A Crosstalk between the Biorhythms and Gatekeepers of Longevity: Dual Role of Glycogen Synthase Kinase-3

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Abstract—This review discusses genetic and molecular pathways that link circadian timing with metabolism, resulting in the emergence of positive and negative regulatory feedback loops. The Nrf2 pathway is believed to be a component of the anti-aging program responsible for the healthspan and longevity. Nrf2 enables stress adaptation by activating cell antioxidant defense and other metabolic processes via control of expression of over 200 target genes in response to various types of stress.

The GSK3 system represents a “regulating valve” that controls fine oscillations in the Nrf2 level, unlike Keap1, which prevents significant changes in the Nrf2 content in the absence of oxidative stress and which is inactivated by the oxidative stress. Furthermore, GSK3 modifies core circadian clock proteins (Bmal1, Clock, Per, Cry, and Rev-erb\textalpha{}). Phosphorylation by GSK3 leads to the inactivation and degradation of circadian rhythm-activating proteins (Bmal1 and Clock) and vice versa to the activation and nuclear translocation of proteins suppressing circadian rhythms (Per and Rev-erb\textalpha{}) with the exception of Cry protein, which is likely to be implicated in the fine tuning of biological clock. Functionally, GSK3 appears to be one of the hubs in the cross-regulation of circadian rhythms and antioxidant defense. Here, we present the data on the crosstalk between the most powerful cell antioxidant mechanism, the Nrf2 system, and the biorhythm-regulating system in mammals, including the impact of GSK3 overexpression and knockout on the Nrf2 signaling. Understanding the interactions between the regulatory cascades linking homeostasis maintenance and cell response to oxidative stress will help in elucidating molecular mechanisms that underlie aging and longevity.

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adaptation and homeostasis maintenance mechanisms, including those providing biorhythm regulation. Interaction between diametrically opposed aging and anti-aging programs determines the shape of survival curves and their alterations in the course of biological and, in the case of humankind, cultural evolution. Over the last 150 years, the human mortality dynamics has drastically changed due to the scientific and technological revolution. These changes have resulted in a substantial lifespan prolongation. The survival curve has become almost rectangular, which clearly distinguishes it from the survival curves of chimpanzees, hunter-gatherers, and even the population of developed European countries in the 18th and 19th centuries (see [1] for details). However, there are also internal factors that influence the lifespan and the pattern of survival curves. They include aging and anti-aging programs, which, in terms of current concepts, represent a set of signaling cascades regulating gene expression [1-6]. If longevity is supported by natural selection (e.g., in the case when “longevity assurance genes” are associated with an adaptive trait and, therefore, become fixed in a population), living organisms can develops special defense and repair systems that slow down chronic phenoptosis. Since lifespan is a stable species-specific trait, similar to the body size and fertility, its duration (i.e., the time of death) and the mechanisms involved should be at least partly programmed in the genome [4, 7, 8]. Long-lived species typically possess more powerful damage repair systems, including antioxidant defense mechanisms. By enabling repair and other restorative processes, such systems should promote aging deceleration and longevity. The baseline activity of these systems and their damage-ameliorating capacity typically decrease with age. Accordingly, the mechanisms that suppress the activity of the anti-aging systems and induce diseases (including age-related disorders), cell aging, or cell death, should be a part of the aging program [1]. According to one of the authors of this work (V.P.S.) [1], transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2, transcriptional factor 2 of the NFE family) is involved in one of the most prominent important cell anti-aging programs (Fig. 1). Nrf2 regulates transcription of the antioxidant and detoxifying enzymes, which form an efficient cell defense system [1-6]. However, the role of Nrf2 is not confined to these activities. Nrf2 is also one of the central regulators of cell homeostasis that controls expression of over 1% of human genes implicated in biotransformation, redox homeostasis, energy metabolism, DNA repair, and proteostasis [2]. Nrf2 strongly influences a wide variety of physiological and pathological processes. In turn, Nrf2 is strictly controlled, mostly through the modulation of its stability. It was also found that such defense systems, apart from being directly controlled by regulators (inhibitors involved in the aging program), can be regulated by proteins participating in circadian, ultradian, and other rhythms, i.e., by the Master Clock (Fig. 1) [1].

In this review, we analyze the data on the molecular mechanisms of interaction between the Nrf2-mediated defense system, GSK3 (as an Nrf2 inhibitor), and circadian clock core proteins within the framework of aging and anti-aging programs.

Fig. 1. Master Clock regulates cellular antioxidant status by acting on the transcription factor Nrf2, which controls the expression of more than 200 cytoprotective enzymes responsible for detoxification and antioxidant defense. The Master Clock promotes body adaptation by regulating circadian rhythms via melatonin and circadian clock proteins. Nrf2 is activated by reactive oxygen species (ROS). Simultaneously, glycogen synthase kinase 3 (GSK3), activated through a variety of signaling pathways, functions as a suppressor by inhibiting Nrf2 activity. Preparations containing lithium salts produce a positive effect by inhibiting GSK3. Gray lines, interactions resulting in the stimulation of Nrf2 activity (and subsequent expression of antioxidant enzymes), including suppression of Nrf2 inhibitors; black lines, interactions resulting in Nrf2 inhibitions; arrows, direct stimulating effect (including catalysis); blunt lines, inhibition. Bach1, BTB domain and CNC homolog 1 protein 1; Keap1, Kelch-like ECH-associated protein-1; β-TrCP, β-transducin repeat-containing protein.
TRANSCRIPTION FACTOR Nrf2

Nrf2 as a factor of the anti-aging programs. Nrf2 is activated by oxidative stressors and electrophilic agents; it enables stress adaptation by upregulating the activity of the cell antioxidant system and other metabolic processes and by controlling expression of over 200 target genes under various types of stress. The products of these genes regulate a large number of defense mechanisms, such as drug detoxification, pentose phosphate pathway, and autophagy [3]. Besides, Nrf2 directly inhibits induced expression of various inflammation-related genes via binding to proximal regulatory regions [10]. Mouse cells with the knocked out Nrf2-encoding gene (Nfe2l2) exhibit higher ROS levels [11] and elevated sensitivity to oxidative stress [12]. With aging, the level of Nrf2 decreases, and the ability of this protein to become activated in response to stress is compromised [1, 2, 6, 13]. The activity of Nrf2 positively correlates with lifespan [2]. In light of all the above-mentioned, Nrf2 is considered as a component of special anti-aging program responsible for healthspan prolongation and longevity [2]. Systems suppressing the Nrf2 activity are components of the aging program [1]. The most important of them are regulatory systems that cause proteasomal degradation of Nrf2. They include the Kelch-like ECH-associated protein 1 (Keap1), GSK3, and β-transducin repeats-containing protein (β-TrCP) (see Figs. 1 and 2).

c-Myc is another Nrf2-suppressing protein [14]. Besides, although Bach1 (BTB domain and CNC

Fig. 2. Molecular mechanisms of circadian rhythms and cytoplasm–nucleus oscillations of the Nrf2 transcription factor controlling expression of cytoprotective enzymes. A characteristic feature of the Nrf2-regulated genes is the presence of ARE sequences in their promoters. At the same time, most circadian clock genes contain the E-box sequence (also present in the Nfe2l2 gene promoter). The gene Bmal1 contains the RORE (RAR-related orphan receptor response element) sequence in the promoter. Dashed lines with arrows, translocation of the corresponding proteins into and out of the nucleus; solid lines with arrows, direct effect, including catalysis; solid line with a blunt end, inhibition; solid line with a break, indirect effect (for example, upregulation of expression of clock proteins and Nrf2 by melatonin); circle with letter "p", phosphate groups attached to the proteins; the presence of two phosphate groups after interaction of the modified protein with one or another kinase indicates that the reaction proceeds by the double phosphorylation mechanism (for more details, see the text). AcT, acetyltransferase; Clock, circadian locomotor output cycles kaput protein; Cry, cryptochrome protein; Per, period protein; Rev-erbα, reverse erythroblastosis virus α protein.
homolog 1 protein 1) is an inhibitor of Nrf2, it competes with it for binding to the antioxidant response elements (AREs) [15] (Fig. 1). Apart from GSK3 mentioned above, other kinases, such as Fyn kinase, may inactivate Nrf2 and initiate its export from the nucleus [16, 17] (Fig. 2).

The activity of Nrf2 in the cell is not maintained at a constant level. The content of this protein constantly oscillates and undergoes circadian and ultradian changes. Activators that protect Nrf2 against proteolysis increase its amount in the cytoplasm; subsequent equilibration of its concentration (i.e., increase in the Nrf2 level in the nucleus) activates transcriptional response of AREs. The increase in the content of Nrf2 and induction of the ARE-containing genes leads to the GSK3 activation. In turn, GSK3 phosphorylates Nrf2 and promotes its proteasomal degradation with the involvement of β-TrCP [18].

Biorhythms are of paramount importance for adaptation; they depend on many factors, including the redox status. Disruption of circadian rhythms is a characteristic aging-related problem. Therefore, maintaining proper operation of circadian clock might be a promising lifespan prolongation strategy [19]. The circadian clock mechanism is based on the Clock and Bmal1 transcription factors. These proteins form heterodimers a via their PAS (Per-Arnt-Sim) domains and induce expression of clock-controlled genes by binding to the E-box sequences in their promoters [20, 21]. The periodicity of expression of circadian rhythm genes is secured by the Per1 and Per2 proteins. They translocate to the nucleus and form stable complexes that also include cryptochrome proteins Cry1 and Cry2. These complexes suppress transcription by binding to the upregulating factors Clock/Bmal1. As a result, the levels of Per1 and Per2 mRNAs and the content of the corresponding proteins periodically increase and decrease with the cycle duration of approximately 24 h [22, 23]. Hence, this mechanism is an example of a negative feedback loop [24, 25]. The protein regulators of circadian rhythms participate in many metabolic pathways, e.g., those involving AMPK (AMP-activated protein kinase), an essential systemic regulator of metabolism [26], which phosphorylates Cry1 and promotes its degradation [27].

**Nrf2 as an oscillator. Regulation of Nrf2 activation in the nucleus and the cytoplasm.** Understanding biorhythm regulation presents serious difficulties since both Nrf2 and ROS have their own rhythms. It was established that stimulation of ARE-dependent gene expression resulting from the action of Nrf2 activators is not mediated by the increase in the Nrf2 stability and its accumulation in the cell. Instead, this stimulatory effect results from the increase in the frequency and decrease in the amplitude of the Nrf2 translocation cycles between the cytoplasm and cell nucleus [28]. In terms of the oscillatory model suggested by Xue et al., GSK3 performs a regulatory role by ensuring Nrf2 inactivation and degradation [28]. According to this model, Keap1 and Nrf2 stay in contact because of the threonine protein phosphatase PGAM5 (protein phosphatase 5 of the phosphoglycerate mutase family) that binds Keap1 and Nrf2 to the outer mitochondrial membrane [29]. Under oxidative (more precisely, electrophilic) stress, Nrf2 dissociates from Keap1, gets phosphorylated by casein kinase 2 (CK2) [3], and translocates to the nucleus with the help of importins α5 and β1 [30]. In the nucleus, Nrf2 activates its target genes by binding to AREs in the gene promoter sequences [3, 14]. Next, Nrf2 is phosphorylated by the Fyn kinase [16], acetylated, and then removed from the nucleus via the export channel of the nuclear membrane (exportin-1/crm1) [28]. GSK3β regulates this process by phosphorylating Nrf2 and causing its degradation with the help of β-TrCP [3, 31, 32]. In the same work [28], Xue et al. demonstrated that Nrf2 retention in the cell nucleus with the aid of the crm1 export channel-blocking leptomycin B results in the downregulation of the ARE-dependent transcriptional activity and this cyclic process has a period of approximately 2 h [28]. According to the above model, this can be related to the acetylation and inactivation of Nrf2 (Fig. 2). Xue et al. estimated [28] that in unstimulated cells; the content of Nrf2 oscillates 2-3 times before the protein degradation, while in the cells exposed to the oxidative stress the number of such oscillations is higher [28].

The oscillatory model that involves Nrf2 suggests an alternative regulatory mechanism. The operation of the nuclear Nrf2 may be regulated by deacetylation, circadian control of the Nfe2l2 gene expression, and conformational changes in the Nrf2/Keap1 complex [28]. It was found that in the absence of oxidative stress, the Keap1/Nrf2 interaction is cyclic. Initially, the complex exists in the open conformation, in which Nrf2 binds to one of the two Keap1 subunits, and then transits to the closed conformation, in which Nrf2 is bound to both Keap1 subunits [33]. The oscillatory model is based on the Nrf2 activation via its release from the Nrf2/Keap1 complex, since the rate of this process influences the oscillation frequency and the cytoprotective transcriptional response. Under the influence of Nrf2 inducers (oxidants and electrophilic agents), Nrf2/Keap1 complexes are fixed in the closed conformation that prevents Nrf2 release. As a result, no regeneration of free Keap1 takes place, and de novo synthesized Nrf2 molecules do not undergo degradation. This model implies the existence of other Nrf2 activation strategies, for example, Nrf2 deacetylation by nuclear deacetylases [28].

**Interaction between biorhythms and Nrf2-based systems.** Nrf2 expression is characterized by a circadian rhythm with a period of 23.7 h [34]. Circadian variations and gender-dependent differences in the levels of the antioxidant gene transcripts can influence the organism’s response to oxidative stress at different times of the day [35]. Early et al. [36] established that deletion of the Bmal1 gene (as well as knockdown) disrupts Nrf2 activity.
in the macrophages, which contributes to the ROS accumulation of ROS and the anti-inflammatory cytokine IL-1β. They also found that Nrf2 knockout reduced lipopolysaccharide (LPS)-induced expression of three primary target genes of Nrf2, namely, Hmox1 (heme oxygenase), Gsr (glutathione reductase), and Nqo1 (NAD(P)H:quinone oxidoreductase 1) compared to Bmal1+/+ macrophages. Like most genes that encode circadian clock core proteins, Nrf2 contains the E-box sequence. The Bmal1/Clock heterodimer binds to this sequence and activates Nrf2 transcription followed by the upregulation of transcription of the target Hmox1, Gsr, and Nqo1 genes. The same authors reported that the baseline ROS level in peritoneal myeloid cells considerably increases in the second half of the day, which inversely correlates with the circadian rhythm of Bmal1 and Nrf2 expression.

In contrast to Bmal1+/+ macrophages, Bmal1−/− macrophages are characterized by a higher base level of ROS, as well as by a higher ROS content after LPS induction (table). The expression of Nrf2 and its capacity to regulate IL-1β in myeloid cells are controlled by the molecular clock.

Pekovic-Vaughan et al. found the level of the Nrf2 protein to undergo rhythmic changes [34]. The rhythmic pattern of Nrf2 expression was also observed in cell lysates and nuclei of Rat1 fibroblasts (table). These findings provide evidence for autonomous, stable rhythmic Nrf2 expression at the cellular level. A mutation in the core part of the E-box sequence of the Nfe2l2 gene promoter completely abolishes its induction by the Clock/Bmal1 complex. In the lungs of wild-type (WT) mice, Nrf2 mRNA exhibits a clear rhythmic expression pattern lacking in the Clock−/− mice. The time-dependent binding of Clock/Bmal1 to the E-box in the Nfe2l2 gene promoter was also demonstrated. These results indicate that the Nfe2l2 gene is directly regulated by the nuclear clock components (both in vitro and in vivo) via the conserved E-box sequence in the promoter. The data obtained in embryonic fibroblasts from with the Nfe2l2-deficient mice indicate direct, Nrf2-dependent rhythmic control of the downstream targets (table).

In the WT mice, Nrf2 induction by D3T (H3–1,2-dithiole-3-thione) results in the activation of the E-box- and D-box-containing clock genes (Rev-ErbA, Rev-ErbB, Dbp, Per3) [37]. Full activation of these genes requires the functioning of the Keap1/Nrf2 signaling pathway, as their activity is significantly attenuated in the Nrf2−/− mice. The loss of Nrf2 causes circadian rhythm disruption in the embryonic fibroblasts of Nrf2−/− mice. This direct effect suggests Nrf2 involvement in the regulation of rhythm amplitude and period length [37] (table). Hence, Nrf2 regulates expression of core proteins and stabilizes circadian rhythms of molecular clock and is responsible for the linking redox potential and general timing [37]. The knockout of the Nrf2 gene in the mouse liver changes the length of the circadian cycle. Nrf2 acts most probably via regulation of the Cry2 and Rev-erbα expression. Nrf2 and clock proteins likely form an inhibitory loop that integrates cell redox signaling within the circadian rhythm [37]. Nrf2 belongs to the CncC (Cap’n’collar) family of transcription factors. It has been revealed recently that constitutive overexpression of CncC proteins in Drosophila positively influences neuronal functions by modifying synaptic mechanisms. Suppression of Keap1, a CncC inhibitor, promotes synaptic function and increases the lifespan [38]. The results obtained by Hansen et al. indicate that the ratio between glutathione reduced and oxidized forms (GSH/GSSG) controls Nrf2 in the cytoplasm, but does not affect Nrf2 binding to AREs in the nucleus [39]. Conversely, since thioredoxin 1 (Trx-1) overexpression does not influence Nrf2 dissociation and translocation to the nucleus, nuclear Trx-1 is assumed to be responsible for the Nrf2 regulation at the level of Nrf2–DNA interaction and not for the cytoplasmic events, such as Nrf2 dissociation and translocation to the nucleus [39].

**GLYCOGEN SYNTHASE KINASE 3 (GSK3)**

Below, we discuss GSK3β protein kinase, its inhibitory effect on Nrf2 and other proteins, and mechanisms of its action in the regulation of cell functions.

GSK3 (ATP:protein phosphotransferase, EC 2.7.1.37) is an intracellular serine/threonine protein kinase (molecular weight, 47 kDa) ubiquitously synthesized in all tissues of an organism [40, 41]. It is represented by two paralogs (α and β), which are routinely referred to as isoforms in the literature, even though the term isoenzymes would be more precise from the biochemical viewpoint. Apart from the common GSK3β form, there is also longer GSK3β form, which is expressed in the brain during its development [42].

The unique role of GSK3β in the regulation of cell functions is related to the fact that it affects the activity of more than 100 proteins. In turn, GSK3β itself is influenced by multiple stimuli. For instance, Akt1 activation results in the phosphorylation and inhibition of GSK3β [43].

GSK3 in involved in most cell processes, such as cell growth, differentiation, and death; it also modulates responses to hormonal, nutritional, and stress stimuli. Stress-induced GSK3β translocation may result in its interaction with mitochondrial proteins, including PI3K-Akt, PGC-1α, HKII, PKCe, respiratory chain components, and mPTP subunits. The mitochondrial pool of GSK3β regulates biogenesis, energetics, permeability, and motility of mitochondria, as well as apoptosis [44]. Some of the essential functions of GSK3β are β-catenin inhibition and involvement in the Wnt signaling pathway that plays a major role in embryogenesis, cell growth and
differentiation [41], neurogenesis, and synaptic plasticity [45]. GSK3β regulates the cell cycle by inhibiting cyclin D1, which is necessary for the cell entry to the S phase [46]. It is also implicated in glucose metabolism regulation via inhibition of insulin receptor substrate (IRS) proteins and kinesins [47].

In all likelihood, GSK3 phosphorylation at serine-9 (S9) is especially prone to oscillations. These oscillations may be rapid (e.g., during neuronal depolarization/polarization) or slow, as in the case of changes in the levels of circulating GSK3-regulating hormones and circadian rhythms in the suprachiasmatic nucleus (SCN) and the liver [48].

GSK3β is constitutively activated by autophosphorylation at Y216 and inactivated by phosphorylation at S9. Active (Y216-phosphorylated) GSK3β accounts for at least a half of the total GSK3β pool in cultured cells [49]. In the mouse brain, GSK3β is predominantly present in...
the active form, whereas the portion of its inactive (S9-phosphorylated) form is insignificant [50]. GSK3β is phosphorylated at Y216 by Pyk2 and Fyn kinases or autokatically [51]. Phosphorylation at S9 inhibits GSK3β activity and represents the primary mechanism of its regulation. Many kinases phosphorylate GSK3β at S9, including protein kinases A, B, and C, PrKG1, ILK, p70S6K, and p90SRK, while protein phosphatase 2A (PP2A) dephosphorylates it [52]. In the brain, the major mechanism of GSK3β regulation is its inhibition via phosphorylation at S389 by the mitogen-activated protein kinase p38 [53].

**GSK3 involvement in the aging program.** Indirect evidence for the GSK3β involvement in the aging program is presented by Krishnankutty et al. [49], who studied three GSK3β fractions in the mouse brain and neurons: the Y216-phosphorylated active enzyme, the inactive S9- and Y216-phosphorylated enzyme, and the unphosphorylated (also inactive) GSK3β. Although the total GSK3β level does not change with age, aging is associated with the decrease in the fraction of inactive, S9-phosphorylated isotype (thus, the relative content of this form is two times lower in 1.5-year-old females compared to the 3-week-old ones) [49].

**GSK3β and cell aging.** The changes in the ratio between the GSK3β isoforms, with the preservation of the total GSK3β level, were observed in the stationary culture of mouse brain primary neurons. The relative content of the S9-phosphorylated (inactive) GSK3β form was maximal (over 30%) after 3 days of culturing without reincoculation and then gradually decreased to 15% after 12 days of culturing. The activity of the Y216-phosphorylated GSK3β, conversely, gradually increased “with age” [49]. Senescent WI-38 human fibroblasts with the population doubling level (PDL) of 58-64, in contrast to middle-age (PDL of 38-41) and young cells (PDL of 26-30), manifested traits typical of aging cells, including increased size, flattened shape, and high senescence-associated β-galactosidase activity [54]. The levels of GSK3α and GSK3β were increased in the nuclei of senescent cells. Lithium (a GSK3 inhibitor) decreases the activity of the enzyme and reduced age-dependent p53 accumulation associated with the senescence state, as well as induced cell transition to the reversible quiescent state. These results indicate that a fraction of the p53 pool activated in aging cells is modulated by the p53 binding to GSK3β in the nucleus, which promotes p53 activity and cell aging [54]. Similarly, the baseline level of phosphorylated (inactive) GSK3 in aged (18 months-old) Syrian hamsters (*Mesocricetus auratus*) is much lower than that in the young (1-3 months-old) animals [55]. In aged hamsters, lithium does not influence the period of the locomotor activity rhythm and the level of GSK3 phosphorylation, unlike its effect in younger hamsters [55]. These data provide indirect evidence for GSK3β as a *bona fide* component of the aging program.

**GSK3β and age-related disorders.** The involvement of GSK3β in the aging program is also confirmed by association of changes in its activity with aging-related diseases (Fig. 3). In neurons, GSK3β selectively phosphorylates microtubule-associated tau protein at the sites that are hyperphosphorylated in the in Alzheimer’s disease (AD) brain [56]. Hyperphosphorylated tau protein exhibits a decreased affinity for the microtubules. It accumulates in a form of helical filaments representing the main components of neurofibrillary tangles and neuropil threads in the AD brain. Neurofibrillary tangles are also detected in patients with amyotrophic lateral sclerosis, Parkinson’s disease, dementia, corticobasal degeneration, trauma-caused brain damage, Down syndrome, post-encephalitis parkinsonism, and Niemann–Pick disease. In the brain tissue of AD patients, the GSK3β level is increased by 50% [56]. GSK3β inhibition improves cognitive symptoms associated with AD and other diseases mentioned above. GSK3β activity is increased in the cellular (growth factor-deprived) and animal (cerebral ischemia-based) models of neurodegeneration [57]. The anti-inflammatory effect of GSK3β is due to the stimulation of IL-1β, IFN-γ, IL-6, and IL-12 production and suppression of IL-10 synthesis [48] via the Toll-like receptors of monocytes [58].

**Mechanism of GSK3 action. Prephosphorylation and GSK3.** Phosphorylation of glycogen synthase and other GSK3 targets requires the prephosphorylation of these substrates by another kinase in position +4 relative to the GSK3 phosphorylation site, which is a widely occurring but not universal consensus S/TXXXS/T sequence [50, 59, 60]. This double modification often results in subsequent ubiquitination and proteasomal degradation mediated by the corresponding adaptor proteins (e.g., F-box proteins).

**Protein degradation. Phosphorylation and ubiquitination.** F-box proteins are responsible for the substrate recognition, each protein recognizing a specific substrate group [61]. Based on the structure of the substrate-recognition region, F-box proteins are subdivided into three categories: proteins with WD40 repeats (Fbxw), proteins with leucine-rich repeats (Fbxl), and proteins with other domains. It is assumed that Fbxl3 is responsible for ubiquitin-dependent degradation of the clock protein Cry;
Fbxl3 mutation in mice results in the prolongation of the circadian period to ~26 h [62, 63]. β-TRCP (also known as Fbw1) recognizes the clock Per protein after its phosphorylation by casein kinase 1 (CK1) (but not by GSK3) [64]. β-TRCP targets frequently contain the DSGXXS degradation motif, the phosphorylation of both serine residues in which significantly promotes target protein recognition by β-TRCP [65]. Since this motif is similar to the consensus sequence for GSK3 (SXXX(X)S), β-TRCP binds many GSK3 substrates. For example, this sequence was identified in β-catenin and Nrf2 [60].

Below, we describe the role of modifications of Nrf2 and circadian clock proteins by GSK3.

**Regulation of Nrf2 activity by GSK3.** GSK3 phosphorylates specific serine residues in the Neh6 domain of Nrf2 with the formation of the degradation domain recognized by the ubiquitin ligase adapter protein Nrf2 with the formation of the degradation domain recog- rylates specific serine residues in the Neh6 domain of Nrf2 [60]. Hence, the rate of ubiquitination and degradation of each of these substrates partly depends on the regulation of their specific prephosphory- lating kinases and GSK3/Ck2 (Fig. 2).

In tumor cells, in which Keap1 (and hence, ubiquitin ligase complex Rbx1/E3/Cul3) cannot interact with Nrf2, GSK3 retains its ability to suppress the Nrf2 activity. In these cells, as well as in the embryonic fibroblasts of Keap1−/− mice, GSK3 inhibition by CT99021 increases Nrf2 activity [31].

**GSK3 and biorhythms.** Circadian rhythms are based on a conserved bioclock system required for the adaptation of behavioral and physiological processes to the 24-h environmental cycles [25, 69]. The regulation by the circadian oscillator of the SCN is exerted via its neuronal links with gonadoliberinergic neurons and a humoral pathway involving melatonin (pineal gland hormone) [69, 70]. Melatonin secretion by the pineal gland is stimulated by light [71]. Different light-sensing systems associated with the SCN differ in the representatives of the Batheyeridae family (characterized by decelerated aging and high longevity quotient), depending on the lifespan and sociality [72].

Melatonin acts via membrane receptors MT1 and MT2 or through the receptor-independent mechanisms, including the Nrf2 signaling [73, 74]. Besides, melatonin binds not only to the plasma membrane receptors but also to the receptor proteins on the nucleus surface; it also operates at the chromatin level, directly affecting protein synthesis. It was demonstrated that the genes for the nuclear receptors Rorα, Rorβ, and Rorγ (the so-called orphan nuclear retinoid receptors Ror/Rzr) are expressed in various organs and tissues, including the hypothalamic SCN, retina, and epiphysis [75]. The ligands of these receptors are cholesterol and its derivatives, but not melatonin [75]. Melatonin stimulates expression of the clock genes via the RORE-elements of the Bmal1 gene [25] and via Nrf2 expression through a chain of intermediate links [74, 75] (line with a break in Fig. 2). Nuclear melatonin receptors exist beyond any doubt [75]. For instance, it was established that melatonin is a ligand of the vitamin D receptor (VDR) in the nucleus, with a K_d of 21.2 ± 1.9 μM [76].

GSK3α and GSK3β are expressed in the hypothala- mic SCN [48]. In mice, the level of GSK3α mRNA is higher than the level of GSK3β mRNA [77]. GSK3 is responsible for the feedback loop that impacts the functioning of molecular clock in the SCN neurons [78]. The expression of GSK3α and emergence of the phosphory- lated form of GSK3β in the SCN are characterized by a circadian rhythm [77].

At the beginning of the nighttime, the GSK activity in rat SCN neurons decreases (the number of cells histochemically stained for the phosphorylated inactive GSK3β form increases and reaches the maximum within
4 h). However, the activity of GSK3β increases towards the end of the night. Immunofluorescent staining of the mouse SCN showed that at the end of the night, light significantly increases GSK3 activity, i.e., decreases the level of phosphorylated GSK3β by as much as 30-60 min after the light pulse [79]. In the control system, the content of phosphorylated GSK3 decreases late at night, whereas the GSK3 activity increases. No decrease in the amount of phosphorylated GSK3 (i.e., no increase in the content of the active GSK3 form) was observed in the experimental system. Therefore, light pulse suppresses GSK3 activity, resulting in the attenuation of its oscillations [80]. Even hippocampal extracts from mice permanently kept in the dark for at least 2 weeks were characterized by a distinct endogenous circadian rhythm in the GSK3β (but not GSK3α) phosphorylation [81]. In Drosophila, the functioning of Shaggy (Sgg), a GSK3 homolog, in small ventral lateral neurons that play a key role in the regulation of general rhythmic locomotor activity of adult individuals, is crucial for maintaining normal rhythms [82].

**GSK3 substrates involved in circadian rhythm regulation.** GSK3 interacts with Per2 in vitro and in vivo, phosphorylates Per2 in vitro, and promotes its translocation to the nucleus (see Fig. 2). It also causes proteasomal degradation of its partner protein Cry2 [48, 83, 84] and phosphorylates Cry2 together with the serine protein kinase DYRK1A by S557 and S553 residues, respectively [24, 85]. GSK3 phosphorylates (i) Bmal1 (S17/T21), resulting in its subsequent ubiquitination and degradation [86], and (ii) Clock (S427/S431) [87] (Fig. 2). The kinase assay of GSK3 activity revealed that this enzyme regulates Clock phosphorylation/degradation by modification of a specific cluster of serine residues (phosphodegragon) [87].

The studies of GSK3β phosphorylation at S9 (which suppresses kinase activity, as mentioned above) demonstrated that the GSK3β activity is maximal at the end of the night until the early morning. This results in the upregulation of the Cry2 phosphorylation at S557, which facilitates rhythmic degradation of this protein [87]. Besides, GSK3 phosphorylates Rev-erbα (protein suppressing Bmal1 expression and, accordingly, Bmal1-induced expression of clock genes); however, this modification causes activation (not degradation) of Rev-erbα and its translocation to the nucleus [88].

Cry2 and Per2 play a prominent role as suppressors of circadian protein expression. GSK3β interacts with Per2 in vitro and in vivo (as mentioned above) [48]. These interactions do not result in the degradation of Per2 (unlike Cry2), but promote its translocation to the nucleus (in contrast to the casein kinase-dependent phosphorylation) (Fig. 2). GSK3β overexpression causes a shift in the Per2 phase, which changes the duration of the period by ~15% (3-4 h) or results in a complete loss of circadian rhythms and generation of extreme phenotypes. Some other molecular mechanisms also regulate the cyclic expression of the Per1 and Per2 genes [23]. The level of the Per proteins is regulated by several factors that promote protein stability and, presumably, ability to translocate to the nucleus. On the other hand, Per phosphorylation by CKIε is responsible for the cytoplasmic degradation of Cry-unbound Per (unlike its GSK3-dependent phosphorylation), thus preventing premature accumulation in the cytoplasm. Per is less stable in the absence of Cry and readily undergoes ubiquitination and proteasomal degradation [22].

The GSK3 ortholog in the Drosophila fruit fly, Sgg, plays a central role in determining the length of the circadian period. Its mutation in Drosophila causes prolongation of the circadian period, whereas upregulation of its activity shortens the period [89]. Sgg phosphorylates Tim (Timeless, an analog of Cry in Drosophila) and regulates nuclear translocation of the Per/Tim heterodimer [89]. It should be noted that Tim and Cry2 form dimers with Per in the clock structures of Drosophila and mice, respectively. It is quite possible that GSK3 contributes to the clock functioning by regulating components that operate together with the Per proteins [24]. GSK3 phosphorylates the core circadian clock proteins (Bmal1, Clock, Per, Cry, and Rev-erbα) in mammals and regulates their stability [25] (Fig. 2).

**Conserved structure of clock proteins and GSK3 in the evolutionary tree.** Using the Ensembl 100 database, we screened for the presence of orthologs of the following genes in vertebrates, Drosophila melanogaster, and the nematode Caenorhabditis elegans: GSK3A (ENSG00000105723), GSK3B (ENSG00000082701), CLOCK (ENSG00000134852), CRY1 (ENSG00000008405), CRY2 (ENSG00000121671), BHLHE41 (DEC2) (ENSG00000123095), and NPS1 (ENSG00000130751) (the human gene identifier according to the Ensembl database is given in parentheses). These genes code for proteins involved in the regulation of biorhythms. The GSK3A gene is highly conserved even in invertebrates (71.27% identical positions in the human and nematode genes) and virtually identical in humans and macaques. The GSK3A gene has no orthologs in birds, some reptiles, and the elephant shark Callorhinichus mili. In particular, no GSK3A ortholog was detected in the common snapping turtle Chelydra serpentina, three-toed box turtle Terrapene carolina triunguis, Agassiz’s desert tortoise Gopherus agassizii, and Goode’s thornscrub tortoise Gopherus evgoodei. No NPS1 ortholog was found in all tested birds and some reptiles, such as the blue-ringed sea krait Laticauda laticaudata, mainland tiger snake Notechis scutatus, Eastern brown snake Pseudonaja textilis, Australian saltwater crocodile Crocodylus porosus, Anole lizard Anolis carolinensis, Argentinian black and white tegu Salvator merianae, Komodo dragon Varanus komodoensis, common snapping turtle C. serpentina, Goode’s thornscrub tortoise G. evgoodei, Chinese soft-shell turtle Pelodiscus sinensis, and three-toed box turtle
**Terrapene carolina triunguis.** No *NPAS1* ortholog was found in the Western clawed frog *Xenopus tropicalis*.

The GSK3β, CLOCK, CRY1, CRY2, and DEC2 genes have orthologs in most vertebrates. Even the *Saccharomyces cerevisiae* yeast contains two GSK3 orthologs, but not orthologs for the other tested genes. *C. elegans* lacks the CRY1, CRY2, and DEC2 orthologs, and *D. melanogaster* has no CRY1 and CRY2 orthologs. Human Clock protein only poorly aligns with the proteins from *C. elegans* and *D. melanogaster* (only 10-20% of positions are identical). Presumably, GSK3α does not play a central role in the circadian rhythm regulation, since it is absent from a large number of species (e.g., birds and many reptiles) and its knockout causes no severe phenotypic disruptions. Instead, this function is fulfilled by GSK3β, which, despite minor variations, is present in animals at all branches of the tree of life. Its knockout results in death at the embryonic stage. Since biorhythm regulation is characteristic of all animals, including reptiles and birds, below we discuss mostly the functions of GSK3β.

**GSK3 inhibitors. Lithium.** Lithium ions prolong the circadian rhythm periods in many species, including unicellular organisms, insects, mice, and humans [77, 90, 91]. Lithium at the concentration of 1-10 mM inhibits GSK3β *in vitro* and *in vivo* in all tested species [92-95]. It decreases the GSK3β/Sgg activity and prolongs the periods of locomotor activity in flies even when they had been permanently kept in the dark (without external light activators) [96]. Lithium at low concentrations (~1 mM) predominantly affects the biorhythm amplitude (presumably, via GSK3β), whereas at high concentrations (~10 mM), it contributes to the prolongation of the period [97].

**SB415286.** Analogous daytime suppression was documented with another GSK3 inhibitor (SB415286; 1 mM), which decreased the frequency of spontaneous neuronal spikes by 66% relative to the control [98].

**Benzofuran-3-yl-(indole-3-yl)maleimides.** Recently, a new generation of GSK3β inhibitors [benzofuran-3-yl-(indole-3-yl)maleimides] has been developed with IC_{50} values within the 4-680 nM range toward human GSK3β. One of them (with IC_{50} of 67 ± 6 nM) is characterized by an acceptable selectivity and solubility at the concentrations of 10-29 μg/kg. In mice, such inhibitors exhibit the antipsychotic activity, analogous to lithium and valproate, which are commonly used to treat bipolar disorder and other manic-depressive states [99].

**Genetic activation/inactivation of GSK3.** The inhibitory phosphorylation of GSK3α and GSK3β in the SCN alternates with a 24-hour period. Transgenic mice with mutations in both GSK3α and GSK3β (GSK3α^{21A/21A}β^{9A/9A}) resulting in the permanent activation of the enzymes have disrupted behavioral rhythms, including significantly decreased rhythm amplitude, prolonged active period, and extended daytime activity perio-

Long-lived species typically possess more efficient/large/robust damage repair systems, including...
antioxidant defense mechanisms. Originally, Frolkis [107] has coined the term antioxidant (vitauct) systems for the systems responsible for damage repair and other restorative processes, since they promote longevity. With aging, the activity of such systems usually decreases below its baseline. Accordingly, the aging program involves (i) systems that inhibit/suppress the anti-aging mechanisms and/or (ii) systems the operation of which results in the development of diseases, including aging-associated disorders, cell aging, or cell death [1, 108]. The antagonistic pleiotropy concept assumes the presence of genes that promote reproductive success at a young age, despite their delayed adverse effects later in life [108]. With aging, the activity of such systems may not decrease but even increase. As for GSK3 and Keap1 responsible for ubiquitin-dependent proteasomal degradation of Nrf2, their activity increases with age [2], while activity of Nrf2 decreases. Taken together, the experimental data discussed in this review demonstrate that Nrf2 and GSK3 are components of antagonistic and actively interacting anti-aging and aging programs, respectively [1].

Robust and, nonetheless, plastic circadian rhythms are characteristic not only of proteins involved in the biorhythm regulation (mostly, transcription factors), but also of their regulator, GSK3, as well as the Nrf2-induced antioxidant system that orchestrates a plethora of proteins in the antioxidant defense mechanisms. Molecular clock is the basis of the regulatory mechanism that enables an organism to prepare for and to respond to daily environmental challenges. The Nrf2 system is induced by oxidants (electrophiles), which initiate the synthesis of antioxidant/detoxifying enzymes that prevent cell damage. Even that the biorhythm hormone melatonin possesses no antioxidant properties per se, it activates Nrf2 [74], albeit indirectly. This provides an additional evidence for the adaptation-promoting function of the circadian clock system.

The situation with Nrf2 is complicated not only by the circadian rhythms in the Nrf2 activity (i.e., its temporal oscillations), but also by the spatial oscillations represented by the nuclear and cytoplasmic Nrf2 pools. Moreover, by orchestrating the antioxidant defense system, Nrf2 counteracts the effect of toxic substances and oxidants and influences expression of circadian clock proteins (table).

GSK3 can be considered as a characteristic representative of the aging programs: unlike the Nrf2 activity, the activity of GSK3 increases with age, both in vivo and in vitro, as well as in diseases. GSK3 is involved in various metabolic pathways, including those associated with aging-associated diseases (type 2 diabetes and cancer) and neurodegenerative disorders. GSK3 is also implicated in the cell death and inflammation. GSK3 inhibitors are presently considered as promising therapeutics for treating the problems mentioned above.

Our interest in GSK3 is dictated by its regulatory influence on Nrf2 that is exerted via at least three different pathways: (i) GSK3 is directly involved in the Nrf2 degradation, as it facilitates Nrf2 ubiquitination and proteasomal cleavage (and not merely inactivation, as in the case of other kinases); (ii) GSK3 phosphorylates Fyn kinase that translocates to the nucleus and modifies Nrf2, resulting in the Nrf2 removal from the nucleus; and (iii) GSK3 phosphorylates proteins Bmal1 and Clock of the positive branch of circadian clock and causes their proteasomal degradation, which decreases Nrf2 expression. It should be noted that promoters of the genes negatively regulating the biorhythms (Cry1, Cry2, and Rev-erbα) and of the Nfe2l2 gene contain the E-box and, therefore, their transcription is upregulated by the Clock/Bmal1 complex [23, 36]. Hence, the GSK3- and β-TrCP-mediated system is a “regulating valve” that controls minor oscillations in the Nrf2 levels and fine-tunes ultradian and circadian (Bmal1-dependent) Nrf2 regulation [3, 36]. Together with the data on circadian and Bmal1-mediated Nrf2 regulation, this indicates that Nrf2 and the clock genes form a regulatory loop that integrates cell redox signals into circadian rhythms [37].

Phosphorylation of various substrates by GSK3 produces a broad spectrum of effects ranging from changes in the enzyme activity to protein translocation, modification of protein–protein interactions, and changes in the protein stability [60]. GSK3-catalyzed phosphorylation of proteins frequently results in their ubiquitination and proteasomal degradation. In the case of Nrf2, Cry2, Clock, and Bmal1, the recognition of the ubiquitinated protein by the proteasome is mediated by adapter proteins, e.g., β-TrCP [60]. These events are often preceded by the phosphorylation of the same protein by another kinase. Protein phosphorylation by GSK3 does not invariably cause protein degradation. On the contrary, phosphorylation of the negative biorhythm regulators Rev-erbα and Per2 by GSK3 increases their stability. Cry is the only negative regulator of circadian biorhythms, whose phosphorylation by GSK3 causes its degradation (similar to the positive regulators Bmal1 and Clock) and not translocation to the nucleus (as is the case with Rev-erbα and Per). Presumably, Cry facilitates adjustment of biological clock to the environmental light rhythm, because it also inhibits Per transcription and regulates the levels of Per protein [22]. Therefore, GSK3 interacts (i) with virtually all core clock proteins (Bmal1, Clock, Per, Cry, Rev-erbα) to produce a wide variety of effects, influencing the length of various phases and (ii) with Nrf2 to regulate the cell antioxidant status [3, 15, 18, 25, 29–32, 36, 37, 89].

Genetic and biomolecular data convincingly demonstrate an importance of feedback loops in gene expression. The biorhythms are also linked with the cell bioenergetics via NAD⁺ metabolism. It was found that the NAD⁺-dependent deacetylase SIRT1 binds to the
Clock/Bmal1 complex with a circadian-type periodicity and regulates circadian transcriptional programs via deacetylating core clock proteins (Bmal1 and Per2) and chromatin-associated proteins [109, 110]. Oxidative stress may reset the molecular clock [111]. Modulation of the pentose phosphate pathway also causes alterations in the rhythmic behavior and affects tissue clocks [112].

Researching proteins located at the intersection of signaling and regulatory pathways (such as clock proteins [20-25, 37, 113] and pineal gland hormone melatonin, its precursors, and metabolites [69-75, 114]) and comparing them in short- and long-lived species using biochemical and bioinformatics tools allows to elucidate molecular mechanisms underlying the processes and phenomena responsible for the timing of ontogeny events and longevity (acute and chronic phenoptosis, neoteny, etc.) [7-9, 115], as well as for the potentially possible suppression of the cytokine storm in COVID-19 [116, 117].

The period of the cell clock rhythm is precisely adjusted by the phosphorylation-based signaling — network that includes multiple protein kinases, with GSK3 being the most universal kinase, at least in terms of the number of substrates involved. Therefore, GSK3 functions as a hub in the cross-regulation network of circadian rhythms and antioxidant defense mechanisms, which constitutes the main subject of this review. Attempts to simultaneously influence both pathways (see pathways directed from the Master Clock to the Nrf2 system in Fig. 1) are exemplified by the development of a preparation representing a hybrid of sulforaphane (well-known Nrf2 activator) and melatonin (circadian clock regulator) [118, 119] and substances that inhibit GSK3 and activate Nrf2 at the same time (2,4-dihydropyran[2,3-c]pyrazoles) [120]. Presumably, such preparations can be used not only for treating neurodegenerative diseases but also for prolonging the healthspan and attaining longevity.

**Ethics declarations.** The authors declare that they have no conflict of interest. This article does not contain any personal data from patients or healthy volunteers.

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