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REVIEW

Transcription factors of the core feedback loop in the molecular circadian clock machinery: internal timekeeping and beyond

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To function more efficiently amid oscillating environmental conditions related to alternating day and night cycles, the circadian clock system developed as an adaptative strategy, serving temporal regulation of internal processes, by anticipating daily recurring changes. At the basis of the circadian clock is a 24-hour oscillation of the expression of clock genes, organized into interconnected self-regulatory transcriptional-translational feedback loops, present throughout the cells of the body, organized into a hierarchical system. Complex combinatorial mechanisms of gene expression regulation at pre-transcriptional, transcriptional, post-transcriptional and post-translational level offer stability and flexibility to the system, responsive to the actual conditions. The core clock genes CLOCK/NPAS2, ARNTL1/ARNTL2, PER1/PER2/PER3 and CRY1/CRY2 encode transcription factors responsible for generating the circadian rhythm in the molecular oscillator machinery, but beyond internal timekeeping, additional functions through gene expression regulation and protein interactions provide them key roles in basic mechanisms like cell cycle control or metabolism, and orchestration of complex physiological or behavioral processes. Elucidation of these intricate regulatory processes, the role of genetic variations as well as clock desynchronization associated with modern lifestyle, promise important medical implications, from a deeper understanding of etiopathology in rare inherited or common adult disorders, to a better management by the application of chronotherapy.

Keywords: circadian clock, transcriptional-translational feedback loop, core clock genes

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The alternating day-and-night cycle induces circadian rhythms of molecular, physiological and behavioral processes, in most if not all organisms on Earth [1,2]. For better energetic fitness amid the oscillating external conditions, an internal timekeeper-the cell-autonomous circadian clock developed, and organized into a hierarchical system, as an adaptative strategy, serving temporal regulation of internal processes by anticipating daily recurring environmental changes [3-6].

At the basis of the circadian clock is a 24-hours oscillation of clock gene expression, delivering maximums and minimums of specific protein levels once every day [3,7]. The molecular circadian rhythm is generated and maintained by the core clock genes organized in regulatory loops controlling themselves and each other, present in the various cells throughout the body [3,8]. Complex combinatorial mechanisms of gene expression regulation at various levels and interlocked regulatory loops ensure both stability and plasticity in varying environmental conditions [9,10]. Organized into a hierarchical system consisting of a central and peripheral clocks, the circadian system in mammals is controlled by the hypothalamic suprachiasmatic nucleus (SCN) master clock that serves as pacemaker generating the rhythm, reset daily by light - the primary environmental zeitgeber, and synchronizing the peripheral slave oscillators in the various organs; a yet not fully elucidated hierarchical multi-oscillator structure is suggested by further extra-SCN food-entrainable and methamphetamine-induced brain oscillators [11,12].

In the recent years, chronobiology underwent a spectacular development, with novel layers of regulatory mechanisms and clock protein functions identified, and genetic variation as well clock desynchronization associated with modern lifestyle recognized as etiopathogenic elements of common adult disorders [6,12-14]. Familiarity with this fundamental molecular mechanism may find utility in the most varied fields of medicine, from opening new directions in research to optimizing health and patient management by application of chronotherapy [15-17].

The core transcriptional-translational feedback loop of the molecular oscillator

The circadian clock is an internal oscillator with a period of 24 hours. It is composed of a small set of clock genes, interacting with each other, organized in auto-regulatory feedback loops similar to the cogwheels of mechanical watches, in which one activation-repression cycle lasts approximately a day, resulting in circadian molecular oscillations; moreover, the circadian clock system is able to respond and adapt to environmental changes, reset by various zeitgebers, most importantly light and food [3,7,8].

Cyclicity of the circadian molecular oscillator is generated by the expression of the core clock genes encoding transcriptional activators and repressors organized in autoregulatory transcriptional–translational feedback loops (TTFLs). In mammals, the core TTFL comprises the transcription factors CLOCK/NPAS2, ARNTL1/ARNTL2, PER1/PER2/PER3 and CRY1/CRY2 (Table I), acting as

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heterodimer complexes, CLOCK/NPAS2:ARNTL representing the positive, and PER:CRY the negative arm. The antiphase oscillation of activator and repressor gene expression lasts about 24 hours, generating a "circa-diem" rhythm, perpetual by moving to-and-from equilibrium. [2,3,8,12]

Though transcription-translation of the core clock proteins is essential for rhythm generation, the complex control of gene expression at various levels by epigenetic, post-transcriptional and post-translational mechanisms are increasingly considered critical, possibly even the main driving force, besides acting as major determinants of period length; actually, only about 25% of circadian mRNA oscillation appears explained by de novo transcription, and the key casein-kinases CK1D and CK1E of clock protein phosphorylation are considered components of the core oscillator [18,23-25]. Post-translational modifications (PTMs) are especially important for protein function and have been well characterized in the recent years [9,10]. Since synthesis happens in the cytoplasm, translocation to the nucleus of clock proteins acting as transcriptional regulators is essential, which depends on heterodimerization and phosphorylation; heterodimerization is based on interaction by the PAS domains characteristic for clock protein structure. Cyclical proteasomal degradation mediated by ubiquitination of the clock proteins is a key mechanism determining period length. Epigenetic mechanisms of rhythmic chromatin remodeling, the role of mRNA processing, RNA interference or even RNA editing involved in the clock function are continuously deciphered [24,26-28].

The positive arm of the core feedback loop consists of the transcriptional activators CLOCK or its paralog NPAS2, and ARNTL1 or its paralog ARNTL2, acting as heterodimers. CLOCK/NPAS2 and ARNTL1/ARNTL2 are bHLH–PAS type transcription factors, in which the highly conserved domains bHLH serve DNA-binding, while PAS allow dimerization with other PAS-domain containing proteins, and thus CLOCK/NPAS2:ARNTL1/ARNTL2 complex formation. Upon phosphorylation and nuclear translocation, CLOCK:ARNTL activates the transcription of target genes by binding in their upstream regulatory sequences to the canonical E/E'-box enhancer elements (5'-CACGT(G/T)-3', or non-canonical motifs) [3,8,29-31]. Within the preferred element, CLOCK/NPAS2 binds to 5'-CAC-3', while ARNTL to the second half [32]. By E/E'-boxes contained in the targeted gene regulatory sequences, CLOCK/NPAS2:ARNTL1/ARNTL2 is considered to regulate approximately 10% of the human genes in a coordinated manner: the clock genes of the circadian molecular oscillator and the clock-controlled genes (CCGs) throughout the genome. It activates the transcription of the clock genes *PER1/PER2/PER3* and *CRY1/CRY2* in the core TTFL, as well as *REVERBA/ROR*, *DBP/TEF/HLF* and *DEC1/DEC2* in the auxiliary TTFLs, following a repressor-precedes-activator pattern [33]. At the level of target gene promoters, DNA-binding of CLOCK:ARNTL, along CBP (CREB-binding protein) recruitment and H3-acetylation, present significant circadian fluctuation, resulting in daily transcriptional oscillation; DNA-binding affinity influenced by the cellular redox state, nutrient and energy status allows entrainment by feeding or neuronal activity [34,35].

Circadian chromatin remodeling, fluctuation of various histone modifications - acetylation or methylation on key H3-lysine positions, enhancer RNAs facilitating the rhythmic binding of transcription factors have been observed mediating a coordinated epigenetic regulation [9]. Chromatin remodeling is closely linked to CLOCK:ARNTL1-activated transcription. The acetyl-transferase activity of CLOCK causes acetylation of histone and nonhistone proteins, including its partner ARNTL1. Histone-acetylation delivers rhythmic circadian chromatin opening and DNA accessibility necessary for transcription; in presence of ARNTL1, CLOCK histone-acetyltransferase activity quadruples. JumonjiC and JARID1A (ARID domain-containing histone lysine demethylase-1a) regulate the process, forming a complex with CLOCK-ARNTL1 at target gene promoters, inhibiting histone-deacetylation [3,18,21,26,27]. Epigenetic changes allow also translation of environmental effects on gene expression. The CLOCK-mediated acetylation and deacetylation especially by the NAD^+-dependent SIRT1

Table I. Transcription factors of the core circadian molecular oscillator

Clock Protein-UniProtKB(18) (alternative names)	Gene-ID Entrez(19)/HGNC(20)/OMIM(21)/Ensembl (22)	Locus (21)
Circadian locomotor output cycles kaput protein/CLOCK-O15516 (BHLHE8/KAT13D-lysine acetyltransferase)	*CLOCK*-9575/2082/601851/ENSG00000134852	4q12
Neuronal PAS domain containing protein 2/NPAS2-Q99743 (BHLHE9/ MOP4/PASD4)	*NPAS2*-4862/7895/603347/ENSG00000170485	2q11.2
Aryl hydrocarbon receptor nuclear translocator-like protein-1/ARNTL(1)-O00327 (Brain and muscle ARNT-like protein 1-BMAL1/BHLHE5/MOP3/PASD3)	*ARNTL(1)*-406/701/602550/ENSG00000133794	11p15.3
ARNTL2-Q8WYA1 (BMAL2/BHLHE6/MOP9/PASD9/CLIF-CYCLE-like factor)	*ARNTL2*-56938/18984/614517/ENSG00000029153	12p11.23
Period Drosophyla homolog or period circadian 1/PER1-O15534	*PER1*-5187/8845/602260/ENSG00000179094	17p13.1
PER2-O15055	*PER2*-8864/8846/603426/ENSG00000132326	2q37.3
PER3-P56645	*PER3*-8863/8847/603427/ENSG00000049246	1p36.23
Cryptochrome circadian regulator 1/CRY1-Q16526	*CRY1*-1407/2384/601933/ENSG00000008405	12q23.3
CRY2-Q49AN0	*CRY2*-1408/2385/603732/ENSG00000121671	11p11.2

bHLH: basic helix-loop-helix; PAS: Per-ARNT-Sim (period-ARNT-single-minded); MOP: member of the PAS-family

appear as central mechanisms by which the clock system orchestrates rhythmic gene expression, adapting it to the actual nutrient and energy supplies [35,36].

The negative arm of the core TTFL is formed by PER1, PER2, PER3 and CRY1, CRY2. PER proteins also contain the evolutionarily conserved PAS motifs of the clock system, allowing homo- and heterodimerization, including PER:CRY formation. The CLOCK/NPAS2:ARNTL1/ARNTL2 complex activates the transcription of *PER* and *CRY* genes by their E-box regulatory sequences. Upon accumulation in the cytoplasm as the result of translation, after heterodimerization and translocation to the nucleus, PER:CRY inhibits the CLOCK:ARNTL-induced transcription, including its own, by various mechanisms. Once PER and CRY transcription is inhibited by the negative feedback mechanism, and the proteins are degraded as a result of PTMs, the CLOCK:ARNTL activator complex is released from the PER:CRY repression, and may restart a new cycle. (Figure 1) Since the cycle lasts approximately 24 hours, a circadian rhythm results [3,7,8,12,29-31].

PER and CRY form large complexes in the cytoplasm, that contain also other proteins necessary for function, including CK1D and CK1E for phosphorylation, splicing factors and helicases that promote transcriptional termination and re-initiation repression [10,18,21]. PERs repress transcription by direct interaction with CLOCK:ARNTL, facilitating recruitment of epigenetic modulators to the E-box elements - first histone deacetylases, and then H3K9 histone methyltransferases, resulting in chromatin remodeling, but also independently of their transcriptional activators by interacting with RNA-binding proteins and helicases recruited to the transcriptional termination site, impeding the release of RNA-polymerase and re-initiation of transcription [18,21,27]. CRYs act independently of PERs and DNA-binding, by protein interactions and epigenetic modifications, repressing expression through histone deacetylation by HDACs. CRY1 can bind to the transactivation domain of ARNTL, at binding sites for CBP and p300 histone acetyl-transferases, suppressing CLOCK:ARNTL transcriptional activation, as a gatekeeper maintaining the transcriptional activator in a repressed state until its opportune activation [7,18,21]. CRY is described as the primary repressor within the core TTFL, binding to CLOCK:ARNTL at the promoter, and blocking transcription without dissociating the complex, while PER alone may have no effect on CLOCK:ARNTL transcription activation, but in the presence of CRY, displaces the activator complex from the promoter [37].

In the SCN neurons, CLOCK level is steady throughout the day, while ARNTL expression is high and low at the beginning of subjective day and night, respectively; thus oscillating CLOCK:ARNTL levels activate transcription of target genes differently dependent on the time of the day. Due to transcriptional activation by CLOCK:ARNTL, CRY accumulates by the end of the day, promoting the formation of stable PER:CRY complexes and nuclear translocation at the beginning of the subjective night [12,37]. While *PER2* promoter activation is followed immediately by transcription, expression of *CRY1* is delayed by about 4 hours. Critical for phase regulation,

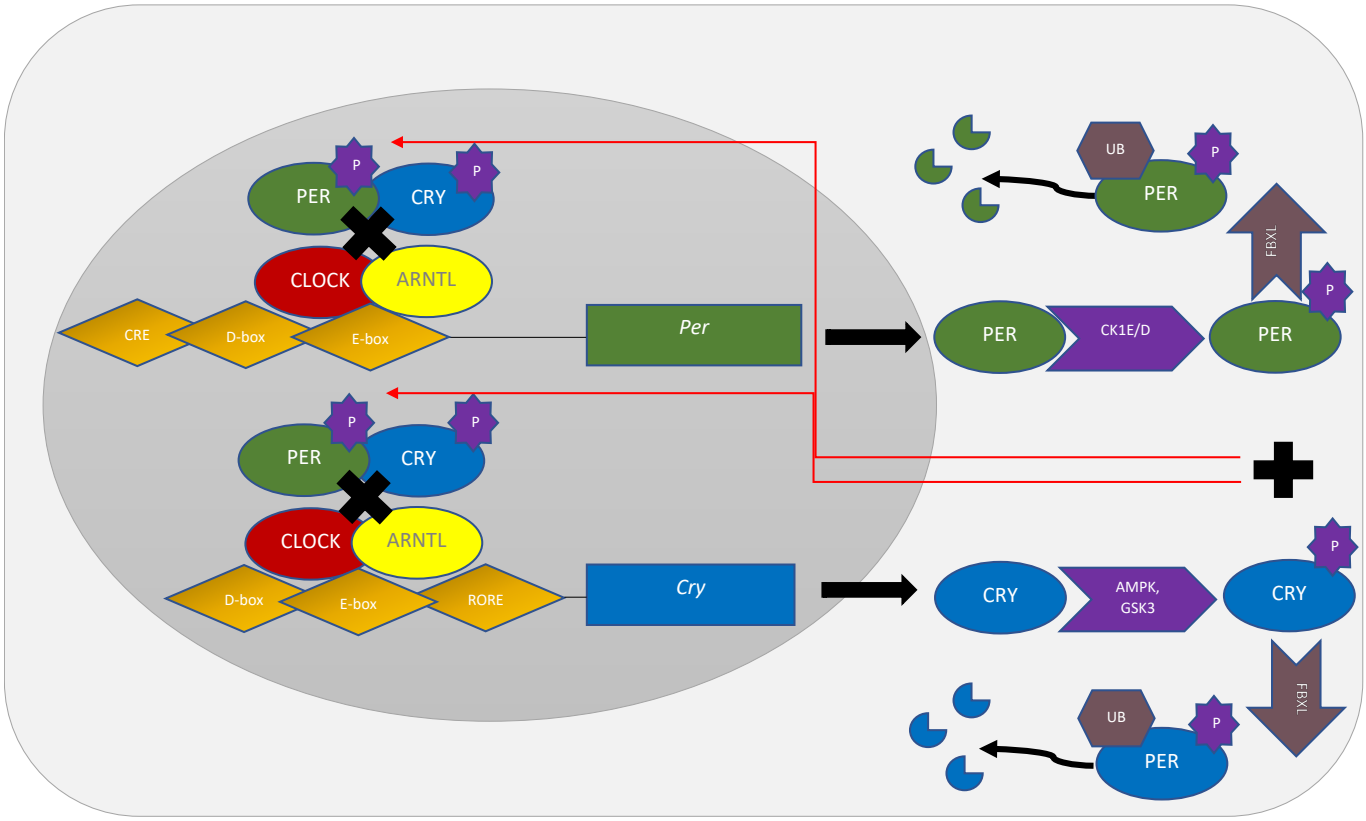

Fig. 1. Core TTFL of the circadian molecular oscillator (P-phosphorylation, UB-ubiquitination)

this results from its regulatory sequence structure characterized by a combination of E and D-boxes in the promoter (day-time elements; regulation through D-box follows a repressor-antiphasic-to-activator mechanism) and RREs in intron 1 (night-time elements), that allow a coordinated modulation by clock proteins within the core and auxiliary TTFLs [27,33,38-40]. Transcriptional regulation by environmental signals of the core TTFL genes is possible by CRE (cAMP response element) and GRE (glucocorticoid responsive element) contained in *PER* and *CRY* regulatory sequences. Photo-entrainment of the master clock – the regulation by light of the molecular oscillators within the SCN neurons - is produced by CREs of *PER1* and *PER2* within the core TTFL.

Stability and adaptability of the circadian system is served by auxiliary TTFLs interconnected with the core regulatory loops (Figure 2). CLOCK/NPAS2:ARNTL activates through E-boxes also the transcription of *REV-ERBalpha/beta* and *RORA/RORB/RORG, DBP, HLFl* and *TEF, DEC1* and *DEC2*. On their turn, though RORE [retinoic acid receptor-related orphan receptor enhancer), RevERBs repress, while their competitor RORs activate the transcription of target genes, including *ARNTL* (and *CLOCK*) in a second feedback loop (Bmal1 loop, antiphasic to the core TTFL). DBP (D-box binding PAR bZIP transcription factor), HLF and TEF activate, while NFIL3 (nuclear factor, interleukin 3 regulated) repress the transcription of target genes containing D-boxes. DEC1 and DEC2 (differentially expressed in chondrocytes 1, 2) constitute the negative elements of the DEC loop, in phase with the core TTFL, repressing transcription through E box elements. Additionally, clock proteins control CCGs throughout the genome in a coordinated circadian manner through their specifically targeted regulatory sequences: E-boxes by CLOCK/NPAS2:ARNTL1/ARNTL2 and DEC1/DEC2, D-boxes by DBP, HLF, TEF and NFIL3, and ROREs by REVERBs and RORs [7,8,12,33].

Post-translational modifications

Novel methodologies and gene expression profiling data suggest that regulation at PTM level may be implicated in 75% of cycling [9]. Core clock protein PTMs are critical for nuclear translocation and stability necessary for function, determination of periodicity and period length. The various modifications include phosphorylation of all components in a daytime-dependent manner, ubiquitination,

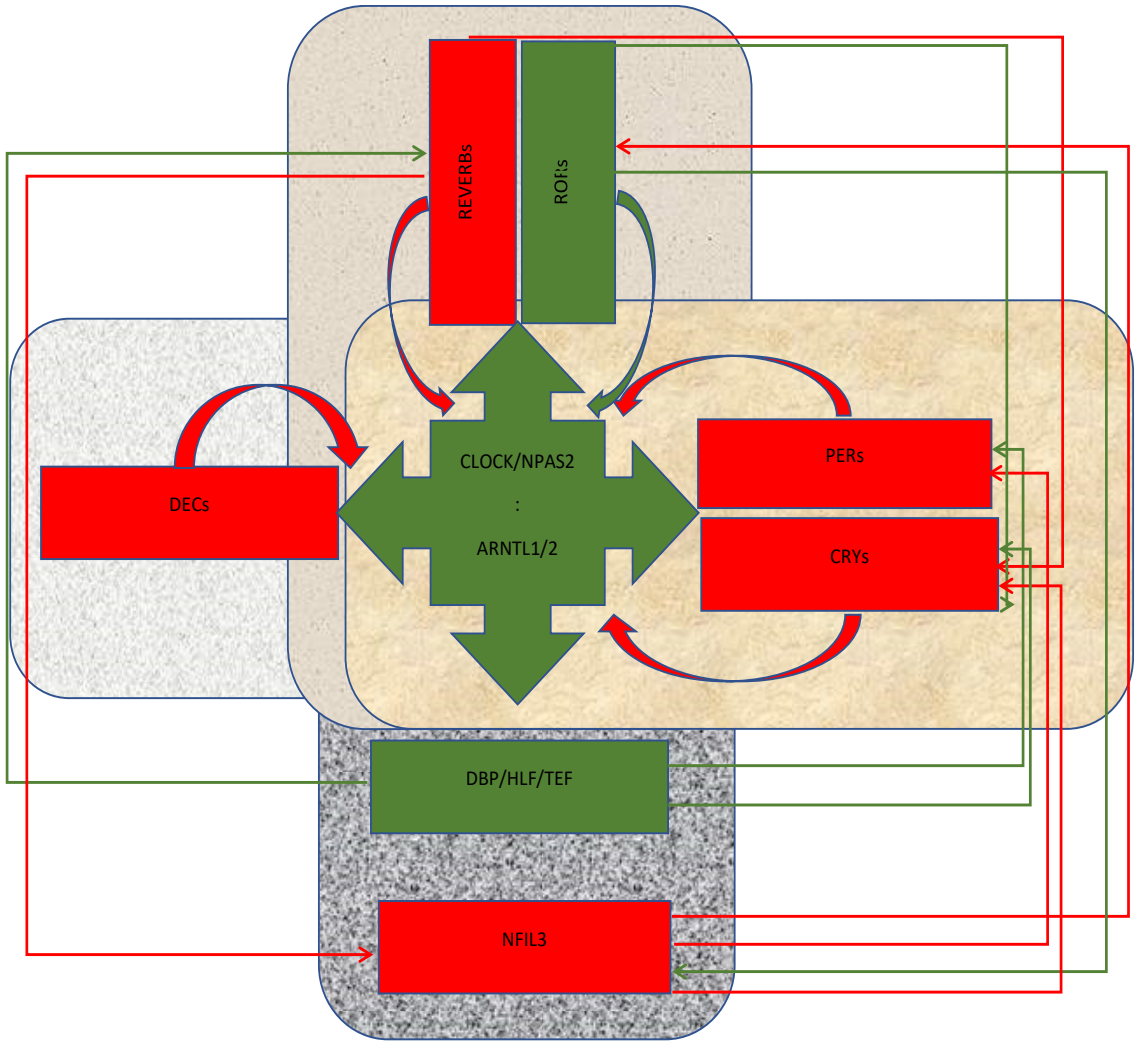

Fig. 2. Interconnected core and auxiliary TTFLs: the central role of CLOCK:ARNTL (green-activation/red-repression)

acetylation of certain components (ARNTL1, PER2), sumoylation, poly-ADP-ribosylation. Post-translational changes target specific amino-acid positions and follow a strict dynamic. They determine cellular sub-compartment localization - cytoplasm-nuclear shuttling, influence function - transcriptional activity, and impact stability-degradation [7,9,10,16,18,21].

CLOCK nuclear shuttling is under circadian regulation, dependent on heterodimerization with ARNTL and phosphorylation. Phosphorylation shows circadian variations, is carried out by CK1D/E, resulting in nuclear localization, enhanced transcriptional activity, and decreased stability with proteasomal degradation; the non-phosphorylated form is characteristic for the cytoplasm. Sumoylation - stimulated by estrogen - enhances transcriptional activity [18,21,41].

ARNTL phosphorylation by CK2 shows circadian variations, peaking at noon, is essential for nuclear localization and activity, while phosphorylation by GSK3B decreases stability, leading to ubiquitination. Sumoylation with rhythmic variations, peaking in the morning, promotes ubiquitin-dependent proteasomal degradation. Acetylation by CLOCK facilitates CRY1-recruitement and transcriptional repression; deacetylation by SIRT1 results in reduced stability [18,21,41].

Nucleo-cytoplasmic shuttling of PER and CRY depends on heterodimerization and phosphorylation. In the nucleus, stability is regulated by phosphorylation-dependent ubiquitination, mediated by E3-ubiqutin ligases, containing βTRCP1 and FBXL21 in case of PER, and FBXL3 and FBXL21 in case of CRY, ultimately regulating periodicity. PER1:PER2 heterodimer formation results in cytoplasmic retention. PER serine-phosphorylation by CK1E/D controls transcriptional repressor function and degradation. Differential phosphorylation events may result in opposite phenotypes - short or long periods. PER2 deacetylation by SIRT1 results in decreased stability. PER ubiquitination with proteasomal degradation is inhibited by CRY1; CRY phosphorylation by CK1E requires interaction with PER. CRY1 phosphorylation by AMPK on various sites is important for CLOCK:ARNTL inhibition and regulation of protein stability. Coordinated ubiquitination at different lysines by FBXL3 or FBXL21 containing E3 ubiquitin ligases (SCF - SKP1-CUL1-F-box protein) regulates the balance between degradation and stabilization: the mainly nuclear SCF(FBXL3) complex mediates degradation, while the mainly cytoplasmic SCF(FBXL21) complex-mediates stabilization, counteracting SCF(FBXL3) [12,18,21,23,41,42].

Core clock transcription factors: intricate roles in internal timekeeping and fundamental biological processes

In addition to generating molecular circadian rhythms and controlling physiological processes as an output of the central and peripheral clocks, clock gene products have additional roles [3]. Besides the core and ancillary TTFLs delivering molecular oscillation and internal timekeeping, the core clock transcription factors control hundreds of CCGs [9,42]. The circadian variation of molecules by transmitting timing information intra- and intercellularly serve physiological optimization - energy use and separation of incompatible processes [12,35]. Novel high throughput analysis showed that in mammals almost half of the protein-coding genes may manifest transcriptional circadian rhythms, mostly in an organ-specific manner, with expression peaking before dusk and dawn ("transcriptional rush hours"). Oscillating genes often cluster together, are longer, and have more splice forms. Oscillation characterizes also the expression of hundreds of non-coding RNA genes [14].

The circadian clock has widespread integrative roles by regulation of physiological and behavioral processes, from DNA repair to sleep-wake cycles - melatonin and glucocorticoid synthesis, from thermoregulation to cognition, from cardiovascular, hepatic and renal function to immunity or fertility. Alterations by genetic variants or environmental desynchronization have similarly widespread implications, from metabolic diseases to cancer or neuropsychiatric disorders [5,6,13,16,43-47].

Cell signaling, energy metabolism and the cellular redox state show circadian variation, glycolysis, fatty acid metabolism, cholesterol biosynthesis are under circadian regulation. The crosstalk between the clock and metabolism is so complex and intricate that discrimination between circadian and metabolic oscillations appears arbitrary; the main links seem redox- and temperature-dependent transcription factors, NAD-dependent enzymes, nutrient-sensing kinases, epigenetic modulators and transcription factors (e.g. CLOCK/NPAS2, SIRT1, AMPK, p300, CBP of those previously mentioned), and the signaling is bidirectional [34,35,43,44,48]. Over half of the nuclear receptors demonstrate tissue-specific circadian rhythms, and the circadian control of nuclear receptor expression may provide key nodal points for orchestrated regulation (e.g. the transcriptional coactivator PGC-1a is a key candidate for integrating clock and metabolic functions) [3,49]. Animal models, epidemiological data and genetic association studies indicate that chronic circadian rhythm disruption associates with metabolic disease risk, while time-restricted feeding may be beneficial; recently, the metabolic syndrome was suggested to be renamed as the "circadian syndrome" [6,13,16,21,41].

CLOCK: transcription factor with acetyl-transferase activity, widespread roles from internal timekeeping to metabolism and behavior

The CLOCK circadian regulator is a transcription factor with lysine acetyl-transferase activity, expressed ubiquitously, constitutively. Besides the activation of clock gene expression within the interlocked TTFLs, it appears to regulate about 150 CCGs [18,19,41]. Animal experi-

ments demonstrated major though not an indispensable role in the circadian clock, besides important functions in metabolism, sleep homeostasis and behavior (clock mutant mice represent a model for human mood and behavior disorders). Clock-null mice show circadian rhythms, even in constant darkness, its paralog NPAS2 being considered to substitute functions in the SCN. In knock-out animals, mania-type hyperactivity and increased cocaine/sucrose reward-value develops, treated by lithium. Overexpression shortens, while mutations lengthen the period and reduce the amplitude, associated with hyperphagia-obesity and impaired glucose tolerance [21,47]. In humans, genetic polymorphisms are described in association with obesity and hypertension (e.g. rs10462028, rs861029, rs57826934), sleep and behavioral changes – insomnia, delayed sleep phase disorder (DSPD), diurnal preference, seasonal depression (rs6832769) [3,21,41]. It is involved in several processes, from hair follicle cycling to spermatogenesis, lipid and lipoprotein metabolism, pancreatic beta-cell development, DNA damage localization and cell-cycle checkpoint control, positive regulation of inflammatory response and NF-kappaβ activity. It drives the circadian rhythm of blood pressure controlling ATP1B1 expression. It interacts among others also with the clock gene CIPC and various transcription factors, the p35/CDK5 complex. CLOCK acetylates histones and nonhistone proteins, and interacts with other acetyl-transferases and deacetylases, allowing widespread epigenetic control of gene expression, but also acetylation of various proteins in a circadian manner (e.g. ASS1 leading to the circadian oscillation of arginine biosynthesis and ureagenesis); by acetylation it represses glucocorticoid-induced transcription [18,19,21,41].

The CLOCK paralog NPAS2 is expressed highly in the brain. It binds heme and HSP90. It is important for sleep homeostasis, clock synchronization with the light cycle when food is plentiful, and shifting the circadian rhythm to food availability when access is restricted. Additionally, NPAS2 is involved in CNS development, memory formation and fear response, mediates the diurnal variation in GABA-receptor expression, and activates MAOA transcription. It is implicated in the cellular response to DNA damage, promoting repair, and inhibiting cell death. It also affects hepatic lipid and drug metabolism. Polymorphisms may associate with anxiety, seasonal depression, diurnal preference (SAD-seasonal affective disorder, MIM:608516) and nonobstructive azoospermia (MIM:603347) [3,18,21,41].

ARNTL – a key element of rhythmicity, circadian clock and metabolism crosstalk, cell cycle control, neurogenesis, immunity, fertility

ARNTL1 is a transcription factor with ubiquitous expression, that may dimerize besides CLOCK/NPAS2 also with HIFs (hypoxia inducible factors). It binds HSP90. It is an essential clock component, necessary for circadian rhythm generation, though some redundancy with ARNTL2 exists. Its loss results in immediate and complete arrhythmicity in constant darkness. In the hypothalamus it appears critical for adaptation to scheduled food restriction. It is the only clock gene resulting in knockout mice in molecular and behavioral arrhythmicity, associated with reduced bodyweight and lifespan. It regulates the circadian expression of CIART, which blocks its binding to the transcriptional co-activator CBP [18,21,41].

ARNTL1 targets an estimated 150 sites in the human genome. Besides transcriptional activator effects shared with CLOCK/NPAS2, ARNTL1 has distinct tissue-specific functions. It is involved in the positive control of Wnt and negative regulation of mTOR signaling – critical elements of linking the clock function with the cell cycle and metabolism [18,21,51]. It positively regulates myogenesis, triglyceride synthesis in the muscle, negatively regulates cold-induced thermogenesis, adipogenesis, while in the pancreas it regulates beta-cell development and insulin secretion. It is involved in the negative regulation of glucocorticoid-receptor signaling, and spermatogenesis or ovarian function by the regulation of key enzymes. It regulates hair growth, and is important for hippocampal neurogenesis. It controls diurnal oscillations of chemokine expression and B-cell differentiation, protection against sepsis, gut defense. It is involved in oxidative stress-induced premature aging, and represents a key link between the circadian clock and the cell cycle-cancer development by regulating expression of CCGs such as *c-Myc*. Genetic variants have been described in association with alterations of sleep pattern and fertility, diseases like obesity, diabetes, hypertension or bipolar disorder [16,18,19,21,41,52].

The Bmal1 loop grants the molecular oscillator stability and the possibility of synchronization according to external and internal cues. Though it is not necessary for rhythm generation, it is crucial for the crosstalk between the circadian clock and metabolism. Suggesting significant functional overlap, REV-ERBs share recognition at over 50% of their DNA-binding sites with ARNTL1 [7,8,12].

ARNTL2 is an ARNTL1 paralog with a 45% identity, differing in the N-terminal region required for nuclear localization, showing widespread constitutive expression. Besides the circadian rhythm, interacting with HIF1A it is involved in the response to hypoxia. Genetic variants (rs4246243, rs4964058, rs111392859) were described in obesity and sleep disturbances [18,21,41].

Period circadian regulators - the light-responsive elements of the core molecular oscillator with key roles in metabolism, vascular and neural functions, DNA damage response

The period circadian regulators PER1, PER2 and PER3 encode the key pacemakers of rhythm generation, representing the light-responsive components of the molecular clock, responsible for photoentrainment. By their PAS-domains, they may form homo- and heterodimers,

competing with TIMELESS for binding CRY1. Various large PER complexes exist that deliver different functions, the core being composed of PER1, PER2, PER3, CRY1, CRY2, CK1D/E. Complexes involved in transcriptional repression contain at least PER2, CDK9, DDX5, DHX9, NCBP1, POLR2A, while those for histone deacetylation and demethylation PER2, HDAC1, SFPQ, SIN3A and PER2, CBX3, TRIM28, SUV39H, respectively. Besides repression of CLOCK/NPAS2:ARNTL-induced transcription, they may act as activators of gene expression in a tissue-specific manner. mRNA and protein levels present circadian oscillation both in central and peripheral clocks critical for rhythm generation, and mutations may shorten, lengthen, or abolish the period [18,21,41].

Though redundancy and compensation among PER-family members exist, all paralogs have distinct and complementary functions, and regulate each other. PER1 functions at post-transcriptional level by protein interactions, regulating the stability of other circadian proteins including the decay of PER2. PER2 controls clock gene expression at transcriptional level, and regulates PER1. While PER1 is broadly expressed in neuronal and non-neuronal populations, PER2 is expressed more in glial and dentate gyrus progenitor cells. Light inducibility characterizes both PER1 and PER2; though overlapping, expression is asynchronous by 4-hours. Mice deficient in both PER1 and PER2 lack circadian rhythms [18,21,41].

PER1 is widely expressed in a circadian pattern, at highest level in skeletal muscles. PER1-containing neurons sustain an electrically excited state without firing, unlike those without it that present daily variations of firing activity. Besides acting as a master circadian rhythm regulator, it has key roles in long-term memory formation, modulation of neuroinflammation through NF-kappa-B-signaling, hair follicle cycling, repression of glucocorticoid receptor-induced transcriptional activation, regulation of genes involved in renal sodium reabsorption. Overexpression in cancer cells causes growth reduction, sensitizes to DNA damage-induced apoptosis. Mutations were associated with different chronotypes (e.g. rs7221412) and sleep disorders, as well as ADHD, hypotrichosis or breast cancer [18,19,21,41].

PER2 is widely expressed, with circadian rhythmicity, at high levels in skeletal muscles and the pancreas, increased by light exposure at night in the SCN and eyes; diurnal oscillations are critical for circadian rhythm generation both in central and peripheral clocks. It is important for mediating clock information to metabolic pathways by interaction with nuclear receptors (e.g. HNF4A, PPARA/G). It is involved in the control of gluconeogenesis, glycogen synthesis, insulin secretion, fatty acid and lipid metabolism, it negatively regulates adipogenesis by blocking PPARG-recruitment, and positively regulates cold-induced thermogenesis. PER2 links the circadian clock and estrogen signaling, attenuating it. It is involved in NO and prostaglandin production, controlling vasoconstriction and the response to ischemia. It links the reciprocal regulation of the clock system and heme synthesis. It contributes to neurogenesis, neuronal impulse transmission by controlling VGLUT1 expression and glutamate uptake, limbic emotional and motivational processes. It controls the cell cycle and DNA damage response by circadian regulation of Cyclin D1/A, Mdm-2, Gadd45α, or transcription of *c-Myc* through its E box [16,18,19,21,53]. Polymorphisms associate with sleep disorders, obesity and tumors, its dysfunction was described in alcoholism and depression. A missense mutation within the CK1E phosphorylation site associates with the autosomal dominant FASPS1 (familial advanced sleep phase syndrome 1, MIM:604348) characterized by a 4-hours advanced onset/offset of sleep, temperature and melatonin rhythms ("morning lark" phenotype) [21,41,54].

The SCN circadian clock is reset by photic signals from the melanopsin-containing intrinsically photosensitive retinal ganglion cells received through the retino-hypothalamic tract. By the neurotransmitter glutamate-induced cAMP level changes leading to binding of CBP to CRE in the clock genes *PER 1, 2* and *DEC1*, photic induction is possible. After light exposure, *PER1* and *DEC1* are transcribed immediately, with a short mRNA half-life, while *PER2* expression appears later, with a high mRNA level, maintained for a longer period demonstrating non-redundant roles [12].

PER3 is not necessary for circadian rhythm maintenance, but important for sleep homeostasis and mood regulation. Expression oscillates in the SCN and eyes, but not induced by light exposure at night. Since expression oscillates during embryogenesis, reset by light after rhythmical mRNA accumulation in oocytes that persists in the embryo, phase inheritance through oogenesis may exist. It binds heme. Two heterozygous missense mutations at conserved residues within the same allele resulting in decreased stability causes FASPS3 (MIM:616882), associated with seasonal depression (Pro415Ala-rs139315125 and His417Arg-rs150812083 appear with a populational frequency of 0.55%). The 54-bp VNTR polymorphism causing the variable number of a 18-aminoacid repeat located in the CK1E phosphorylation site with at least two different alleles (PER3.4/PER3.5) influences the sleeping and feeding behavior. With important populational differences, approximately 50 and 10% are PER3.4- and PER3.5-homozygous, characterized by evening and morning preference [18,19,21,41,47].

Cryptochromes: gatekeepers of activation within the molecular oscillator˝and modulators of metabolism, DNA-damage repair

Cryptochromes found in all species are highly conserved blue light-sensitive flavoproteins derived from photolyases, involved in the circadian rhythm and possibly magnetic field-sensing in some species. In higher eukaryotes, the photolyase activity repairing UV light-induced DNA

damage is absent, while photoreceptor role in mammalian peripheral tissues remains controversial. They contain cofactor binding sites for FAD (flavin adenine dinucleotide) and MTHF (5,10-methenyltetrahydrofolate) [18,21,41].

In the core TTFL loop, CRY1 and CRY2 function as light-independent inhibitors of CLOCK:ARNTL-induced transcription, with a central role in circadian rhythmicity. They are widely expressed, rhythmically also in constant darkness - with a maximum during the light and a minimum in the dark phase within the eye and SCN. Homozygous *CRY1* and *CRY2* double-mutant mice have normal circadian locomotor activity and feeding rhythms when exposed to light-dark cycles, but abolished rhythmicity of body temperature, oxygen consumption, heart rate and decreased insulin secretion [18,21].

In maintaining period length and circadian rhythmicity, the balance between CRY1 and CRY2 appears critical. With an amino-acid sequence identity of 73%, they have redundant and specific functions in defining the pace of the master clock. They inhibit CLOCK:ARNTL probably by direct contacts between CRY1-ARNTL and CRY2-CLOCK, respectively. CRY1 is a stronger repressor, lengthening the period, while CRY2 dispensable for rhythm generation, is necessary for synchrony by intercellular network development, and critical for the central clock period by opposing CRY1; in total darkness, $Cry1^{-/-}$ mice have a faster and $Cry2^{-/-}$ a slower clock [18,21,41].

CRY1 is able alone to sustain circadian rhythms, appearing essential for rhythm generation in the retina. By interacting with various proteins, it has several functions: it interacts directly with clock genes PER and TIMELESS, histone deacetylases blocking transcription, nuclear receptors repressing their function (e.g. AR, NR3C1/GR, RORA/C, PPARG/D/A, VDR). It is involved in the transcriptional regulation of genes involved in metabolic pathways (e.g. LEP), circadian regulation of cAMP-signaling, negative regulation of G protein-coupled receptor signaling. By various mechanisms, it has key roles in the modulation of metabolism or insulin response, with negative effect on gluconeogenesis and lipid storage. It mediates the clock-controlled activation of ATR, central for DNA-damage signaling. Polymorphisms are associated with altered sleep pattern, brain development and function, diseases like winter depression, retinitis pigmentosa. The autosomal dominant DSPD (MIM:614163) by exon-skipping caused gain-of-function mutation with increased nuclear localization produces period lengthening with sleep-onset insomnia and waking difficulty (0.6% of the population, rs184039278) [18,20,21,41].

CRY2 expressed highest in muscles, interacts with clock proteins as well as key nuclear receptors similar to CRY1, mediates the circadian control of cAMP-signaling, modulates metabolism, represses glucocorticoid receptor-induced transcriptional activation, and is involved in the control of sodium-dependent phosphate transport. Mutations may associate with sleep and mood disorders, cognitive deficit (rs12805422) and metabolic problems (e.g. rs11038428, rs11605924) [18,19,21,41,47].

In conclusion, the core elements-transcription factors of the molecular oscillator machinery are responsible for circadian rhythm generation in the central and peripheral clocks, with a certain amount of redundancy-paralogs and interconnected loops, multilevel control for flexibility. Besides the molecular oscillation and internal timekeeping, responsive to environmental effects, additional functions by gene expression regulation and protein interactions render them key roles in basic mechanisms like cell cycle control or metabolism, and orchestration of complex physiological or behavioral processes. Elucidation of these intricate regulatory processes promises important medical implications, from a deeper understanding of etiopathology to a better clinical management.

Author's contribution

KCs - Conception and design of the manuscript, acquisition, analysis and interpretation of data, drafting, revising of the manuscript, final approval of the version to be published.

Conflict of interest

None to declare.

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