Another improvement to the study could have been to fully understand and classify the mode of action of each of the selected mutations. To achieve this goal, one can do an electrophoretic mobility shift assay (EMSA) to detect the tandem heterodimer formation of CLOCK:BMAL1. Chromatin immunoprecipitation can also be done, in order to identify activators or repressors binding sites with CLOCK exon 19.

6 References

Anafi, R.C., Lee, Y., Sato, T.K., Venkataraman, A., Ramanathan, C., Kavakli, I.H., Hughes, M.E., Baggs, J.E., Growe, J., Liu, A.C., Kim, J., and Hogenesch, J.B. (2014). Machine learning helps identify CHRONO as a circadian clock component. *PLoS Biol* 12**,** e1001840.

Antoch, M.P., Song, E.J., Chang, A.M., Vitaterna, M.H., Zhao, Y., Wilsbacher, L.D., Sangoram, A.M., King, D.P., Pinto, L.H., and Takahashi, J.S. (1997). Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell* 89**,** 655-667.

Aryal, R.P., Kwak, P.B., Tamayo, A.G., Gebert, M., Chiu, P.L., Walz, T., and Weitz, C.J. (2017). Macromolecular Assemblies of the Mammalian Circadian Clock. *Mol Cell* 67**,** 770-782 e776.

Bording, T., Abdo, A.N., Maier, B., Gabriel, C., and Kramer, A. (2019). Generation of Human CRY1 and CRY2 Knockout Cells Using Duplex CRISPR/Cas9 Technology. *Front Physiol* 10**,** 577.

Cao, X., Yang, Y., Selby, C.P., Liu, Z., and Sancar, A. (2021). Molecular mechanism of the repressive phase of the mammalian circadian clock. *Proc Natl Acad Sci U S A* 118.

Debruyne, J.P., Weaver, D.R., and Reppert, S.M. (2007). CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nat Neurosci* 10**,** 543-545.

Etchegaray, J.P., Lee, C., Wade, P.A., and Reppert, S.M. (2003). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature* 421**,** 177-182.

Finger, A.M., Dibner, C., and Kramer, A. (2020). Coupled network of the circadian clocks: a driving force of rhythmic physiology. *FEBS Lett* 594**,** 2734-2769.

Finger, A.M., and Kramer, A. (2021). Mammalian circadian systems: Organization and modern life challenges. *Acta Physiol (Oxf)* 231**,** e13548.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. *Science* 280**,** 1564-1569.

Haeussler, M., Schonig, K., Eckert, H., Eschstruth, A., Mianne, J., Renaud, J.B., Schneider-Maunoury, S., Shkumatava, A., Teboul, L., Kent, J., Joly, J.S., and Concordet, J.P. (2016). Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol* 17**,** 148.

Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y., and Honma, K. (2002). Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature* 419**,** 841-844.

Hou, Z., Su, L., Pei, J., Grishin, N.V., and Zhang, H. (2017). Crystal Structure of the CLOCK Transactivation Domain Exon19 in Complex with a Repressor. *Structure* 25**,** 1187-1194 e1183.

J. J. . De Mairan, “Observation Botanique,” Hist. l’Academie R. des Sci., 1729.

Katada, S., and Sassone-Corsi, P. (2010). The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. *Nat Struct Mol Biol* 17**,** 1414-1421.

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W., and Takahashi, J.S. (1997). Positional cloning of the mouse circadian clock gene. *Cell* 89**,** 641-653.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proc Natl Acad Sci U S A* 68**,** 2112-2116.

Kristensen, M., Nierenberg, A.A., and Ostergaard, S.D. (2018). Face and predictive validity of the ClockDelta19 mouse as an animal model for bipolar disorder: a systematic review. *Mol Psychiatry* 23**,** 70-80.

Laible, M., and Boonrod, K. (2009). Homemade site directed mutagenesis of whole plasmids. *J Vis Exp*.

Lee, E., Cho, E., Kang, D.H., Jeong, E.H., Chen, Z., Yoo, S.H., and Kim, E.Y. (2016). Pacemaker-neuron-dependent disturbance of the molecular clockwork by a Drosophila CLOCK mutant homologous to the mouse Clock mutation. *Proc Natl Acad Sci U S A* 113**,** E4904-4913.

Maier, B., Lorenzen, S., Finger, A.M., Herzel, H., and Kramer, A. (2021). Searching Novel Clock Genes Using RNAi-Based Screening. *Methods Mol Biol* 2130**,** 103-114.

Maier, B., Wendt, S., Vanselow, J.T., Wallach, T., Reischl, S., Oehmke, S., Schlosser, A., and Kramer, A. (2009). A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. *Genes Dev* 23**,** 708-718.

Michael, A.K., Harvey, S.L., Sammons, P.J., Anderson, A.P., Kopalle, H.M., Banham, A.H., and Partch, C.L. (2015). Cancer/Testis Antigen PASD1 Silences the Circadian Clock. *Mol Cell* 58**,** 743-754.

Mitchell, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 245**,** 371-378.

Partch, C.L., Green, C.B., and Takahashi, J.S. (2014). Molecular architecture of the mammalian circadian clock. *Trends Cell Biol* 24**,** 90-99.

Patke, A., Murphy, P.J., Onat, O.E., Krieger, A.C., Ozcelik, T., Campbell, S.S., and Young, M.W. (2017). Mutation of the Human Circadian Clock Gene CRY1 in Familial Delayed Sleep Phase Disorder. *Cell* 169**,** 203-215 e213.

Rey, G., Cesbron, F., Rougemont, J., Reinke, H., Brunner, M., and Naef, F. (2011). Genome-wide and phase-specific DNA-binding rhythms of BMAL1 control circadian output functions in mouse liver. *PLoS Biol* 9**,** e1000595.

Sanjana, N.E., Shalem, O., and Zhang, F. (2014). Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 11**,** 783-784.

Shimomura, K., Kumar, V., Koike, N., Kim, T.K., Chong, J., Buhr, E.D., Whiteley, A.R., Low, S.S., Omura, C., Fenner, D., Owens, J.R., Richards, M., Yoo, S.H., Hong, H.K., Vitaterna, M.H., Bass, J., Pletcher, M.T., Wiltshire, T., Hogenesch, J., Lowrey, P.L., and Takahashi, J.S. (2013). Usf1, a suppressor of the circadian

Clock mutant, reveals the nature of the DNA-binding of the CLOCK:BMAL1 complex in mice. *Elife* 2**,** e00426.

S. H. Yoo, Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J. and Menaker, M., and Takahashi, J.S. "PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues," Proc. Natl. Acad. Sci. U. S. A., 2004.

Spencer, S., Torres-Altoro, M.I., Falcon, E., Arey, R., Marvin, M., Goldberg, M., Bibb, J.A., and Mcclung, C.A. (2012). A mutation in CLOCK leads to altered dopamine receptor function. *J Neurochem* 123**,** 124-134.

Steeves, T.D., King, D.P., Zhao, Y., Sangoram, A.M., Du, F., Bowcock, A.M., Moore, R.Y., and Takahashi, J.S. (1999). Molecular cloning and characterization of the human CLOCK gene: expression in the suprachiasmatic nuclei. *Genomics* 57**,** 189-200.

Takahata, S., Ozaki, T., Mimura, J., Kikuchi, Y., Sogawa, K., and Fujii-Kuriyama, Y. (2000). Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. *Genes Cells* 5**,** 739-747.

Tian, L., Li, X., Ding, Y., Li, M., Tang, Y., and Li, D. (2022). The CLOCK protein regulates insulin secretion related with L-type calcium channels in rat pancreatic beta cells. *Biochem Biophys Res Commun* 589**,** 116-122.

Turek, F.W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., Mcdearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D.R., Eckel, R.H., Takahashi, J.S., and Bass, J. (2005). Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* 308**,** 1043-1045.

Vitaterna, M.H., King, D.P., Chang, A.M., Kornhauser, J.M., Lowrey, P.L., Mcdonald, J.D., Dove, W.F., Pinto, L.H., Turek, F.W., and Takahashi, J.S. (1994). Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* 264**,** 719-725.

Weile, J., and Roth, F.P. (2018). Multiplexed assays of variant effects contribute to a growing genotype-phenotype atlas. *Hum Genet* 137**,** 665-678.

Yoshitane, H., Takao, T., Satomi, Y., Du, N.H., Okano, T., and Fukada, Y. (2009). Roles of CLOCK phosphorylation in suppression of E-box-dependent transcription. *Mol Cell Biol* 29**,** 3675-3686.

Zhao, W.N., Malinin, N., Yang, F.C., Staknis, D., Gekakis, N., Maier, B., Reischl, S., Kramer, A., and Weitz, C.J. (2007). CIPC is a mammalian circadian clock protein without invertebrate homologues. *Nat Cell Biol* 9, 268-275.

Statutory Declaration

I, Ashraf Abdo, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic **Mutational scanning of CLOCK exon 19 transactivation domain, Mutationsscanning der Transaktivierungsdomäne von CLOCK Exon 19**, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts, which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

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My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.

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Declaration of your own contribution to the top-journal publication for a PhD or MD/PhD degree

Ashraf N. Abdo contributed the following to the below listed publication:

Publication 1: Ashraf N. Abdo, Carola Rintisch, Christian H. Gabriel, Achim Kramer. Mutational scanning identified amino acids of the CLOCK exon 19-domain essential for circadian rhythms. Acta Physiologica, 2022.

Contributions:

Ashraf N. Abdo performed most of the experiments and data analysis for this publication (Figure 1 D, E, F), supporting figures (1-4) and supporting tables (2-4), including bioluminescence recordings, site-directed mutagenesis related cloning, virus productions, all western blotting, co-transactivation assay. Ashraf N. Abdo did all data analysis and figure presentation in the publication.

Carola Rintisch performed experiments related to sgRNA design, cloning and transduction to create the CLOCK-knockout cell line.

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Achim Kramer supervised and oversaw the project and contributed with reagents, materials and analytical tools.

Manuscript writing and submission was done by Ashraf N. Abdo and Achim Kramer.

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

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ACTA PHYSIOLOGICA

REGULAR PAPER

Mutational scanning identified amino acids of the CLOCK exon 19-domain essential for circadian rhythms

Ashraf N. Abdo | **Carola Rintisch** | **Christian H. Gabriel** | **Achim Kramer**

Laboratory of Chronobiology, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Charité–Universitätsmedizin Berlin, Berlin, Germany

Correspondence
Ashraf N. Abdo and Achim Kramer, Laboratory of Chronobiology, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Charité–Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany.
Email: ashraf.abdo@charite.de (A. N. A.) and achim.kramer@charite.de (A. K.)

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Abstract

Aim: In the mammalian circadian clock, the CLOCK/BMAL1 heterodimer binds to E-box enhancer elements in the promoters of its target genes to activate transcription. The classical *Clock* mice, the first circadian mouse mutant discovered, are behaviourally arrhythmic. In this mutant, CLOCK lacks a 51 amino acid domain corresponding to exon 19 (CLOCKΔ19), which is required for normal transactivation. While the importance of this CLOCK domain for circadian rhythms is well established, the exact molecular mechanism is still unclear.

Methods: Using CRISPR/Cas9 technology, we created a *CLOCK* knockout – *CLOCK* rescue system in human circadian reporter cells and performed systematic mutational scanning to assess the functionality of individual amino acids within the CLOCK exon 19-domain.

Results: *CLOCK* knockout cells were arrhythmic, and circadian rhythms could be rescued by introducing wild-type CLOCK, but not CLOCKΔ19. In addition, we identified several residues, whose mutation failed to rescue rhythms in *CLOCK* knockout cells. Many of these are part of the hydrophobic binding interface of the predicted dimer of the CLOCK exon 19-domain.

Conclusion: Our data not only indicate that CLOCK/BMAL1 oligomerization mediated by the exon 19-domain is important for circadian dynamics but also suggest that the exon 19-domain provides a platform for binding coactivators and repressors, which in turn is required for normal circadian rhythms.

KEYWORDS
circadian clock, *Clock* knockout, CLOCK/BMAL1, CLOCKΔ19, CRISPR/Cas9, mutational scanning

1 | INTRODUCTION

The circadian clock is an endogenous oscillator that regulates daily physiological and behavioral rhythms. The suprachiasmatic nucleus (SCN) in the hypothalamus is considered the master clock. The SCN is synchronized to the environmental light-dark cycle and relays timing information to peripheral clocks in the rest of the body.[1] In humans, the misalignment between the circadian clock and environmental cues can not only cause a range of

sleep disorders but is also considered to be a risk factor for many common diseases.[2]

The mammalian molecular circadian clock consists of a transcriptional-translational feedback loop that has positive and negative arms.[3] In the positive arm of the loop, the CLOCK/BMAL1 heterodimer binds to the E-box enhancer elements in the promoters of its target genes, thus activating gene expression of their own inhibitors *CRYs* and *PERs*.[4,5] Upon CRY and PER translation, they form a multiprotein complex, get phosphorylated and translocated into the nucleus, where they inhibit CLOCK/BMAL1 transactivation activity by at least two distinct mechanisms.[6] After PER/CRY degradation, CLOCK/BMAL1 can resume transcriptional activation and a new cycle starts. CLOCK interacting protein, circadian (CIPC), CHRONO as well as DEC proteins form additional negative transcriptional regulatory loops.[7–9] Chromatin modifying enzymes, such as histone acetyl transferases (eg, p300 and CREB-binding protein (CBP)) and methyl transferases (eg, MLL1) play a regulatory activating role for CLOCK/BMAL1 activity.[10–12]

From *Drosophila melanogaster* to human cell lines, mutation studies have shaped our understanding of clock proteins' structure and function providing insight into the role of individual amino acids. Changes in a single amino acid can result in misfolding of the entire protein, thermodynamic instability, alterations of enzymatic activity or posttranslational modifications. The very first clock gene identified (*Period*) was discovered by a mutation study in *Drosophila*.[13] The first clock gene identified in mammals (*Clock*) was also discovered by a random mutagenesis technique. Vitaterna and colleages performed a forward genetic screen in mice using N-ethyl-N-nitrosourea (ENU), which led to the discovery of the *Clock* gene.[14] Phenotypically, they identified a mouse with a prolongation of the circadian period under constant dark conditions and named the corresponding allele *Clock*. Homozygous *Clock* mice were arrhythmic in constant conditions. Genetically, King and colleages identified a deletion of exon 19 in the *Clock* gene due to a mutation of an RNA splicing site.[15]

Clock exon 19 deletion constitutes an antimorphic dominant-negative mutation. Dominant mutations in coding regions alter protein properties and functionality. The 51 amino acids encoded by *Clock* exon 19 are important for CLOCK/BMAL1-mediated transactivation.[5] This CLOCK domain was also shown to be critical for binding of repressors such as CIPC[8] and activators such as MLL1[12] as well as PER in *Drosophila*.[16] In addition, CLOCKΔ19 was found to be hypophosphorylated attenuating CLOCK degradation. CIPC stimulated CLOCK phosphorylation, and it was therefore suggested that the phosphorylation state of CLOCK correlates with its activity.[17]

Structural insights into the *Clock* exon 19 encoded 51 amino acids came from a crystal structure with the CLOCK repressor CIPC. The exon 19 corresponding CLOCK domain co-crystallized in a 2:1 stoichiometry with CIPC, in which the CLOCK domain formed an extended coiled-coil and directly interacted with CIPC.[18] This structure provided initial clues to the possible function of individual amino acids of this CLOCK domain. However, this has not been systematically investigated so far, although it is important not only for a comprehensive understanding of the structural basis of circadian rhythmicity, but also, especially in view of increasing genome sequencing, for an informed analysis of circadian rhythm disorders in humans. The exon 19 homolog of CLOCK is exon 17 in humans.[19] Note that for historic reasons, we will also call human *CLOCK*, which lacks amino acids corresponding to exon 17, CLOCKΔ19.

Here, we systematically investigated the role of individual amino acids of the CLOCKΔ19 domain for CLOCK function and circadian rhythm dynamics in human cells. Using a newly developed *CLOCK* knockout and *CLOCK* rescue system in combination with a mutational scanning approach, we identified critical amino acids that recapitulated the CLOCKΔ19 phenotype, and others, whose mutation shortened the period. Using these data and the results of a CLOCK/BMAL1 transactivation assay, we propose that CLOCK exon 19-domain residues promote a dimerization of CLOCK/BMAL1 complexes, which is potentially required for the interacting with DNA, coactivators and repressors. Overall, our results lead to a better molecular understanding of the mechanism of circadian rhythm generation.

2 | RESULTS

To analyse the functional significance of individual CLOCK residues for circadian rhythm generation, we developed a *CLOCK* knockout – *CLOCK* rescue system. To this end, we first generated a human cell line (U-2 OS – an established circadian clock model[20]) that lacked a functional CLOCK protein. We followed our recently published workflow to create and analyse circadian gene knockout cells using CRISPR/Cas9 technology.[21] We designed single-guide RNAs (sgRNAs) targeting intronic regions upstream of exon 6 and downstream of exon 7 to induce a frameshift in the open reading frame of the *CLOCK* gene by exon deletion (Figure 1A). The corresponding oligonucleotides were integrated into the lentiCRISPRv2 plasmid,[22] lentiviruses were produced, U-2 OS cells harbouring a *Bmal1*-luciferase reporter[20] were transduced, and single clones were selected. To analyse whether the intended genomic deletions had occurred, we

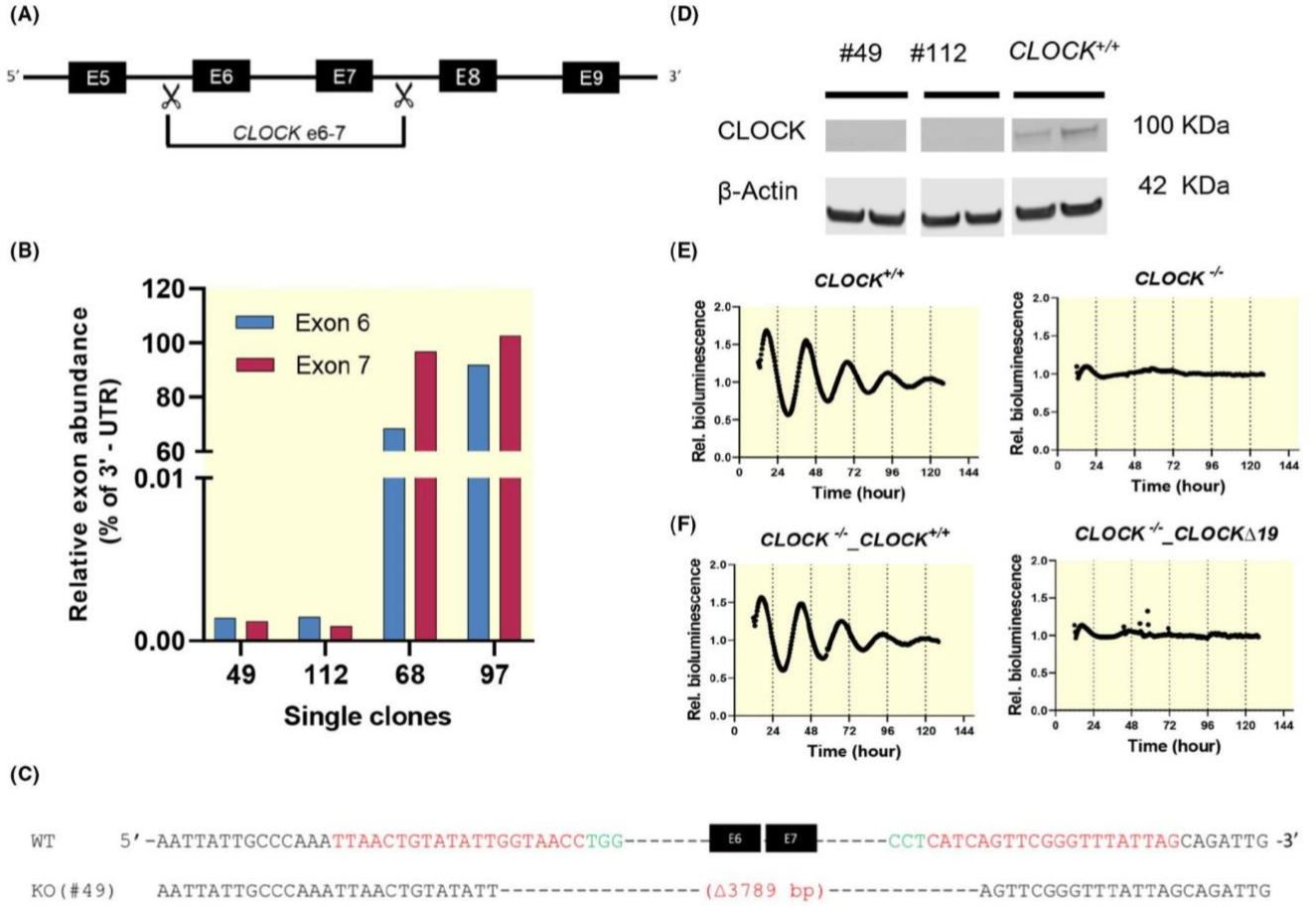


FIGURE 1 Generation of U-2 OS *CLOCK* knockout cells via CRISPR/Cas9 and characterization of CLOCKΔ19 circadian phenotype. (A) Schematic overview of the CRISPR/Cas9 exon deletion strategy showing sgRNAs targeting exon 6 and 7 boundaries in U-2 OS cells. (B) Genomic abundance of the targeted exons (relative to *CLOCK* 3'-UTR) in indicated single clones determined by qPCR. (C) Sequences of the genomic regions of single-cell clone #49 confirming deletion of the targeted exons. SgRNA sequences are indicated in red and the PAM sequence in green. (D) CLOCK protein was not detected by Western blotting in candidate knockout cell clones compared with wild-type clones. (E) Representative detrended bioluminescence time series of *Bmal1*-luciferase reporter activity from wild-type cells (left) and *CLOCK* knockout cells (clone #49, right). Clone #112 showed similar results (not shown). (F) Representative detrended bioluminescence time series of *CLOCK* knockout cells lentivirally transduced with either wild-type CLOCK (left) or CLOCKΔ19 (right)

performed qPCR in individual cell clones using primers targeting the deleted region. In two of 12 analysed clones (#49 and #112) we did not detect any PCR product with primers targeting exons 6 and 7 (Figure 1B). Genomic sequencing confirmed the deletion of 3789 bases correlating with the target region of the deleted exons 6 and 7 (Figure 1C). Because deletion of exons 6 and 7 leads to a premature STOP codon, no full-length CLOCK protein should be produced. In contrast to wild-type clones, we, indeed, could not detect CLOCK protein by western blotting in the two tested putative knockout clones (#49 and #112) (Figure 1D). Together, these results indicate that clones #49 and #112 represent *CLOCK* knockout cells.

To characterize the circadian phenotype of the *CLOCK* knockout clones, we synchronized the cells using dexamethasone and measured the *Bmal1*-luciferase activity using live-cell bioluminescence recordings over several days. In contrast to wild-type cells, both knockout clones showed an arrhythmic phenotype (Figure 1E) as expected from in vivo[23] and *CLOCK* knockdown experiments.[20] In addition, the overall bioluminescence intensity (magnitude) was much higher in the knockout clones compared with the wild-type cell line, likely due to a decreased expression of the *BMAL1* repressor *REV-ERBα*, whose expression is activated by CLOCK/BMAL1. In *CLOCK* knockout cells *REV-ERBα* expression is predicted to be low, which in turn leads to an increased *Bmal1*-luciferase expression (Figure S1A). Taken together, these data demonstrate successful development of *CLOCK* knockout reporter cells that served as a basis for our rescue experiments.

To test whether the arrhythmic phenotype of our *CLOCK* knockout cells is indeed caused by the lack of

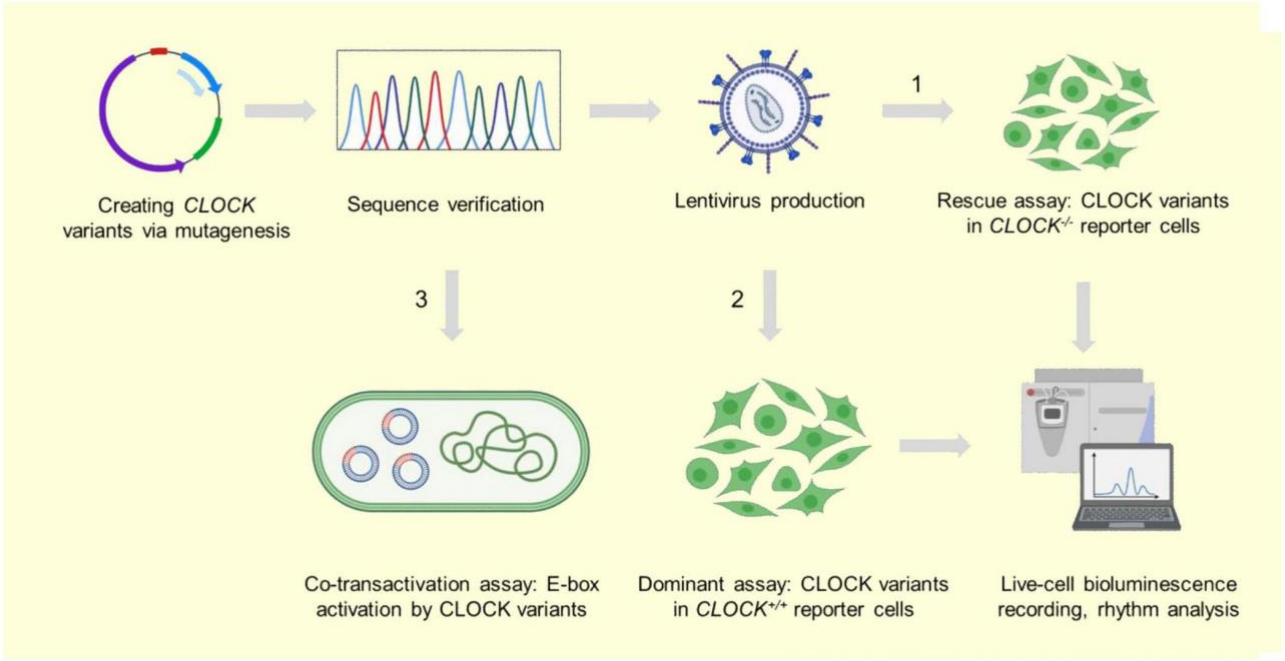


FIGURE 2 Workflow to create and analyse the alanine substitutions for each residue coded in *CLOCK* exon 19 (exon 17 in humans) using site-directed mutagenesis. Primers carrying the mutated codon were used PCR-amplification of the whole plasmid carrying *CLOCK* coding sequence driven by the CMV promoter. After sequence verification of the successful mutagenesis, lentiviral particles were produced to transduce *CLOCK*-knockout cells harbouring a *Bmal1*-luciferase reporter. Cells synchronized with dexamethasone and bioluminescence rhythms were recorded for several days. Data were analysed, and CLOCK variants with altered functionality were selected and tested for dominant behaviour in wild-type U-2 OS reporter cells (2). Finally, selected CLOCK variants were tested for their ability (together with BMAL1) to activate transcription from E-box enhancer elements

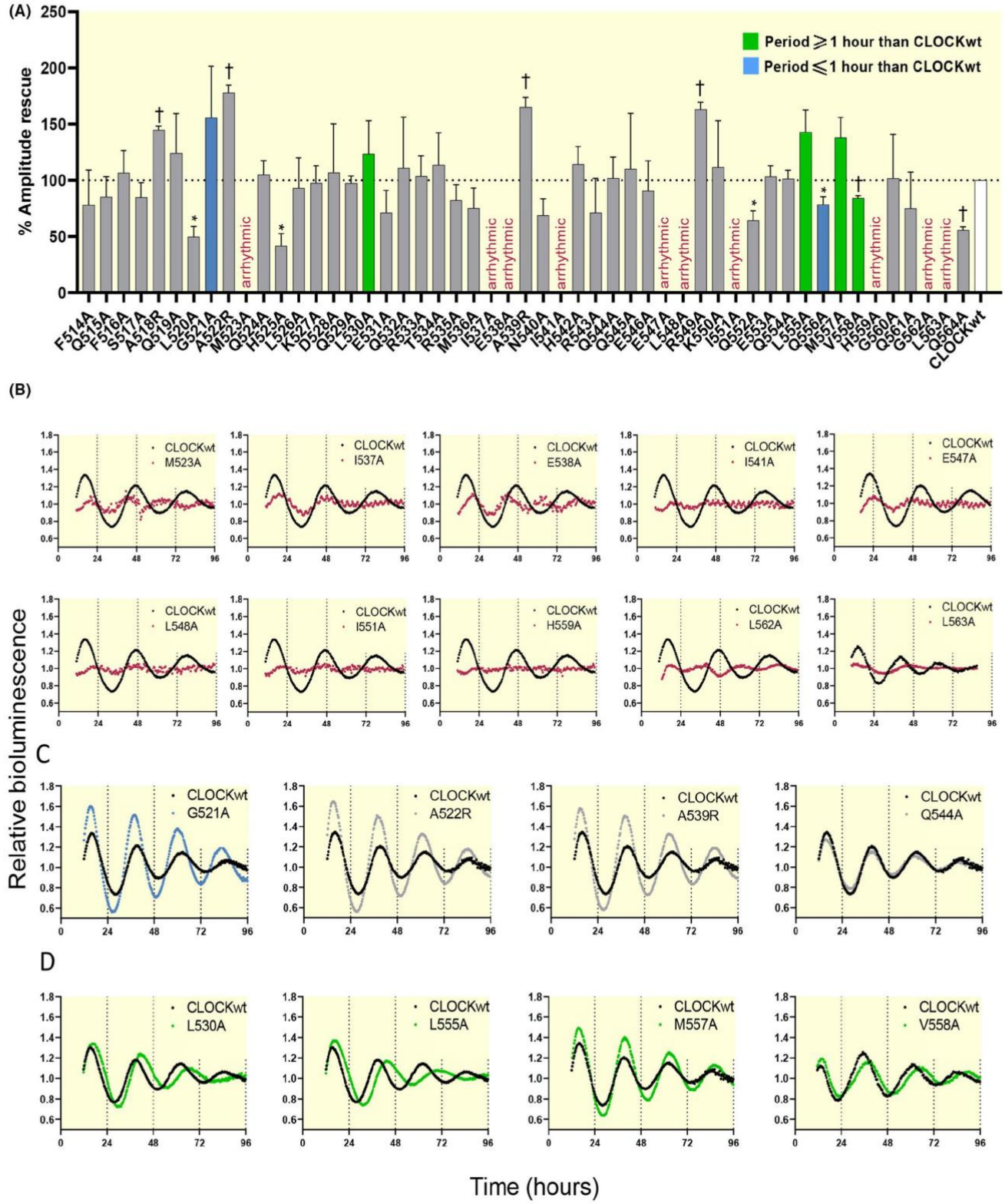
functional CLOCK protein, we then analysed whether constitutive expression of wild-type CLOCK could rescue circadian rhythms in *CLOCK* knockout cells. To this end, we lentivirally transduced our *CLOCK* knockout reporter cells with a plasmid expressing wild-type human CLOCK or CLOCKΔ19 from a CMV promoter. We found that expression of wild-type CLOCK but not CLOCKΔ19 (Figure S2A) rescued circadian rhythms in *CLOCK* knockout cells (Figure 1F) and also decreased the high bioluminescence magnitude to normal levels (Figure S1B), suggesting that this rescue system is suitable to study the functional significance of CLOCK variants for circadian rhythm generation.

With the aim of identifying residues within CLOCK responsible for the CLOCKΔ19 phenotype, we used site-directed mutagenesis of the human *CLOCK* gene exon 17 (corresponding to exon 19 in mice) to create a library of all 51 hCLOCK single amino acid variants constitutively expressed using a CMV promoter (Figure 2). To this end, we individually mutated 48 CLOCK residues to alanine and 3 CLOCK alanine residues to arginine. Successful mutations were confirmed using Sanger sequencing, lentiviral particles were produced, and *CLOCK* knockout reporter cells were transduced. For each CLOCK variant, three independent cell lines were

FIGURE 3 Alanine scanning mutagenesis for *CLOCK* exon 17 (corresponding to exon 19 in mice) reveals critical residues for CLOCK functionality. (A) Percentage of the amplitude rescue of CLOCK knockout cells lentivirally transduced with plasmids expressing either wild-type CLOCK or 51 CLOCK variants with each residue corresponding to CLOCK exon 17 (exon 19 in mice) replaced by alanine (or arginine). Wild-type CLOCK rescue was set to 100%. Variants with blue or green bars showed a ≥1 h shorter or longer period compared with wild-type CLOCK rescue respectively. Time series were classified as arrhythmic, if the sine-wave curve fitting error calculated by Chronostar[37] exceeded 0.3. Mean ± SD of three independent experiments is plotted ($^{*}P < .05$, $^{\dagger}P < .01$; one-sample *t*-test). (B) Representative detrended bioluminescence time series from cell lines expressing CLOCK variants (red) that could not rescue the arrhythmic phenotype of CLOCK knockout in contrast to wild-type CLOCK (black). (C) Representative detrended bioluminescence time series from a cell line expressing CLOCK variants (blue) that rescued the arrhythmic phenotype of CLOCK knockout with a higher amplitude and a ≥1 h shorter period. (D) Representative detrended bioluminescence time series from cell lines expressing CLOCK variants (green) that rescued the arrhythmic phenotype of CLOCK knockout with a ≥1 h longer period

created, and live-cell bioluminescence recordings were performed for 5 days after dexamethasone synchronization. Bioluminescence time-series were analysed, and periods, relative amplitudes, magnitudes and sine-wave-curve fitting error were extracted.

To quantify the degree of rescue of circadian rhythmicity, the percentage of amplitude rescue was determined with wild-type CLOCK rescue set to 100% (Figure 3A, Table S3). Clones with a sine-wave-curve fitting error of more than 0.3 were classified as arrhythmic.



We identified 10 CLOCK variants that failed to rescue circadian rhythms in *CLOCK* knockout cells, although they were expressed, namely M523A, I537A, E538A, I541A, E547A, L548A, I551A, H559A, G562A and L563A (Figure 3A,B, Figure S2B) indicating that these amino acids are essential for the functionality of CLOCK protein. Concordantly, the bioluminescence magnitudes of 6 out of these 10 CLOCK variant-expressing cells were comparable or higher than of *CLOCK* knockout cells (Figure S3, Table S3). Remarkably, we identified 2 CLOCK variants that promoted a ≥1 h shorter period, one of which also with a ~twofold higher amplitude than the wild-type CLOCK (G521A) (Figure 3A,C). In addition, we identified 4 CLOCK variants that resulted in a ≥1 h longer period upon rescue (L530A, L555A, M557A and V558A), however with inconsistent effects on the amplitude (Figure 3A,D, Table S3). Moreover, rescue with four CLOCK variants (A518R, A522R, A539R, R549A), including all those, where wild-type alanine was replaced by arginine, resulted in significantly higher amplitudes and with others (L520A, H525A, Q552A and Q564A) in significantly lower amplitudes without affecting the circadian period. Together, these data not only identify crucial residues responsible for CLOCK functionality but also suggest that this CLOCK domain has multiple functions, the disruption of which results in distinct circadian phenotypes.

In mice, CLOCKΔ19 has a dominant-negative effect, that is, it leads to long-period rhythms even in the presence of wild-type CLOCK.[15] To test, whether this is also the case in human cells, we transduced wild-type U-2 OS reporter cells with lentiviruses harbouring CLOCKΔ19 and found that it indeed caused a disruption of circadian rhythms, whereas the expression of wild-type CLOCK had no effect (Figure 4A). Furthermore, we explored whether any of the CLOCK variants also act dominantly potentially recapitulating the effect of exon 17 (exon 19 in mice) deletion. To this end, we expressed several identified CLOCK variants covering the whole range of rescue phenotypes in wild-type reporter cells. Mutations of residues A522 and A539 were tested for increasing the amplitude (Figure 4B), mutations of residues L530, L555 and V558 were tested for lengthening the period (Figure 4C), and mutations of residues M523, I537, E538, I541, E547, L548, I551, H559, G562 and L563 were tested for inducing arrhythmicity (Figure 4D). Whereas wild-type CLOCK expression did not show any significant effects on circadian rhythms compared with non-transduced cells (Figure 4A), the variants that previously increased the amplitudes (A522R, A539R) acted dominantly with shortening the period by about 1 hour but not with increasing the amplitudes (Figure 4C,E). However, mutations that previously lengthened the periods (L530A, L555A, V558A) did not show significant period lengthening or amplitude effects. CLOCK variants, which did not rescue circadian rhythms of CLOCK knockout cells, had differential effects on wild-type cells. Some had no effects, while E547A and L548A acted dominantly lengthening the period by about 1 hour, and reducing the amplitudes by up to 60% (Figure 4D,F). These data again suggest that this CLOCK domain has multiple functions, with residues having differential roles for CLOCK functionality.

To directly characterize the CLOCK residues encoded by exon 17 (exon 19 in mice) for their ability to modulate CLOCK/BMAL1 transactivation, we cotransfected HEK293 cells with a six E-box-luciferase reporter construct, BMAL1 and CLOCK wild-type or selected variants and analysed the resulting luciferase activity. As expected, CLOCK or BMAL1 alone did not transactivate and CLOCKΔ19/BMAL1 showed a reduced transactivation.[5] CLOCK variants that previously showed a high amplitude rescue (A522R, A539R) in *CLOCK* knockout cells activated transcription from E-boxes in a similar manner as wild-type CLOCK, while some but not all CLOCK variants that previously showed period lengthening or arrhythmicity showed a reduced transactivation activity (Figure 5). L530A and L555A (long-period variants), but not V558A, showed decreased transactivation similar to CLOCKΔ19, and similar to the variants L548A, H559A and L563A, which were unable to rescue rhythmicity in *CLOCK* knockout cells. These data suggest that amino acids at CLOCK positions 530, 548, 555, 559 and 563 are crucial for normal transactivation, while those at positions 537 and 551 may have different roles within the negative feedback loop.

3 | DISCUSSION

The Takahashi laboratory discovered *Clock* as the first clock allele in mammals, and this – along with insights from the fly clock – laid the foundation for the molecular biology era of chronobiology.[14,15,24] Since then, the *Clock* mutant mouse model has been extensively used and underlined the importance of an intact clock for physiology and behaviour. The corresponding protein to the mutant allele, CLOCKΔ19, lacks a 51 amino acid domain within the glutamine-rich C-terminal region of the protein[15] and showed a severely reduced capacity to activate transcription of its target genes[5] (Figure 5). Because glutamine-rich regions of transcription factors are associated with transactivation,[25] and early experiments showed no decrease of CLOCKΔ19's ability to bind to its heterodimerization partner BMAL1 or to DNA,[5] it has been speculated that the glutamine residues of exon 19 would be critical for CLOCK activity.[15]

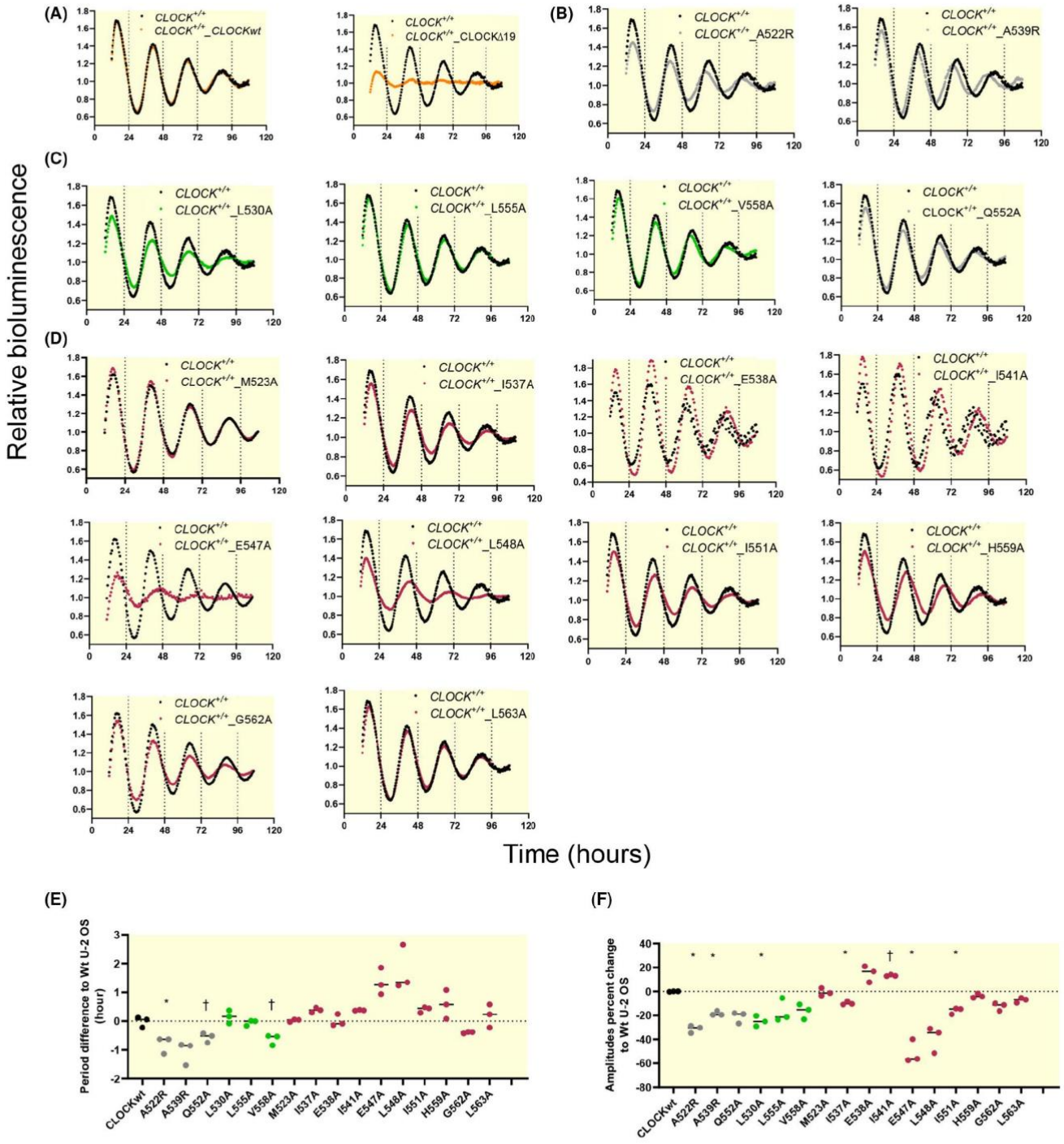


FIGURE 4 CLOCKΔ19 and specific CLOCK variants act dominantly in *CLOCK* wild-type reporter cells. (A) Detrended bioluminescence time series of wild-type *Bmal1*_luc reporter cells upon expression of CLOCKΔ19 (right) or CLOCKwt (left). (B) Detrended bioluminescence time series of wild-type *Bmal1*-luciferase reporter cells upon expression of CLOCK variants that rescue rhythms of *CLOCK* knockout cells with a high amplitude. (C) Detrended bioluminescence time series of wild-type *Bmal1*-luciferase reporter cells upon expression with CLOCK variants that lengthened the period upon rescue in CLOCK knockout cells. (D) Bioluminescence time series of wild-type *Bmal1*-luciferase reporter cells upon expression with CLOCK variants that did not rescue circadian rhythms of *CLOCK* knockout cells. (E and F) Quantifications of period difference and amplitude from experiments shown in (B-D) compared with wild-type reporter cells from three different experiments ($^{*}P < .05$, $^{\dagger}P < .01$; one-sample *t*-test)

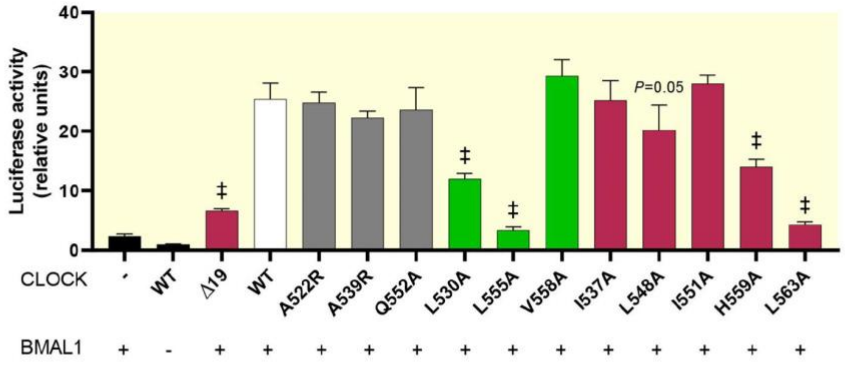


FIGURE 5 CLOCK variants show different transactivation activities. CLOCK/BMAL1-mediated transactivation of a six E-boxes containing luciferase construct in HEK293 cells. The spacing between E-boxes is 12 nucleotides. Shown are means ± SD from three independent experiments. Data were analysed using one-way ANOVA with Dunnett's multiple-comparison test ($^{‡}P < .0001$, compared with CLOCKwt/BMAL1 activation)

To better understand the molecular basis of the *Clock* phenotype on the amino acid level and to transfer knowledge accumulated in mouse models to the human circadian clock, we developed a *CLOCK* knockout – *CLOCK* rescue system in a human reporter cell line. This *CLOCK* knockout cell line is arrhythmic, but rhythmicity can be rescued by expressing wild-type CLOCK but not CLOCKΔ19 in these cells making it an ideal system for systematically analysing the functionality of CLOCK mutant variants. Thus, we performed a single amino acid mutational scanning of the exon 19-domain of CLOCK. However, if glutamine residues would be important for transactivation, we expected that we would probably not see an effect of single amino acid exchanges because clusters of glutamines are required for transactivation. Indeed, none of the 12 glutamine residues turned out to be particularly important for circadian rhythmicity (Figure 3). In contrast, we found several other positions that upon mutation phenocopied the arrhythmic phenotype of the *Clock* mutation.

Interestingly, in most of the 10 CLOCK variants that could not rescue the rhythmicity of the *CLOCK* knockout cell line (analogous to *Clock* homozygous mice), a hydrophobic amino acid was exchanged to alanine (M523A, I537A, I541A, L548A, I551A and L563A). Mutation of four additional hydrophobic residues (L530A, L555A, M557A and V558A) induced a long circadian period (analogous to *Clock* heterozygous mice). Remarkably, the central hydrophobic amino acids (L530, I537, I541, L548, I551, L555) are all highly conserved across species from *Drosophila* to *Homo sapiens*[18] advocating a conserved role for those residues in CLOCK protein function.

In the crystal structure of the mouse exon 19-domain, it was present in a 2:1 stoichiometry with its binding partner CIPC, and showed a coiled-coiled dimer[18] suggesting that oligomers of the CLOCK/BMAL1 heterodimer could also exist in vivo. The dominant-negative effect of CLOCKΔ19 may, therefore, at least in part be caused by the inhibition of CLOCK/BMAL1 oligomer formation. Indeed, an elegant study of Takahashi laboratory showed that the exon 19-domain of CLOCK is required for cooperative binding of CLOCK/BMAL1 to tandem E-boxes, and that the affinity of CLOCKΔ19/BMAL1 to DNA is reduced.[26] However, the relevance of these discoveries for circadian dynamics has never been tested.

The hydrophobic residues within the exon 19 that we identified to be critical for circadian rhythm generation are very likely required for the exon 19-domain dimer formation that has been observed in the crystal structure and probably also for BMAL1/CLOCK-CLOCK/BMAL1 formation in vivo. In the crystal structure, the two exon 19-domain helices run antiparallel to each other with a leucine-zipper-like arrangement of the conserved hydrophobic amino acids (L530, I537, I541, L548, I551, L555) (Figure S4). For example, isoleucine 537 makes a hydrophobic contact to leucine 548, and isoleucine 541 interacts with isoleucine 541 of the other helix. Mutating these residues to alanine abolished circadian rhythmicity, and we propose that this is due to a prevention of the CLOCK/BMAL1 oligomer formation. Interestingly, in PASD1 – an evolutionarily related protein to CLOCK – the coiled-coil 1 (CC1) domain is remarkably similar to the CLOCK exon 19-domain and L520, L530, L548 and L555 of CLOCK are conserved in PASD1. PASD1 acts as a repressor of CLOCK/BMAL1 activity, but not upon deletion of its CC1 domain[27] suggesting that the PASD1 CC1 domain inhibits CLOCK exon 19-domain dimerization and thus efficient transactivation.

How does CLOCK/BMAL1 oligomerization regulate transactivation activity? There are at least three scenarios that are not mutually exclusive. (a) CLOCK/BMAL1 oligomerization increases binding strength to DNA through cooperativity.[26] Indeed, many CLOCK/BMAL1 target genes contain tandem E-boxes, whose spacing requirements (6-7 nucleotides) suggest structural constraints.[28] Because

the six E-box-luciferase reporter construct we used in our cotransactivation assay (Figure 5) does not fulfil these structural requirements (12 nucleotides spacing between E-boxes), this assay is probably less sensitive towards an alteration of DNA binding. For example, the CLOCK variant I537A (which likely affects exon 19-domain dimerization, Figure S4) showed a transactivation activity similar to wild-type CLOCK (Figure 5), although it cannot rescue rhythms of CLOCK knockout cells (Figure 3A) and the bioluminescence magnitude suggests a lower transactivation capacity (Figure S2). Future experiments will reveal whether and to what extent DNA binding is modulated by our CLOCK mutations. (b) CLOCK/BMAL1 oligomerization may create binding interfaces for interacting proteins such as coactivators (eg, MLL1) or repressors (eg, CIPC). With our data, we cannot discriminate between these two first scenarios; further experiments are needed, for example, to quantify binding affinities between CLOCK/BMAL1 heterodimers, between CLOCK/BMAL1 and DNA as well as between CLOCK/BMAL1 and interacting proteins. (c) CLOCK/BMAL1 heterodimers alone can activate transcription to a certain extent, and some of our mutations affect this by modulating the interaction to coactivators or repressors. For example, CLOCK variant L563A cannot transactivate from our six E-box-luciferase reporter and is not able to rescue rhythms of CLOCK knockout cells, but does not act dominantly in wild-type cells indicating that a defect in coactivator binding is dominating over any additional effect of this mutation on dimerization. Another CLOCK variant, E538A also is unable to rescue rhythms, but shows wild-type like transactivation capacity inferred from its bioluminescence magnitude (Figure S3) suggesting a mechanistically different cause for the arrhythmicity, maybe an inability to bind repressors. In contrast, the CLOCK variant A522R (where we introduced a positively charged amino acid often found in transactivating domains) rescues rhythms in *CLOCK* knockout cells with a higher amplitude and shows a shorter period in wild-type cells consistent with the shorter period of *Clock* mutant mice with several additional rescue copies of wild-type *Clock*.[24] We, therefore, conclude that this CLOCK variant probably shows increased coactivator binding.

The *Clock* mutant mice suffer from several metabolic pathologies such as hyperlipidaemia, hepatic steatosis, hyperglycaemia and hyperinsulinemia, probably because of the altered circadian output rhythms, which cause disruption in the diurnal feeding rhythm[29] or through the regulation of insulin secretion.[30] In addition, *Clock* mutant mice show an altered dopamine system and are thus widely used as a model for bipolar disorder.[31,32] It will be interesting to assess whether these different pathologies can be recapitulated in single amino acid mutant mice and whether the differential mechanistic impacts of our mutations provide insights into the pathogenesis of these diseases.

In summary, this study sheds light on the residues of CLOCK exon 19 essential for circadian rhythms. Our data not only indicate that CLOCK/BMAL1 oligomerization mediated by the exon 19-domain is highly relevant for circadian dynamics, but also suggest that the exon 19-domain is a platform for coactivator and repressor binding, which in turn is required for normal rhythms. Given the known studies of inherited forms of circadian disorders in humans,[33] it is reasonable to speculate that some of the circadian disorders in humans are due to altered functionality of CLOCK variants. As genome sequencing has become much easier in the last decade, variants of unclear significance are increasingly being identified.[34] For example, missense mutations in *CLOCK* exon 17 have been described that affect almost all CLOCK residues (Table S4), and their role in circadian rhythms can only be elucidated by systematic analyses of such variants. Our study will contribute to this.

4 | MATERIALS AND METHODS

4.1 | Single-guide RNAs targeting *CLOCK*

sgRNAs were designed using CRISPOR tool,[35] purchased as DNA oligonucleotides from IDT (Integrated DNA Technologies), phosphorylated using T4 polynucleotide kinase (NEB M0201S) and ligated into the lentiCRISPR v2 plasmid (Addgene #52961).[22] Sequences for sgRNA are given in Table S1.

4.2 | Site-directed mutagenesis

Mutations were created in *CLOCK* exon 17 (exon 19 in mice) as described.[36] To this end, oligonucleotides were designed to substitute each amino acid with alanine, and the three wild-type alanine residues at positions 518, 522 and 539 with arginine (Table S2). The whole plasmid was amplified with conditions as described.[36] The parent plasmid template was digested using the enzyme DpnI (NEB #R0176S). Successful mutagenesis was verified by DNA sequencing.

4.3 | Lentivirus production

Human embryonic kidney 293T cells were used to produce lentiviruses as described.[20] HEK293T cells were plated at 70% confluence and transfected using CalPhos

kit (Takara Bio, #631312) with packaging and lentiviral expression plasmids (psPAX and pMD2G plasmids Addgene, #12260 and Addgene, #12259 respectively). The next day, the medium was replaced and supernatants containing viral particles were harvested and filtered the following day.

4.4 | Viral transduction

U-2 OS *Bmal1*-luciferase reporter cells were plated at 50%-70% confluency and transduced with the lentiviral supernatant and protamine sulphate (8 μg/ml) in a 24-well plate. Cells were incubated for 2 days before replacing the culture medium with selection antibiotic (10 μg/ml). Selection was performed for 2 days with puromycin (for *CLOCK* knockout cell generation) or at least 4 days with blasticidine (for all other experiments).

4.5 | Bioluminescence recordings

Live cell bioluminescence recordings were performed as described.[20] Cells were plated in a 96-well plate, circadian rhythms were synchronized using dexamethasone (1 μM) for 30 min. Cells were washed with pre-warmed PBS and cultured in a reporter medium, that is, a phenol-red-free medium supplemented with 10% foetal calf serum, penicillin-streptomycin and D-luciferin (250 μM). Bioluminescence recordings were performed using a 96-well plate luminometer (TopCount, Perkin Elmer) for 5 days. Circadian parameters such as period, amplitude, magnitude and fitting error were calculated using the software Chronostar.[37] Amplitude reference was set to 24 hours. The rhythmicity of the cells was assessed using the sine-wave curve fitting error parameter of Chronostar, and time series with a fitting error of more than 0.3 were considered arrhythmic.

4.6 | Cotransactivation assay

HEK293 cells were plated at around 60% confluency in a 96-well plate 1 day before transfection. Cells were grown in DMEM with 10% calf serum and 1% penicillin-streptomycin. Cells were transfected using lipofectamine 2000 (1 mg/ml) according to the manufacturer's protocol with equal amounts of DNA. Cells were transduced with constructs expressing firefly luciferase reporter driven by six E-box elements (12.5 ng), 75 ng of *mBMAL1* and either 75 ng of pLenti vectors harbouring *hCLOCK*, *hCLOCKΔ19* or *hCLOCK* variants. For normalization, 0.5 ng of a vector expressing *Renilla* luciferase was cotransfected in all wells. The total amount of transfected cDNA was 175 ng in each transfection reaction. Amounts of transfected DNA were adjusted using the pDEST51 vector expressing *lacZ*. After 48 hours, cells were lysed and harvested in 50 μl passive lysis buffer provided in the Dual-Luciferase Reporter Assay System kit (Promega). 5 μl of each lysate was measured by adding 25 μl of LARII and 25 μl of Stop&Glow reagents. The Orion II luminometer (Berthold Detection System) was used to measure luciferase signals. Data from each well were analysed using firefly luciferase activity normalized to *Renilla* luciferase activity.

4.7 | Western blotting

Cells were plated in a 6-well plate at 60%-70% confluency and harvested the next day in RIPA lysis buffer (1× PBS, 1% Igepal CA-630, 0.5% Na deoxicholate, 0.1% SDS) containing protease inhibitor cocktail (1:100). Protein concentrations were measured using Qubit Protein Assay Kits (Life technologies Q33211) according to the manufacturer's protocol. Equal amounts of protein were separated by SDS-PAGE using 4%-12% Bis-Tris gels (Invitrogen). Proteins were then transferred to a nitrocellulose membrane. Blocking was done for 1 hour at room temperature using PBST with 5% non-fat dry milk. Primary antibodies for ß-actin (1:100,000, Sigma #A5441) and CLOCK (1:1000, Cell Signalling #5157) were added to PBST and incubated overnight at 4°C. The next day, membranes were incubated for 1 hour at room temperature with goat-anti-mouse secondary antibodies IgG-HRP (1:1000 in TBS-T, Santa Cruz #D0116) and donkey-anti-rabbit IgG-HRP (1:1000 dilution in TBS-T, Santa Cruz #D0615). Signal detection was done using a chemiluminescence assay with Super Signal West Pico substrate.

All the material submitted is conform with good publishing practice in physiology.[38]

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Ashraf N. Abdo and Carola Rintisch performed experiments; Ashraf N. Abdo, Carola Rintisch, Christian H. Gabriel and Achim Kramer designed experiments and analysed data; Ashraf N. Abdo and Achim Kramer wrote the paper and Achim Kramer oversaw the project.

DATA AVAILABILITY STATEMENT

Processed data that support the findings of this study are available in the Supporting Information of this article. Raw data are available from the corresponding author upon reasonable request.

ORCID

Ashraf N. Abdo https://orcid.org/0000-0002-8977-0495
Achim Kramer https://orcid.org/0000-0001-9671-6078

REFERENCES

1. Finger AM, Dibner C, Kramer A. Coupled network of the circadian clocks: a driving force of rhythmic physiology. *FEBS Lett*. 2020;594(17):2734-2769.
2. Finger AM, Kramer A. Mammalian circadian systems: organization and modern life challenges. *Acta Physiol (Oxf)*. 2021;231(3):e13548.
3. Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. *Trends Cell Biol*. 2014;24(2):90-99.
4. Aryal RP, Kwak PB, Tamayo AG, et al. Macromolecular assemblies of the mammalian circadian clock. *Mol Cell*. 2017;67(5):770-782 e776.
5. Gekakis N, Staknis D, Nguyen HB, et al. Role of the CLOCK protein in the mammalian circadian mechanism. *Science*. 1998;280(5369):1564-1569.
6. Cao X, Yang Y, Selby CP, Liu Z, Sancar A. Molecular mechanism of the repressive phase of the mammalian circadian clock. *Proc Natl Acad Sci U S A*. 2021;118(2):e2021174118.
7. Anafi RC, Lee Y, Sato TK, et al. Machine learning helps identify CHRONO as a circadian clock component. *PLoS Biol*. 2014;12(4):e1001840.
8. Zhao W-N, Malinin N, Yang F-C, et al. CIPC is a mammalian circadian clock protein without invertebrate homologues. *Nat Cell Biol*. 2007;9(3):268-275.
9. Honma S, Kawamoto T, Takagi Y, et al. Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature*. 2002;419(6909):841-844.
10. Takahata S, Ozaki T, Mimura J, Kikuchi Y, Sogawa K, Fujii-Kuriyama Y. Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. *Genes Cells*. 2000;5(9):739-747.
11. Etchegaray JP, Lee C, Wade PA, Reppert SM. Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature*. 2003;421(6919):177-182.
12. Katada S, Sassone-Corsi P. The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. *Nat Struct Mol Biol*. 2010;17(12):1414-1421.
13. Konopka RJ, Benzer S. Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*. 1971;68(9):2112-2116.
14. Vitaterna MH, King DP, Chang AM, et al. Mutagenesis and mapping of a mouse gene, clock, essential for circadian behavior. *Science*. 1994;264(5159):719-725.
15. King DP, Zhao Y, Sangoram AM, et al. Positional cloning of the mouse circadian clock gene. *Cell*. 1997;89(4):641-653.
16. Lee E, Cho E, Kang DH, et al. Pacemaker-neuron-dependent disturbance of the molecular clockwork by a Drosophila CLOCK mutant homologous to the mouse Clock mutation. *Proc Natl Acad Sci U S A*. 2016;113(33):E4904-4913.
17. Yoshitane H, Takao T, Satomi Y, Du NH, Okano T, Fukada Y. Roles of CLOCK phosphorylation in suppression of E-box-dependent transcription. *Mol Cell Biol*. 2009;29(13):3675-3686.
18. Hou Z, Su L, Pei J, Grishin NV, Zhang H. Crystal structure of the CLOCK transactivation domain exon19 in complex with a repressor. *Structure*. 2017;25(8):1187-1194.e3.
19. Steeves TDL, King DP, Zhao Y, et al. Molecular cloning and characterization of the human CLOCK gene: expression in the suprachiasmatic nuclei. *Genomics*. 1999;57(2):189-200.
20. Maier B, Wendt S, Vanselow JT, et al. A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. *Genes Dev*. 2009;23(6):708-718.
21. Bording T, Abdo AN, Maier B, Gabriel C, Kramer A. Generation of human CRY1 and CRY2 knockout cells using duplex CRISPR/Cas9 technology. *Front Physiol*. 2019;10:577.
22. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods*. 2014;11(8):783-784.
23. DeBruyne JP, Weaver DR, Reppert SM. CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nat Neurosci*. 2007;10(5):543-545.
24. Antoch MP, Song E-J, Chang A-M, et al. Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell*. 1997;89(4):655-667.
25. Mitchell PJ, Tjian R. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science*. 1989;245(4916):371-378.
26. Shimomura K, Kumar V, Koike N, et al. Usf1, a suppressor of the circadian Clock mutant, reveals the nature of the DNA-binding of the CLOCK:BMAL1 complex in mice. *Elife*. 2013;2:e00426.
27. Michael A, Harvey S, Sammons P, et al. Cancer/testis antigen PASD1 silences the circadian clock. *Mol Cell*. 2015;58(5):743-754.
28. Rey G, Cesbron F, Rougemont J, Reinke H, Brunner M, Naef F. Genome-wide and phase-specific DNA-binding rhythms of BMAL1 control circadian output functions in mouse liver. *PLoS Biol*. 2011;9(2):e1000595.
29. Turek FW, Joshu C, Kohsaka A, et al. Obesity and metabolic syndrome in circadian Clock mutant mice. *Science*. 2005;308(5724):1043-1045.
30. Tian L, Li X, Ding Y, Li M, Tang Y, Li D. The CLOCK protein regulates insulin secretion related with L-type calcium channels in rat pancreatic beta cells. *Biochem Biophys Res Commun*. 2022;589:116-122.
31. Spencer S, Torres-Altoro MI, Falcon E, et al. A mutation in CLOCK leads to altered dopamine receptor function. *J Neurochem*. 2012;123(1):124-134.
32. Kristensen M, Nierenberg AA, Ostergaard SD. Face and predictive validity of the ClockDelta19 mouse as an animal model for bipolar disorder: a systematic review. *Mol Psychiatry*. 2018;23(1):70-80.

33. Patke A, Murphy PJ, Onat OE, et al. Mutation of the human circadian clock gene CRY1 in familial delayed sleep phase disorder. *Cell*. 2017;169(2):203-215.e13.
34. Weile J, Roth FP. Multiplexed assays of variant effects contribute to a growing genotype-phenotype atlas. *Hum Genet*. 2018;137(9):665-678.
35. Haeussler M, Schönig K, Eckert H, et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol*. 2016;17(1):148.
36. Laible M, Boonrod K. Homemade site directed mutagenesis of whole plasmids. *J Vis Exp*. 2009;(27):1135.
37. Maier B, Lorenzen S, Finger AM, Herzel H, Kramer A. Searching novel clock genes using RNAi-based screening. *Methods Mol Biol*. 2021;2130:103-114.
38. Persson PB. Good publication practice in physiology 2019. *Acta Physiol (Oxf)*. 2019;227(4):e13405.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Mutational scanning identified amino acids of the CLOCK exon 19-domain essential for circadian rhythms

Ashraf N. Abdo*, Carola Rintisch, Christian H. Gabriel and Achim Kramer*

Charité –Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Laboratory of Chronobiology, Charitéplatz 1, 10117 Berlin, Germany

* correspondence: ashraf.abdo@charite.de or achim.kramer@charite.de

Supporting Information

Supporting Figures 1-4
Supporting Tables 1-4

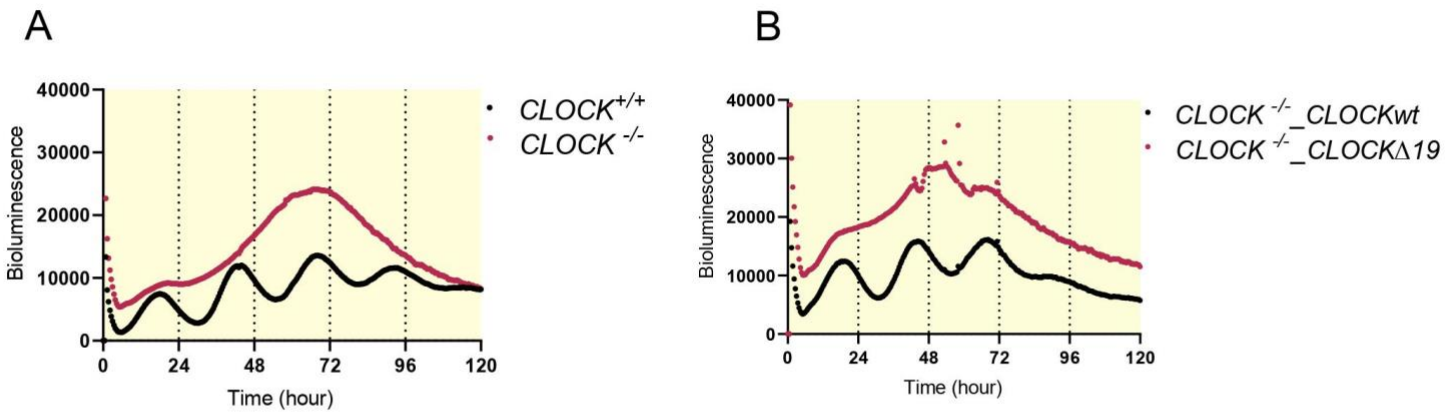

Figure S1. (A) Representative bioluminescence time series of wild-type U-2 OS cells (black) and *CLOCK* knockout cells (red). Note the higher overall bioluminescence (magnitude) of the knockout cells. **(B)** Representative bioluminescence time series of *CLOCK* knockout cells lentivirally transduced with either CLOCKwt (black) versus the CLOCKΔ19 (red).

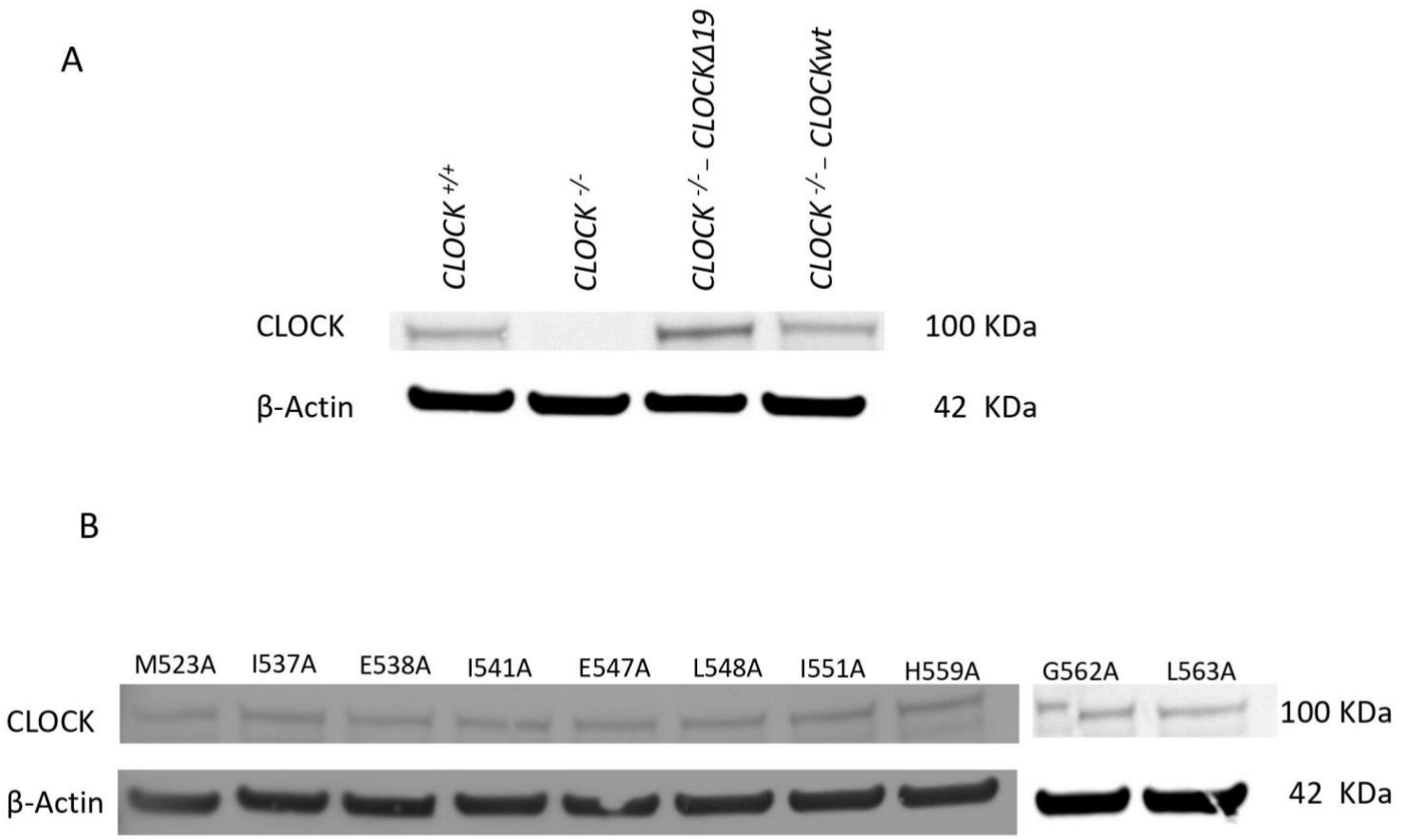

Figure S2. (A) CLOCK protein in indicated cell lines detected by western blotting. **(B)** Expression of selected CLOCK variants in *CLOCK* knockout cells lentivirally transduced with indicated mutant constructs.

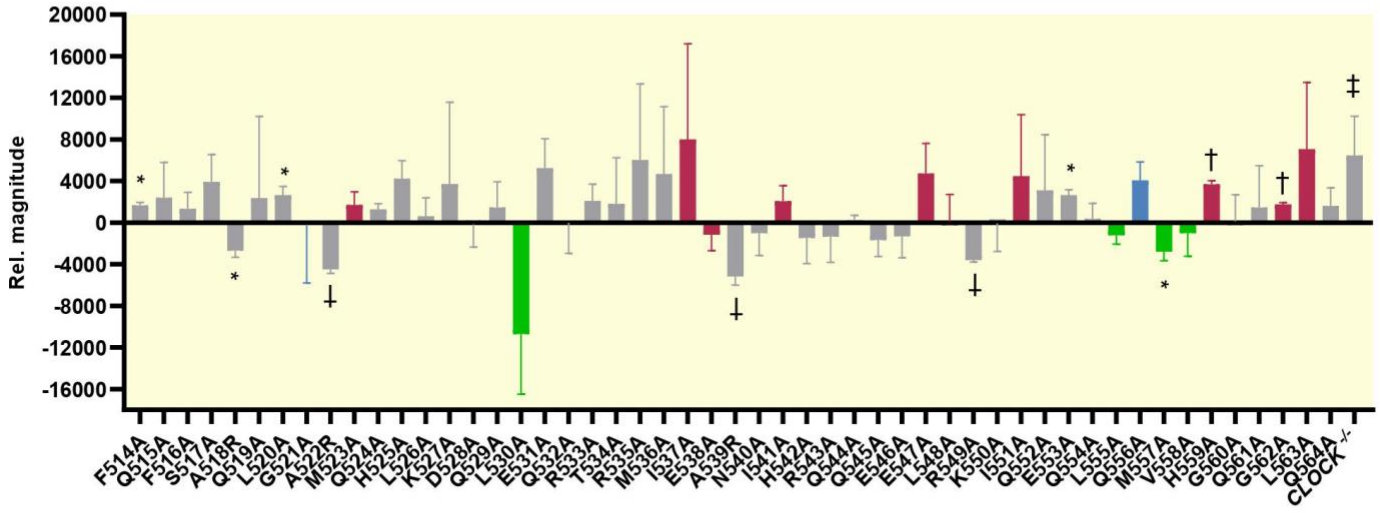

Figure S3. Relative magnitudes of bioluminescence time series of *CLOCK* knockout cells lentivirally transduced with indicated CLOCK variants. Mean ± SD of three independent experiments is shown. ($^{‡}p<0.001$, $^{†}p<0.01$, $^{*}p<0.05$, one-way ANOVA with Dunnett's multiple comparisons test to CLOCKwt).

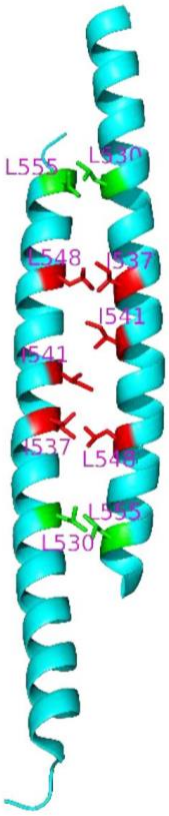

Figure S4. CLOCK exon 19-domain crystal structure (PDB 5VJI) depicting the hydrophobic residues forming the coiled-coil interaction critical for CLOCK functionality.

Name	Sequence
sgRNA_i7-8	CTAATAAACCCGAACTGATGAGG
sgRNA_i5-6	CCAGGTTACCAATATACAGTTAA

Table S1: sgRNA for creating *CLOCK* knockout cells using CRISPR/Cas9 technology by targeting regions flanking *CLOCK* exon 6 and 7.

Name	Sequence
F514A_Fw	cacaaggcatgtcccagGCAcagttttcagctcaattagg
F514A_Rev	cctaattgagctgaaaactgTGCctgggacatgccttgtg
Q515A_Fw	aaggcatgtcccagtttGCAttttcagctcaattaggagc
Q515A_Rev	gctcctaattgagctgaaaaTGCaaactgggacatgcctt
F516A_Fw	gcatgtcccagtttcagGCAtcagctcaattaggagcc
F516A_Rev	ggctcctaattgagctgaTGCctgaaactgggacatgc
S517A_Fw	ccagtttcagtttGCAgctcaattaggagcc
S517A_Rev	ggctcctaattgagcTGCaaactgaaactgg
A518R_Fw	gtcccagtttcagttttcacggcaattagg
A518R_Rev	gttgcatggctcctaattgccgtgaaaact
Q519A_Fw	ccagtttcagttttcagctGCAttaggagccatgcaac
Q519A_Rev	gttgcatggctcctaaTGCagctgaaaactgaaactgg
L520A_Fw	ttcagttttcagctcaaGCAggagccatgcaacat
L520A_Rev	atgttgcatggctccTGCttgagctgaaaactgaa
G521A_Fw	agttttcagctcaattaGCAgccatgcaacat
G521A_Rev	atgttgcatggcTGCtaattgagctgaaaact
A522R_Fw	Cagttttcagctcaattaggacgcatgcaa
A522R_Rev	gtctttcagatgttgcatgcgtcctaattg
M523A_Fw	tcagctcaattaggagccGCAcaacatctgaaagaccaa
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Q524A_Rev	aattggtctttcagatgTGCcatggctcctaattgagct
H525A_Fw	tcaattaggagccatgcaaGCActgaaagaccaattggaacaac
H525A_Rev	gttgttccaattggtctttcagTGCttgcatggctcctaattga
L526A_Fw	tcaattaggagccatgcaacatGCAaaagaccaattggaacaac
L526A_Rev	gttgttccaattggtctttTGCatgttgcatggctcctaattga
K527A_Fw	gagccatgcaacatctgGCAgaccaattggaacaac
K527A_Rev	gttgttccaattggtcTGCcagatgttgcatggctc
D528A_Fw	atgcaacatctgaaaGCAcaattggaacaacggacac
D528A_Rev	gtgtccgttgttccaattgTGCtttcagatgttgcat
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L530A_Fw	aacatctgaaagaccaaGCAgaacaacggacacgcatg
L530A_Rev	catgcgtgtccgttgttcTGCttggtctttcagatgtt
E531A_Fw	gaccaattgGCAcaacggacacgcat
E531A_Rev	atgcgtgtccgttgTGCcaattggtc
Q532A_Fw	tgaaagaccaattggaaGCAcggacacgcatgatagaa
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I537_Fw	gaacaacggacacgcatgGCAgaagcaaatattcatcg
I537_Rev	cgatgaatatttgcttcTGCcatgcgtgtccgttgttc
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L548A_Rev	tgaagttgttcttgaatttttctTGCttcttcttgttgccgatgaat
R549A_Fw	atcggcaacaagaagaactaGCAaaaattcaagaacaacttcagatg
R549A_Rev	catctgaagttgttcttgaattttTGCtagttcttcttgttgccgat
K550A_Fw	cggcaacaagaagaactaagaGCAattcaagaacaacttcagatg
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Q552A_Rev	ccatggaccatctgaagttgttcTGCaatttttcttagttcttcttg
E553A_Fw	ctaagaaaaattcaaGCAcaacttcagatggtccatggt
E553A_Rev	accatggaccatctgaagttgTGCttgaatttttcttag
Q554A_Fw	ctaagaaaaattcaagaaGCActtcagatggtccatggtcaggg
Q554A_Rev	ccctgaccatggaccatctgaagTGCttcttgaatttttcttag
L555A_Fw	ctaagaaaaattcaagaacaaGCAcagatggtccatggtcagggg

L555A_Rev	cccctgaccatggaccatctgTGCttgttcttgaatttttcttag
Q556A_Fw	aaaattcaagaacaacttGCAatggtccatggtcaggggct
Q556A_Rev	agcccctgaccatggaccatTGCaagttgttcttgaatttt
M557A_Fw	caagaacaacttcagGCAgtccatggtcaggggct
M557A_Rev	agcccctgaccatggacTGCctgaagttgttcttg
V558A_Fw	aacaacttcagatgGCAcatggtcaggggct
V558A_Rev	agcccctgaccatgTGCcatctgaagttgtt
H559A_Fw	caacttcagatggtcGCAggtcaggggctgcag
H559A_Rev	ctgcagcccctgaccTGCgaccatctgaagttg
G560A_Fw	cagatggtccatGCAcaggggctgcagat
G560A_Rev	atctgcagcccctgTGCatggaccatctg
Q561A_Fw	ttcagatggtccatggtGCAgggctgcagatgtttt
Q561A_Rev	aaaacatctgcagcccTGCaccatggaccatctgaa
G562A_Fw	agatggtccatggtcagGCActgcagatgtttt
G562A_Rev	aaaacatctgcagTGCctgaccatggaccatct
L563A_Fw	gtccatggtcaggggGCAcagatgtttttgcaacaatc
L563A_Rev	gattgttgcaaaaacatctgTGCcccctgaccatggac
Q564A_Fw	gtccatggtcaggggctgGCAatgtttttgcaacaatc
Q564A_Rev	gattgttgcaaaaacatTGCcagcccctgaccatggac

Table S2: Primer sequences carrying mutated codon for each position of human *CLOCK* exon 17 (homologue of exon 19 in mice).

CLOCK variant	Δ Period	SD	% Amplitude rescue	SD	Sine fitting error	Δ Magnitude	SD
F514A	0.6	0.60	78	31	0.247	**1639***	315
Q515A	0.3	0.49	85	18	0.206	2379	3414
F516A	**0.5****	0.06	107	20	0.136	1315	1603
S517A	-0.3	0.25	85	13	0.198	3898	2651
A518R	0.3	0.11	**145****	3	0.039	**-2674***	669
Q519A	**-0.7***	0.19	124	35	0.105	2368	7831
L520A	-0.6	0.66	**50***	9	0.249	**2629***	842
G521A	-1.2	0.95	156	46	0.069	-175	5628
A522R	0.0	0.07	**178****	7	0.030	**-4486****	394
M523A	-	-	-	-	0.387	1702	1268
Q524A	**-0.1***	0.03	105	13	0.131	1257	578
H525A	0.7	0.48	**42***	11	0.094	4208	1754
L526A	0.7	0.33	93	27	0.198	597	1776
K527A	-0.2	0.37	98	15	0.095	3716	7875
D528A	0.3	0.32	107	43	0.172	-153	2196
Q529A	**0.6***	0.15	97	6	0.129	1446	2500
L530A	**2.6****	0.36	123	30	0.096	-10685	5799
E531A	0.2	0.71	71	20	0.198	5223	2850
Q532A	**-0.7***	0.13	111	45	0.119	-16	2940
R533A	**-0.5***	0.12	104	18	0.098	2089	1605
T534A	-0.4	0.83	114	29	0.097	1798	4453
R535A	-0.7	0.42	82	14	0.212	6029	7297
M536A	0.1	0.65	75	18	0.235	4676	6461
I537A	-	-	-	-	0.447	7987	9213
E538A	-	-	-	-	0.348	-1153	1545
A539R	0.0	0.06	**165****	9	0.027	**-5152****	855
N540A	-0.2	0.42	69	15	0.278	-994	2162
I541A	-	-	-	-	0.502	2053	1489
H542A	-0.1	0.20	114	16	0.087	-1453	2480
R543A	-0.2	1.01	71	30	0.216	-1315	2502
Q544A	-0.3	0.13	102	19	0.078	228	474
Q545A	0.1	0.14	110	50	0.095	-1644	1617
E546A	0.2	0.41	90	27	0.137	-1311	2075
E547A	-	-	-	-	0.317	4729	2894
L548A	-	-	-	-	0.672	124	2585
R549A	**0.6*****	0.02	**163****	7	0.037	**-3577****	211
K550A	-0.1	0.23	111	42	0.069	-61	2701
I551A	-	-	-	-	0.384	4461	5920
Q552A	0.7	0.60	**64***	9	0.252	3099	5364
E553A	-0.4	0.90	103	10	0.146	**2627***	540
Q554A	**-0.4***	0.14	101	8	0.247	342	1507
L555A	**3.3****	0.34	143	20	0.062	-1187	875
Q556A	-1.2	1.07	**78***	7	0.113	4051	1777
M557A	1.4	1.71	138	18	0.055	**-2763***	893

V558A	**1.0***	0.25	**84****	2	0.130	-1004	2222
H559A	-	-	-	-	0.678	**3669****	365
G560A	**-0.8***	0.31	102	39	0.078	115	2572
Q561A	-0.4	0.38	75	32	0.164	1441	4030
G562A	-	-	-	-	0.313	**1753****	182
L563A	-	-	-	-	0.495	7059	6430
Q564A	-0.4	0.17	**56****	3	0.238	1609	1735
CLOCK$^{-/-}$	-	-	-	-	0.585	**6446*****	3787

Table S3: Period difference, % amplitude rescue and magnitude difference relative to wild-type *CLOCK* as well as error of sine wave curve fitting. If the error of the sine wave curve fitting was higher than 0.3 (marked in red), time series were considered arrhythmic and rhythm parameters were not calculated. Shown are means and SD of three independent experiments (n=9 for *CLOCK* $^{-/-}$).

SNP name	Position	Amino acid variation	Nucleotide exchange	Allele frequency (%)
rs1249659019	514	Phe→Cys	A→C	0.0007
rs1278388353	518	Ala→Thr	G→A	0.0004
rs745374367	519	Gln→Glu	G→C	0.0007
rs756941305	519	Gln→His	T→C / T→G	0.0007
rs1372516916	520	Leu→Phe	T→G	0.0007
rs1301971877	521	Gly→Arg	C→T	0.0004
rs1387354726	523	Met→Arg	A→C	0.0012
rs1392759691	525	His→Tyr	G→A	0.0004
rs141240333	525	His→Gln	A→T	0.0050
rs34812164	528	Asp→Glu	G→A / G→C	0.1473
rs150012219	529	Gln→Lys	G→C / G→T	0.0043
rs1428754930	530	Leu→Trp	A→C	0.0004
rs1248972337	533	Arg→Trp	G→A / G→T	0.0007
rs767621413	533	Arg→Gln	C→T	0.0008
rs761998791	535	Arg→Cys	G→A / G→C	0.0007
rs529037715	536	Met→Val	T→C	0.0004
rs958536060	536	Met→Arg	A→C / A→G / A→T	0.0004
rs1200200735	538	Glu→Lys	C→T	0.0004
rs1320960358	541	Ile→Thr	A→G	0.0004
rs3762836	542	His→Leu	T→A / T→C	0.0020 (ALFA)
rs775779441	543	Arg→Gln	C→T	0.0007
rs746419884	546	Glu→Gln	C→G	0.0021
rs1390611889	550	Lys→Arg	T→C	0.0007
rs1279304508	552	Gln→Lys	G→T	0.0008 (TOPMED)
rs1400686387	552	Gln→Arg	T→C	0.0004
rs202127369	557	Met→Ile	C→T	0.0004
rs780830912	559	His→Arg	T→C	0.0007
rs756851458	560	Gly→Asp	C→T	0.0004
rs139335390	561	Gln→Arg/Pro	T→A / T→C / T→G	0.0008
rs758071702	563	Leu→Arg	A→C	0.0004
rs752522729	564	Gln→Arg	T→C / T→G	0.0008

Table S4: Single nucleotide polymorphisms (SNP) in exon 17 of human *CLOCK*. Allele frequency is given according to the Genome Aggregation Database (gnomAD) unless otherwise stated.

List of publications

- Abdo, A. N., Rintisch, C., Gabriel, C. H., & Kramer, A. (2022). Mutational scanning identified amino acids of the CLOCK exon 19-domain essential for circadian rhythms. Acta Physiologica, e13794.
  Impact factor 6.311

- Börding, T., Abdo, A. N., Maier, B., Gabriel, C., & Kramer, A. (2019). Generation of human CRY1 and CRY2 knockout cells using duplex CRISPR/Cas9 technology. Frontiers in physiology, 10, 577.
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My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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Thanks to Allah, for always blessing the steps that I took or will do.