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EDITORIAL

Mutational scanning of the CLOCKΔ19 domain identifies amino acids modulating circadian clock dynamics and co-regulator binding

A new cell culture–based knock-out and rescue system published in this issue provides functional insight into specific amino acids of the circadian clock protein CLOCK (Circadian Locomotor Output Cycles Kaput).[1] By systematically mutating individual amino acids in the functionally important Δ19 domain,[2] Abdo et al[1] identify amino acids critical for CLOCK/BMAL1 oligomerization and circadian dynamics, including amino acids important for co-regulator binding. These results provide a basis for a more detailed molecular understanding of the mechanism of circadian rhythm generation.

Clock came up by chemical mutagenesis screening as the first gene component reported of the mammalian circadian clock.[2,3] Chemical mutagenesis deleted exon 19 of the murine *Clock* gene by mutating an RNA splice site. The resulting protein from this mutated gene is termed CLOCKΔ19. Hypophosphorylization attenuates its degradation.[4] Hence, mice with this mutation overexpress CLOCKΔ19 protein, which prolongs the circadian period ending in arhythmicity in constant darkness. The 51 amino acids lacking in CLOCKΔ19 (Figure 1, Δ19) affect CLOCK/BMAL1-mediated transactivation of E-box containing promoters and allow extended coiled-coil dimer formation. The latter structure is an antiparallel leucine zipper (Figure 1, purple gradients) that can co-crystallize with a protein called CLOCK interacting protein circadian (CIPC).[5] CLOCK coil interaction with CIPC may influence CLOCK:BMAL1 activity by CLOCK phosphorylation and degradation. The Δ19 domain may be involved in forming CLOCK:BMAL1 super-complexes present on several E-boxes (eg tandem E-boxes) to form a macromolecular complex recognized by regulatory proteins for activation or repression. However, the exact role of the Δ19 domain regulating CLOCK:BMAL1-mediated transcription is not understood.

In the novel work presented in this issue by Abdo et al[1] the authors systematically mutate every single amino acid in the Δ19 domain to alanine (48 residues) or to arginine (3 Ala residues) and investigated their influence on cell circadian rhythmicity. After site directed mutagenesis, the CLOCK variants were transfected into human *Bmal1*-luciferase reporter cells lacking the *Clock* gene using a lentiviral system. Cells were synchronized and bioluminescence rhythms were recorded for several days. Circadian period assessment identified the point mutation rescuing the CLOCKΔ19 phenotype. The data were set in relation to the rescue by wild-type CLOCK. Interestingly, some of the CLOCK variants were not able to rescue arhythmicity of *Clock* knock-out reporter cells (Figure 1, highlighted in yellow). Thus, those are the essential amino acids for CLOCK functionality. Other variants promoted a more than 1 hour shorter period (Figure 1, highlighted in blue) or a more than 1 hour longer period (Figure 1, highlighted in grey), suggesting multiple functions of the Δ19 domain.

CLOCK variant overexpression in wild-type cells ($Clock^{+/+}$) caused diverse clock period effects. Those lengthening the *Clock* knock-out cells' period did not lengthen the period in wild-type cells, whereas those not rescuing circadian rhythms of *Clock* knock-out cells had differential effects on wild-type cells, again indicating multiple functions of the Δ19 domain.

In a co-transactivation assay, Abdo et al[1] tested CLOCK variant abilities to activate a reporter construct containing six E-boxes coupled to a luciferase reporter. Co-transfection with BMAL1 revealed several amino acids in the Δ19 domain crucial for transactivation (Figure 1, amino acids with red lettering). Others played no role in this process (Figure 1, amino acids with blue lettering) suggesting other functions for these amino acids in the negative feedback loop.

Taken together, Abdo et al[1] show that leucine zipper amino acids are most important for CLOCK function (Figure 1). This view is supported by conservation of these amino acids across species[5] (Figure 1, amino acids in bold). Interestingly, apparently important amino acids for period length determination are located at the N- or C-term of the leucine zipper. Accordingly, these amino acids may regulate CLOCK protein stability. This could occur

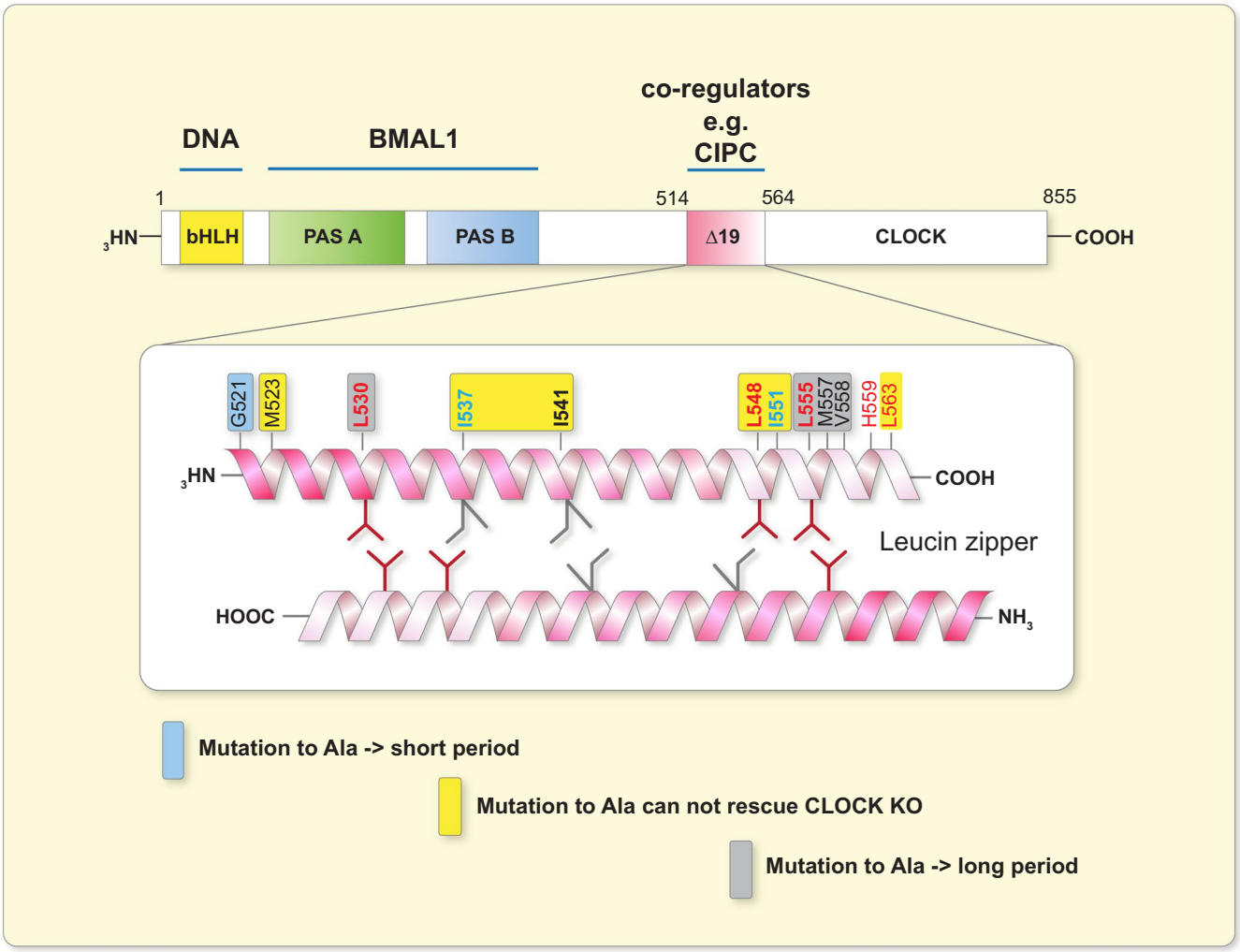

FIGURE 1 Scheme of the mouse CLOCK protein and its known structural domains. The CLOCK protein interacts via its basic helix-loop-helix (bHLH) domain with DNA and via its PAS A and PAS B domains with BMAL1. The Δ19 domain, encoded by exon 19 of the mouse Clock gene is able to form a leucine zipper structure consisting of two antiparallel CLOCK entities (enlarged in graded pink colours). To this structure co-regulators, such as CIPC, can bind and modulate CLOCK function. Bold letters indicate conservation across species, red letters indicate amino acids crucial for normal transactivation and blue letters indicate amino acids with other roles than transactivation in the negative feedback loop

either via binding of kinases and/or co-regulators such as CIPC,[5] MLL1,[6] PASD1[7] and other so far unidentified factors. The tools shared by Abdo et al[1] can be adapted to identify novel functionally important interacting partners of the CLOCKΔ19 domain to unravel circadian clock machinery.

CONFLICT OF INTEREST

The author has declared no conflict of interest.

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