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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** | **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

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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

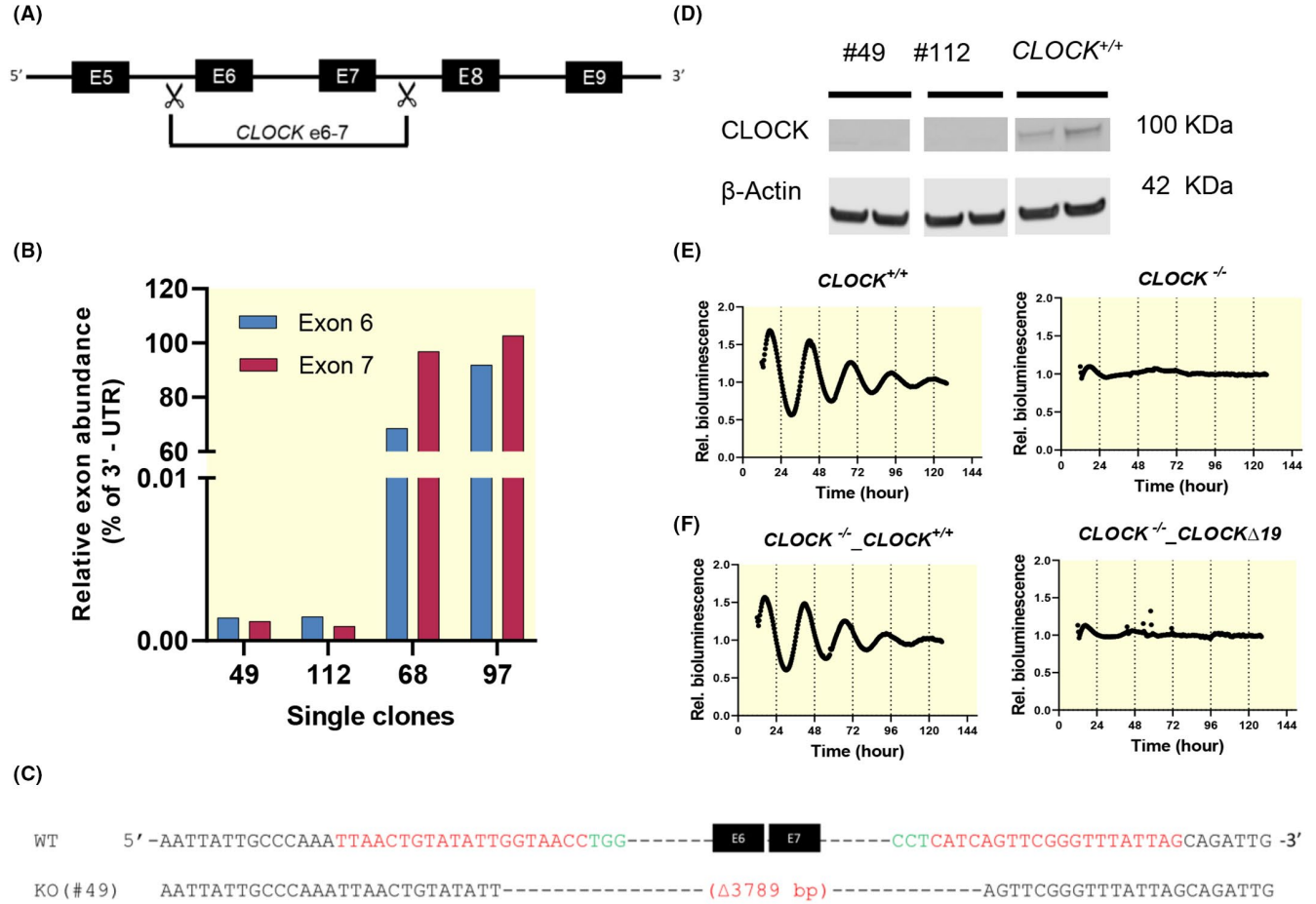


FIGURE 1 Generation of U-2 OS *CLOCK* knockout cells via CRISPR/Cas9 and characterization of CLOCKΔ19 circadian phenotype. (A) Schematic overview of the CRISPR/Cas9 exon deletion strategy showing sgRNAs targeting exon 6 and 7 boundaries in U-2 OS cells. (B) Genomic abundance of the targeted exons (relative 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

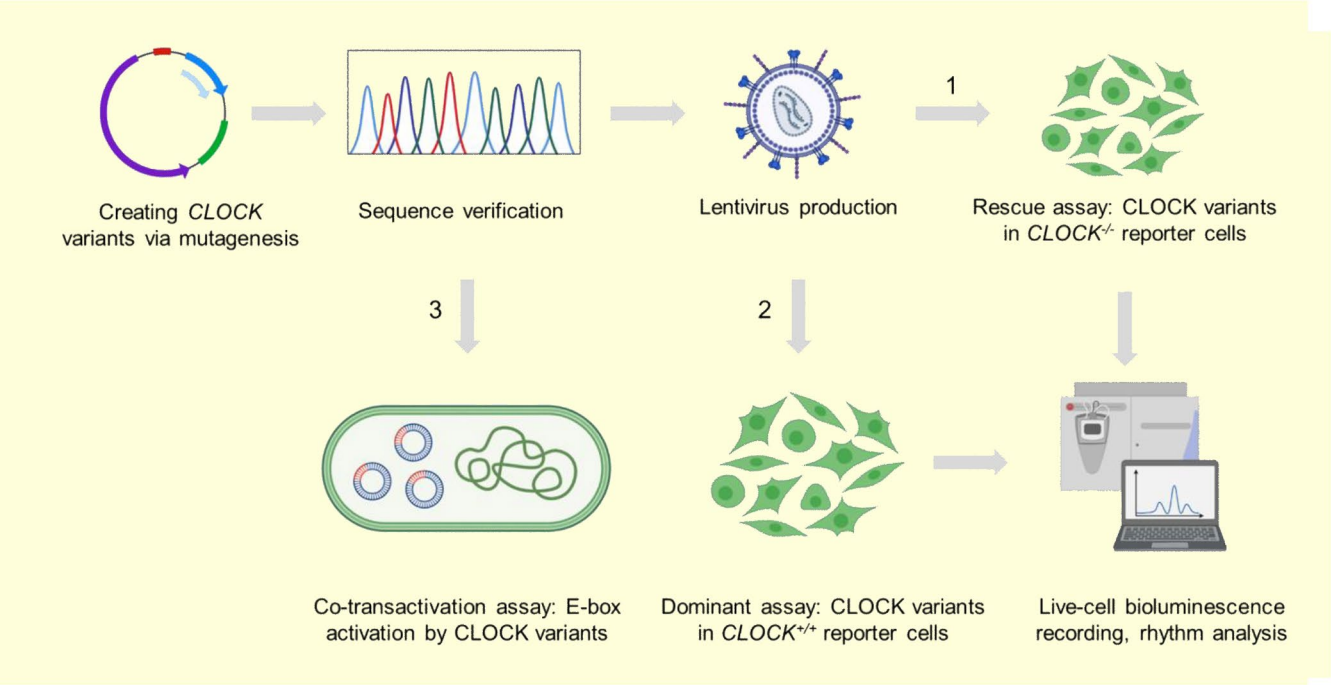


FIGURE 2 Workflow to create and analyse the alanine substitutions for each residue coded in *CLOCK* exon 19 (exon 17 in humans) using site-directed mutagenesis. Primers carrying the mutated codon were used PCR-amplification of the whole plasmid carrying *CLOCK* coding sequence driven by the CMV promoter. 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

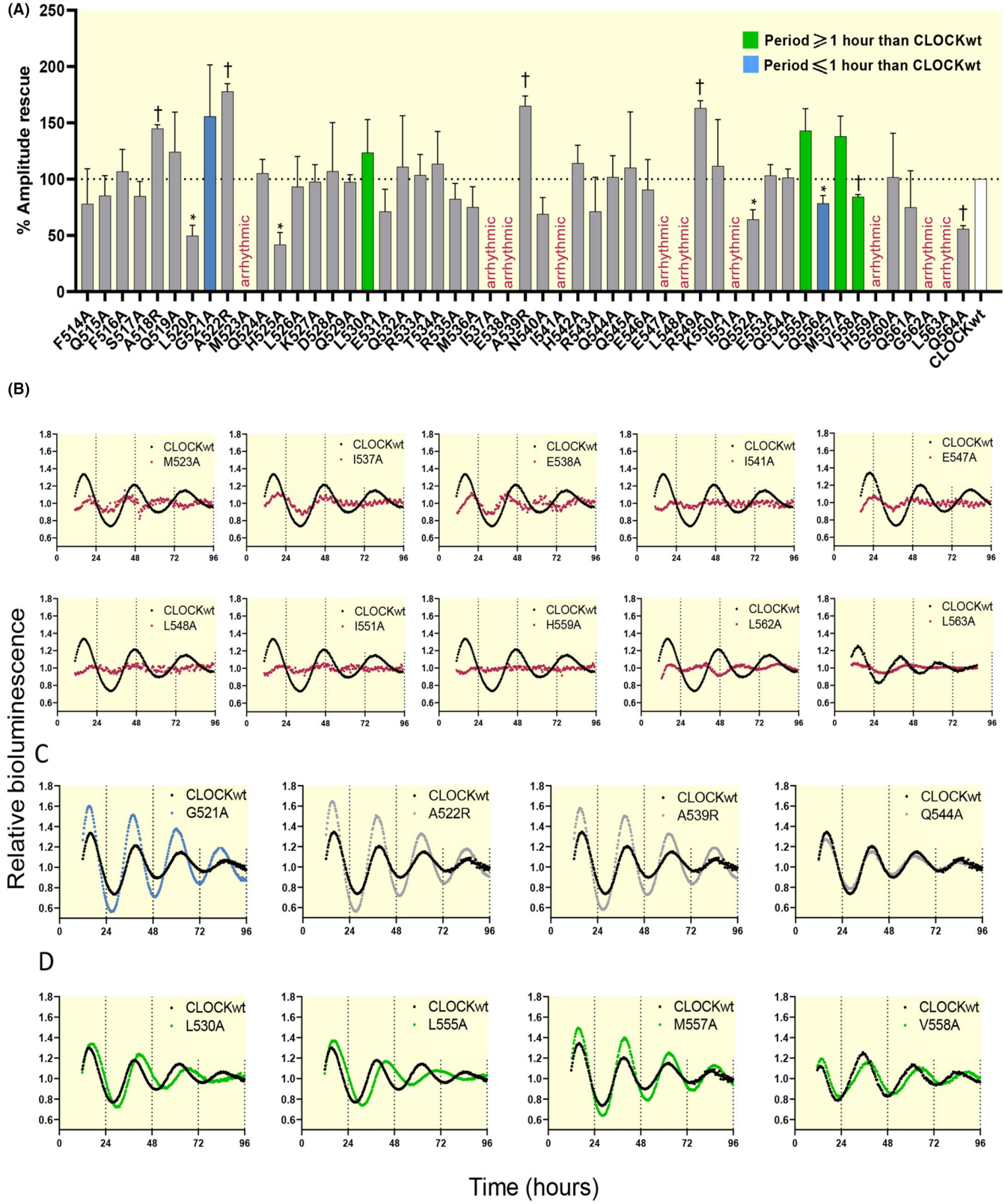
functional CLOCK protein, we then analysed whether constitutive expression of wild-type CLOCK could rescue circadian rhythms in *CLOCK* knockout cells. To this end, we lentivirally transduced our *CLOCK* knockout reporter cells with a plasmid expressing wild-type human CLOCK or CLOCKΔ19 from a CMV promoter. 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]

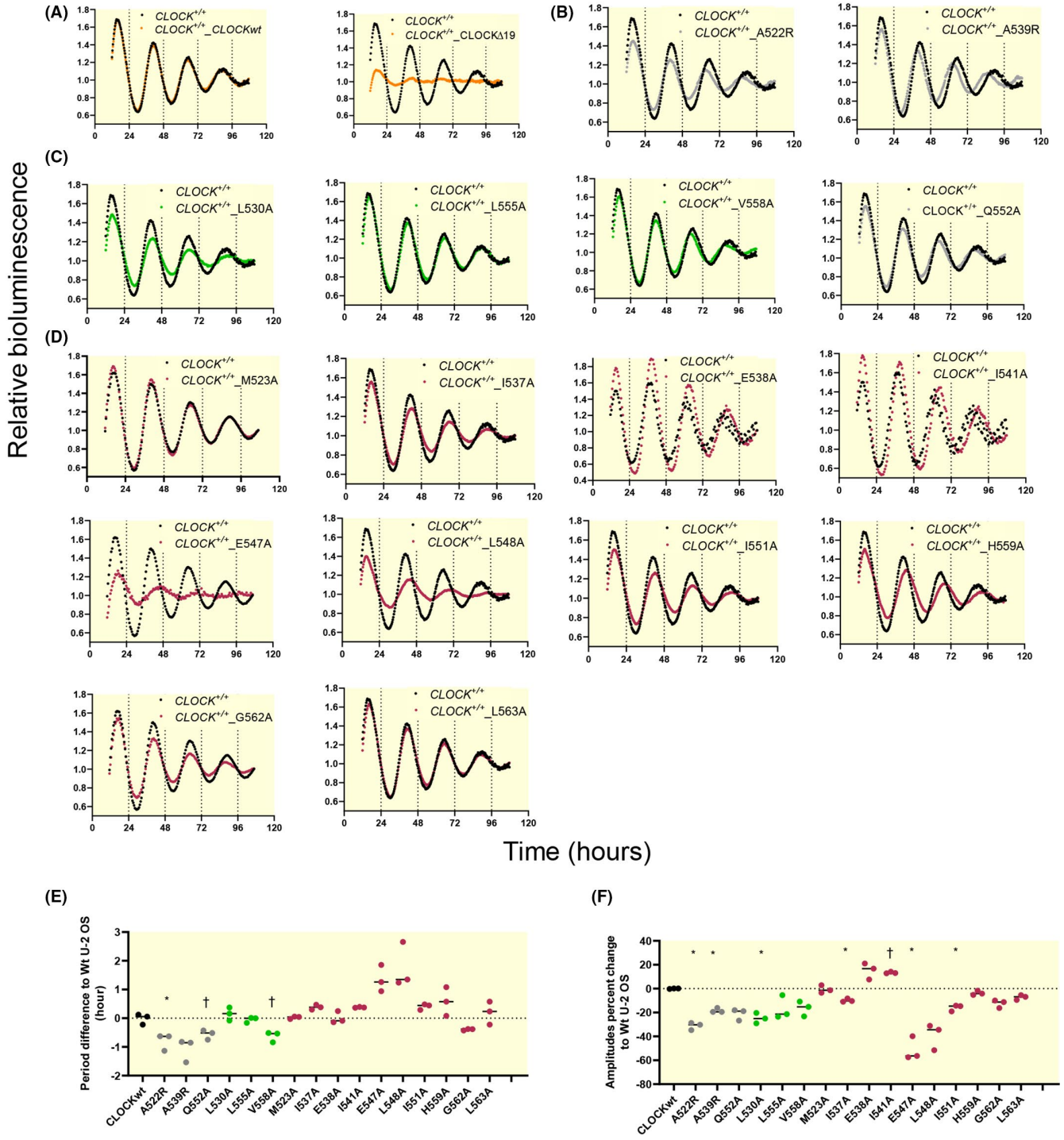


FIGURE 4 CLOCKΔ19 and specific CLOCK variants act dominantly in *CLOCK* wild-type reporter cells. (A) Detrended bioluminescence time series of wild-type *Bmal1*_luc reporter cells upon expression of CLOCKΔ19 (right) or CLOCKwt (left). (B) Detrended bioluminescence time series of wild-type *Bmal1*-luciferase 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)

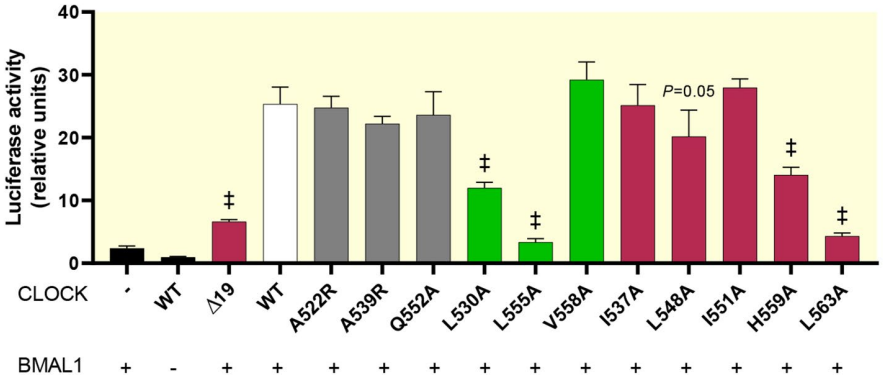


FIGURE 5 CLOCK variants show different transactivation activities. CLOCK/BMAL1-mediated transactivation of a six E-boxes containing luciferase construct in HEK293 cells. The spacing between E-boxes is 12 nucleotides. Shown are means ± SD from three independent experiments. Data were analysed using one-way 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

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SUPPORTING INFORMATION

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

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