Watching the Clock in Glioblastoma

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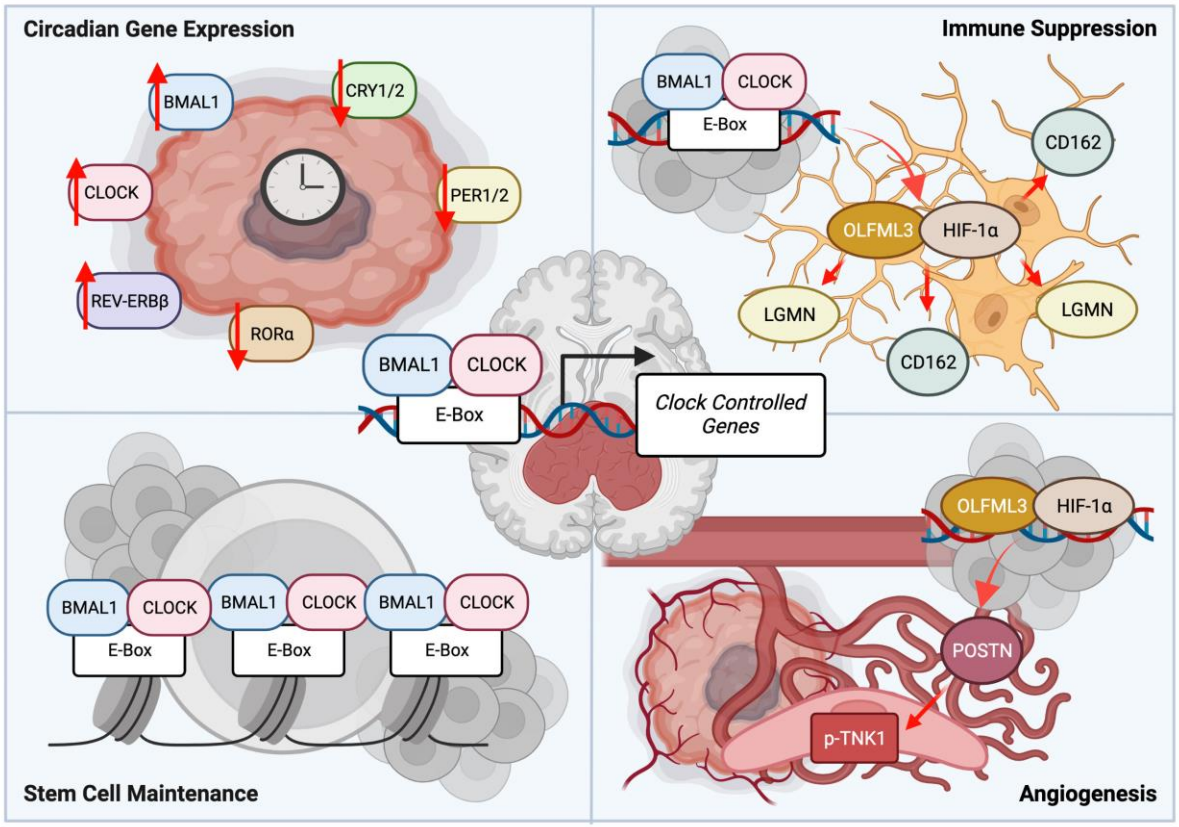
Abstract:

Glioblastoma (GBM) is the most prevalent malignant primary brain tumor, accounting for 14.2% of all diagnosed tumors and 50.1% of all malignant tumors, and the median survival time is approximately 8 months irrespective of whether a patient receives treatment without significant improvement despite expansive research[1]. Recently, important roles for the circadian clock in GBM tumorigenesis have been reported. Positive regulators of circadian-controlled transcription, Brain and Muscle ARNT-Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK), are highly expressed also in GBM and correlated with poor patient prognosis. BMAL1 and CLOCK promote maintenance of GBM stem cells (GSCs) and establishment of a pro-tumorigenic tumor microenvironment (TME), suggesting that targeting the core clock proteins may augment GBM treatment. Here, we review findings that highlight the critical role the circadian clock plays in GBM biology and the strategies by which the circadian clock can be leveraged for GBM treatment in the clinic moving forward.

Keywords: Glioblastoma, Circadian Rhythm, Chronomedicine, Chronotherapy, Circadian Pharmacology

Graphical abstract



Key Points:

- Circadian clock proteins play an indispensable role in GBM tumor biology, including that of GSCs and the GBM TME.
- Targeting the circadian clock either through chronomedicine, chronotherapy, or circadian pharmacology can be combined with current standard-of-care to improve upon GBM patient outcomes and survival times.

INTRODUCTION

Circadian rhythms in biological functions exist across a spectrum of organisms and have been adapted through evolution to coincide with the Earth's rotation about its axis that generates a 24-hour cycle in light and resulting environmental cues. Organisms receive these cues, resulting in downstream signaling that either simulates or suppresses biological functions depending on the time of day[2]. Humans harbor melanopsin in their retina, which is activated by blue wavelengths of visible light and transmits signals to the "master clock", situated in the suprachiasmatic nucleus (SCN), which comprises two nuclei of approximately 10,000 neurons within the hypothalamus of the brain[3–5]. The SCN will then signal to various sites in the body, such as other locations of the brain, as well as the liver, heart, digestive tract, and muscles, otherwise known as the "peripheral clocks". Peripheral clocks feed back to the master clock via hormones, metabolites, or other circulating factors as well as through time-of-day signaling via sleep, activity, or food intake (Figure 1)[6]. Disruption of circadian clocks due to modern lifestyles (i.e., shift work, illumination, or jet lag) or modulation of circadian genes via other environmental factors, mutations, or drugs contribute to disease onset and progression, but also treatment of such diseases[7].

There has been an emerging picture in recent years of how circadian biology plays a role in tumorigenesis and understanding of this crosstalk opens the door for leveraging circadian medicine in GBM treatment[8]. GBM was among the first cancers characterized by The Cancer Genome Atlas (TCGA), but the deep molecular characterization has failed to translate into effective precision medicine[9]. Standard-of-care for newly diagnosed GBM includes maximal, safe surgical resection, followed by concurrent radiation and temozolomide (TMZ) chemotherapy, then adjuvant TMZ[10]. Upon the inevitable recurrence of GBM, no consistent, effective treatments have been defined. Given the central role of the brain in global circadian rhythm control, molecular circadian clock of brain tumors has recently undergone investigation. Here, we explore the emergence of circadian clock genes as major players in GBM pathogenesis.

THE CIRCADIAN CLOCK

Circadian Transcription Translation Feedback Loops

Circadian rhythms in biological processes are generated by a network of proteins that govern timing of gene transcription. The mammalian circadian clock is controlled by two master clock regulators: BMAL1 and CLOCK. The BMAL1 and CLOCK heterodimer (BMAL1::CLOCK) binds to the E-box enhancer element of clock-controlled gene (CCG) promoters, thereby regulating their transcription[11]. BMAL1::CLOCK generates feedforward loops by promoting the transcription of its own positive regulators, Retinoic Acid Related Orphan Receptor$\alpha/\beta/\gamma$ (ROR$\alpha/\beta/\gamma$), and negative feedback loops through negative regulators, Cryptochrome 1/2 (CRY1/2), Period 1/2 (PER1/2), and Nuclear Receptor Subfamily 1 Group D 1/2 (NR1D1/2). Collectively, these proteins generate the mammalian circadian transcription-translation feedback loop (TTFL). In the primary loop of the TTFL, CRY1/2 and PER1/2 form a heterodimer repressor complex (CRY1/2::PER1/2), which sequesters BMAL1::CLOCK and directly inhibits its transcriptional activity. In the secondary TTFL, ROR$\alpha/\beta/\gamma$ promotes the transcription of *BMAL1* while REV-ERBα/β, which is encoded by NR1D1/2, indirectly inhibits the transcription of *BMAL1* by blocking the Retinoic Acid-Related Orphan Receptor Response Element (RORE) binding site of the *BMAL1* promoter (Figure 2)[12–14].

Post-Translational Modifications of Circadian Regulators

Circadian clock proteins also undergo post-translational modifications, which govern their subcellular localization as well as their degradation and/or stabilization, to fine tune rhythmicity of clock-controlled gene expression and biological processes so that the clock period runs over the course of roughly 24 hours, rather than the few hours more typical of a TTFL[15]. Subcellular localization of circadian regulators is incredibly important given that it

dictates transcriptional output of CCGs and controls the transcription of BMAL1 and transcriptional activity of BMAL1::CLOCK. Many CCG gene products are also regulated through post-translational modifications and/or rhythmic degradation[16].

*BMAL1 and CLOC*K: Phosphorylation of BMAL1::CLOCK via Glycogen Synthase Kinase-3 β (GSK-3β) leads to their degradation[17,18]. SUMOylation of BMAL1 promotes subsequent ubiquitination, therefore leading to its degradation[19]. Cyclin Dependent Kinase 5 (CDK5) phosphorylates CLOCK to accelerate its turnover[20]. Degradation of the BMAL1::CLOCK complex following DNA binding is necessary for activation of CCG transcription via their E-boxes[21]. By contrast, O-GlcNAcylation stabilizes BMAL1 and CLOCK by competing with phosphorylation at the same sites, which would otherwise lead to ubiquitination and proteasomal degradation (Figure 3A)[22].

PER1/2: Casein Kinase 1 δ/ε (CK1δ/ε) and Casein Kinase 2 (CK2) phosphorylate PER1/2, thereby signaling for proteasomal degradation. CK1 and CK2 display differential modes of regulation; CK1-mediated phosphorylation at the N-terminus occurs at night when PER levels are falling, while CK2-mediated phosphorylation occurs during the day when PER is accumulating[23]. Phosphorylation of PER1/2 leads to the Skp-Cullin-F-box (SCF) complex containing β-TrCP1/2 to ubiquitinate and target the proteins for proteasomal degradation[24,25]. CK1δ/ε also mediates PER1/2 nuclear localization via phosphorylation of the FASP site. Whether CK1-mediated phosphorylation of PER1/2 leads to protein degradation or stabilization and subsequent nuclear import is dependent upon two factors: sequence and phosphorylation site[23,26]. Dimerization of PER1/2 and CRY1/2 also enhances the nuclear localization of PER1/2 and, consequently, the heterodimer (Figure 3B)[27].

CRY1/2: Phosphorylation of CRY1/2 affects their stabilization and protein turnover. AMP-activated Protein Kinase (AMPK) phosphorylates CRY1/2, which signals the E3 ubiquitin ligase F-box and Leucine Rich Repeat Protein 3 (FBXL3) to ubiquitinate CRY1/2[28]. Such events lead to proteasomal mediated degradation of CRYs[29]. FBXL3 enters the nucleus to target CRY1/2[30] to terminate the transcriptional repressive activity of CRYs. However, there are multiple mechanisms that can affect FBXL3-mediated degradation of CRY1/2. FBX21, a homolog of FBXL3, also ubiquitinates CRY1/2 but at different sites on the protein, counteracting FBXL3-mediated degradation, thereby stabilizing CRYs instead[31]. Ubiquitin Specific Protease 7 (USP7) antagonizes the activity of FBXL3 and other E3 ubiquitin ligases by deubiquitinating and stabilizing both CRY1/2 in the absence of DNA damage. Under DNA damage conditions, however, only CRY1 is deubiquitinated and stabilized while CRY2 is still subjected to FBXL3-mediated degradation[31,32]. The dimerization of CRY1/2::PER1/2 can also inhibit FBXL3 targeting of CRY1/2. PER and FBXL3 both interact with CRY at overlapping locations and PER can therefore exclude FBXL3 from the protein complex and protect CRY1/2 from degradation (Figure 3C)[33].

REV-ERBα: Little is known regarding post-translational modifications of the secondary mammalian TTFL molecular members, but REV-ERBα is ubiquitinated and targeted for degradation by F-box and WD Repeat Domain Containing 7 (FBXW7). This ubiquitination event is dependent upon CDK1-mediated phosphorylation of REV-ERBα prior to recognition of FBXW7. The CDK1-FBXW7-REV-ERBα axis is a key regulator of *Bmal1* transcription and oscillation[34]. GSK-3β also phosphorylates and stabilizes REV-ERBα (Figure 3D)[35].

THE CIRCADIAN CLOCK AND GBM DEVELOPMENT

GBM was the among first cancers characterized by The Cancer Genome Atlas (TCGA), but the deep molecular characterization has failed to translate into effective precision medicine[33]. Standard-of-care for newly diagnosed GBM includes maximal surgical resection, followed by concurrent radiation and temozolomide (TMZ) chemotherapy, then adjuvant TMZ[34]. Upon the inevitable recurrence of GBM, no consistent, effective treatments have been defined. Given the central role of the brain in global circadian rhythm control, molecular circadian clock of brain tumors has recently undergone investigation. Here, we explore the emergence of circadian clock genes as major players in GBM pathogenesis.

Circadian Gene Expression in GBM

The World Health Organization (WHO) classification of GBM (grade IV glioma) integrates key defining molecular alterations, including mutations of Isocitrate Dehydrogenase 1 or 2 (IDH1/2), ATRX (alpha-thalassemia/mental retardation, X-linked), TERT (Telomerase Reverse Transcriptase), or Histones 3.3 and 3.1, as well as chromosome 1p and 19q co-deletion. IDH1/2 mutations portend favorable outcomes for glioma patients relative to gliomas with wildtype IDH[36]. Alterations of TERT, C228T and C250T mutations in particular, have been found to occur in about 80-90% of all GBMs, regardless of IDH1/2 mutational status[37]. O^6-Methylguanine-DNA Methyltransferase (MGMT) encodes a suicide repair enzyme for aberrant alkylation, which can be induced by alkylator chemotherapies, like TMZ. Methylation of the MGMT promoter reduces its transcription, which, in turn, diminishes DNA damage response mechanisms and, therefore, confers sensitivity to TMZ[38]. Thus, MGMT promoter methylation serves as both a positive prognostic marker for patient survival and predictive marker for TMZ response. While the collective molecular markers used in clinical management for glioma patients are useful in classifying tumors and predicting patient survival, additional markers may be useful to guide patient

management. Circadian clock components could potentially be utilized as novel biomarkers in GBM, and their expression levels and/or "circadian clustering" may inform patient survival and/or response to treatment[39].

BMAL1: Analysis of TCGA data revealed that high expression of *BMAL1* correlated with shorter survival times, and *BMAL1* mRNA levels positively correlated with glioma grade[40]. *BMAL1* levels are much higher in GBM tissues than surrounding non-tumor tissue, but this difference was not observed when examining low grade gliomas. Increased expression of *BMAL1* positively correlated with and drive the high expression levels of Hypoxia Inducible Factor 1 α (HIF-1α), Angiopoietin 2 (ANG2), and Vascular Endothelial Growth Factor (VEGF) in grade III glioma and GBM tumor tissues. Tumor tissues with high levels of *BMAL1* across all glioma grades had increased microvascular density and severity of edema was positively correlated with *BMAL1* mRNA levels[41]. Such findings suggest that *BMAL1* and clock-mediated transcriptional control could inform patient prognosis and response to anti-angiogenesis therapies.

CLOCK: CLOCK is amplified in about 5% of GBM cases and 2.8% of low-grade glioma cases[42]. *CLOCK* is expressed at high levels in grade III gliomas and GBM compared to normal, healthy brain tissue and grade I and II glioma tissues as well as in multiple GBM cell lines (U87MG, T98G, A172, and U251 cells) compared to normal astrocyte cell lines[43]. siRNA targeting of *CLOCK* led to a reduction in glioma cell growth, survival, colony formation, and cell migration and increased sensitivity to serum starvation. Algorithmic modeling predicted that the microRNA (miRNA) miR-124 binds to the 3'-UTR of *CLOCK*. Luciferase reporter assays using both wildtype and mutant *CLOCK* 3'UTRs confirmed that mi-R-124 represses *CLOCK* luciferase activity in two different GBM lines, suggesting that miR-124 directly targets CLOCK. Conversely, downregulation of miR-124 contributes to high

expression levels of *CLOCK* in glioma cells. Furthermore, *CLOCK* knockdown or *miR-124* overexpression in T98G cells decreased NF-κB activity and p-p65 levels and attenuated mRNA and proteins levels of multiple NF-κB target genes. This data suggests that miR-124 modulates the NF-κB pathway by affecting *CLOCK*, thereby playing a key role in cell cycle, apoptosis, and cellular transformation programming in GBM[44].

CRY1/2: Expression of CRY1/2 is much lower in glioma tissue compared to surrounding healthy tissue. CRY1 levels were not significant differences between low (grade I and II) and high grades of glioma (grade III and GBM) but CRY2 levels were different these between glioma grades[45]. *Cry2* mRNA and protein expression are dysregulated and rhythmic in the C6 GBM rat model. Irradiation induces high expression of *Cry2* and disrupts the rhythmicity of Cry2 expression. High Cry2 levels positivity correlate with cell proliferation but negatively correlate with apoptosis. High expression of *CRY2* correlated with longer survival times, as would be expected for a negative regulator of BMAL1::CLOCK activity, and results from TUNEL assays following irradiation suggest that CRY2 sensitizes cells to apoptosis following irradiation[40,46]. These findings suggest that leveraging either CRY 1 or 2, but especially CRY2, can be beneficial in GBM treatment.

PER1/2: Like *CRY1/2*, *PER1/2* is decreased in glioma tissue compared to surrounding non-cancerous cells from glioma samples. However, *PER2* did not display significant differences in expression level when comparing between low- or high-grade gliomas, but *PER1* levels were significantly lower in low grade gliomas[47]. An analysis of SNPs in clock genes revealed that the rs2289591 variant in PER1 was associated with overall glioma risk, including risk of GBM[48]. Despite analysis of expression levels in tumor tissue, analysis in cell and animal models have uncovered mechanistic insights into PER1/2 function in treatment response. High expression of both *Per1/2* in a rat model of GBM correlated with increased sensitivity to

irradiation in tumor tissue but not healthy tissue. Moreover, *Per1/2* levels are negatively correlated with cell proliferation and positively correlated with apoptosis. PER1 increases apoptosis of GBM cells exposed to X-rays by promoting *chk2-P53* signaling[49]. *Per2* deficiency in mutant mice models increased risk for cancer development following gamma radiation and expression of core cell cycle genes, such as *Cyclin D1*, and tumor suppressive genes, such as *p53*, were deregulated in these mice[50].

RORα: RORα is expressed at low levels in gliomas and its overexpression is associated with increased survival rates in both TCGA and Chinese Glioma Genome Atlas (CGGA) data. In GBM, low *RORα* levels are associated with poor prognosis. *RORα* is significantly lower in Grade II-IV glioma tissue tumor tissue compared to healthy tissue. Overexpression of *RORα* decreased cell proliferation and induced cell cycle arrest in T98G and GSC4D GBM and GSC cell lines and inhibited tumorigenesis *in vivo*. Gene Set Enrichment Analysis (GSEA) studies found that low *RORα* expression levels were associated with the tumor necrosis factor-mediated (TNF) signaling pathway and glioma specimens exhibited a negative correlation between *RORα* and *TNF-α*. RORA mediated inhibition of TNF-α led to downstream inhibition of the NF-κB signaling pathway, all of which contributes to the anti-proliferative effects of RORA in glioma. The authors also reported that miR-18a negatively regulates *RORα* expression by binding to its 3'-UTR and mrR-18a also activates the TNF-α and NF-κB pathways[51]. Given that RORA promotes *BMAL1* transcription, these studies contradict studies delineating the role of BMAL1 and the negative regulators of the clock in GBM and GSCs. This suggests that the role of the core clock is still not well defined.

REV-ERBα/β: REV-ERBα is more abundant in healthy, non-neoplastic tissues while REV-ERBβ is the major variant in numerous cancer cell lines, including liver, prostate, melanoma,

and colon cancers[52]. Increasing expression levels of NR1D2 (*REV-ERBβ)* correlates with increasing human glioma grade in patient samples. Additionally, *NR1D2* levels were higher in these GBM cell lines compared to healthy, noncancerous astrocytes. Depletion of *NR1D2* via siRNA decreased cell migration, invasion, and viability and increased G_1 phase population of cells in GBM cell lines but not in human astrocytes. Epithelial-mesenchymal transition (EMT) and focal adhesion (FA) genes were found to be a REV-ERBβ target genes, which suggests that NR1D2/REV-ERBβ may be a novel target for GBM therapy by inhibiting the migration and invasion of GBM cells[53].

GBM Stem Cells

The presence of GSCs has remained a difficult challenge in treating GBM and preventing tumor recurrence. The specific origins of GSCs are still disputed and purification of these cells remain challenging but technological advances of methods, such as single-cell RNA-sequencing and lineage tracing have provided further evidence of GSCs in GBM tumors. This heterogeneous population of cells have functional characteristics and contribute to chemoradio-resistance, promoting angiogenesis, and suppressing immune responses[54].

GSCs have been reported to be uniquely sensitive to modulation of the circadian clock[40,42]. Loss of *BMAL1* and *CLOCK* or reduction of *BMAL1* or BMAL1::CLOCK activity either through genetic targeting or pharmacological treatment specifically attenuated stemness genes and metabolic genes, induced apoptosis and cell cycle arrest, and inhibited proliferation in GSCs. A similar response to reduction of BMAL1::CLOCK activity was not observed in differentiated GSCs nor normal neural stem cells (NSCs). Such genetic or pharmacological intervention extended survival after inoculation of GSCs into mice and lowered tumor burden (Figure 4A). Chromatin Immunoprecipitation followed by deep

sequencing (ChIP-seq) results suggest BMAL1::CLOCK-driven transcriptional reprogramming and upregulation, which could explain the high sensitivity to circadian targeting in GSCs. BMAL1 preferentially bound to metabolic genes, encoding proteins involved in the glycolysis and the Citric Acid (TCA) cycles, suggesting GSCs rely on BMAL1::CLOCK transcriptional activity to drive the upregulation of metabolic output and usage in GSCs. *In vitro* and *in vivo* experiments highlight the potential of pharmacologic modulators of REV-ERBs and CRYs with not only monotherapy efficacy but also synergistic anti-tumor activity in combination. These findings support the conclusion that the positive loop of circadian clock proteins plays an oncogenic role in GSC biology (Figure 4B)[40].

GBM Tumor Microenvironment

Besides contributing to an increase in cancer risk, circadian disruption promotes remodeling of the TME that in turn drives cancer growth and proliferation. In murine melanoma, mice were subjected to a circadian rhythm disruption protocol that inverted the rhythmicity of macrophages and decreased the ratio of M1/M2 macrophages, thereby promoting tumor growth. Proinflammatory cytokines IL-1β and IL-6 lost variation in time of day cycling while TNF-α cycling was antiphase under circadian disruption conditions compared to 12-hour light/dark cycles. Circadian disruption led to an overall increase in leukocyte counts and ablated a time of day-dependent difference in levels. Circadian disruption can facilitate tumorigenesis and tumor growth by altering immune response patterns[55]. In breast cancer metastasis, circulating tumor cells (CTCs) generated during sleep have the highest metastatic potential compared to those generated during wake/active phases. Single cell RNA-sequencing found that these sleep phase generated-CTCs have a significant increase in expression of cell division and mitosis genes and circadian-related hormones dexamethasone, testosterone, and insulin contribute to the generation of these CTCs[56]. Thus, disruption of sleep or exposure to light at all times of the day that may result

from cancer treatments and hospital stays during such treatments can have a detrimental effect on patient prognosis and response to therapy.

Circadian clock proteins not only modulate the tumor but also the TME, allowing for crosstalk and feed-forward action of the primary tumor cells, cancer stem cells, and TME-related cells to promote cancer proliferation, angiogenesis, inflammation, and immune evasion[57,58]. *Bmal1*, *Clock*, and *Cry1* expression have been correlated with DCs, neutrophil cells, and CD4+ T cells, respectively, in clear renal cell carcinoma (RCC). In thoracic cancer, BMAL1 and CLOCK is associated with the infiltration of CD8+ T cells. Meanwhile, in metastatic melanoma, BMAL1 is lined to T-cell activation, differentiation, and exhaustion markers[57,58]. In GBM specifically, high *CLOCK* expression correlated with higher microglia and hematopoietic stem cells and lower activated CD8+ T cells and dendritic cells signatures. BMAL1::CLOCK regulates Olfactomedin-Like 3 (*OLFML3*) transcription, as heterodimer binding to the *OLFML3* promoter detected in ChIP-PCR experiments, and knockdown of either *BMAL1* or *CLOCK* in GSC lines, led to a decrease in *OLFML3* and, consequently, suppressed the migration of microglia (Figure 5A). OLFML3 was previously detailed to be part of a network of genes that are microglia-specific and upregulated during microglia maturation and is continually expressed by mature microglia[60]. Depletion of either *BMAL1*, *CLOCK*, and/or *OLFML3* extended overall survival and decreased intratumoral microglia density in GBM PDX mice[42]. Legumain (LGMN) was recently identified as an additional BMAL1::CLOCK transcriptional target that functions as a novel CLOCK-regulated chemokine in GSCs via OLFML3. LGMN was previously reported to be upregulated in microglia following spinal cord injury in zebrafish[61]. OLMFL3 upregulates *LGMN* via HIF-1α mediated signaling, and LGMN increases *CD162* levels to promote microglia migration (Figure 5B). Inhibition of the CLOCK/OLFML3/HIF-1α/LGMN/CD162 axis via pharmacological targeting (using a *REV-ERB* agonist) was able to synergize with anti-Programmed Cell Death Protein 1 (PD-1) therapy to extend survival in *in* vivo mouse

models[61]. BMAL1 also forms a heterodimer with HIF-1α (BMAL1::HIF-1α) and CRY1/2 negatively regulates the activity of BMAL1::HIF-1α heterodimers as well as HIF-1α's protein levels, suggesting that CRYs may also play a key role in microglia migration and the immunosuppressive GBM TME[63]. These findings highlight an additional mechanism in which circadian clock components drives an immune suppressive TME via upstream transcriptional control of microglia migration programs, thereby contributing to another "hallmark" of GBM biology.

A recent study also highlights the role of BMAL1::CLOCK in promoting angiogenesis by modulating OLFML3 in GSCs. There have been previous studies that underscore the role of GSCs in angiogenesis by excreting pro-angiogenic factors and extracellular vesicles; however, Pang et al., build upon this story by identifying OLFML3 as a modulator of HIF-1α that results in the upregulation of Periostin (POSTN). POSTN then activates TANK-Binding Kinase 1 (TBK1) (p-TBK1) signaling in endothelial cells, resulting in blood vessel formation (Figure 5C). [64–66]. Although TBK1 is involved in other pro-tumorigenic signaling pathways and GSC self-renewal, TBK1 was previously reported to promote angiogenesis by upregulating Vascular Endothelial Factor (VEGF) and inducing human umbilical vein endothelial cell (HUVEC) proliferation, supporting the notion that TBK1 is also critical for angiogenesis in tumor tissue[67,68].

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leukocyte counts and ablated a time of day-dependent difference in levels. Circadian disruption can facilitate tumorigenesis and tumor growth by altering immune response patterns[60]. In breast cancer metastasis, circulating tumor cells (CTCs) generated during sleep have the highest metastatic potential compared to those generated during wake/active phases. Single cell RNA-sequencing found that these sleep phase generated-CTCs have a significant increase in expression of cell division and mitosis genes and circadian-related hormones dexamethasone, testosterone, and insulin contribute to the generation of these CTCs[61]. Thus, disruption of sleep or exposure to light at all times of the day that may result from cancer treatments and hospital stays during such treatments can have a detrimental effect on patient prognosis and response to therapy.

MODULATING THE CIRCADIAN CLOCK FOR TREATMENT

As the circadian clock components and CCGs have been linked to all hallmarks of cancer and disruption of circadian rhythms can pose higher risk for several cancer types, the idea of leveraging the circadian clock as a novel approach in addressing cancer treatment have been tested both in preclinical and clinical studies.

Chronotherapy

Light is the most influential factor that affects our circadian rhythms given that light stimulation drives signaling of the SCN master clock. However, as mentioned, other factors, such as food consumption and activity can also affect circadian rhythms via influencing our peripheral clocks, resulting in feedback to the master clock. If disruption of these clocks due to modern lifestyles can drive cancer risk, consideration of these “timing factors”, if you will, could also provide therapeutic benefits or curb treatment side effects for cancer patients.

Light therapy has been employed in randomized clinic trials of cancer patients that assessed fatigue following chemotherapy and/or radiation treatment for breast cancer patients and survivors. In one randomized study of newly diagnosed stage I-III breast cancer patients, patients who were exposed to dim red light for 30 minutes in the morning throughout their first four rounds of chemotherapy experienced an increase in fatigue compared to patients exposed to bright white light, who did not exhibit any significant changes from base line fatigue levels. Although overall fatigue did not improve due to bright light therapy, worsening of overall fatigue can be prevented and bright light treatment could be an intervention tool for breast cancer patients undergoing chemotherapy[69]. Bright light therapy improved fatigue as well as sleep quality, latency, duration, and disruption in breast cancer survivors one to three years following chemotherapy and/or radiotherapy treatment[70]. Fatigue is the most common symptom following chemotherapy treatment that leads patients to discontinue their treatment. Even if the effectiveness of treatments is not changed or improved due to light therapy, if unfavorable side effects could be lessened, such as fatigue, this will be beneficial for quality of life and survival due to continuation of treatment for all cancer patients undergoing chemotherapy, including GBM patients.

Chronomedicine

As different genes and physiological processes cycle throughout the day and peak within a 24-hour period, it is not coincidental that cancer driver genes and disease symptoms also cycle and peak at designated times of the day. Generally, patients are instructed to take their medications once a day or several times a day, but the time of day is usually not specified. Chronomedicine has been a new avenue of exploration in personalized and precision medicine as it combines understanding of circadian biology, disease phenotypes, and a drug's mechanism of action to improve upon efficacy and patient outcome. Research into chronomedicine has been focused on determining whether a patient would benefit from

a time-of-day based treatment regimen. This coincides with findings that many drugs on the market directly target a CCG and that most clinical studies interrogating chronomedicine in treatment of a diverse number of diseases, not limited to cancer, have been evaluated and indicated a time-of-day increase in favorable physiological responses and efficacy of treatments with decrease in toxicity[71,72].

Radiation and chemotherapy have remained at the forefront of oncology treatment. The potency of chemoradiation application for cancer treatment relies solely on its ability to pose damage to DNA. Since circadian machinery monitors the cell cycle and DNA damage status, it is understandable that there is therapeutic potential for employing chronomedicine in chemoradiation application especially when patient biomarkers can inform better or worse response to DNA damage agents. A retrospective study performed at Washington University School of Medicine found that patients given temozolomide (TMZ) in the morning had longer overall survival compared to patients given TMZ in the evening. Categorization of MGMT-methylation status of patients in this study displayed improvement in overall survival was more pronounced in MGMT-methylated patients[73]. However, a recent systematic review of literature from 1946 to 2022 have shown that timing of TMZ administration may not provide such a robust signal as originally hoped[74]. Pre-clinical studies indicated that cancerous glioma tissue was more affected by radiation at zeitgeber time (ZT) 0, when *Per2* expression is high, versus ZT8. This time difference in the quantity of apoptotic cells was not observed in noncancerous brain tissue. These findings suggest that timing radiation therapy could potentially decrease detrimental effects on healthy tissue without jeopardizing antitumor effects[75]. All in all, there is a need for larger, randomized controlled trials that will need to be conducted to determine the therapeutic value of timing chemoradiation for GBM patients.

Results from oncology-based chronomedicine studies to date have also highlighted that potentially it is not so much as clock time as it is a person's individual chronotype that would be most effective in taking into consideration for future patient care. Although two separate clinical glioma studies investigating time of day administration of radiation or TMZ did not show differences in patient outcomes, these studies did exemplify that timing chemoradiation is feasible, which introduces potential new treatment plans for patients of all cancer types that considers timing of chemoradiation treatment [76,77]. Endogenous clocks can vary from person to person as one individual's phase can vary relative to external time from another's. Such differences in an individual's chronotype have allowed coining of the terms such as "morning larks" or "night owls". Ideally, if a patient's chronotype could be determined, it could generate an improved personalized treatment plan that considers the best time relative to a patient's chronotype in which drug application be the most effective in its effects, whether that be at a time that where symptoms are exacerbated, a gene target is peaking, or is even the least disruptive to a patient's sleep-wake cycle. This notion is supported by pre-clinical data from Slat et al., that indicates that circadian time of treatment of TMZ affected sensitivity to treatment in GBM tumor cells and the maximal DNA damage response, apoptosis, and growth inhibition occurred when *Bmal1* peaked in expression[78].

Circadian Pharmacology

Circadian clock compounds have been applied in numerous disease and behavioral disorder studies, but in recent years they have been investigated as a potential novel therapeutic option for cancer patients given the emergence of the circadian clock's role in cancer biology (Figure 6). Here we discuss the results of studies interrogating the therapeutic potential of various families of circadian clock compounds that can negatively regulate *BMAL1* and BMAL1::CLOCK function to date in different cancer types:

REV-ERB Agonists: GSK4112 was the first REV-ERB agonist discovered via fluorescence resonance energy transfer (FRET) assays[79]. SR9009 and SR9011 were developed thereafter and had increased plasma and brain exposure, allowing for *in vivo* analysis of REV-ERB agonist function. These agonists function by increasing recruitment of NCoR and HDAC3 to REV-ERBs, thereby increasing REV-ERB-mediated repression of *BMAL1* transcription[80]. Functional and molecular assays have been utilized to validate the increase in repressor activity and effect on *BMAL1* transcription and mRNA levels[81]. SR9009 and SR9011 have been studied in numerous disease models including multiple cancer types[81–82]. Both SR9009 and SR9011 relative to vehicle were able to decrease self-renewal, stemness, survival, migration, and clock gene expression in GSCs but not healthy or differentiated GBM cells[40]. Although researchers have demonstrated via gene knockout or knockdown experiments that many of these SR9009 and SR9011 activities are dependent on REV-ERBs, other studies have reported some REV-ERB-independent effects of SR9009[81,82,84,85]. These results suggest that REV-ERB agonists remain promising as an anti-cancer treatment but new scaffolds that do not display any off-target effects need to be developed and/or REV-ERBs must be validated as the target in the disease and cell models of interest where the compounds are being applied.

CRY Stabilizers: KL001 was the first CRY stabilizer identified through a library screen of compounds that affect the rhythmicity of U2OS *Bmal1-dLuc* luciferase reporter. KL001 treatment resulted in period lengthening and amplitude reduction in a dose-dependent response in U2OS cells harboring either a *Bmal1-dLuc* or *Per2-dLuc* reporter. KL001 interacts with both CRYs and stabilizes the proteins by inhibiting FBXL3-mediated degradation of the compounds through binding to CRYs in their Flavin Adenine Dinucleotide (FAD) binding pocket[86]. KL001 decreased self-renewal, stemness, survival, migration, and clock gene expression in GSCs but not healthy or differentiated GBM cells. Furthermore, combination treatments of both KL001 and REV-ERB agonists SR9009 and SR9011

displayed synergistic effects, suggesting that targeting both negative limbs of the circadian TTFLs can have a dramatic increase in targeting GSCs[40]. SHP656, another CRY stabilizer that was developed based on KL001 to increase bioavailability and brain penetration, also significantly reduced GSC cell proliferation compared to differentiated GBM cells or non-cancerous cells. SHP656 furthermore prolonged the survival of mice bearing tumors induced from two different patient derived GSC lines, suggesting the therapeutic potential of CRY stabilizers in targeting GBM[40]. Isoform-specific CRY stabilizers have recently been developed[87,88]. Leveraging novel CRY isoform-specific compounds may also provide additional therapeutic benefits given the aforementioned differences in CRY1 or 2 expression in GBM tissue[45].

CK1/2 Inhibitors: Although yet to be investigated in GBM models, CK1/2 inhibitors are promising candidates in preventing PER1/2 degradation and affecting circadian rhythms and can perhaps potentiate the anti-tumor effects of PERs in GBM. Longdaysin and PF-670462 are two different CK1 inhibitors that affect circadian rhythms following treatment. Their effects are likely due to inhibiting CK1-mediated phosphorylation of PERs and their subsequent degradation[89]. Longdaysin inhibited the Wnt/β-Catenin signaling pathway and decreased colony formation, migration, invasion, and sphere formation of breast cancer cells. *In vivo* applications of longdaysin prevented growth of triple negative breast cancer xenografts[90]. PF-670462 blocked interactions between chronic lymphocytic leukemia (CLL) cells with the TME and was able to decrease progression and delay onset of CLL and increase overall survival *in vivo*[91]. The CK2 inhibitor GO289 also affects circadian rhythms and prevent phosphorylation and degradation of PER. GO289 is specific for CK2 given that it does not interact at the hinge region that is highly conserved across kinases. Furthermore, it was able to decrease growth of multiple RCC cell lines and a mouse MLL-AF9 acute myeloid leukemia (AML) cells[92].

ROR Agonists: ROR-targeting compounds have not been investigated in the context of GBM but leveraging them to inhibit immunosuppression may also prove to anti-tumorigenic by modulating the GBM TME. SR1078, a RORα/γ agonist, increased CD8+ T cell responses and suppressed NF-κB activity in the Jurkat leukemic cell line[93]. The RORγ specific agonists LYC-53772 and LYC-54143 were reported to block immunosuppression and differentiation of Th17 cells and increase levels of cytokines, therefore having anti-tumor activity by both increasing immune activation and decreasing immune suppression. Furthermore, LYC-54143 was able to enhance the cytotoxic activity of Tc17 cells and increased the CAR-T cell-mediated targeting of K562 cancer cells *in vitro* while also inhibiting both MC38 colorectal and 4T1 breast tumor growth *in vivo*[94].

CONCLUSION

The average survival time of GBM has only improved incrementally despite the laborious efforts of scientists and physicians and there is a dire need to improve upon the available effective treatments for GBM patients. There is evidence that circadian components play an oncogenic role in the pathogenesis of GBM and could be used as novel GBM biomarkers, but there is still much to be uncovered for there to be a path forward in the clinic. The emerging picture seems to be that combining circadian clock compounds may improve upon the efficacies of current standard-of-care. A troubling aspect of GBM chemotherapy using temozolomide treatment is the change in MGMT methylation status and/or the inevitable selection for high MGMT-expressing cells that confers resistance to TMZ[95]. The application of circadian compounds may help to overcome gained resistance to TMZ by targeting stem cells that survive chemoradiation. If circadian clock compounds can target GSCs that are resistant to TMZ and radiation, this suggests that other cell types that are also chemoradio-resistant could also be sensitive to circadian targeting. We can also

utilize these compounds to target the TME either alone or in combination with other lines of treatment, such as immune therapy or targeted anti-angiogenic therapy. In doing so, it may increase GBM tumor susceptibility to immune targeting and decrease the secretion of pro-angiogenic factors and growth of blood vessels in the TME. Combining such pharmacological tools with timing of chemoradiation and light therapy, could not only improve upon patient survival but also curb the effects on normal brain tissue and promote patient quality of life during and after treatment. A recent study in a GBM Drosophila model revealed that tumor-bearing flies have a disrupted circadian rhythm that is driven by neurodegeneration, which suggests a vicious cycle and relationship between the tumor and brain function and that proper readjustment of the internal clock can be beneficial in delaying GBM progression and associated neurodegenerative effects[96]. Albeit that these observations provide much needed optimism in the field, there is still much to uncover regarding modulating circadian machinery in treatment and management of GBM and the results from many preclinical and clinical studies on cancer types can also be applied in designing and aiding future GBM studies.

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FIGURE LEGENDS

Figure 1. The Master and Peripheral Clocks in Humans

Multiple environmental cues known as zeitgebers provide organisms with time-of-day information and result in downstream signaling that entrains the circadian clock. The most influential zeitgeber is light, which signals via the retina to the master clock or SCN. The SCN will then direct other locations and peripheral clocks through the body, including the brain, lungs, heart, muscles, liver, and digestive tract via both the endocrine and neuronal systems. The peripheral clocks are further entrained through non-photic zeitgebers such as food, sleep, activity, hormones, metabolites, and other circulating factors and can maintain rhythm independent of signaling from the SCN. Peripheral clocks also provide the SCN with feedback and all in all, they work together to generate 24-hour rhythms in physiological functions.

Figure 2. The Mammalian Circadian Oscillator

The core of the circadian TTFL consists of BMAL1 and CLOCK, which form a heterodimer and binds to the E-box motif of CCG promoters to regulate their transcription. BMAL1::CLOCK also regulate the transcription of their own positive and negative regulators. In the core, or primary, loop, CRY1/2 and PER1/2 form a heterodimer that inhibits the transcriptional activity of BMAL1::CLOCK. In the secondary loop, RORα/β/γ acts upon the RORE element of the *BMAL1* promoter to promote *BMAL1* transcription, whereas REV-ERBα/β binding to the RORE element blocks RORα/β/γ and inhibits BMAL1 transcription.

Figure 3. Post-Translational Modifications of Core Clock Proteins

(A) SUMOylation of BMAL1 promotes its ubiquitination, therefore leading to proteasomal degradation. CDK5 phosphorylates CLOCK, also leading to proteasomal degradation. O-GlcNAcylation stabilizes BMAL1 and CLOCK by competing with phosphorylation at the same sites. In contrast, phosphorylation of BMAL1::CLOCK via GSK-3β leads to their degradation.

(B) CK1 phosphorylates PER1/2 at night while CK2 phosphorylates PER1/2 during the day. Phosphorylation of PER1/2 leads to ubiquitination via the SCFβ-TrCP1/2 complex, targeting the proteins for proteasomal degradation. CK1 also mediates PER1/2 nuclear localization via phosphorylation of the FASP site. Dimerization of PER1/2 and CRY1/2 enhances their localization to the nucleus to affect transcription.

(C) AMPK phosphorylates CRY1/2, which then signals FBXL3 to ubiquitinate CRY1/2, leading to their proteasomal mediated degradation and termination of CRYs' transcriptional repressive activity. FBXL3 can also enter the nucleus to target CRY1/2. However, PER and FBXL3 both interact with CRY at overlapping locations and PER can therefore exclude FBXL3 and protect CRY1/2 from degradation. FBX21 also ubiquitinates CRY1/2 but competes with FBXL3 and stabilizes CRYs. USP7 antagonizes the activity of FBXL3 by deubiquitinating and stabilizing CRY1/2.

(D) CDK1 phosphorylates REV-ERBα and this event is followed by FBXW7-mediated ubiquitination and degradation of REV-ERBα. Additionally, GSK-3β also phosphorylates REV-ERBα but such event stabilizes the protein and prevents its proteasomal degradation.

Figure 4. Circadian Clock Control of GSCs

(A) Genetic targeting or pharmacological treatment targeting in GSCs of either BMAL1 or BMAL1::CLOCK transcriptional activity in both *in vivo* and *in vitro* models resulted in an increase in apoptosis, cell cycle arrest, and overall survival in mice while decreasing levels of stemness and metabolic genes, cell proliferation, and tumor burden.

(B) Compared to control NSCs, GSCs have a more open chromatin landscape that allows for increased BMAL1::CLOCK binding at the E-box of CCGs that control glycolysis, TCA cycle, and stem cell maintenance genes in GSCs, conferring active transcription and sensitivity to circadian clock targeting.

Figure 5. The Clock and the GBM TME

(A) High expression of *CLOCK* in GBM is correlated with higher levels of microglia and hematopoietic stem cells present in the GBM TME. BMAL1::CLOCK regulate the transcription of *OLFML3* in GSCs, which increases microglia migration.

(B) OLFML3 and HIF-1α work alongside BMAL1::CLOCK to drive an increase in LGMN expression in GSCs and, consequently, microglia migration. LGMN is also highly expressed in the microglia population of GBM patient samples, and it drives the expression of *CD162*, further promoting microglia migration in the TME.

(C) BMAL1::CLOCK upregulates *OLFML3*. OLFLM3 promotes HIF-1α -mediated transcription of *POSTN*. POSTN is then secreted from GSCs to endothelial cells to activate TBK1 (p-TBK1) and promote angiogenesis.

Figure 6. Mechanism of Action of Circadian Clock Compounds Targeting GBM

REV-ERB agonists promote the recruitment of NCoR and HDAC3 to REV-ERBs, which therefore increases REV-ERB-mediated repression of *BMAL1* transcription. ROR agonists potentiate the transcriptional effects of RORs. CRY stabilizers inhibit ubiquitination of CRY1/2 via the E3 ubiquitin ligase FBXL3, thereby preventing it's signaling for proteasome degradation, resulting in CRY stabilization. CK1 and CK2 inhibitors prevent CK1/2-mediated phosphorylation and resulting degradation of PER1/2.

Figure 1

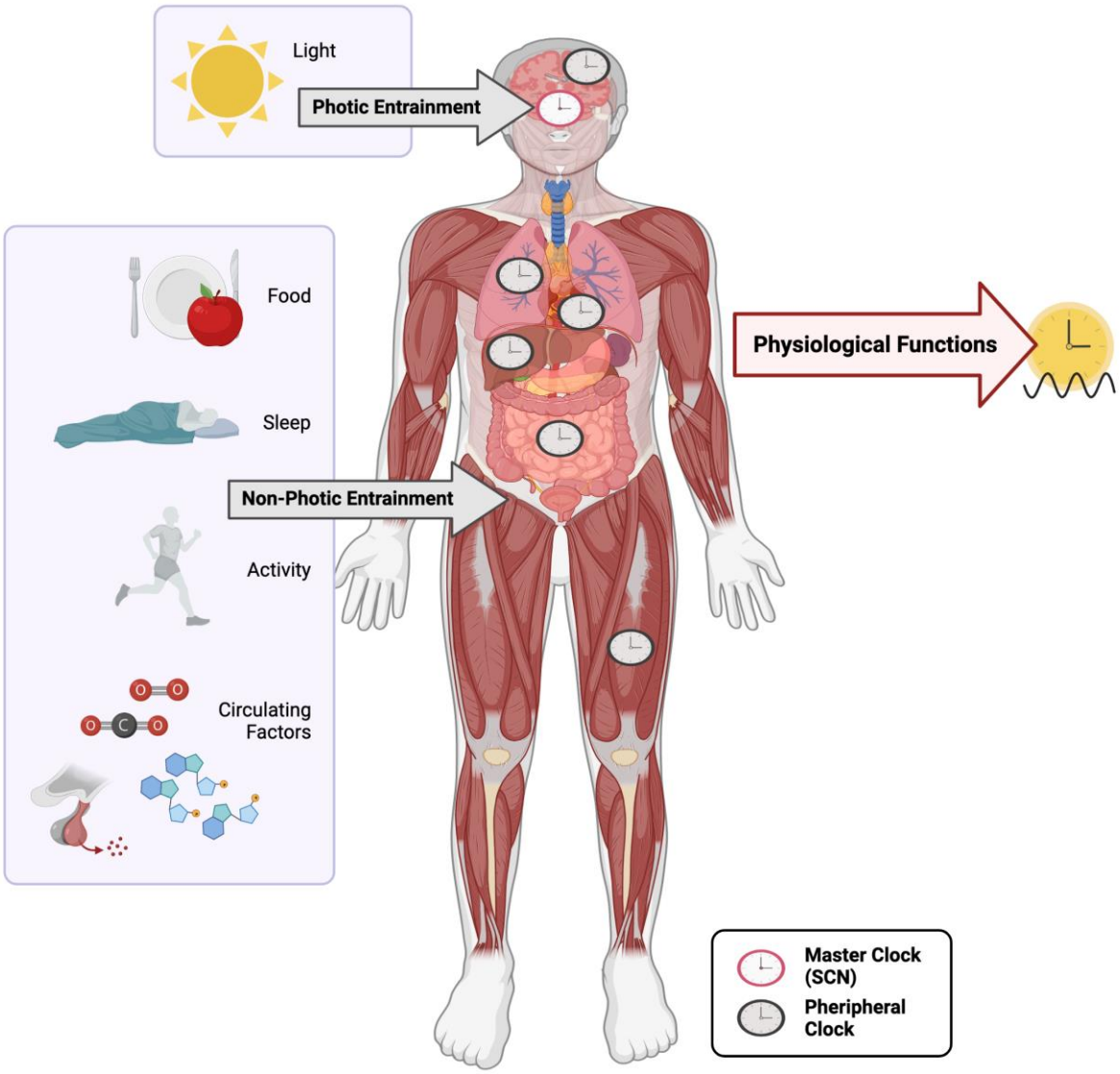


Figure 2

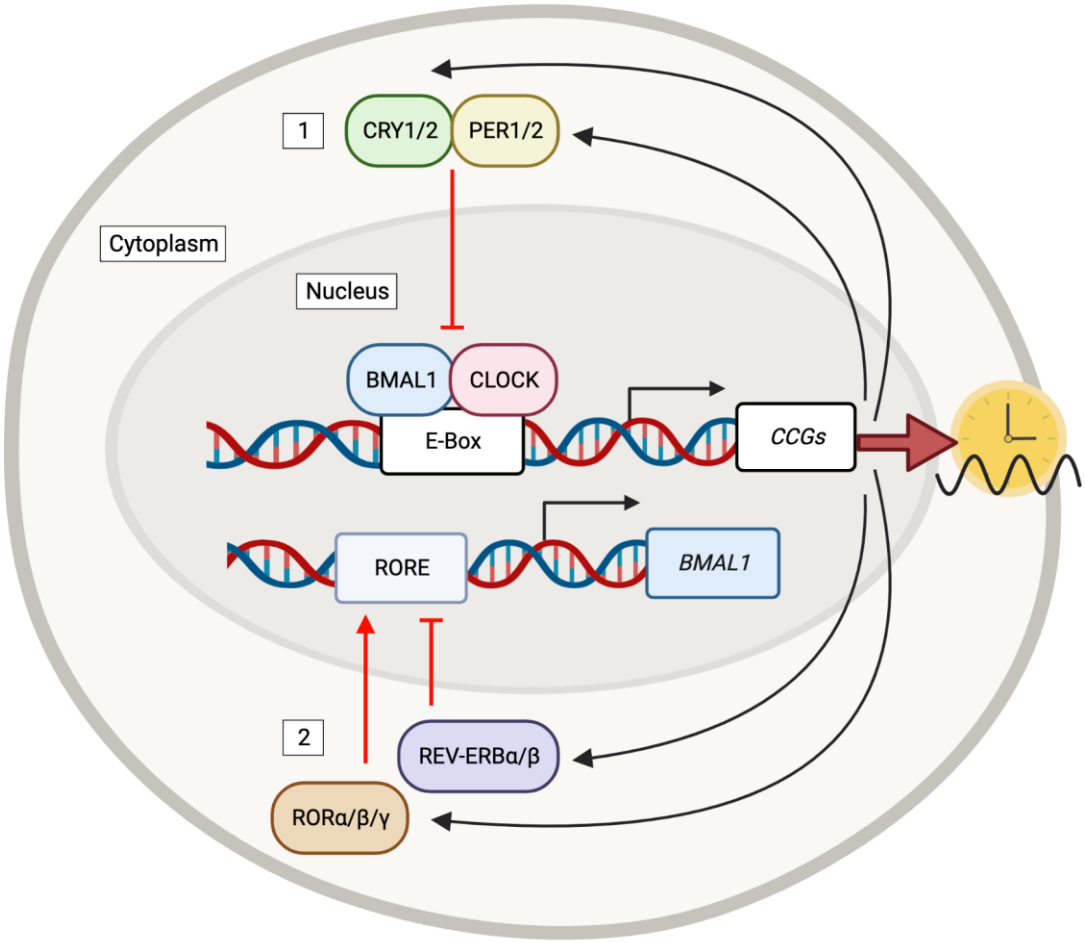
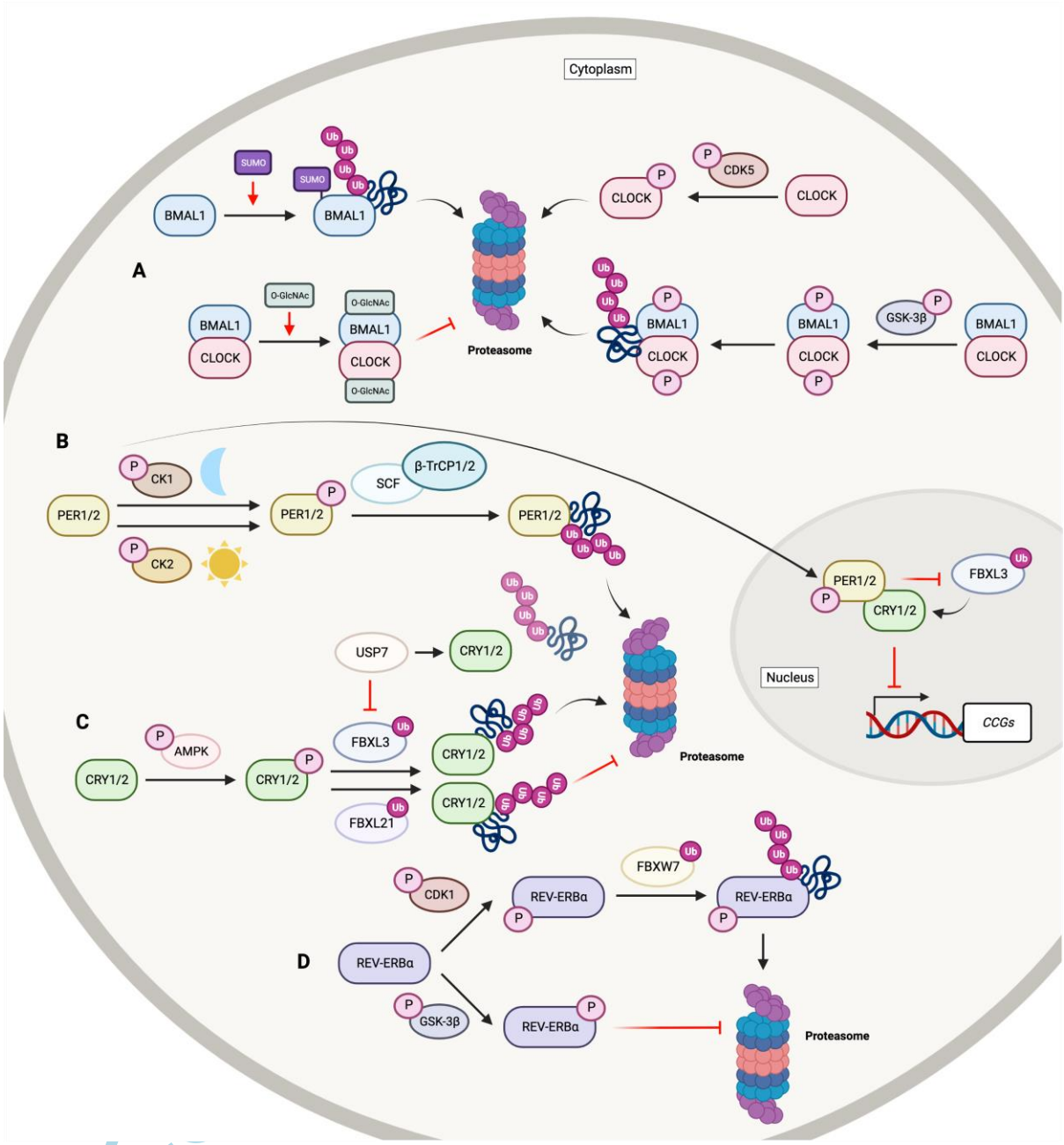


Figure 3



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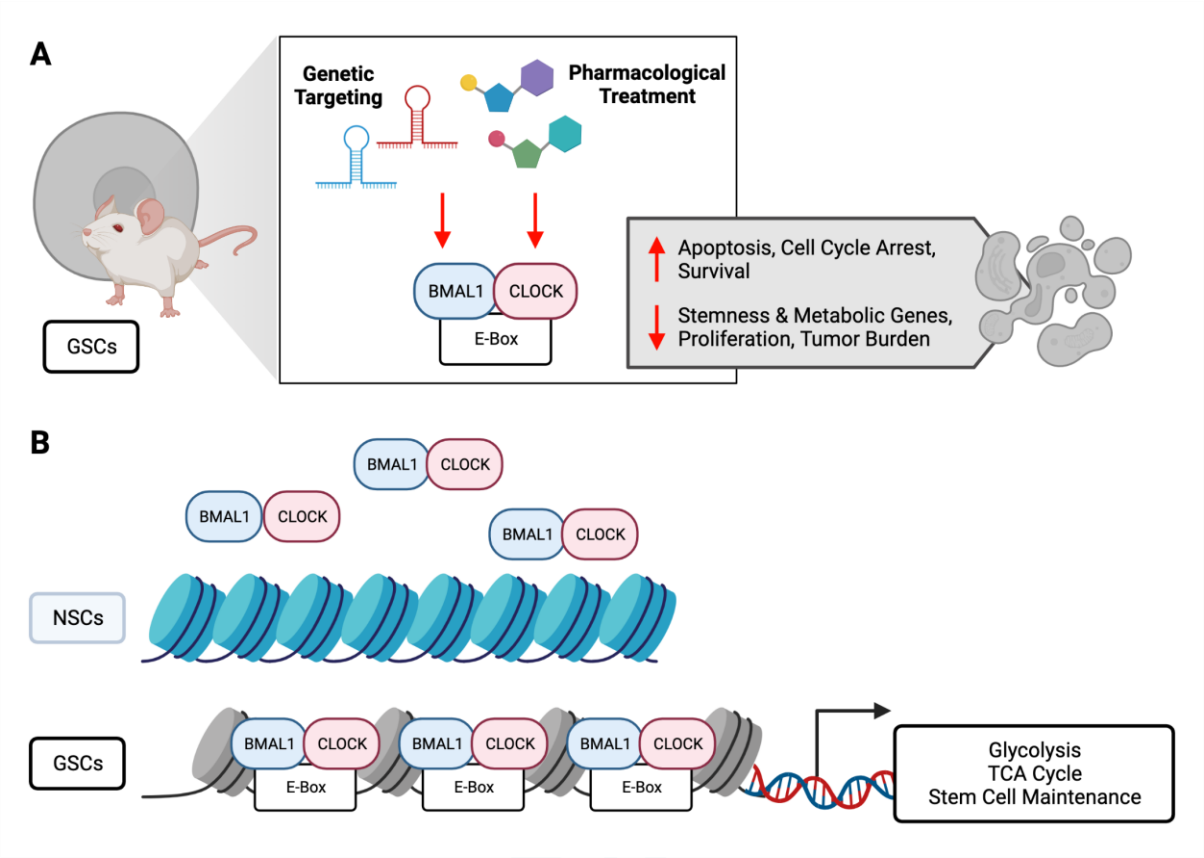

Figure 5

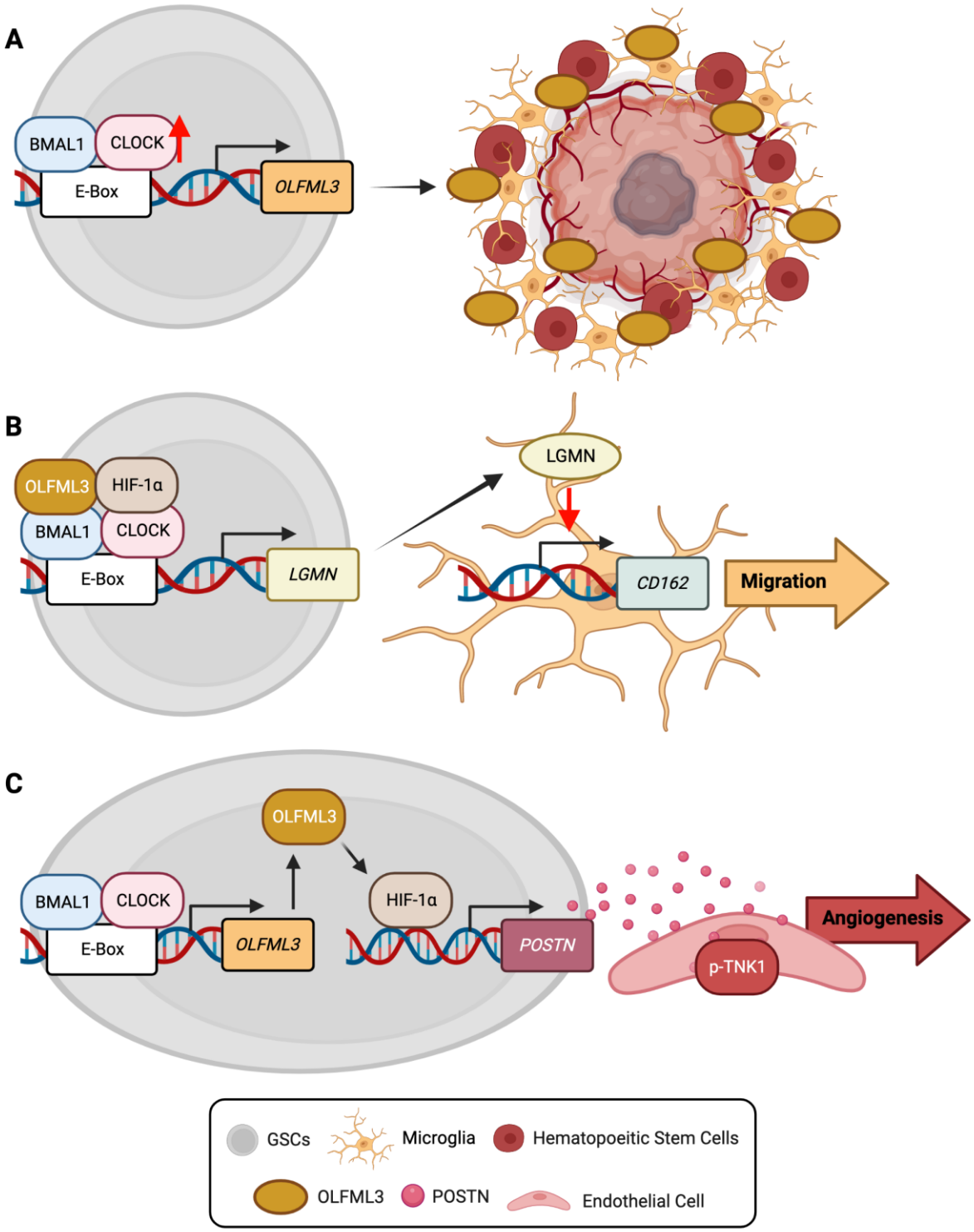

Figure 6